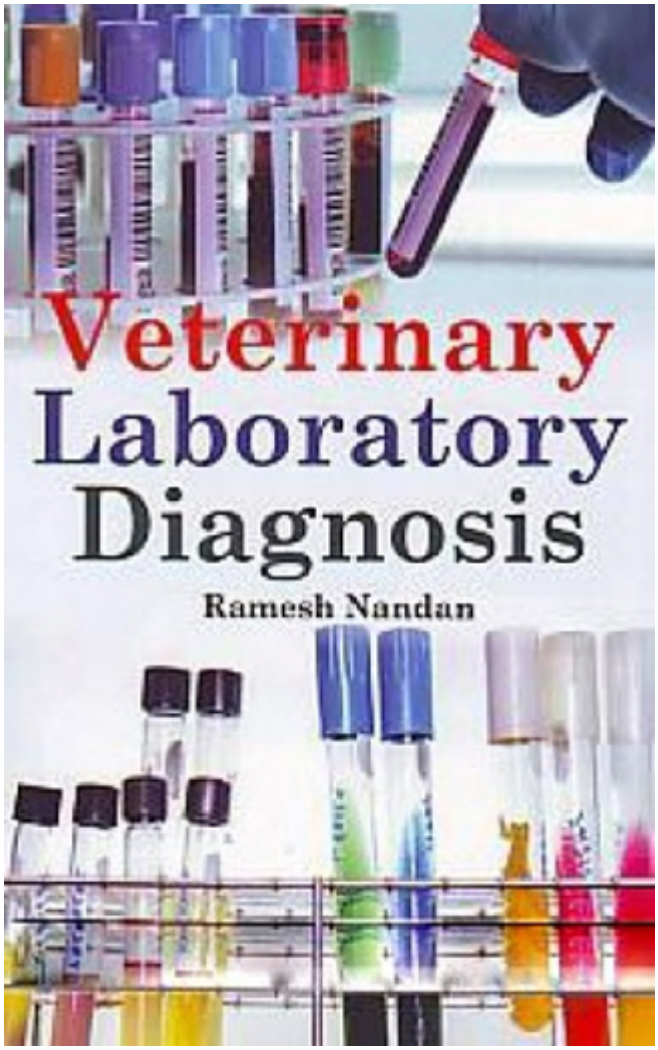
Veterinary
Laboratory
Diagnosis
Ramesh Nandan

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Veterinary Laboratory Diagnosis

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Preface

Laboratory Diagnosis or Clinical pathology is a subspecialty of pathology that deals with the use of laboratory methods (clinical chemistry, microbiology, hematology and emerging subspecialties such as molecular diagnostics) for the diagnosis and treatment of disease. Just as your physician will check your vital signs, weight, and other conditions when you visit the doctor, veterinarian will also want to check vital signs and obtain basic medical information about pets and livestock. In addition to checking pet's weight, looking at pet's eyes, checking its ears, routine examination of the mouth and teeth, and observing the pet's movements, there are other simple tests that are often performed. The veterinarian will use a stethoscope to listen for abnormal heart, lung, or digestive system sounds that may indicate problems with these organs. The veterinarian may gently press on the pet's gums with a finger and then release the pressure to determine how long it takes for the capillaries in the gums to refill. A longer than normal capillary refill time may indicate that the pet is going into, or is already in, shock. Long refill times also occur in certain heart diseases. The colour of the gums can also indicate problems such as jaundice (a sign of liver disease), shock, or anemia.

Veterinarians use their hands to check for the size and location of internal organs such as the liver, spleen, kidneys, and urinary bladder. This is called abdominal palpation. They will also check for enlargement of lymph nodes located throughout the body. If the pet has a specific problem at the time of the examination, the veterinarian may perform additional tests that are not generally part of a routine physical examination. For example, examination of a dog with suspected vision problems might include tests that assess overall vision, examination with an ophthalmoscope and various stains, and determination of the pressure within the eye. Similarly, examination of a lame horse might include a hands-on examination of the affected leg, blood and biochemical tests, muscle biopsy, and various types of imaging techniques. Early warning is the rapid detection of the introduction of, or sudden increase in, any

disease of livestock which has the potential of developing to epidemic proportions and/or causing serious socio-economic consequences or public health concerns. It embraces all initiatives and is mainly based on disease surveillance, reporting and epidemiological analysis. These lead to improved awareness and knowledge of the distribution and behaviour of disease outbreaks and infection, allow forecasting of the source and evolution of the disease outbreaks and the monitoring of the effectiveness of disease control campaigns. The rapid and accurate diagnosis of diseases can only be assured in fully equipped laboratories that have a range of standardized diagnostic reagents, experienced staff and a sufficient throughput of diagnostic specimens to maintain expertise. It should be noted that development of diagnostic expertise for exotic disease using tests that require handling the live agent should only be attempted in microbiologically high-security laboratories. Laboratory diagnostic procedures should not be done without prior detailed clinical-epidemiological investigations as otherwise the lab results will be too undetermined. It is important to always include the entire herd and also to bear in mind that the unborn animals play a significant role with regard to the chain of infection. In other words, it pays off to plan for the long term – i.e. to include the age structure and the pregnant animals in all considerations.

This book is designed to indicate which tests are most clinically useful for the dog, cat, horse, and ruminant, with suggestions for diagnostic applications and interpretations of results. Students of veterinary science and animal health practitioners will this book valuable in their study and practice.

— Ramesh Nandan

Chapter 1: Laboratory Tests in Animal Health

Blood Test

A blood test is a laboratory analysis performed on a blood sample that is usually extracted from a vein in the arm using a needle, or via fingerprick. Blood tests are used to determine physiological and biochemical states, such as disease, mineral content, drug effectiveness, and organ function. They are also used in drug tests. Although the term *blood test* is used, most routine tests (except for most haematology) are done on blood plasma instead of blood cells.

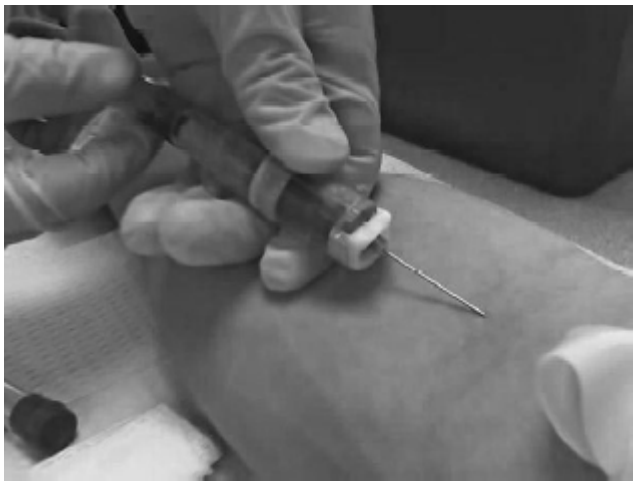


Figure: *A venipuncture performed using a vacutainer*

A blood test is a laboratory analysis performed on a blood sample that is usually extracted from a vein in the arm using a needle, or via fingerprick. Blood tests are used to determine physiological and biochemical states, such as disease, mineral content, drug effectiveness, and organ function. They are also used in drug tests. Although the term *blood test* is used, most routine tests (except for most haematology) are done on blood plasma instead of blood cells.

Extraction

Venipuncture is useful as it is a relatively non-invasive way to obtain cells and extracellular fluid (plasma) from the body for analysis. Since blood flows throughout the body, acting as a medium for providing oxygen and nutrients, and drawing waste products back to the excretory systems for disposal, the state of the bloodstream affects, or is affected by, many medical conditions. For these reasons, blood tests are the most commonly performed medical tests.

If only a few drops of blood are needed, a fingerstick is performed instead of drawing blood from a vein.

Phlebotomists, laboratory practitioners and nurses are those charged with patient blood extraction. However, in special circumstances, and emergency situations, paramedics and physicians sometimes extract blood. Also, respiratory therapists are trained to extract arterial blood for arterial blood gases.

Types of Blood Tests

Biochemical Analysis

A basic metabolic panel measures sodium, potassium, chloride, bicarbonate, blood urea nitrogen (BUN), magnesium, creatinine, glucose, and sometimes includes calcium. Blood tests focusing on cholesterol levels can determine LDL and HDL cholesterol levels, as well as triglyceride levels.

Some blood tests, such as those that measure glucose, cholesterol, or for determining the existence or lack of STD, require fasting (or no food consumption) eight to twelve hours prior to the drawing of the blood sample.

For the majority of blood tests, blood is usually obtained from the patient's vein. However, other specialized blood tests, such as the arterial blood gas, require blood extracted from an artery. Blood gas analysis of arterial blood is primarily used to monitor carbon dioxide and oxygen levels related to pulmonary function, but it is also used to measure blood pH and bicarbonate levels for certain metabolic conditions.

While the regular glucose test is taken at a certain point in time, the glucose tolerance test involves repeated testing to determine the rate at which glucose is processed by the body.

White blood cells (including T-cells) are often counted.

Normal Ranges

Test	*Low*	*High*	*Unit*	*Comments*
Sodium (Na)	136	145	mmol/L	
Potassium (K)	3.5	5.0	mmol/L	
Urea	2.5	6.4	mmol/L	BUN - blood urea nitrogen
Urea	15	40	mg/dL	
Creatinine - male	62	115	mmol/L	
Creatinine - female	53	97	mmol/L	
Creatinine - male	0.7	1.3	mg/dL	
Creatinine - female	0.6	1.2	mg/dL	
Glucose (fasting)	3.9	5.8	mmol/L	glycosylated hemoglobin
Glucose (fasting)	70	120	mg/dL	

Molecular Profiles

- Protein electrophoresis (general technique—not a specific test)
- Western blot (general technique—not a specific test)
- Liver function tests
- Polymerase chain reaction (DNA). DNA profiling is today possible with even very small quantities of blood: this is commonly used in forensic science, but is now also part of the diagnostic process of many disorders.
- Northern blot (RNA)
- Sexually transmitted diseases

Cellular Evaluation

- Full blood count (or “complete blood count”)
- Hematocrit and MCV (“mean corpuscular volume”)
- Erythrocyte sedimentation rate (ESR)

- Cross-matching. Determination of blood type for blood transfusion or transplants
- Blood cultures are commonly taken if infection is suspected. Positive cultures and resulting sensitivity results are often useful in guiding medical treatment.

Future Alternatives

Saliva tests

In 2008, scientists announced that the more cost effective saliva testing could eventually replace some blood tests, as saliva contains 20% of the proteins found in blood.

Microemulsion

February 2011: Canadian researchers have developed a microchip for blood tests. It is called microemulsion, a droplet of blood captured inside a layer of another substance. It can control the exact size and spacing of the droplets. The new test could improve the efficiency, accuracy and speed of laboratory tests while also doing it cheaply. The microchip costs $25, whereas the robotic dispensers currently in use cost around $10,000.

SIMBAS

March 2011: A team of researchers from UC Berkeley, DCU and University of Valparaíso have developed lab-on-a-chip that can diagnose diseases within 10 minutes without the use of external tubing and extra components. It is called Self-powered Integrated Microfluidic Blood Analysis System (SIMBAS). It uses tiny trenches to separate blood cells from plasma (99 percent of blood cells were captured during experiments). Researchers used plastic components, to reduce manufacturing costs. Laboratory examination of blood

Physicians rely upon laboratory analysis to obtain measurements of many constituents of the blood, information useful or necessary for the detection and recognition of disease.

Hemoglobin contains a highly coloured pigment that interferes with the passage of a beam of light. To measure hemoglobin concentration, blood is diluted and the red blood cells (erythrocytes) broken down to yield a clear red solution. A photoelectric instrument is used to measure

the absorbance of transmitted light, from which hemoglobin concentration can be calculated.

Changes in the hemoglobin concentration of the blood are not necessarily directly paralleled by changes in the red cell count and the hematocrit value, because the size and hemoglobin concentration of red cells may change in disease. Therefore, measurements of the red cell count and the hematocrit value may provide useful information as well. Electronic particle counters for determining red cell, white cell (leukocyte), and platelet counts are widely used. Only a drop of blood is needed for the analyses, which are completed within a minute.

Adequate examination of the blood cells requires that a thin film of blood be spread on a glass slide, stained with a special blood stain (Wright stain), and examined under the microscope. Individual red cells, white cells, and platelets are examined, and the relative proportions of the several classes of white cells are tabulated. The results may have important diagnostic implications.

In iron-deficiency anemia, for example, the red cells look paler than normal because they lack the normal amount of hemoglobin; in malaria the diagnosis is established by observing the malarial parasites within the red cells. In pneumonia and many infections, the proportion of neutrophilic leukocytes is usually increased, while in others, such as pertussis (whooping cough) and measles, there is an increase in the proportion of lymphocytes.

Chemical analyses measure many of the constituents of plasma. Often serum rather than plasma is used, however, since serum can be obtained from clotted blood without the addition of an anticoagulant. Changes in the concentrations of chemical constituents of the blood can indicate the presence of disease.

For example, quantitative determination of the amount of sugar (glucose) in the blood is essential for the diagnosis of diabetes, a disease in which the blood sugar tends to be elevated. Nitrogenous waste products, in particular urea, tend to accumulate in persons with diseased kidneys that are unable to excrete these substances at a normal rate. An increase in the concentration of bilirubin in the serum often reflects a disorder of the liver and bile ducts or an increased rate of destruction of hemoglobin.

Tests can be performed manually using an individual procedure for each analysis; however, the auto analyzer, a completely automated machine, increases the number of chemical analyses that can be

performed in laboratories. A dozen analyses may be made simultaneously by a single machine employing a small amount of serum.

The serum is automatically drawn from a test tube and is propelled through plastic tubing of small diameter. As the serum specimen advances, it is divided; appropriate reagents are added; chemical reactions occur with formation of a product that can be measured with a photoelectric instrument; and the result appears as a written tracing from which serum concentration of various substances can be read directly.

The data acquired by the machine may be fed automatically into a computer and the numerical results printed on a form that is submitted to a physician. Many of the available analyses are not performed routinely but are invaluable in special circumstances.

In cases of suspected lead poisoning, for example, detection of an elevated level of lead in the blood may be diagnostic. Some analytical procedures have specific diagnostic usefulness. These include assays for certain hormones, including measurement of the thyroid hormone in the serum of patients suspected of having thyroid disease.

Other important laboratory procedures are concerned with immunologic reactions of the blood. Careful determinations of the blood groups of the patient and the blood donor, and cross matching of the cells of one with the serum of the other to ensure compatibility, are essential for the safe transfusion of blood.

The Rh type of a pregnant woman is regularly determined and is necessary for the early detection of fetal-maternal incompatibility and for proper prevention or treatment of erythroblastosis fetalis (hemolytic disease of the newborn).

The diagnosis of certain infectious diseases depends upon the demonstration of antibodies in the patient’s serum.

Many other kinds of blood examination yield useful results. Enzymes normally present in the muscle of the heart may be released into the blood when the heart is damaged by a coronary occlusion (obstruction of the coronary artery) with consequent tissue death. Measurement of these enzymes in the serum is regularly performed to assist in diagnosis of this type of heart disease.

Damage to the liver releases other enzymes, measurement of which aids in evaluation of the nature and severity of liver disease. Inherited abnormalities of proteins are increasingly recognized and identified by use of sophisticated methods.

Accurate diagnosis of hemophilia and other bleeding disorders is made possible by investigations of the coagulation mechanism. Measurements of the concentration of folic acid and vitamin B_{12} in the blood provide the basis for diagnosis of deficiencies of these vitamins.

Stool Test

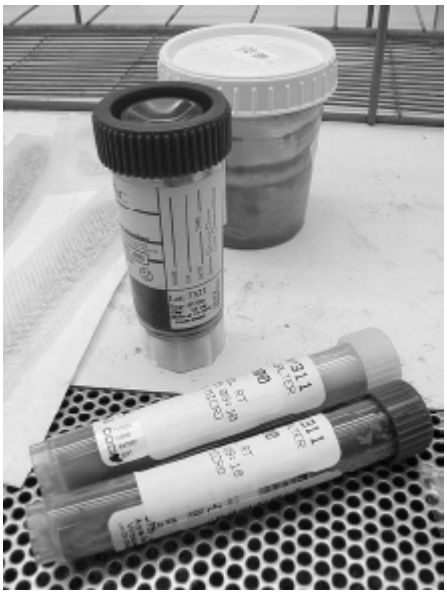


Figure: *Transport vials filled with human feces for stool testing. Yellow and blue tops for parasite testing, red top for stool cultures and the white top was provided by the patient with the sample.*

A stool test involves the collection and analysis of fecal matter to diagnose the presence or absence of a medical condition.

Fecal Occult Blood Test

One of the most common stool tests, the fecal occult blood test can be used to diagnose many conditions that cause in bleeding in the gastrointestinal system including colorectal cancer or stomach cancer.

Microbiology Tests

Parasitic diseases such as ascariasis, hookworm, strongyloidiasis and whipworm can be diagnosed by examining stools under a microscope for the presence of worm larvae or eggs. Some bacterial diseases can be detected with a stool culture. Toxins from bacteria such as *Clostridium difficile* ('C. diff.') can also be identified. Viruses such as rotavirus can also be found in stools.

Chemical Tests

A fecal pH test may be used determine lactose intolerance or the presence of an infection. Steatorrhea can be diagnosed using a Fecal fat test that checks for the malabsorption of fat.

Faecal elastase levels are becoming the mainstay of pancreatitis diagnosis.

Feces

Feces, faeces, or fæces (see spelling differences), also known as excrement, is a waste product from an animal's digestive tract expelled through the anus or cloaca during a process called *defecation*.

Etymology

The word *faeces* is the plural of the Latin word *fæx* meaning "dregs". There is no singular form in the English language, making it a plurale tantum. There are many colloquial terms for feces, of which some are considered profanity (such as *shit* and *crap*) while others (such as *poo*, *poop*, *number two*, *deuce*, *doodoo*, *dookie* and *doody*) are not. Terms such as *dung*, *scat*, *spoor* and *droppings* are normally used to refer to animal feces.

Stool is a common term normally used in reference to human feces. For example, in medicine to diagnose the presence or absence of a medical condition, a stool sample is sometimes requested for testing purposes. The term "stool" can also be used for that of nonhuman species.

Ecology

After an animal has digested eaten material, the remains of that material are expelled from its body as waste. Though it is lower in energy than the food it came from, feces may still contain a large amount of energy, often 50% of that of the original food. This means that of all food eaten, a significant amount of energy remains for the decomposers of ecosystems. Many organisms feed on feces, from bacteria to fungi to insects such as dung beetles, which can sense odors from long distances. Some may specialize in feces, while others may eat other foods as well. Feces serve not only as a basic food, but also a supplement to the usual diet of some animals. This is known as coprophagia, and occurs in various animal species such as young elephants eating their mother's feces to gain essential gut flora, or by other animals such as dogs, rabbits, and monkeys.

Feces and urine, which reflect ultraviolet light, are important to raptors such as Kestrels, which are able to locate their prey by their middens and territorial markers.

Seeds may also be found in feces. Animals that eat fruit are known as frugivores. The advantage for a plant in having fruit is that animals will eat the fruit and unknowingly disperse the seed in doing so. This mode of seed dispersal is highly successful, as seeds dispersed around the base of a plant are unlikely to succeed and are often subject to heavy predation. Provided the seed can withstand the pathway through the digestive system, it is not only likely to be far away from the parent plant, but is even provided with its own fertilizer.

Organisms which subsist on dead organic matter or *detritus* are known as detritivores, and play an important role in ecosystems by recycling organic matter back into a simpler form which plants and other autotrophs may once again absorb. This cycling of matter is known as the biogeochemical cycle. To maintain nutrients in soil it is therefore important that feces return to the area from which they came, which is not always the case in human society where food may be transported from rural areas to urban populations and then feces disposed of into a river or sea.

Human Feces

In humans, defecation may occur (depending on the individual and the circumstances) from once every two or three days to several times a day. Extensive hardening of the feces may cause prolonged interruption in the routine and is called constipation.

Human fecal matter varies significantly in appearance, depending on diet and health. Normally it is semisolid, with a mucus coating. Its brown coloration comes from a combination of bile and bilirubin, which comes from dead red blood cells.

In newborn babies, fecal matter is initially yellow/green after the meconium. This coloration comes from the presence of bile alone. In time, as the body starts expelling bilirubin from dead red blood cells, it acquires its familiar brown appearance, unless the baby is breast feeding, in which case it remains soft, pale yellowish, and not completely malodorous until the baby begins to eat significant amounts of other food.

Throughout the life of an ordinary human, one may experience many types of feces. A “green” stool is from rapid transit of feces

through the intestines (or the consumption of certain blue or green food dyes in quantity), and "clay-like" appearance to the feces is the result of a lack of bilirubin.

Bile overload is very rare, and not a health threat. Problems as simple as serious diarrhea can cause blood in one's stool. Black stools caused by blood usually indicate a problem in the intestines (the black is digested blood), whereas red streaks of blood in stool are usually caused by bleeding in the rectum or anus.

Food may sometimes make an appearance in the feces. Common undigested foods found in human feces are seeds, nuts, corn and beans, mainly because of their high dietary fiber content. Beets may turn feces different hues of red. Artificial food coloring in some processed foods such as highly colorful packaged breakfast cereals can also cause unusual feces coloring if eaten in sufficient quantities.

Laboratory examination of feces, usually termed as stool examination or stool test, is done for the sake of diagnosis, for example, to detect presence of parasites such as pinworms and/or their eggs (ova) or to detect disease spreading bacteria.

Personal Hygiene

Cultures employ a variety of personal cleansing practicing after elimination.

- In Western and East Asian societies, the use of toilet paper is widespread. Other paper products were also used before the advent of flush toilets.
- Some European countries use a bidet for additional cleaning.
- In South Asia and Southeast Asia, showers are provided for use in toilets.
- In Islam, washing of the anus with water using the left hand is part of the prescribed ritual ablutions.
- In India, the anus is also washed with water using the left hand.
- In the United Kingdom, the Indian toilet was adapted as the "WC" (water closet) and widely deployed in England during the reign of Queen Victoria. London suffered numerous outbreaks of food poisoning resulting from workers handling food after using the toilet. Cleansing of the anus was an arbitrary practice left to personal choice and facilities available.

- In Ancient Rome, a communal sponge was employed. It was rinsed in a bucket of salt water after use.
- In Japan, flat sticks were used in ancient times, being replaced by toilet paper as the country became more Westernized. Toilets that include built-in bidets have now become widely popular in private homes; these can be very sophisticated appliances, allowing users to adjust the temperature, direction and force of water jets, and offering warm air to dry the anus and surrounding regions. The toilet flushes automatically when the buttocks leave the seat.

Health Issues

Consistency and shape of stools may be classified medically according to the Bristol Stool Scale. Pica, a disorder where non-food items are eaten, can cause unusual stool. Intestinal parasites and their ova (eggs) can sometimes be visible to the naked eye.

Odor

The distinctive odor of feces is due to bacterial action. Gut flora produce compounds such as indole, skatole, and thiols (sulfur-containing compounds), as well as the inorganic gas hydrogen sulfide. These are the same compounds that are responsible for the odor of flatulence. Consumption of foods with spices may result in the spices being undigested and adding to the odor of feces. The perceived bad odor of feces has been hypothesized to be a deterrent for humans, as consumption or touching it may result in sickness or infection. Of course, human perception of the odor is a subjective matter; an animal that eats feces may be attracted to its odor.

Pets

Pets can be trained to use litter boxes or wait to be allowed outside and defecate there. Training can be done in several ways, especially dependent on species. An example is crate training for dogs. Several companies market carpet cleaning products aimed at pet owners.

Uses

Human feces may be used as fertilizer (Humanure) in the form of biosolids (treated sewage sludge). The feces of animals are often used as fertilizer; see manure and guano. Some animal feces, especially those of camel, bison and cattle, is used as fuel when dried out. Animal dung,

besides being used as fuel, is occasionally used as a cement to make adobe mudbrick huts or even in throwing sports such as cow pat throwing or camel dung throwing contests. Kopi Luwak or Civet coffee, is coffee made from coffee berries which have been eaten by and passed through the digestive tract of the Asian Palm Civet (*Paradoxurus hermaphroditus*).

Fewmets for the use of feces in venery, or Hunting in the Middle Ages, and Kumalak for the use of feces in fortune-telling in Central Asia. Dog feces were used in the tanning process of leather during the Victorian era. Collected dog feces were mixed with water to form a substance known as "bate". Enzymes in the dog feces helped to relax the fibrous structure of the hide before the final stages of tanning.

Social Implications

Feces has always been associated with the lowest people among society, the social outcasts, the pariahs, and the social discards. The Caste system in India was created along the lines of profession and the dalits (untouchables) were left to do work related to human emissions. They did such work as clean and pick feces from streets, clean toilets, work with dead bodies. Such practices are prevalent even today in the rural and small villages of India. Even in western cultures, feces is associated with social discards. The usage of the word "shit" in modern English has its roots in the use of the word to denote something that is of little value, or having a negative value.

Animal Feces

The feces of animals often have special names. For example:

- Non-human animals generally –
  - As bulk material – dung
  - Individually – droppings
- Cattle –
  - Bulk material – cow dung
  - Individual droppings – cow pats, meadow muffins etc.
- Deer (and formerly other quarry animals) – fewmets.
- Wild carnivores – scat.
- Otter – spraint.
- Birds (individual) – droppings (also include urine as white crystals of uric acid).

- Seabirds or bats (large accumulations) – guano.
- Herbivorous insects, such as caterpillars and leaf beetles – frass.
- Earthworms, lugworms etc. – worm castings (feces extruded at ground surface).
- Feces when used as fertilizer (usually mixed with animal bedding and urine) – manure.
- Horses – horse manure, roadapple.

Chapter 2: Fecal Occult Blood Test and Guaiac Screening

The stool guaiac test or guaiac fecal occult blood test (gFOBT) is one of several methods that detect the presence of fecal occult blood (FOB). Fecal occult blood is blood present in the feces that is not visibly apparent. The term guaiac denotes the name of the paper surface used in the test which has a phenolic compound, alpha-guaiaconic acid, that is extracted from the wood resin of Guaiacum trees.

Methodology

The stool guaiac test involves fasting from iron supplements, red meat (the blood it contains can turn the test positive), certain vegetables (which contain a chemical with peroxidase properties that can turn the test positive), and vitamin C and citrus fruits (which can turn the test falsely negative) for a period of time before the test. It has been suggested that cucumber, cauliflower and horseradish, and often other vegetables, should be avoided for three days before the test.

In testing, feces are applied to a thick piece of paper attached to a thin film coated with guaiac. Either the patient or medical professional smears a small fecal sample on to the film. The fecal sample is obtained by catching the stool and transferring a sample with an applicator. Digital rectal examination specimens are also used but this method is discouraged for colorectal cancer screening due to very poor performance characteristics .

Both sides of the test card can be peeled open, to access the inner guaiac paper. One side of the card is marked for application of the stool and the other is for the developer fluid.

After applying the feces, one or two drops of hydrogen peroxide are then dripped on to the other side of the film, and it is observed for a rapid blue color change.

When the hydrogen peroxide is dripped on to the guaiac paper, it oxidizes the alpha-guaiaconic acid to a blue colored quinone. Normally,

when no blood and no peroxidases or catalases from vegetables are present, this oxidation occurs very slowly. Heme, a component of hemoglobin found in blood, catalyzes this reaction, giving a result in about two seconds. Therefore, a positive test result is one where there is a quick and intense blue color change of the film.

Analytical Interpretation

The guaiac test can often be false-positive which is a positive test result when there is in fact no source of bleeding. This is particularly common if the recommended dietary preparation is not followed, as the heme in red meat or the peroxidase or catalase activity in vegetables, especially if uncooked, can cause analytical false positives.

Vitamin C can cause analytical false negatives due to its anti-oxidant properties inhibiting the color reaction.

If the card has not been promptly developed, the water content of the feces decreases, and this can reduce the detection of blood. Although rehydration of stored samples can reverse this effect this is not recommended because the test becomes unduly analytically sensitive and thus much less specific.

Some stool specimens have a high bile content that causes a green color to show after applying the developer drops. If entirely green, such samples are negative, but if questionably green to blue, such samples are designated positive.

The package insert guidelines from the manufacturers, for example Hemoccult SENSA, recommend that nonsteroidal anti-inflammatory drugs (NSAID), such as ibuprofen and aspirin, and iron supplements be discontinued for at least several days before the tests. There is a concern that these agents may irritate the body and cause biologically positive tests even in the absence of a more substantial illness, but there is some doubt about how frequently this occurs with NSAID medication. Although both iron and bismuth containing products such as antacids and antidiarrheals can cause dark stools that are occasionally confused as containing blood, actual bleeding from iron is unusual.

There is no consensus on whether to stop warfarin before a guaiac test. Even when using anticoagulants a high proportion of positive guaiac tests were found to be due to diagnosable lesions, suggesting anticoagulants may not cause bleeding unless there is an abnormality.

Clinical Application

The article fecal occult blood (FOB) provides an expanded consideration of the clinical application of FOB tests generally, including other clinical methods, and the comments here are those that relate specifically to the guaiac gFOBT method.

One major use of stool testing for blood is detection of colorectal cancer. However, other possible positive results include: gastroesophageal cancer, GI bleeds, diverticulae, hemorrhoids, anal fissures, colon polyps, ulcerative colitis, Crohn's disease, celiac disease, GERD, esophagitis, peptic ulcers, gastritis, inflammatory bowel disease, vascular ectasias, portal hypertensive gastropathy, aortoenteric fistulas, hemobilia, endometriosis, and trauma.

The stool guaiac test was originally the principal colon cancer screening technology available, but modern tests which look for globin or DNA are now also available. Several recent colon cancer screening guidelines have recommended replacing any older low-sensitivity, guaiac-based fecal occult blood testing (gFOBT) with either newer high-sensitivity guaiac-based fecal occult blood testing (gFOBT) or fecal immunochemical testing (FIT), which tests for globin rather than the heme detected by the guaiac method. The US Multisociety Task Force (MSTF) looked at 6 studies that compared high sensitivity gFOBT (Hemoccult SENSA) to FIT, and concluded that there were no clear difference in overall performance between these methods, and a similar recommendation was made by the National Guideline Clearinghouse (NGC).

Results of a single fecal sample should be interpreted cautiously, as there is a high rate of false negativity associated with the test. Using three cards, each on different days, is recommended to improve sensitivity. The Centers for Disease Control and Prevention (CDC) in a 2006–2007 survey found extensive inappropriate use of low sensitivity gFOBT and of single specimens; it is unclear if these widespread suboptimal approaches have since declined. The Current Procedural Terminology (CPT) coding was changed in January 2006 to include CPT code 82270, which indicates that consecutive collection of three stool samples has occurred, either as three single cards or a single triple card. Since January 2007, the US Medicare program reimburses for colorectal cancer screening with gFOBT only when this code is used.

The stool guaiac test method may be preferable to fecal immunochemical testing (FIT) if there is a clinical concern about possible gastric or proximal upper intestinal bleeding. However,

although heme breakdown is less than globin during intestinal transit, false negative results can be seen with the stool guaiac tests due to degradation of the peroxidase-activity. This can cause false negative results in upper gastrointestinal bleeding sources, or in right colon adenomas and cancers that have comparable blood losses to positively testing left colon lesions.

A positive gFOBT with subsequent negative colonoscopy may lead to an upper endoscopy. It is unclear whether this is an effective intervention if there is a positive gFOBT but no anemia. Endoscopy when there is a positive gFOBT along with iron deficiency anemia, or iron deficiency anemia on its own, has a higher rate of finding problems.

Fecal Examination in Dogs

A fecal examination is the microscopic evaluation of feces. The test is indicated for pets with diarrhea, straining, lack of appetite or vomiting. Annual fecal examinations are recommended on all animals as part of a yearly health exam. Fecal examinations are also recommended on all puppies and kittens.

There is no contraindication to performing this test. Negative results help determine health or may exclude the presence of disease and gastrointestinal parasites.

What Does a Fecal Examination Reveal or Demonstrate?

Fecal examinations are primarily performed to detect microscopic gastrointestinal parasites, such as roundworms, hookworms, whipworms, Giardia, coccidia and tapeworms. Some abnormal parasites known as spirochetes or flagellates can also be detected.

A positive test result indicates gastrointestinal parasitic disease. Negative results from one fecal sample may be misleading. Some parasites do not shed eggs consistently so some samples may be negative even though the animal actually has a parasitic infection. Repeated fecal examinations may be necessary to detect some elusive parasites.

How is a Fecal Examination Done?

In order to perform a fecal examination, a fecal sample needs to be obtained. The easiest way to do this would be to pick up a sample of feces after the pet has eliminated. Fresh samples will give the most information.

Fecal samples can also be obtained by other means. There are special fecal loops commercially available. This long narrow wand with a loop at the end is inserted into the rectum. If feces is present, a sample will be obtained in the loop. Another method of obtaining a fecal sample is for the veterinarian to use an exam glove and place a finger in the rectum. Fecal material will adhere to the glove and can be evaluated under a microscope.

After obtaining a fecal sample, the feces is placed in a tube or commercially manufactured fecal container. Special fecal flotation fluid is then added to the tube with the feces and the combination is stirred.

This is then allowed to sit for about 20 minutes. The hope is that any parasitic eggs present in the feces will float to the top of the fluid. After 20 minutes, the top layer of fluid is placed on a microscope slide and examined under a microscope. Each parasite has a characteristic appearance that can be detected by an experienced technician or veterinarian.

The fecal examination generally takes about 30 to 40 minutes and is often performed in your veterinarian's office. Some veterinarian's choose to submit the fecal sample to a veterinary diagnostic laboratory. In these situations, tests results may take 1 to 2 days to obtain.

Is a Fecal Examination Painful?

A fecal examination is not painful. If a fecal loop or digital exam is used to obtain the sample, discomfort may be involved.

Is Sedation or Anesthesia Needed for a Fecal Examination?

Sedation or anesthesia is not needed for a fecal examination.

Fecal Occult Blood

Fecal Occult Blood (FOB) refers to blood in the feces that is not visibly apparent. A fecal occult blood test (FOBT) checks for hidden (occult) blood in the stool (feces). Newer tests look for globin, DNA, or other blood factors including transferrin, while conventional stool guaiac tests look for heme.

Purpose

Fecal occult blood testing (FOBT), as its name implies, aims to detect subtle blood loss in the gastrointestinal tract, anywhere from the mouth to the colon. Positive tests ("positive stool") may result from

either upper gastrointestinal bleeding or lower gastrointestinal bleeding and warrant further investigation for peptic ulcers or a malignancy (such as colorectal cancer or gastric cancer). The test does not directly detect colon cancer but is often used in clinical screening for that disease, but it can also be used to look for active occult blood loss in anemia or when there are gastrointestinal symptoms.

Nomenclature

In 2007 the nomenclature of overt, obscure and occult bleeding was clarified.

The different methods of testing for "fecal occult blood" as broadly considered actually test for particular components of blood or for aberrantly expressed cellular markers from the intestinal mucosa.

Methodology

There are four methods in clinical use for testing for occult blood in feces. These look at different properties, such as antibodies, heme, globin, or porphyrins in blood, or at DNA from cellular material such as from lesions of the intestinal mucosa.

- *Fecal Immunochemical Testing (FIT), and immunochemical fecal occult blood test (iFOBT):* FIT products utilize specific antibodies to detect globin. FIT screening is more effective in terms of health outcomes and cost compared with guaiac FOBT. The FIT tests are superior to low sensitivity gFOBT for colorectal cancer screening. Although FIT may be a consideration to replace gFOBT in colon cancer screening, high sensitivity gFOBT, such as Hemoccult SENSA, remains an accepted option alongside FIT in recent guidelines, being assessed as having similar overall performance characteristics to FIT. The number of fecal samples submitted for FIT may affect the clinical sensitivity and specificity of the methodology. This methodology can be adapted for automated test reading and to report quantitative results, which are potential factors in design of a widescale screening strategy. FOBT may have a role in monitoring gastrointestinal conditions such as ulcerative colitis.
- *Stool guaiac test for fecal occult blood (gFOBT):* The stool guaiac test involves smearing some feces on to some absorbent paper that has been treated with a chemical. Hydrogen peroxide is then dropped on to the paper; if trace amounts of blood are present, the paper will change color in one or two seconds. This

method works as the heme component in hemoglobin has a peroxidase-like effect, rapidly breaking down hydrogen peroxide. In some settings such as gastric or proximal upper intestinal bleeding the guaiac method may be more sensitive than tests detecting globin because globin is broken down in the upper intestine to a greater extent than is heme. There are various commercially available gFOBT tests which have been categorized as being of low or high sensitivity, and only high sensitivity tests are now recommended in colon cancer screening. Optimal clinical performance of the stool guaiac test depends on preparatory dietary adjustment.

- *Fecal porphyrin quantification:* Hemo Quant, unlike gFOBT and FIT, permits precise quantification of hemoglobin, and is analytically validated with gastric juice and urine, as well as stool samples. The heme moiety of intact hemoglobin is chemically converted by oxalic acid and ferrous oxalate or ferrous sulfate to protoporphyrin, and the porphyrin content of both the original sample and of the sample after hemoglobin conversion to porphyrin is quantified by comparative fluorescence against a reference standard; the specificity for hemoglobin is increased by subtracting the fluorescence of a sample blank prepared with citric acid to correct for the potential confounding effect of existing non-specific substances. Precise quantification measurement has been very useful in many clinical research applications.
- *Fecal DNA test:* The Pre-Gen-Plus test extracts human DNA from the stool sample and tests it for alterations that have been associated with cancer. The test looks at 23 individual DNA alterations, including 21 specific point alterations in the APC, KRAS and p53 genes, as well as testing BAT26, a gene involved in microsatellite instability (MSI). and a proprietary DNA Integrity Assay (DIA).

Additional methods of looking for occult blood are being explored, including transferrin dipstick and stool cytology.

Test Performance

Reference Standards

The estimates for test performance characteristics are based on comparison with a variety of reference methods including 51-chromium studies, analytical recovery studies in spiked stool samples, analytical recovery after ingestion of autologous blood, rarer studies of carefully quantified blood instilled at bowel surgery as well as other research approaches. Additionally, clinical studies look at variety of additional factors.

Gastrointestinal Blood Loss in Health

In healthy people about 0.5 to 1.5 ml of blood escapes blood vessels into the stool each day. Significant amounts of blood can be lost without producing visible blood in the stool, estimated as 200 ml in the stomach, 100 ml in the duodenum, and lesser amounts in the lower intestine. Tests for occult blood identify lesser blood loss.

Clinical Sensitivity and Specificity

Stool guaiac test for fecal occult blood (gFOBT) sensitivity varies depending on the site of bleeding. Moderately sensitive gFOBT can pick up a daily blood loss of about 10 ml (about two teaspoonfuls), and higher sensitivity gFOBT can pick up lesser amounts, sometimes becoming positive at about 2 ml. The sensitivity of a single stool guaiac test to pick up bleeding has been quoted at 10 to 30%, but if a standard three tests are done as recommended the sensitivity rises to 92%. Further discussion of sensitivity and sensitivity issues that relate particularly to the guaiac method is found in the stool guaiac test article.

Fecal Immunochemical Testing (FIT) picks up as little as 0.3 ml but because it does not detect occult blood from the stomach and upper small intestine the test threshold doesn’t cause undue false positives from normal upper intestinal blood leakage and it is much more specific for bleeding from the colon or lower gastrointestinal tract. The detection rate of the test decreases if the time from sample collection to laboratory processing is delayed.

Fecal porphyrin quantification by Hemo Quant can be false positive due to exogenous blood and various porphyrins. Hemo Quant is the most sensitive test for upper gastrointestinal bleeding and therefore may be most appropriate fecal occult blood test to use in the evaluation of iron deficiency Advised to stop red meat and aspirin for 3 days prior to specimen collection False positives can occur with myoglobin, catalase, or protohemes and in certain types of porphyria

The DNA based PreGen-Plus was four times more sensitive than fecal blood testing, including detection of early stage disease, when treatment is most effective. Sensitivity increased to 51.6% compared to 12.9%. Additional clinical trials of the PreGen-Plus method are underway to more fully characterize its clinical performance.

Expanding the range of DNA testing by looking at additional known genetic markers, such as CTNNB1, or by analyzing epigenetically methylated genes such as MLH1 which is very common in serrated polyps with microsatellite instability (MSI) and in proximal colon tumours that have poorer differentiation, does not appear to appreciably increase the sensitivity of the method because CTNNB1 mutations are infrequent in sporadic colorectal cancer, and because BAT26 alterations and lack of MLH1 expression show a high degree of overlap.

Clinical Application

The stool guaiac test for hidden (occult) blood in the stool can be done at home or in the doctor's office, or can be performed on samples submitted to a clinical laboratory. Testing kits are available at pharmacies in some countries without a prescription, or a health professional may order a testing kit for use at home. If a home fecal occult blood test detects blood in the stool it is recommended to see a health professional to arrange further testing.

Sources of Gastrointestinal Bleeding

Gastrointestinal bleeding has many potential sources, and positive results usually result in further testing for the bleeding site, usually looking for lower gastrointestinal bleeding before upper gastrointestinal bleeding causes unless there are other clinical clues. Colonoscopy is usually preferred to computerized tomographic colonography.

An estimated 1–5% of large tested populations have a positive fecal occult blood test. Of those, about 2–10% have cancer, while 20– 30% have adenomas.

A positive test can result from upper gastrointestinal bleeding or lower gastrointestinal bleeding. The common causes are:

- 2–10%: cancer (colorectal cancer, gastric cancer)
- 20–30% adenoma or polyps
- Bleeding peptic ulcer
- Angiodysplasia of the colon

- Sickle cell anemia

In the event of a positive fecal occult blood test, the next step in the workup is a form of visualization of the gastrointestinal tract by one of several means:

1. Sigmoidoscopy, an examination of the rectum and lower colon with a lighted instrument to look for abnormalities, such as polyps.
2. Colonoscopy, a more thorough examination of the rectum and entire colon.
3. Virtual colonoscopy
4. Endoscopy refers to upper gastrointestinal endoscopy. It is sometimes performed with chromoendoscopy, a method that assists the endoscopist by enhancing the visual difference between cancerous and normal tissue, either by marking the abnormally increased DNA content (toluidine blue) or failing to stain the tumor, possibly due to decreased surface glycogen on tumor cells(Lugol). Infrared fluorescent endoscopy and ultrasonic endoscopy can interrogate vascular abnormalities such as esophageal varices.
5. Double contrast barium enema: a series of x-rays of the colon and rectum.

Stool Color

Although red or black stools can be an indication of bleeding, a dark or black color can be due to black licorice, blueberries, iron supplements, lead, Pepto-bismol, and a red color can come from natural or artificial coloring such as red gelatin, popsicles, Kool-Aid, and large amounts of beets.

Colorectal Cancer Screening

Screening methods for colon cancer depend on detecting either precancerous changes such as certain kinds of polyps or on finding early and thus more treatable cancer. The extent to which screening procedures reduce the incidence of gastrointestinal cancer or mortality depends on the rate of precancerous and cancerous disease in that population. gFOBT and flexible sigmoidoscopy screening have each shown benefit in randomized clinical trials. Evidence for other colon cancer screening tools such as iFOBT or colonoscopy is substantial and guidelines have

been issued by several advisory groups but does not include randomized studies.

Guaiac FOB testing of average risk populations may reduce the mortality associated with colon cancer by about 25%. It is not always cost effective to screen a large population.

If colon cancer is suspected in an individual (such as in someone with an unexplained anemia) fecal occult blood tests may not be clinically helpful. If a doctor suspects colon cancer, more rigorous investigation is necessary, whether or not the test is positive.

The 2009 recommendations of the American College of Gastroenterology (ACG) suggest that colon cancer screening modalities that are also directly preventive by removing precursor lesions should be given precedence, and prefer a colonoscopy every 10 years in average-risk individuals, beginning at age 50.

The ACG suggests that cancer detection tests such as any type of FOB are an alternative that is less preferred and which should be offered to patients who decline colonoscopy or another cancer prevention test. However, two other recent guidelines, from the US Multisociety Task Force (MSTF) and the US Preventive Services Task Force (USPSTF) while permitting immediate colonoscopy as an option, did not categorize it as preferred.

The ACG and MSTF also included CT colonography every 5 years, and fecal DNA testing as considerations. All three recommendation panels recommended replacing any older low-sensitivity, guaiac-based fecal occult blood testing (gFOBT) with either newer high-sensitivity guaiac-based fecal occult blood testing (gFOBT) or fecal immunochemical testing (FIT). MSTF looked at 6 studies that compared high sensitivity gFOBT (Hemoccult SENSA) to FIT, and concluded that there were no clear difference in overall performance between these methods. In colon cancer screening, using only one sample of feces collected by a doctor performing a digital rectal examination is strongly discouraged.

Iron Deficiency Anemia

An extensive literature has examined the clinical value of FOBT in iron deficiency anemia.

Gastrointestinal Disease and Medications

Conditions such as ulcerative colitis or certain types of relapsing infectious diarrhea can vary in severity over time, and FOBT may assist in assessing the severity of the disease. Medications associated with gastrointestinal bleeding such as Bortezomib are sometimes monitored by FOBT.

Alcoholism

Several aspects of FOBT in alcoholism warrant further discussion.

Outpatient Clinics

Several studies have reported clinical benefit from gFOBT testing including urology and gynecology clinics.

Inpatient Guaiac Testing

Several studies have questioned the traditional Admission Screening Guaiac (ASG). The utility of following stool guaiac in ICU settings is also questioned.

Testing of Upper Gastrointestinal or Aerodigestive Tract Secretions for Occult Blood

The use of tests for occult blood in disorders of the mouth, nasal passages, esophagus, lungs and stomach, while analogous to fecal testing, is often discouraged, due to technical considerations including poorly characterized test performance characteristics such as sensitivity, specificity, and analytical interference. However, chemical confirmation that coloration is due to blood rather than coffee, beets, medications, or food additives can be of significant clinical assistance.

A related concept to colon cancer screening by FOBT, based on most neoplasms affecting the surface epithelium and losing small amounts of blood but no visible blood loss, is screening in populations at high risk for esophageal or gastric cancers by testing for blood by swallowing a small capsule that is recovered after 3 to 5 minutes by gentle retrieval by means of an attached nylon thread.

Regulatory Impact

Regulations from the Joint Commission may have unintentionally decreased digital rectal examination and FOBT in hospital settings such as Emergency Departments.

Fecal Occult Blood in Marathon Runners

Gastrointestinal (GI) complaints and low intensity GI bleeding frequently occur in marathon runners. Strenuous exercise, particularly in elite athlete runners and less frequently in other exercise activities, can cause acute incapacitating gastrointestinal symptoms including heartburn, nausea, vomiting, abdominal pain, diarrhea and gastrointestinal bleeding.

Approximately one third of endurance runners experience transient but exercise limiting symptoms, and repetitive gastrointestinal bleeding occasionally causes iron deficiency and anaemia. Runners can sometimes experience significant symptoms including hematemesis.

Exercise is associated with extensive changes in gastrointestinal (GI) tract physiology, including diversion of blood flow from the GI tract to muscle and lungs, decreased GI absorption and small intestinal motility, increased colonic transit, neuroimmunoendocrine changes in hormones and peptides such as vasoactive intestinal peptide, secretin and peptide-histidine-methionine.

Substantial changes occur in stress hormones including cortisol, in circulating concentrations and metabolic behavior of various leucocytes, and in immunoglobulin levels and major histocompatibility complex expression. Symptoms can be exacerbated by dehydration or by pre-exercise ingestion of certain foods and hypertonic liquids, and lessened by adequate training.

Ingestion of 800 mg of cimetidine 2 hr before running a marathon did not significantly affect the frequency of gastrointestinal symptoms or occult gastrointestinal bleeding. Conversely, 800 mg of cimetidine 1 hr before the start and again at 50 miles of a 100-mile running race substantially decreased GI symptoms and postrace guaiac test positivity but did not affect race performance.

Additional studies have reviewed the effect of cimetidine and of PPI.

Role of endoscopy in marathon runners with positive FOBT.

This is a different process than march hemoglobinuria.

Urine Test Strip

A urine test strip or dipstick is a basic diagnostic instrument used to determine pathological changes in the urine in standard urinalysis. A standard urine test strip may comprise up to 10 different chemical pads

or reagents which react (change colour) when immersed in, and then removed from, a urine sample.

The test can be read between 60 and 120 seconds after dipping. Routine testing of the urine with multiparameter strips is the first step in the diagnosis of a wide range of diseases.

Characteristics

Consist of a ribbon made of plastic or paper of about 5 millimetre wide, plastic strips have pads impregnated with chemicals which react with the compounds present in urine producing a characteristic color. On paper strips, the reactants are absorbed directly thereon. Paper strips are often specific to a single reaction (e.g. pH measurement), while the strips with pads allow several determinations simultaneously.

There are strips with different objectives, there are qualitative strips that's only determine if the sample is positive or negative, and there semiquantitative ones that in addition to providing a positive or negative reaction approaching a quantitative result, in the latter color reactions are approximately proportional to the concentration of substance in the sample.

The reading of the results obtained by comparing the colors with a color scale provided by the manufacturer, no equipment needed.

This type of analysis is very common in the control and monitoring of diabetic patients. The test can be read from a few minutes to 30 minutes after immersion of the strip in the urine (depending on the brand of product that you are using).

Semiquantitative values can be reported, usually expressed as trace, 1+, 2+, 3+ and 4+. In the test areas also provides an estimate in milligrams per deciliter. Automated readers of test strips also provide units of the International System of Units. They are used in the physicochemical stage of a urinalysis to determine glucose bilirubin, acetone, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocytes, or to reaffirm the suspicion of infection by different pathogens.

Technique

The test method consists of immersing the test strip completely in a well mixed sample of urine for a short period of time, then extracted from the container supporting the edge of the strip over the mouth of the container to remove excess urine.

The strip is left to stand for the time necessary for the carrying out reactions (usually 1 to 2 minutes), and finally compares the colors that appear with the chromatic scale provided by the manufacturer.

Improper technique can produce false results, for example, leukocytes and erythrocytes precipitate at the bottom of the container and can not detect if the sample is not mixed, and in the same way, an excess of urine in the strip after being extracted, may cause spillage of the pads, causing the reagents of adjacent pads mixes and distorting the colors. To ensure that this does not occur it is recommended to dry the edge of the strip on absorbent paper.

Diseases Identified with a Urine Test Strip

With the aid of routine examinations early symptoms of the following three groups are identified:

- Diseases of the kidneys and the urinary tract
- Carbohydrate metabolism disorders (diabetes mellitus)
- Liver diseases and haemolytic disorders

Diseases of the Kidneys and Urinary Tract

Screening parameters: Many renal and urinary tract diseases may be asymptomatic for a long period of time. Routine urinalysis is recommended as a basic yet fundamental step in identifying renal damage and / or urinary tract disease at an early stage, especially in high risk populations such as diabetics, the hypertensive, African Americans, Polynesians, and those with a family history.

Specific Kidney & Urinary Tract Diseases Able to be Identified

Chronic kidney disease, Glomerulonephritis

Carbohydrate Metabolism Disorders

- Glucose - Identified as Glycosuria
- Ketones - Identified as Ketonuria (also see ketoacidosis and ketosis)

Around 30–40% of type I diabetics and around 20% of type II diabetics suffer in time from a nephropathy, and early recognition of diabetes is therefore of major significance for the further state of health of these patients.

Specific Carbohydrate Metabolism Disorders Able to be Identified

Diabetes Mellitus

Liver diseases and haemolytic disorders

- Urobilinogen - Identified as Urobilinogenuria
- Bilirubin - Identified as Bilirubinuria

In many liver diseases the patients often show signs of pathology only at a late stage. Early diagnosis allows appropriate therapeutic measures to be instituted in good time, avoiding consequential damage and further infections.

Specific Liver Diseases and Haemolytic Disorders Able to be Identified

Liver Disease, (accompanied by Jaundice), Cirrhosis

Uses for Urine Test Strips

Urine test strips can be used in many areas of the healthcare chain including screening for routine examinations, treatment monitoring, self-monitoring by patients and/or general preventive medicine.

Screening

Urine test strips are used for screening both in hospitals and in general practice. The aim of screening is early identification of likely patients by examination of large groups of the population. The importance of screening for diabetes and kidney disease amongst high risk populations is becoming very high.

Treatment Monitoring

Treatment monitoring with the aid of urine test strips allows a health professional to check on the results of the prescribed therapy, and if necessary to introduce any changes into the course of therapy.

Self-monitoring by Patients

Self monitoring with urine test strips under the guidance of a health professional is an effective method for monitoring the disease state. This applies particularly to diabetics, where the idea of self monitoring of the metabolic status (determinations of glucose and ketones) is self-evident.

General Preventive Medicine

Unsolicited self testing has become a popular measure in recent years as various urine test strips become available via pharmacy and online stores. Self monitoring for frequent urinary tract infections is a popular example as sufferers monitor their own urine on a daily basis and discuss the results with their health professional.

Veterinary

In veterinary medicine, especially in cats and dogs, the test strip can be used for urinalysis.

Chapter 3: Animal Health Urine Test Strip

In many cultures urine was once regarded as a mystical fluid, and in some cultures it is still regarded as such to this day. Its uses have included wound healing, stimulation of the body's defences, and examinations for diagnosing the presence of diseases.

It was only towards the end of the 18th century that doctors interested in chemistry turned their attention to the scientific basis of urinalysis and to its use in practical medicine.

- 1797 - Carl Friedrich Gärtner(1772–1850) expressed a wish for an easy way of testing urine for disease at the patient's bedside.
- 1797 - William Cumberland Cruikshank (1745–1800) described for the first time the property of coagulation on heating, exhibited by many urines.
- 1827 - English physician Richard Bright describes the clinical symptom of nephritis in "Reports of Medical Cases."
- 1840 - The arrival of chemical urine diagnostics aimed at the detection of pathological urine constituents
- 1850 - Parisian chemist Jules Maumene (1818–1898) develops the first "test strips" when he impregnated a strip of merino wool with "tin protochloride" (stannous chloride). On application of a drop of urine and heating over a candle the strip immediately turned black if the urine contained sugar.
- 1883 - English physiologist George Oliver (1841–1915) markets his "Urinary Test Papers"
- Approx. 1900 - Reagent papers become commercially obtainable from the chemical company of Helfenberg AG.
- 1904 - A test for the presence of blood by a wet-chemical method using benzidine became known.
- Approx. 1920 - Viennese chemist Fritz Feigl (1891–1971) publishes his technique of "spot analysis".

- 1930s - Urine diagnostics makes major progress as reliability improves and test performance becomes progressively easier.
- 1950s - Urine test strips in the sense used today were first made on industrial scale and offered commercially.
- 1964 - The company Boehringer Mannheim, today a top leader on the world market under the name of Roche, launched its first Combur test strips. Even though the test strips have changed their external appearance little since the 1960s, they now contain a number of revolutionary innovations. New impregnation techniques, more stable color indicators, and the steady improvement in color gradation have all contributed to the fact that the use of urine test strips has now become established in clinical and general practice as a reliable diagnostic instrument. The parameter menu offered has steadily grown longer in the intervening decades.

Ascorbic Acid Interference

Ascorbic acid (vitamin C) is known to interfere with the oxidation reaction of the blood and glucose pad on common urine test strips. Some urine test strips are protected against the interference with iodate, which eliminates ascorbic acid by oxidation.

Automated Urine Test Strip Analyzers

Automatic analysis of urine test strips using automated urine test strip analyzers is a well established practice in modern day urinalysis.

Urinary Sediment Urinary Cast

During routine screening, if a positive test for leukocytes, blood, protein, nitrite, and a pH greater than 7 is identified, the urine sediment be microscopically analyzed to further pinpoint a diagnosis.

Urinary casts are cylindrical structures produced by the kidney and present in the urine in certain disease states. They form in the distal convoluted tubule and collecting ducts of nephrons, then dislodge and pass into the urine, where they can be detected by microscopy.

They form via precipitation of Tamm-Horsfall mucoprotein which is secreted by renal tubule cells, and sometimes also by albumin in conditions of proteinuria. Cast formation is pronounced in environments favoring protein denaturation and precipitation (low flow, concentrated

salts, low pH). Tamm-Horsfall protein is particularly susceptible to precipitation in these conditions.

Casts were first described by Henry Bence Jones (1813-1873).

As reflected in their cylindrical form, casts are generated in the small distal convoluted tubules and collecting ducts of the kidney, and generally maintain their shape and composition as they pass through the urinary system. Although the most common forms are benign, others indicate disease. All rely on the inclusion or adhesion of various elements on a mucoprotein base—the hyaline cast. "Cast" itself merely describes the shape, so an adjective is added to describe the composition of the cast. Various casts found in urine sediment may be classified as follows.

Acellular Casts

Hyaline Casts

The most common type of cast, hyaline casts are solidified Tamm-Horsfall mucoprotein secreted from the tubular epithelial cells of individual nephrons. Low urine flow, concentrated urine, or an acidic environment can contribute to the formation of hyaline casts, and, as such, they may be seen in normal individuals in dehydration or vigorous exercise. Hyaline casts are cylindrical and clear, with a low refractive index, so that they can easily be missed on cursory review under brightfield microscopy, or in an aged sample where dissolution has occurred. On the other hand, phase contrast microscopy leads to easier identification. Given the ubiquitous presence of Tamm-Horsfall protein, other cast types are formed via the inclusion or adhesion of other elements to the hyaline base.

Granular Casts

The second-most common type of cast, granular casts can result either from the breakdown of cellular casts or the inclusion of aggregates of plasma proteins (e.g., albumin) or immunoglobulin light chains. Depending on the size of inclusions, they can be classified as fine or coarse, though the distinction has no diagnostic significance. Their appearance is generally more cigar-shaped and of a higher refractive index than hyaline casts. While most often indicative of chronic renal disease, these casts, as with hyaline casts, can also be seen for a short time following strenuous exercise.

Waxy Casts

Thought to represent the end product of cast evolution, waxy casts suggest the very low urine flow associated with severe, longstanding kidney disease such as renal failure. Additionally, due to urine stasis and their formation in diseased, dilated ducts, these casts are significantly larger than hyaline casts. While cylindrical, they also possess a higher refractive index and are more rigid, demonstrating sharp edges, fractures, and broken-off ends. Waxy casts also fall under the umbrella of "broad" casts, a more general term to describe the wider cast product of a dilated duct. It is seen in chronic renal failure.

In nephritic syndrome many additional types of casts include broad and waxy casts if the condition is chronic (this is referred to as a telescopic urine with the presence of many casts) condition is chronic (this is referred to as a telescopic urine with the presence of many casts).

Fatty Casts

Formed by the breakdown of lipid-rich epithelial cells, these are hyaline casts with fat globule inclusions, yellowish-tan in color. If cholesterol or cholesterol esters are present, they are associated with the "Maltese cross" sign under polarized light. They are pathognomonic for high urinary protein nephrotic syndrome.

Pigment Casts

Formed by the adhesion of metabolic breakdown products or drug pigments, these casts are so named due to their discoloration. Pigments include those produced endogenously, such as hemoglobin in hemolytic anemia, myoglobin in rhabdomyolysis, and bilirubin in liver disease. Drug pigments, such as phenazopyridine, may also cause cast discoloration.

Crystal Casts

Though crystallized urinary solutes, such as oxalates, urates, or sulfonamides, may become enmeshed within a ketanaline cast during its formation, the clinical significance of this occurrence is not felt to be great.

Cellular Casts

Red Blood Cell Casts

The presence of red blood cells within the cast is always pathological, and is strongly indicative of glomerular damage, which can occur in glomerulonephritis from various causes or vasculitis, including Wegener's granulomatosis, systemic lupus erythematosus, post-streptococcal glomerulonephritis or Goodpasture's syndrome. They can also be associated with renal infarction and subacute bacterial endocarditis. They are a yellowish-brown color and are generally cylindrical with sometimes ragged edges; their fragility makes inspection of a fresh sample necessary. They are usually associated with nephritic syndromes or urinary tract injury.

White Blood Cell Casts

Indicative of inflammation or infection, the presence of white blood cells within or upon casts strongly suggests pyelonephritis, a direct infection of the kidney. They may also be seen in inflammatory states, such as acute allergic interstitial nephritis, nephrotic syndrome, or post-streptococcal acute glomerulonephritis. White cells sometimes can be difficult to discern from epithelial cells and may require special staining. Differentiation from simple clumps of white cells can be made by the presence of hyaline matrix.

Bacterial Casts

Given their appearance in pyelonephritis, these should be seen in association with loose bacteria, white blood cells, and white blood cell casts. Their discovery is likely rare, due to the infection-fighting efficiency of neutrophils, and the possibility of misidentification as a fine granular cast.

Epithelial Cell Casts

This cast is formed by inclusion or adhesion of desquamated epithelial cells of the tubule lining. Cells can adhere in random order or in sheets and are distinguished by large, round nuclei and a lower amount of cytoplasm. These can be seen in acute tubular necrosis and toxic ingestion, such as from mercury, diethylene glycol, or salicylate. In each case, clumps or sheets of cells may slough off simultaneously, depending of the focality of injury. Cytomegalovirus and viral hepatitis are organisms that can cause epithelial cell death as well.

Urine Sediment

Microscopic examination of urine sediment should be part of a routine urinalysis. For centrifugation, 3-5 mL of urine is transferred to a conical centrifuge tube. Urine is centrifuged at 1,500-2,000 rpm for ~5 min. The supernatant is decanted, leaving ~0.5 mL of urine and sediment in the tip of the conical tube. The sediment is resuspended by tapping the tip of the conical tube against the table several times. A few drops of the sediment are transferred to a glass slide, and a cover slip is applied. Examination of unstained urine is recommended for routine samples. Microscopic examination is performed at 100¥ (for crystals, casts, and cells) and 400× (for cells and bacteria) magnifications. Contrast of the sample is enhanced by closing the iris diaphragm and lowering the condenser of the microscope. Stains such as Sedistain and new methylene blue can be used to aid in cell identification but tend to dilute the specimen and introduce artifacts such as stain precipitate and crystals.

Red Blood Cells: In an unstained preparation, RBC are small and round and have a slight orange tint and a smooth appearance. Normal urine should contain <5 RBC/field at 400× magnification. Increased RBC in urine (hematuria) indicates hemorrhage somewhere in the urogenital system; however, sample collection by cystocentesis or catheterization may induce hemorrhage.

White Blood Cells: WBC are slightly larger than RBC and have grainy cytoplasm. Normal urine should contain <5 WBC/field at 400¥ magnification. Increased WBC (pyuria) can occur due to inflammation, infection, trauma, or neoplasia. Catheterization or collection of voided urine may introduce a few WBC from the urogenital tract.

Epithelial Cells: Transitional epithelial cells, a common urine contaminant derived from the bladder and proximal urethra, resemble WBC but are larger. They have a greater amount of grainy cytoplasm and a round, centrally located nucleus. In a voided urine sample, squamous epithelial cells may be observed. They are large, oval to cuboidal in shape, and may or may not contain a nucleus. Occasionally, neoplastic transitional cells may be observed in an animal with a transitional cell carcinoma. Neoplastic squamous cells may be observed in an animal with a squamous cell carcinoma.

Cylindruria (Casts): Casts are elongated, cylindrical structures formed by mucoprotein ongealing within renal tubules and may contain cells. Hyaline casts have parallel sides and rounded ends and are composed of mucoprotein. They may occur with fever, exercise, and

renal disease. Epithelial cellular casts form from entrapment of sloughed tubular epithelial cells in the mucoprotein; they may be observed with renal disease. Granular casts are thought to represent degenerated epithelial cellular casts. Waxy casts have a granular appearance, and are thought to arise from longstanding granular casts. They typically have sharp borders with broken ends. Other cellular casts include erythrocyte casts and WBC casts. Erythrocyte casts form because of renal hemorrhage. WBC casts occur because of renal inflammation, as with pyelonephritis. Fatty casts are not common, but can be observed with disorders of lipid metabolism, such as diabetes mellitus. A few hyaline or granular casts are considered normal. However, presence of cellular casts or other casts in high numbers indicates renal damage, and may be one of the earliest laboratory abnormalities noted with toxic damage to renal epithelial cells (e.g., gentamicin, amphotericin B).

Infectious Organisms: The presence of bacteria in urine collected by cystocentesis indicates infection. Small numbers of bacteria from the lower urogenital tract may contaminate voided samples or samples collected by catheterization and do not indicate infection. Bacterial rods are most easily identified in urine sediment. Particles of debris may be mistaken for bacteria. Suspected bacteria can be confirmed by staining urine sediment with Gram's stain; however, aerobic culture is best to confirm a bacterial urinary tract infection. Rarely, yeast and fungal hyphae and parasitic ova may be observed in urine sediment. Their presence is not always associated with clinical disease. Parasitic ova observed include Stephanus dentatus, Capillaria plica, C felis, and Dioctophyma renale. Additionally, microfilariae of Dirofilaria immitis may be observed in urine sediment.

Crystals: Many urine sediments contain crystals. The type of crystal present depends on urine pH, concentration of crystallogenic materials, urine temperature, and length of time between urine collection and examination. Crystalluria is not synonymous with urolithiasis and is not necessarily pathologic. Furthermore, uroliths may form without observed crystalluria. Struvite crystals are commonly observed in canine and feline urine. Struvite crystalluria in dogs is not a problem unless there is a concurrent bacterial urinary tract infection with a urease-producing microbe. Without an infection, struvite crystals in dogs will not be associated with struvite urolith formation. However, some animals (e.g., cats) do form struvite uroliths without a bacterial urinary tract infection. In these animals, struvite crystalluria may be pathologic. Struvite crystals appear typically as "coffin-lids" or "prisms"; however, they may be

amorphous. Calcium oxalate crystalluria occurs less commonly in dogs and cats; if persistent, it may indicate an increased risk for calcium oxalate urolith formation.

However, calcium oxalate and calcium carbonate crystalluria is common in healthy horses and cattle. Calcium oxalate dihydrate crystals appear as squares with an "X" in the middle or "envelope-shaped." Calcium oxalate monohydrate crystals are "dumb-bell" shaped. An unusual form of calcium oxalate crystals is typically seen in association with ethylene glycol toxicity (*Ethylene Glycol Toxicity: Introduction*). These crystals occur in neutral to acidic urine. They are small, flat, and colorless, and are shaped like "picket fence posts." Ammonium acid urate crystals suggest liver disease (e.g., portosystemic shunt).

These crystals occur in acidic urine and are yellow-brown spheres with irregular, spiny projections; however, they may also be amorphous. Certain species, such as birds and reptiles, and certain breeds of dogs, specifically Dalmatians, can normally have ammonium acid urate crystalluria. Cystine crystals are 6-sided and of variable size. They occur in acidic urine. Presence of cystine crystals represents a proximal tubular defect in amino acid reabsorption. Cystinuria has been reported to occur in many breeds of dogs and rarely in cats. Dachshunds, Newfoundlands, English Bulldogs, and Scottish Terriers have a high incidence of cystine urolithiasis. Bilirubin crystals occur with bilirubinuria; however, they may be normal in small numbers in dogs.

Lipids: Fat droplets are commonly present in urine from dogs and cats and may be mistaken for RBC. However, they often vary in size and tend to float on a different plane of focus than the remainder of the sediment. They are not considered to be pathologic.

Spermatozoa: Spermatozoa may be observed normally in urine collected from male dogs.

Plant Material: Occasionally, plant material may be observed in urine samples collected by voiding. When present, they indicate contamination of the urine sample and are not pathologic.

Urine Chemistries

Urine must be at room temperature for accurate measurement of USG and for chemical analysis. These tests are usually done prior to centrifugation; however, if urine is discolored or turbid, it may be

beneficial to perform these tests on supernatant (*see* urine sediment, *Urine Sediment*).

Specific Gravity: The USG is determined using a refractometer designed for veterinary samples, which includes a scale calibrated specifically for cat urine. USG for species other than cats should be determined using the scale for dogs. In healthy animals, USG is highly variable, depending on fluid and electrolyte balance of the body. Interpretation of USG, therefore, depends on the clinical presentation and serum chemistry findings.

An animal that is dehydrated or has other causes of prerenal azotemia will have hypersthenuric urine with a USG >1.025-1.040 (depending on species). Dilute urine in a dehydrated or azotemic animal is abnormal and could be caused by renal failure, hypo- or hyperadrenocorticism, hypercalcemia, diabetes mellitus, hyperthyroidism, diuretic therapy, or diabetes insipidus. Glucosuria increases the refractive index of urine, resulting in an increased USG despite increased urine volume.

Semiquantitative, Colorimetric Reagent Strips: Reagent strips such as Multistix or Chemstrip can be used to perform several semiquantitative chemical evaluations simultaneously. They are used routinely to determine urine pH, protein, glucose, ketones, bilirubin/ urobilinogen, and occult blood. Some reagent strips include test pads for leukocyte esterase (for detection of WBC), nitrite (for detection of bacteria), and USG; these are not valid in animals and should not be used. Reagent strips are adversely affected by moisture and have a limited shelf life. Bottles should be kept tightly capped, and unused strips should be discarded after their expiration date.

Urine pH: Urine pH is typically acidic in dogs and cats and alkaline in horses and ruminants, but varies depending on diet, medications, or presence of disease. Reagent strip colorimetric test pads for pH determination are accurate to within ~0.5 pH units. For example, a reading of 6.5 means the actual pH is likely to be between 6.0 and 7.0. A bacterial urinary tract infection with a urease-producing microbe will result in alkaluria. Urine pH will affect crystalluria because some crystals, such as struvite, form in alkaline urine, while other crystals, such as cystine, form in acidic urine.

Protein: The protein test pad detects primarily albumin in urine. Proteinuria can be seen with inflammation, hemorrhage, or glomerular

disease. A positive reaction must be interpreted in light of USG, pH, and urine sediment examination.

For example, a trace amount of protein in concentrated urine is less significant than a trace amount of protein in dilute urine. Alkaluria will give a false positive reaction. Likewise, presence of other proteins, such as Bence-Jones proteins, will give false negative results. Proteinuria can be measured using sulfosalicylic acid precipitation, which detects albumin and globulins. If proteinuria is present with an inactive urine sediment, its significance can be verified and quantitated by dividing the urine protein concentration by the urine creatinine concentration (urine protein to urine creatinine ratio; UP:UC). Interpretation of a UP:UC is as follows: <0.5:1 is normal, 0.5-1.0:1.0 is questionable, and >1.0:1.0 is abnormal. It is important to ensure that hematuria, pyuria, and infection are not present before determining a UP:UC because inflammation and hemorrhage result in significant proteinuria.

Glucose: Glucosuria is not present normally because the renal threshold for glucose is >180 mg/dL in most species and >240 mg/dL in cats. With euglycemia, the amount of filtered glucose is less than the renal threshold and all of the filtered glucose is reabsorbed in the proximal renal tubules. Glucosuria can result from hyperglycemia (due to diabetes mellitus, excessive endogenous or exogenous glucocorticoids, or stress) or from a proximal renal tubular defect (such as primary renal glucosuria or Fanconi syndrome). If glucosuria is present, blood glucose concentration should be determined.

Ketones: The ketone test pad detects acetate and acetoacetate, but not a-hydroxybutyrate. Ketonuria is associated with primary ketosis (ruminants), ketosis secondary to diabetes mellitus (small animals), and occasionally with prolonged fasting or starvation. A false positive reaction can occur with presence of reducing substances in urine.

Bilirubin/Urobilinogen: Presence of conjugated bilirubin in urine will result in a positive reaction. A tablet test, Ictotest, can be used to detect bilirubin as well. Bilirubinuria occurs when conjugated bilirubin exceeds the renal threshold as with liver disease or hemolysis. In dogs with concentrated urine, a small amount of bilirubin can be normal. Pigmenturia may result in a false positive reaction. Urobilinogen, formed from bilirubin by intestinal microflora, is absorbed into the portal circulation and is excreted renally. A small amount of urinary urobilinogen is normal. Increased urinary urobilinogen occurs with hyperbilirubinemia; a negative test may be observed with biliary

obstruction. However, the test is not specific enough to be clinically useful.

Occult Blood: The occult blood test pad uses a "pseudoperoxidase" method to detect intact RBC, hemoglobin, and myoglobin. A positive reaction can be due to hemorrhage (hematuria), intravascular hemolysis (hemoglobinuria), or myoglobinuria. The latter 2 processes can be distinguished by examination of plasma—plasma will appear pink to red after intravascular hemolysis, while myoglobin is rapidly cleared from plasma, resulting in clear plasma. As with other colorimetric test pads, discolored urine may yield false positive results. A positive result should be interpreted with microscopic examination of urine sediment.

The Litmus Milk Test, Atlas p. 64.

The Litmus Milk test is a rather complex test containing milk, litmus (a pH indicator), lactose, and casein (a milk protein) all of which can be metabolized by some bacteria. Litmus milk also contains sulfur and many nutrients that are found in milk. It is an undefined and differential media that is used to help differentiate and identify Enterobacteria, Clostridium, and Lactic acid bacteria.

A number of results may be obtained in the Litmus Milk test, but there are four main reactions: lactose fermentation, litmus reduction, casein coagulation, and protein hydrolysis. Some or all of these reactions may occur at the same time, and many of them have multiple or similar sub-reactions (for example, casein coagulation can form a solid clot (as in an acid clot) or casein coagulation can occur as the casein is converted into a solid curd in the bottom and a liquid whey on the top (as in curd formation).

Four reactions can occur in Litmus Milk:	*Litmus Color*	*pH*	*Notes*
1. lactose fermentation (without gas) (or fermentation with gas production)	pink (pink)	4.5 and below (4.5 and below)	acid (acid with bubbles or fissures)
2. reduction of litmus	white	any	white starts in tube bottom
3. casein protein coagulation	any	any	solid (clot or curd, in lower tube)
4. casein protein hydrolysis (partial) (or nearly complete hydrolysis)	blue (clear & dark)	8.3 & above (8.3 & above)	basic (especially at tube top) (basic and media is thin)
There can also be no reaction and any combination of the above reactions (see Table 6-2, Atlas p. 64)	purple shades	4.6 to 8.2	near neutral pH
	other	pH uncertain	

As you might have noticed, it is rather complex, for further details, see Atlas, p64-65. It is required that you be able to use and interpret the data in Table 6-2 and Fig 6-50 (Atlas, p. 64) when provided with the table and/or figures.

Procedure:

1. Obtain a tube. (If you do not have a lab partner, it is recommended that you repeat this test with a second tube.)
2. Label each tube.
3. Use your wire loop to aseptically inoculate the Litmus Milk tube with your unknown bacteria. Add a lot of bacteria as some species do not grow well in this medium.
4. Remind your instructor to set up the control uninoculated tubes (they will be useful for comparing with your tube). Place your tubes in the rack for this test, they will be incubated for one week at 35-37 degrees C.
5. The tubes should be observed in 2-3 days to check for the color of the media (or the pH). Continue incubating the tubes for a total of one week. The media color should be checked because at this early date, the litmus will not be totally reduced. When the litmus is totally reduced, it is difficult determining the pH.
6. Record your results of media color after day 2-3, and all your results including color after 1 week.

Results: Obtain your tubes after one week of incubation, checking for gas bubbles before you handle the tubes as these disappear after handling. Obtaining results for such a complex test can be tricky if several reactions occur together. Follow this guide for assistance:

- First, determine if there is white in the tube. This is from reduction of litmus and it can make determining the pH difficult.
- Second, determine the color of the media, use your observations on day 2 or 3 if needed. Then follow the steps below.
- Third, after determining the color, determine if the media has solidified.
- Fourth, look for specific characteristics of each reaction, such as gas production in a clot, etc. See the steps below.
- First, determine if there is white in the tube, especially at the bottom (check that is not sedimented milk).
    - If there is no white in the tube, there is no reduction of litmus. (Result so far is No Reduction which is usually not even recorded.)
    - If there is white in the tube, you have reduction of litmus. If the uninocculated controls have the same amount of white in the bottom, assume it is sedimented milk. If in doubt, use a marker to mark the area of white and reincubate the tube. If

the area of white expands with time, you have litmus reduction. If the litmus has been totally reduced and you have no color other than white, and you did not make an observation on day 2 or 3, then it will be difficult to proceed with this test. (Result so far is Reduction which may occur with any of the below results.)

- Second, determine if the tube is (or in the case of reductionwas pink).
  - If the tube is pink, it means that lactose fermentation has occurred resulting in the production of acid end products (result, an Acid reaction).
  - If the tube is pink, check for bubbles or check for fissures or breaks in any clotted milk. The bubbles usually rise to the surface and pop at the first disturbance, so they are difficult to see. If any of these are present, there is fermentation with the production of gas (result, Acid and Gas).
  - If the tube is pink, check to see if any of the tube contents are solid, especially in the tube bottom. The acids that accumulate from fermentation may cause casein to clot. Usually, one can tip the tube and immediately tell if the tube is solid or not. If needed, you can flame a loop and gently stick it into the media to test if any of it is solid. Keep the tube sterile in case it needs to incubated further, but be careful not to damage the loop! If there is a solid pink clot, coagulation has occurred (result, is an Acid Clot, with or without gas). (Note, an acid clot always occurs with an acid reaction which is probably why it is not called “an acid and an acid clot.”)
  - If there is an acid clot, it is easier to observe gas production from fermentation due to the presence fissures in the clot. If enough gas is produced it can break up the clot and some call it stormy fermentation, but we will keep things simple and just call it gas production.
- Third, determine if the tube is or was blue.
  - If the tube is blue (especially at the top), the media has become basic from the partial hydrolysis and degradation of proteins, especially casein, which tends to release ammonia. (Result is a Basic reaction.)

- If the tube is blue, look to see if there is any solid media especially in the bottom of the tube (see the discussion above for an Acid Clot). If there is a blue solid, it may only be in the bottom with a fluid whey on top, then there is coagulation of casein (result, a Basic reaction and a Curd).
- If the tube is or was blue, check to see if there is any clarification of the media, where the milk has become thin, as if watered down. The top may appear as a transparent fluid that is dark, or brownish (or grey if there is reduction). The curd appears to be partially or fully dissolved. If so, this is digestion of protein that is nearing complete hydrolysis and peptonization. (Result is Digestion or Peptonization which always occurs with a Basic reaction, with or without curd.)

- Fourth, determine if the tube is (or in the case of reduction, was purple).
  - If the tube is purple, then the pH of the media is near neutral.
  - If the tube is purple, check to see if there is a solid, especially in the bottom of the tube (see the discussion above for an Acid Clot). If there is a solid, then there is coagulation of casein by enzymes released by your unknown without an acid or a basic reaction (result is a Curd).
  - If the tube is purple and if none of the above have occurred, then there is no change in the tube. If there is no change, sterilize a loop, cool it, stick it the litmus milk, and streak it across a general purpose agar plate to ensure that there is bacterial growth in the tube.
    - § If few colonies grow on the plate, then the strain of bacteria did not grow in the litmus milk (result is Little or No Growth).
    - § If there are many colonies, then the results of the experiment are valid (result is No Change).

Note to Instructors

Students often struggle with the results of this experiment. As the pH of the tube must be known to determine the result and instructors will not likely have observed the tube after about two days of growth, it is handy to use some pH paper when attempting to assist students in interpreting the results, especially when multiple reactions are occurring at the same time. To keep from contaminating the tubes in case further

incubation is required, one can remove a small sample of solution from the tube using a sterile pipette or loop and transferring it to the pH paper. Once the pH is known and the reduction of litmus has been determined, most of the above results can easily be distinguished, except for maybe digestion. In determining if digestion has occurred, first realize that a basic reaction must come first, where protein has been partially hydrolyzed. One can determine if there is a basic reaction by referring to the pH. If there is a basic reaction, next determine if there is a basic reaction and a curd by looking for solids. Lastly, look for signs of digestion where the proteins are nearing complete hydrolysis.

Lactose Intolerance

Lactose intolerance, also called lactase deficiency and hypolactasia, is the inability to digest lactose, a sugar found in milk and to a lesser extent milk-derived dairy products.

Lactose intolerant individuals have insufficient levels of lactase, the enzyme that metabolizes lactose into glucose and galactose, in their digestive system. In most cases this causes symptoms such as abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi (rumbling stomach) and/or vomiting after consuming significant amounts of lactose. Some studies in the U.S. and elsewhere suggest that milk consumption by lactose intolerant individuals may be a significant cause of irritable bowel syndrome.

Most mammals normally become lactose intolerant after weaning, but some human populations have developed lactase persistence, in which lactase production continues into adulthood. It is estimated that 75% of adults worldwide show some decrease in lactase activity during adulthood. The frequency of decreased lactase activity ranges from 5% in northern Europe through 71% for Sicily to more than 90% in some African and Asian countries. This distribution is now thought to have been caused by recent natural selection favoring lactase persistent individuals in cultures that rely on dairy products. While it was first thought that this would mean that populations in Europe, India and Africa that had high frequencies of lactase persistence shared a single mutation, it has now been shown that lactase persistence is caused by several independently occurring mutations.

Terminology

"Lactose intolerance" primarily refers to a syndrome having one or more symptoms upon the consumption of food substances containing lactose. Individuals may be lactose intolerant to varying degrees, depending on the severity of these symptoms. "Lactose malabsorption" refers to the physiological concomitant of lactase deficiency (i.e. the body does not have sufficient lactase capacity to digest the amount of lactose ingested). A medical condition with similar symptoms is fructose malabsorption.

Lactase deficiency has a number of causes, and is therefore classified as one of three types:

- Primary lactase deficiency is genetic, only affects adults and is caused by the absence of a lactase persistence allele. It is the most common cause of lactose intolerance as a majority of the world's population lacks these alleles.
- Secondary, acquired, or transient lactase deficiency is caused by an injury to the small intestine, usually during infancy, from acute gastroenteritis, diarrhea, chemotherapy, intestinal parasites or other environmental causes.
- Congenital lactase deficiency is a very rare, autosomal recessive genetic disorder that prevents lactase expression from birth. It is particularly common in Finland. People with congenital lactase deficiency are thus unable to digest lactose from birth, and they are unable to digest breast milk.

Lactose intolerance is not an allergy, because it is not an immune response, but rather a problem with digestion caused by lactase deficiency. Milk allergy is a separate condition, with distinct symptoms that occur when the presence of milk proteins trigger an immune reaction.

Symptoms

The principal symptom of lactose intolerance is an adverse reaction to products containing lactose (primarily milk), including abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi (rumbling stomach) and vomiting (particularly in adolescents). These appear thirty minutes to two hours after consumption. The severity of symptoms typically increases with the amount of lactose consumed, and most lactose intolerant people can tolerate a certain level of lactose in their diet without ill effect.

Nutritional Implications

While dairy products can be a significant source of nutrients in some societies, there is no evidence that lactose intolerance has any adverse impact on nutrition where consumption is the norm among adults. Congenital lactase deficiency (CLD), where the production of lactase is inhibited from birth, can be dangerous in any society because of infants' nutritional reliance on breast milk during their first months. Before the 20th century, babies born with CLD were not expected to survive, but these death rates can now be lowered using soybean-derived infant formulas and manufactured lactose-free dairy products. Beyond infancy, individuals affected by CLD usually have the same nutritional concerns as any lactose intolerant adult.

Causes

Lactose intolerance is a consequence of lactase deficiency, which may be either genetic or environmentally induced, depending on whether it is primary, secondary, or congenital. In any case symptoms are caused by insufficient levels of the enzyme lactase in the lining of the duodenum.

Lactose, a disaccharide molecule found in milk and dairy products, cannot be directly absorbed through the wall of the small intestine into the bloodstream so, in the absence of lactase, passes intact into the colon. Bacteria in the colon are able to metabolise lactose and the resulting fermentation produces copious amounts of gas (a mixture of hydrogen, carbon dioxide and methane) that causes the various abdominal symptoms. The unabsorbed sugars and fermentation products also raises the osmotic pressure of the colon, resulting in an increased flow of water into the bowels (diarrhea).

Diagnosis

To assess lactose intolerance, intestinal function is challenged by ingesting more dairy products than can be readily digested. Clinical symptoms typically appear within 30 minutes, but may take up to two hours, depending on other foods and activities. Substantial variability in response (symptoms of nausea, cramping, bloating, diarrhea, and flatulence) is to be expected, as the extent and severity of lactose intolerance varies among individuals.

It is important to distinguish lactose intolerance from milk allergy, an abnormal immune response (usually) to milk proteins. This may be

done in diagnosis by giving lactose-free milk, producing no symptoms in the case of lactose intolerance, but the same reaction as to normal milk if it is a milk allergy. An intermediate result might suggest that the person has both conditions. Since lactose intolerance is the normal state for most adults worldwide, it is not considered a disease and a medical diagnosis is not normally required. However, if confirmation is necessary, four tests are available.

Hydrogen Breath Test

In a hydrogen breath test, after an overnight fast, 25 grams of lactose (in a solution with water) is swallowed. If the lactose cannot be digested, enteric bacteria metabolize it and produce hydrogen, which, along with methane, if produced, can be detected on the patient's breath by a clinical gas chromatograph or compact solid-state detector. The test takes about 2 to 3 hours to complete.

Blood Test

In conjunction, measuring blood glucose level every 10 to 15 minutes after ingestion will show a "flat curve" in individuals with lactose malabsorption, while the lactase persistent will have a significant "top", with a typical elevation of 50% to 100%, within one to two hours. However, due to the need for frequent blood sampling, this approach has been largely replaced by breath testing.

After an overnight fast, blood is drawn and then 50 grams of lactose (in aqueous solution) is swallowed. Blood is then drawn again at the 30 minute, 1-hour, 2-hour, and 3-hour mark. If the lactose cannot be digested, blood glucose levels will rise by less than 20 mg/dL.

Stool Acidity Test

This test can be used to diagnose lactose intolerance in infants, for whom other forms of testing are risky or impractical. The infant is given lactose to drink. If the individual is tolerant, the lactose is digested and absorbed in the small intestine; otherwise it is not digested and absorbed and it reaches the colon. The bacteria in the colon, mixed with the lactose, cause acidity in stools. Stools passed after the ingestion of the lactose are tested for level of acidity. If the stools are acidic, the infant is intolerant to lactose.

Intestinal Biopsy

An intestinal biopsy can confirm lactase deficiency following discovery of elevated hydrogen in the hydrogen breath test. Modern techniques have enabled a test to be performed at the patient's bedside, identifying the presence/absence of the lactase enzyme in conjunction with upper gastrointestinal endoscopy. However, for research applications such as mRNA measurements, a specialist laboratory is required.

Management

Lactose intolerance is not considered a condition that requires treatment in societies where the diet contains relatively little dairy. However, those living among societies that are largely lactose-tolerant may find lactose intolerance troublesome. Although there are still no methodologies to reinstate lactase production, some individuals have reported that their intolerance varies over time, depending on health status and pregnancy About 44% of lactose intolerant women regain the ability to digest lactose during pregnancy. This might be caused by slow intestinal transit and intestinal flora changes during pregnancy.

Lactose intolerance is not usually an absolute condition: The reduction in lactase production, and the amount of lactose that can therefore be tolerated, varies from person to person. Since lactose intolerance poses no further threat to a person's health, the condition is managed by minimizing the occurrence and severity of symptoms. Berdanier and Hargrove recognise four general principles in dealing with lactose intolerance — avoidance of dietary lactose, substitution to maintain nutrient intake, regulation of calcium intake and use of enzyme substitute.

Avoiding Lactose-containing Products

Since each individual's tolerance to lactose varies, according to the U.S. National Institutes of Health (NIH), "Dietary control of lactose intolerance depends on people learning through trial and error how much lactose they can handle." Label reading is essential, as commercial terminology varies according to language and region.

Lactose is present in two large food categories — conventional dairy products, and as a food additive in dairy and non dairy products.

Dairy Products

Lactose is a water-soluble substance. Fat content and the curdling process affect tolerance of foods. After the curdling process lactose is found in the water-based portion (along with whey and casein), but not in the fat-based portion. Dairy products that are "reduced-fat" or "fat-free" generally have slightly higher lactose content. Low-fat dairy foods also often have various dairy derivatives added, such as milk solids, increasing the lactose content.

Milk

Human milk has a high lactose content, around 9%. Unprocessed cow milk is about 4.7% lactose. Unprocessed milk from other bovids contains a similar fraction of lactose (goat milk 4.7%, buffalo 4.86%, yak 4.93%, sheep 4.6%)

Butter

The butter-making process separates the majority of milk's water components from the fat components. Lactose, being a water soluble molecule, will largely be removed, but will still be present in small quantities in the butter unless it is also fermented to produce cultured butter. Clarified butter, however, contains very little lactose and is safe for most LI patients.

Yogurt, Frozen Yogurt and Kefir

People can be more tolerant of traditionally made yogurt than milk, because it contains lactase produced by the bacterial cultures used to make the yogurt. Frozen yogurt, if cultured similarly to its unfrozen counterpart, will contain similarly reduced lactose levels. However, many commercial brands contain milk solids, increasing the lactose content.

Cheeses

Traditionally made hard cheese, such as Emmental, and soft ripened cheeses may create less reaction than the equivalent amount of milk because of the processes involved. Fermentation and higher fat content contribute to lesser amounts of lactose. Traditionally made Emmental or Cheddar might contain 10% of the lactose found in whole milk. In addition, the traditional aging methods of cheese (over two years) reduces their lactose content to practically nothing. Commercial cheese brands, however, are generally manufactured by modern processes that do not have the same lactose reducing properties, and as no regulations

mandate what qualifies as an "aged" cheese, this description does not provide any indication of whether the process used significantly reduced lactose.

Sour *Cream*

If made in the traditional way, this may be tolerable, but most modern brands add milk solids.

Examples of Lactose Levels in Foods

As industry standardization has not been established concerning lactose content analysis methods (non-hydrated form or the mono-hydrated form), and considering that dairy content varies greatly according to labeling practices, geography and manufacturing processes, lactose numbers may not be very reliable. The following table contains a guide to the typical lactose levels found in various foods.

Dairy product	*Serving size*	*Lactose content*	*Percentage*
Milk, regular	250 ml	12 g	4.80%
Milk, reduced fat	250 ml	13 g	5.20%
Yogurt, plain, regular	200 g	9 g	4.50%
Yogurt, plain, low-fat	200 g	12 g	6.00%
Cheddar cheese	30 g	0.02 g	0.07%
Cottage cheese	30 g	0.1 g	0.33%
Butter	1 tsp (5.9ml)	0.03 g	0.51%
Ice cream	50 g	3 g	6.00%

Lactose in Non-dairy Products

Lactose (also present when labels state lactoserum, whey, milk solids, modified milk ingredients, etc.) is a commercial food additive used for its texture, flavour and adhesive qualities, and is found in foods such as processed meats (sausages/hot dogs, sliced meats, pates), gravy

stock powder, margarines, sliced breads, breakfast cereals, potato chips, processed foods, medications, pre-prepared meals, meal replacement (powders and bars), protein supplements (powders and bars) and even beers in the milk stout style. Some barbecue sauces and liquid cheeses used in fast-food restaurants may also contain lactose.

Kosher products labeled *pareve* or *fleishig* are free of milk. However, if a "D" (for "Dairy") is present next to the circled "K", "U", or other hechsher, the food likely contains milk solids, although it may also simply indicate that the product was produced on equipment shared with other products containing milk derivatives.

Alternative Products

Plant-based milks and derivatives are inherently lactose free — soy milk, rice milk, almond milk, coconut milk, hazelnut milk, oat milk, hemp milk, peanut milk, horchata.

The dairy industry has created low-lactose or lactose-free products to replace regular dairy products for those with lactose intolerance.

Lactase Supplementation

When lactose avoidance is not possible, or on occasions when a person chooses to consume such items, then enzymatic lactase supplements may be used.

Lactase enzymes similar to those produced in the small intestines of humans are produced industrially by fungi of the genus *Aspergillus*. The enzyme, a-galactosidase, is available in tablet form in a variety of doses, in many countries without a prescription. It functions well only in high-acid environments, such as that found in the human gut due to the addition of gastric juices from the stomach. Unfortunately, too much acid can denature it, and it therefore should not be taken on an empty stomach. Also, the enzyme is ineffective if it does not reach the small intestine by the time the problematic food does. Lactose-sensitive individuals can experiment with both timing and dosage to fit their particular needs.

While essentially the same process as normal intestinal lactose digestion, direct treatment of milk employs a different variety of industrially produced lactase. This enzyme, produced by yeast from the genus *Kluyveromyces*, takes much longer to act, must be thoroughly mixed throughout the product, and is destroyed by even mildly acidic

environments. Its main use is in producing the lactose-free or lactose-reduced dairy products sold in supermarkets.

Enzymatic lactase supplementation may have an advantage over avoiding dairy products, in that alternative provision does not need to be made to provide sufficient calcium intake, especially in children.

Rehabituation to Dairy Products

For healthy individuals with secondary lactose intolerance, it may be possible in some cases for the bacteria in the large intestine to adapt to an altered diet and break down small quantities of lactose more effectively by habitually consuming small amounts of dairy products several times a day over a period of time. Reintroducing dairy in this way to people who have an underlying or chronic illness, however, is not recommended, as certain illnesses damage the intestinal tract in a way which prevents the lactase enzyme from being expressed.

Some studies indicate that environmental factors — more specifically, the consumption of lactose — may "play a more important role than genetic factors in the etio-pathogenesis of milk intolerance", but some other publications suggest that lactase production does not seem to be induced by dairy/lactose consumption.

Lactase Persistence

Lactase persistence is the phenotype associated with various autosomal dominant alleles prolonging the activity of lactase beyond infancy; conversely, lactase non-persistence is the phenotype associated with primary lactase deficiency (see above). Among mammals, lactase persistence is unique to humans — it evolved relatively recently (in the last 10,000 years) among some populations, and the majority of people worldwide remain lactase non-persistent. For this reason lactase persistence is of some interest to the fields of anthropology and human genetics, which typically use the genetically derived persistence/non-persistence terminology.

Recognition of the extent and genetic basis of lactose intolerance is relatively recent. Though its symptoms were described as early as Hippocrates (460-370 B.C.), until the 1960s the prevailing assumption in the medical community was that tolerance was the norm and intolerance either the result of milk allergy, an intestinal pathogen, or else was psychosomatic (it being recognised that some cultures did not practice

dairying, and people from those cultures often reacted badly to consuming milk). There were two reasons for this perception. Firstly, many Western countries have a predominantly European heritage, and so have low frequencies of lactose intolerance, and have an extensive cultural history of dairying.

Therefore, tolerance actually was the norm in most of the societies investigated by medical researchers at that point. Secondly, within even these societies lactose intolerance tends to be under-reported: genetically lactase non-persistent individuals can tolerate varying quantities of lactose before showing symptoms, and their symptoms differ in severity. Most are able to digest a small quantity of milk, for example in tea or coffee, without suffering any adverse effects. Fermented dairy products, such as cheese, also contain dramatically less lactose than plain milk.

Therefore, in societies where tolerance is the norm, many people who consume only small amounts of dairy or have only mild symptoms, may be unaware that they cannot digest lactose. Eventually, however, it was recognised that in the United States lactose intolerance is correlated with race. Subsequent research revealed that intolerance was the worldwide norm, and that the variation was genetic. However, as yet there is no comprehensive understanding of either the global distribution of lactase persistence, the number of alleles that cause it, or the reasons for its recent selection.

Testing Mare Milk to Predict Foaling

For several years, we used an idea that I got from another breeder in testing the mare's milk to predict foaling time. I cannot remember who the person is to give them credit, but I remember that they had used this method for decades with excellent success. Usually, a mare will foal within 48 hours of getting colostrum. So, the idea is to know when there is colostrum to get a better idea the mare's foaling timetable.

[Colostrum: milk secreted just prior to parturition (mare giving birth) and for a few days afterwards, characterized by high protein and antibody content.]

Please note that when testing the milk you must keep in mind all of the other factors that let you know when your mare is close to foaling, such as the waxing over of the nipple, sunken croup, restlessness, irritability, etc.

The Mare's Milk

A pregnant mare's milk prior to foaling will consist of three different variables: Taste, Color and Consistency.

Taste will range from bland (no taste), salty, and sweet. The Color will be from a dark yellow to a skim milk white, and the Consistency will fluctuate from thin and runny like milk to sticky and tacky. A mare's milk will bounce around and fluctuate between all of these ranges from hour to hour and day to day.

Colostrum is sticky. It is very tacky and very sweet and has a white color. You may get every combination of Taste, Color and Consistency in what seems to be a random order. We have found that most of the time when a mare is at the end of her gestation, the milk can go in any order with colostrum being the end result. The changes in the mare's milk may happen in a matter of hours or days, or over several weeks.

The most common sequence and combination for the pregnant mare's milk transition in our experience has been:

- runny, salty
- not quie as thin and runny, cloudy and bland
- maybe thicker, tacky, "milk white" sweet colostrum

Milking the Mare

Make sure that the mare is comfortable with you handling her udder. Some mares get more sensitive in the udder the closer they are to foaling so always use common sense and extreme caution. It helps to plan ahead and gently handle the mare's udder, to desensitize her before attempting to milk her. The training for handling the mare's udder is obviously critical to safe and successful milking. The process also helps prepare the mare for a foal suckling.

Milking a mare would be similar to milking a cow. If you do not have that experience (as I hadn't) I'll try to explain the process. Grasp above the nipple between the pads of your index finger and your thumb. You then gently squeeze and roll your fingers down toward the nipple. This is not a pinching process. Imagine that you are trapping some milk above the nipple, then motioning it down toward the nipple. The movement will often tug the nipple down slightly, similar to a suckling foal. You should be able to get between a few drops to a steady stream of milk, but all you need is a few drops of milk that you catch with your other hand..

This is something that will take practice, but remember that the mare's udder is sensitive so minimal pressure should be applied as

necessary to accomplish the task. You will find that some mares are more difficult to milk, but persistence is the key.

Testing the Milk

When you go to test the mare's milk, you only need a few drops. A black or white surface is most reliable for determining color. After you collect the milk, wipe any droplets off of the mare's nipple. It will harden in time and you might mistake it for wax. With these few drops you can look at the color of the milk, check consistency, and taste the milk with the tip of your tongue.

You can roll the taste around (I've heard that the tongue's salty receptors in more to the rear of your tongue) and get a good feel for the taste and then spit it out. (I'm not sure that the raw milk is sanitary to swallow.) You can rinse your mouth with water if the thought of the milk residue in your mouth is unpleasant to you.

To check the consistency, take your other hand's index finger and dip it in the milk. Press your thumb to the milk on your index finger to see if it is tacky or stringy (leaves a string between the two fingers when you pull them apart.)

Colostrum is a milky color, very sweet and very sticky and stringy. (Not necessarily thick. I've had some mares have a gel type milk that is very thick, but it is not colostrum.) The first time that you taste colostrum, you will notice the sweetness and it will stand out in your mind. The mare will probably foal within 48 hours from the time she produces the colostrum.

Timing

Remember that not every mare has a full-to-bursting udder before foaling. I start testing the milk as soon as I can express any fluid. We try to check the mare's milk at least 2 times a day; then more frequently as the changes in the milk and other signs indicate the mare is getting closer. I make notes on every test to try to detect patterns.

We have foaled about 5 mares a year. Since we have started using this method, we have predicted every foaling. I've found that once there is colostrum, a mare usually foals within 24 hours. If we find colostrum at the evening feed milk check, we usually have a foal by morning. I've only had one mare change from colostrum for a few hours back to bland. She foaled about 36 hours from the first change to colostrum.

I was told that some mares will get their colostrum and foal within an hour…and, of course, there are those mares who never get colostrum and milk.

In these cases - as with every pregnant mare - all of the factors to predict a mare's foaling should be taken into consideration. Testing the mare's milk simply makes it easier to predict timing for foaling since it is a fairly reliable method..

Subsequent Note: We no longer milk the mares. Milking is reported to cause discomfort and possibly contractions. Instead we observe the mare more closely to monitor visible obvious and subtle physical changes, including changes in her behavior.

Synovial Fluid

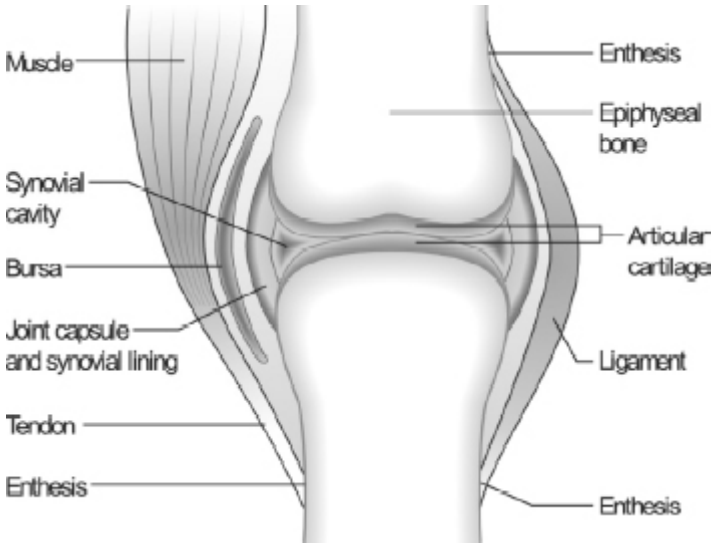


Figure: *A typical joint*

Synovial fluid is a viscous, non-Newtonian fluid found in the cavities of synovial joints.

With its yolk-like consistency ("synovial" partially derives from *ovum*, Latin for egg), the principal role of synovial fluid is to reduce friction between the articular cartilage of synovial joints during movement.

Overview

The inner membrane of synovial joints is called the synovial membrane and secretes synovial fluid into the joint cavity. The fluid contains hyaluronic acid secreted by fibroblast-like cells in the synovial membrane and interstitial fluid filtered from the blood plasma. This fluid

forms a thin layer (roughly 50 im) at the surface of cartilage and also seeps into microcavities and irregularities in the articular cartilage surface, filling all empty space. The fluid in articular cartilage effectively serves as a synovial fluid reserve. During movement, the synovial fluid held in the cartilage is squeezed out mechanically to maintain a layer of fluid on the cartilage surface (so-called *weeping lubrication*). The functions of the synovial fluid include:

- reduction of friction - synovial fluid lubricates the articulating joints
- shock absorption - as a dilatant fluid, synovial fluid is characterized by the rare quality of becoming more viscous under applied pressure; the synovial fluid in diarthrotic joints becomes thick the moment shear is applied in order to protect the joint and subsequently thins to normal viscosity instantaneously to resume its lubricating function between shocks
- nutrient and waste transportation - the fluid supplies oxygen and nutrients and removes carbon dioxide and metabolic wastes from the chondrocytes within the surrounding cartilage

Composition

Synovial tissue is sterile and composed of vascularized connective tissue that lacks a basement membrane. Two cell types (type A and type B) are present: Type A is derived from blood monocytes, and it removes the wear-and-tear debris from the synovial fluid. Type B produces synovial fluid. Synovial fluid is made of hyaluronic acid and lubricin, proteinases, and collagenases. Synovial fluid exhibits non-Newtonian flow characteristics; the viscosity coefficient is not a constant and the fluid is not linearly viscous. Synovial fluid has thixotropic characteristics; viscosity decreases and the fluid thins over a period of continued stress.

Normal synovial fluid contains 3–4 mg/ml hyaluronan (hyaluronic acid), a polymer of disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine joined by alternating beta-1,4 and beta-1,3 glycosidic bonds. Hyaluronan is synthesized by the synovial membrane and secreted into the joint cavity to increase the viscosity and elasticity of articular cartilages and to lubricate the surfaces between synovium and cartilage.

Synovial fluid contains lubricin secreted by synovial cells. Chiefly, it is responsible for so-called boundary-layer lubrication, which reduces

friction between opposing surfaces of cartilage. There also is some evidence that it helps regulate synovial cell growth.

Its functions are:

reducing friction by lubricating the joint, absorbing shocks, and supplying oxygen and nutrients to and removing carbon dioxide and metabolic wastes from the chondrocytes within articular cartilage.

It also contains phagocytic cells that remove microbes and the debris that results from normal wear and tear in the joint.

Chapter 4: Animal Diseases and Lab Diagnosis

Collection

Synovial fluid may be collected by syringe in a procedure termed arthrocentesis, also known as joint aspiration.

Many synovial fluid types are associated with specific diagnoses:

- Noninflammatory (Group I)
  - Osteoarthritis, degenerative joint disease
  - Trauma
  - Rheumatic fever
  - Chronic gout or pseudogout
  - Scleroderma
  - Polymyositis
  - Systemic lupus erythematosus
  - Erythema nodosum
  - Neuropathic arthropathy (with possible hemorrhage)
  - Sickle-cell disease
  - Hemochromatosis
  - Acromegaly
  - Amyloidosis
- Inflammatory (Group II)
  - Rheumatoid arthritis
  - Reactive arthritis
  - Psoriatic arthritis
  - Acute rheumatic fever
  - Acute gout or pseuodgout
  - Scleroderma
  - Polymyositis

- Systemic lupus erythematosus
- Ankylosing spondylitis
- Inflammatory bowel disease arthritis
- Infection (viral, fungal, bacterial) including Lyme disease
- Acute crystal synovitis

- Septic (Group III)
  - Pyogenic bacterial infection
  - Septic arthritis
- Hemorrhagic
  - Trauma
  - Tumors
  - Hemophilia/coagulopathy
  - Scurvy
  - Ehlers-Danlos syndrome
  - Neuropathic arthropathy

Cracking Joints

When the two articulating surfaces of a synovial joint are separated from one other, the volume within the joint capsule increases and a negative pressure results.

The volume of synovial fluid within the joint is insufficient to fill the expanding volume of the joint and gases dissolved in the synovial fluid (mostly carbon dioxide) are liberated and quickly fill the empty space, leading to the rapid formation of a bubble. This process is known as cavitation. Cavitation in synovial joints results in a high frequency 'cracking' sound.

Synovial Fluid Analysis

How is it Used?

Synovial fluid analysis may be ordered to help diagnose the cause of joint inflammation, pain, swelling, and fluid accumulation. Diseases and conditions affecting one or more joints and the synovial fluid can be divided into four main categories:

***Infectious Diseases*:** Those caused by bacteria, fungi, or viruses. They may originate in the joint or spread there from other places in the body. These conditions include acute and chronic septic arthritis.

***Bleeding*:** Bbleeding disorders and/or joint injury can lead to blood in the synovial fluid. Commonly present in people with untreated blood clotting disorders such as hemophilia or von Willebrand Disease.

Infectious Diseases:

- Conditions that cause crystal formation and accumulation such as gout (needle-like uric acid [monosodium urate] crystals) and pseudogout (calcium pyrophosphate dihydrate crystals). Typically affect the feet and legs.
- Conditions that cause joint inflammation, such as synovitis, or other immune responses. These may include autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus.

Degenerative diseases – such as osteoarthritis

When is it Ordered?

Synovial fluid analysis may be ordered when a doctor suspects that a person has a condition or disease involving one or more of their joints. It may be ordered when someone has some combination of the following signs and symptoms:

- Joint pain
- Redness over the joint
- Joint inflammation and swelling
- Synovial fluid accumulation

It may sometimes be ordered to monitor a person with a known joint condition.

It may sometimes be ordered to monitor a person with a known joint condition.

What Does the Test Result Mean?

Synovial fluid usually contains a small amount of glucose and protein and may have a few white blood cells (WBCs) and red blood cells (RBCs).

There are a variety of joint abnormalities including osteoarthritis, rheumatoid arthritis, gout, and infection (septic arthritis) that can cause inflammation, swelling, an accumulation of synovial fluid, and sometimes bleeding into one or more joints. These conditions can limit mobility and, if left untreated, may permanently damage the joints.

Results of tests performed on a sample of synovial fluid may include:

Physical characteristics – the normal appearance of a sample of synovial fluid is usually:

- Straw colored
- Clear
- Moderately viscous – drops of it from a syringe needle will form a "string" a few inches long.

Changes in the physical characteristics may provide clues to the disease present, such as:

- Less viscous fluid may be seen with inflammation.
- Cloudy synovial fluid may indicate the presence of microorganisms, white blood cells, or crystals.
- Reddish synovial fluid may indicate the presence of blood, but an increased number of red blood cells may also be present in cloudy synovial fluid.

Chemical tests – tests that may be performed on synovial fluid samples may include:

- Glucose—typically a bit lower than blood glucose levels; may be significantly lower with joint inflammation and infection.
- Protein—increased with bacterial infection
- Lactate dehydrogenase—increased LD (LDH) level may be seen in rheumatoid arthritis, infectious arthritis, or gout.
- Uric acid—increased with gout

Microscopic Examination: Normal synovial fluid has small numbers of white blood cells (WBCs) and red blood cells (RBCs) but no microorganisms or crystals present.

Laboratories may examine drops of the synovial fluid and/or use a special centrifuge (cytocentrifuge) to concentrate the fluid's cells at the bottom of a test tube. Samples are placed on a slide, treated with special stain, and an evaluation of the different kinds of cells present is performed.

- Total cell counts—number of WBCs and RBCs in the sample;increased WBCs may be seen with infections and with conditionssuch as gout and rheumatoid arthritis.
- A WBC differential determines the percentages of different types of WBCs. An increased number of neutrophils may be seen with

bacterial infections. Greater than 2% eosinophils may suggest Lyme disease.

- Synovial fluid is evaluated under polarized light to recognize the presence of crystals and to distinguish the types of crystals that are present. Needle-like monosodium urate crystals are associated with gout and calcium pyrophosphate crystals are associated with pseudogout.

Infectious disease tests – in addition to chemistry tests, other tests may be performed to look for microorganisms if infection is suspected.

- Gram stain allows for the direct observation of bacteria or fungi under a microscope. There should be no microorganisms present in synovial fluid.
- Culture and susceptibility testing is ordered to determine what type of microorganisms are present. If bacteria are present, susceptibility testing against certain antibiotics can be performed to guide antimicrobial therapy.
- If there are no microorganisms present, it does not rule out an infection; they may be present in small numbers or their growth may be inhibited because of prior antibiotic therapy.
- Other tests for infectious diseases that are less commonly ordered include AFB smear and culture. This test for the presence of mycobacteria may help diagnose tuberculosis. Molecular test methods for *Mycobacteria tuberculosis* are more sensitive and specific than traditional cultures and may also be performed.

Is There Anything Else I Should Know

A blood or urine uric acid or blood glucose may be ordered to compare concentrations with those in the synovial fluid.

If a doctor suspects that a patient may have a systemic infection, then a blood culture may be ordered in addition to the synovial fluid analysis.

Joint injury, surgery, and joint replacement can increase the risk of developing an infection in a joint.

Bone Marrow

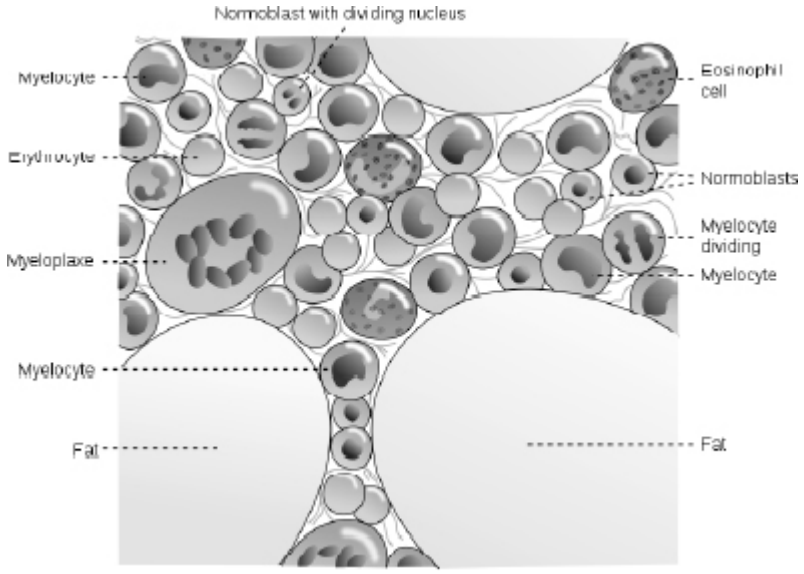


Figure: *A simplified illustration of cells in bone marrow*

Bone marrow (Latin: *medulla ossium*) is the flexible tissue found in the interior of bones. In humans, bone marrow in large bones produces new blood cells. On average, bone marrow constitutes 4% of the total body mass of humans; in an adult weighing 65 kilograms (140 lb), bone marrow accounts for approximately 2.6 kilograms (5.7 lb). The hematopoietic compartment of bone marrow produces approximately 500 billion blood cells per day, which use the bone marrow vasculature as a conduit to the body's systemic circulation. Bone marrow is also a key component of the lymphatic system, producing the lymphocytes that support the body's immune system.

Marrow Types

There are two types of bone marrow: medulla ossium rubra (*red marrow*), which consists mainly of hematopoietic tissue, and medulla ossium flava (*yellow marrow*), which is mainly made up of fat cells. Red blood cells, platelets and most white blood cells arise in red marrow. Both types of bone marrow contain numerous blood vessels and capillaries. At birth, all bone marrow is red. With age, more and more of it is converted to the yellow type; only around half of adult bone marrow is red. Red marrow is found mainly in the flat bones, such as the pelvis, sternum, cranium, ribs, vertebrae and scapulae, and in the cancellous ("spongy") material at the epiphyseal ends of long bones such as the femur and humerus. Yellow marrow is found in the medullary cavity, the hollow interior of the middle portion of long bones. In cases of severe blood loss, the body can convert yellow marrow back to red marrow to increase blood cell production.

Stroma

The *stroma* of the bone marrow is all tissue not directly involved in the primary function of hematopoiesis. Yellow bone marrow makes up the majority of bone marrow stroma, in addition to smaller concentrations of stromal cells located in the red bone marrow. Though not as active as parenchymal red marrow, stroma is indirectly involved in hematopoiesis, since it provides the hematopoietic microenvironment that facilitates hematopoiesis by the parenchymal cells. For instance, they generate colony stimulating factors, which have a significant effect on hematopoiesis. Cells that constitute the bone marrow stroma are:

- fibroblasts (reticular connective tissue)
- macrophages
- adipocytes
- osteoblasts
- osteoclasts
- endothelial cells, which form the sinusoids. These derive from endothelial stem cells, which are also present in the bone marrow.

Macrophages contribute especially to red blood cell production, as they deliver iron for hemoglobin production.

Bone Marrow Barrier

The blood vessels of the bone marrow constitute a barrier, inhibiting immature blood cells from leaving the marrow. Only mature blood cells contain the membrane proteins required to attach to and pass the blood vessel endothelium. Hematopoietic stem cells may also cross the bone marrow barrier, and may thus be harvested from blood.

Mesenchymal Stem Cells

The bone marrow stroma contain mesenchymal stem cells (MSCs), also called *marrow stromal cells*. These are multipotent stem cells that can differentiate into a variety of cell types. MSCs have been shown to differentiate, in vitro or in vivo, into osteoblasts, chondrocytes, myocytes, adipocytes and beta-pancreatic islets cells. MSCs can also transdifferentiate into neuronal cells.

Red Marrow Parenchyma

In addition, the bone marrow contains hematopoietic stem cells, which give rise to the three classes of blood cells that are found in the circulation: white blood cells (leukocytes), red blood cells (erythrocytes), and platelets (thrombocytes).

Compartmentalization

Biological compartmentalization is evident within the bone marrow, in that certain cell types tend to aggregate in specific areas. For instance, erythrocytes, macrophages, and their precursors tend to gather around blood vessels, while granulocytes gather at the borders of the bone marrow.

Lymphatic Role

The red bone marrow is a key element of the lymphatic system, being one of the primary lymphoid organs that generate lymphocytes from immature hematopoietic progenitor cells. The bone marrow and thymus constitute the primary lymphoid tissues involved in the production and early selection of lymphocytes. Furthermore, bone marrow performs a valve-like function to prevent the backflow of lymphatic fluid in the lymphatic system.

Diseases Involving the Bone Marrow

The normal bone marrow architecture can be displaced by malignancies, aplastic anemia, or infections such as tuberculosis, leading to a decrease in the production of blood cells and blood platelets. In addition, cancers of the hematologic progenitor cells in the bone marrow can arise; these are the leukemias.

Exposure to radiation or chemotherapy will kill many of the rapidly dividing cells of the bone marrow, and will therefore result in a depressed immune system. Many of the symptoms of radiation sickness are due to damage to the bone marrow cells.

To diagnose diseases involving the bone marrow, a bone marrow aspiration is sometimes performed. This typically involves using a hollow needle to acquire a sample of red bone marrow from the crest of the ilium under general or local anesthesia.

Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cells, usually derived

from bone marrow, peripheral blood, or umbilical cord blood. It is a medical procedure in the fields of hematology and oncology, most often performed for patients with certain cancers of the blood or bone marrow, such as multiple myeloma or leukemia. In these cases, the recipient's immune system is usually destroyed with radiation or chemotherapy before the transplantation. Graft-versus-host disease is a major complication of HSCT.

Hematopoietic stem cell transplantation remains a risky procedure with many possible complications; it has traditionally been reserved for patients with life-threatening diseases. While occasionally used experimentally in nonmalignant and nonhematologic indications such as severe disabling auto-immune disease and cardiovascular disease, the risk of fatal complications appears too high to gain wider acceptance.

History

Georges Mathe, a French oncologist, performed the first bone marrow transplant in 1959 on five Yugoslavian nuclear workers whose own marrow had been damaged by irradiation caused by a Criticality accident at the Vinea Nuclear Institute, but all of these transplants were rejected. Mathe later pioneered the use of bone marrow transplants in the treatment of leukemia.

Stem cell transplantation was pioneered using bone-marrow-derived stem cells by a team at the Fred Hutchinson Cancer Research Center from the 1950s through the 1970s led by E. Donnall Thomas, whose work was later recognized with a Nobel Prize in Physiology or Medicine. Thomas' work showed that bone marrow cells infused intravenously could repopulate the bone marrow and produce new blood cells. His work also reduced the likelihood of developing a life-threatening complication called graft-versus-host disease.

The first physician to perform a successful human bone marrow transplant on a disease other than cancer was Robert A. Good at the University of Minnesota in 1968.

Indications

Many recipients of HSCTs are multiple myeloma or leukemia patients who would not benefit from prolonged treatment with, or are already resistant to, chemotherapy. Candidates for HSCTs include pediatric cases where the patient has an inborn defect such as severe combined immunodeficiency or congenital neutropenia with defective stem cells, and also children or adults with aplastic anemia who have lost

their stem cells after birth. Other conditions treated with stem cell transplants include sickle-cell disease, myelodysplastic syndrome, neuroblastoma, lymphoma, Ewing's Sarcoma, Desmoplastic small round cell tumor, chronic granulomatous disease and Hodgkin's disease. More recently non-myeloablative, or so-called "mini transplant," procedures have been developed that require smaller doses of preparative chemo and radiation. This has allowed HSCT to be conducted in the elderly and other patients who would otherwise be considered too weak to withstand a conventional treatment regimen.

Number of Procedures

A total of 50,417 first hematopoietic stem cell transplants were reported as taking place worldwide in 2006, according to a global survey of 1327 centers in 71 countries conducted by the Worldwide Network for Blood and Marrow Transplantation. Of these, 28,901 (57%) were autologous and 21,516 (43%) were allogeneic (11,928 from family donors and 9,588 from unrelated donors). The main indications for transplant were lymphoproliferative disorders (54.5%) and leukemias (33.8%), and the majority took place in either Europe (48%) or the Americas (36%). In 2009, according to the world marrow donor association, stem cell products provided for unrelated transplantation worldwide had increased to 15,399 (3,445 bone marrow donations, 8,162 peripheral blood stem cell donations, and 3,792 cord blood units).

Graft Types

Autologous

Autologous HSCT requires the extraction (apheresis) of haematopoietic stem cells (HSC) from the patient and storage of the harvested cells in a freezer. The patient is then treated with high-dose chemotherapy with or without radiotherapy with the intention of eradicating the patient's malignant cell population at the cost of partial or complete bone marrow ablation (destruction of patient's bone marrow function to grow new blood cells). The patient's own stored stem cells are then returned to his/her body, where they replace destroyed tissue and resume the patient's normal blood cell production. Autologous transplants have the advantage of lower risk of infection during the immune-compromised portion of the treatment since the recovery of immune function is rapid.

Also, the incidence of patients experiencing rejection (graft-versus-host disease) is very rare due to the donor and recipient being the same individual. These advantages have established autologous HSCT as one of the standard second-line treatments for such diseases as lymphoma. However, for others such as Acute Myeloid Leukemia, the reduced mortality of the autogenous relative to allogeneic HSCT may be outweighed by an increased likelihood of cancer relapse and related mortality, and therefore the allogeneic treatment may be preferred for those conditions. Researchers have conducted small studies using non-myeloablative hematopoietic stem cell transplantation as a possible treatment for type I (insulin dependent) diabetes in children and adults. Results have been promising; however, as of 2009 it was premature to speculate whether these experiments will lead to effective treatments for diabetes.

Allogeneic

Allogeneic HSCT involves two people: the (healthy) donor and the (patient) recipient. Allogeneic HSC donors must have a tissue (HLA) type that matches the recipient. Matching is performed on the basis of variability at three or more loci of the HLA gene, and a perfect match at these loci is preferred. Even if there is a good match at these critical alleles, the recipient will require immunosuppressive medications to mitigate graft-versus-host disease. Allogeneic transplant donors may be *related* (usually a closely HLA matched sibling), *syngeneic* (a monozygotic or 'identical' twin of the patient - necessarily extremely rare since few patients have an identical twin, but offering a source of perfectly HLA matched stem cells) or *unrelated* (donor who is not related and found to have very close degree of HLA matching). Unrelated donors may be found through a registry of bone marrow donors such as the National Marrow Donor Program.

People who would like to be tested for a specific family member or friend without joining any of the bone marrow registry data banks may contact a private HLA testing laboratory and be tested with a mouth swab to see if they are a potential match. A "savior sibling" may be intentionally selected by preimplantation genetic diagnosis in order to match a child both regarding HLA type and being free of any obvious inheritable disorder. Allogeneic transplants are also performed using umbilical cord blood as the source of stem cells. In general, by transplanting healthy stem cells to the recipient's immune system, allogeneic HSCTs appear to improve chances for cure or long-term

remission once the immediate transplant-related complications are resolved.

A compatible donor is found by doing additional HLA-testing from the blood of potential donors. The HLA genes fall in two categories (Type I and Type II). In general, mismatches of the Type-I genes (i.e. HLA-A, HLA-B, or HLA-C) increase the risk of graft rejection. A mismatch of an HLA Type II gene (i.e. HLA-DR, or HLA-DQB1) increases the risk of graft-versus-host disease. In addition a genetic mismatch as small as a single DNA base pair is significant so perfect matches require knowledge of the exact DNA sequence of these genes for both donor and recipient. Leading transplant centers currently perform testing for all five of these HLA genes before declaring that a donor and recipient are HLA-identical.

Race and ethnicity are known to play a major role in donor recruitment drives, as members of the same ethnic group are more likely to have matching genes, including the genes for HLA.

Sources and Storage of Cells

To limit the risks of transplanted stem cell rejection or of severe graft-versus-host disease in allogeneic HSCT, the donor should preferably have the same human leukocyte antigens (HLA) as the recipient. About 25 to 30 percent of allogeneic HSCT recipients have an HLA-identical sibling. Even so-called "perfect matches" may have mismatched minor alleles that contribute to graft-versus-host disease.

Bone Marrow

Figure: *Bone marrow harvest.*

In the case of a bone marrow transplant, the HSC are removed from a large bone of the donor, typically the pelvis, through a large needle that reaches the center of the bone. The technique is referred to as a bone marrow harvest and is performed under general anesthesia.

Peripheral Blood Stem Cells

Peripheral blood stem cells are now the most common source of stem cells for allogeneic HSCT. They are collected from the blood through a process known as apheresis. The donor's blood is withdrawn through a sterile needle in one arm and passed through a machine that removes white blood cells. The red blood cells are returned to the donor. The peripheral stem cell yield is boosted with daily subcutaneous injections of Granulocyte-colony stimulating factor, serving to mobilize stem cells from the donor's bone marrow into the peripheral circulation.

Amniotic Fluid

It is also possible to extract hematopoietic stem cells from amniotic fluid for both autologous or heterologous use at the time of childbirth.

Umbilical Cord Blood

Umbilical cord blood is obtained when a mother donates her infant's Umbilical Cord and Placenta after birth. Cord blood has a higher concentration of HSC than is normally found in adult blood. However, the small quantity of blood obtained from an Umbilical Cord (typically about 50 mL) makes it more suitable for transplantation into small children than into adults. Newer techniques using ex-vivo expansion of cord blood units or the use of two cord blood units from different donors allow cord blood transplants to be used in adults.

Cord blood can be harvested from the Umbilical Cord of a child being born after preimplantation genetic diagnosis (PGD) for human leucocyte antigen (HLA) matching (see PGD for HLA matching) in order to donate to an ill sibling requiring HSCT.

Storage of HSC

Unlike other organs, bone marrow cells can be frozen (cryopreserved) for prolonged periods, without damaging too many cells. This is a necessity with autologous HSC because the cells must be harvested from the recipient months in advance of the transplant treatment. In the case of allogeneic transplants, fresh HSC are preferred, in order to avoid cell loss that might occur during the freezing and

thawing process. Allogeneic cord blood is stored frozen at a cord blood bank because it is only obtainable at the time of childbirth. To cryopreserve HSC, a preservative, DMSO, must be added, and the cells must be cooled very slowly in a controlled-rate freezer to prevent osmotic cellular injury during ice crystal formation. HSC may be stored for years in a *cryofreezer,* which typically uses liquid nitrogen.

Conditioning Regimens

Myeloablative Transplants

The chemotherapy or irradiation given immediately prior to a transplant is called the conditioning or preparative regimen, the purpose of which is to help eradicate the patient's disease prior to the infusion of HSC and to suppress immune reactions. The bone marrow can be *ablated* with dose-levels that cause minimal injury to other tissues. In allogeneic transplants a combination of cyclophosphamide with busulfan or total body irradiation is commonly employed. This treatment also has an immunosuppressive effect that prevents rejection of the HSC by the recipient's immune system.

The post-transplant prognosis often includes acute and chronic graft-versus-host disease that may be life-threatening. However in certain leukemias this can coincide with protection against cancer relapse owing to the *graft versus tumor* effect. *Autologous* transplants may also use similar conditioning regimens, but many other chemotherapy combinations can be used depending on the type of disease.

Non-myeloablative Allogeneic Transplants

This is a newer treatment approach using lower doses of chemotherapy and radiation, which are too low to eradicate all the bone marrow cells of a recipient. Instead, non-myeloablative transplants run lower risks of serious infections and transplant-related mortality while relying upon the *graft versus tumor* effect to resist the inherent increased risk of cancer relapse. Also significantly, while requiring high doses of immunosuppressive agents in the early stages of treatment, these doses are less than for conventional transplants. This leads to a state of mixed chimerism early after transplant where both recipient and donor HSC coexist in the bone marrow space.

Decreasing doses of immunosuppressive therapy then allows donor T-cells to eradicate the remaining recipient HSC and to induce the graft

versus tumor effect. This effect is often accompanied by mild graft-versus-host disease, the appearance of which is often a surrogate for the emergence of the desirable graft versus tumor effect, and also serves as a signal to establish an appropriate dosage level for sustained treatment with low levels of immunosuppressive agents.

Because of their gentler conditioning regimens, these transplants are associated with a lower risk of transplant-related mortality and therefore allow patients who are considered too high-risk for conventional allogeneic HSCT to undergo potentially curative therapy for their disease. These new transplant strategies are still somewhat experimental, but are being used more widely on elderly patients unfit for myeloablative regimens and for whom the higher risk of cancer relapse may be acceptable.

Engraftment

After several weeks of growth in the bone marrow, expansion of HSC and their progeny is sufficient to normalize the blood cell counts and reinitiate the immune system. The offspring of donor-derived hematopoietic stem cells have been documented to populate many different organs of the recipient, including the heart, liver, and muscle, and these cells had been suggested to have the abilities of regenerating injured tissue in these organs. However, recent research has shown that such lineage infidelities does not occur as a normal phenomenon.

Complications

HSCT is associated with a high treatment-related mortality in the recipient (10% or higher), which limits its use to conditions that are themselves life-threatening. Major complications are veno-occlusive disease, mucositis, infections (sepsis), graft-versus-host disease and the development of new malignancies.

Infection

Bone marrow transplantation usually requires that the recipient's own bone marrow be destroyed ("myeloablation"). Prior to "engraftment" patients may go for several weeks without appreciable numbers of white blood cells to help fight infection. This puts a patient at high risk of infections, sepsis and septic shock, despite prophylactic antibiotics. However, antiviral medications, such as acyclovir and valacyclovir, are quite effective in prevention of HSCT-related outbreak of herpetic infection in seropositive patients. The immunosuppressive

agents employed in allogeneic transplants for the prevention or treatment of graft-versus-host disease further increase the risk of opportunistic infection. Immunosuppressive drugs are given for a minimum of 6-months after a transplantation, or much longer if required for the treatment of graft-versus-host disease.

Transplant patients lose their acquired immunity, for example immunity to childhood diseases such as measles or polio. For this reason transplant patients must be re-vaccinated with childhood vaccines once they are off immunosuppressive medications.

Veno-occlusive Disease

Severe liver injury can result from hepatic veno-occlusive disease (VOD). Elevated levels of bilirubin, hepatomegaly and fluid retention are clinical hallmarks of this condition. There is now a greater appreciation of the generalized cellular injury and obstruction in hepatic vein sinuses, and hepatic VOD has lately been referred to as sinusoidal obstruction syndrome (SOS). Severe cases of SOS are associated with a high mortality rate. Anticoagulants or defibrotide may be effective in reducing the severity of VOD but may also increase bleeding complications. Ursodiol has been shown to help prevent VOD, presumably by facilitating the flow of bile.

Mucositis

The injury of the mucosal lining of the mouth and throat is a common regimen-related toxicity following ablative HSCT regimens. It is usually not life-threatening but is very painful, and prevents eating and drinking. Mucositis is treated with pain medications plus intravenous infusions to prevent dehydration and malnutrition.

Graft-versus-host Disease

Graft-versus-host disease (GVHD) is an inflammatory disease that is unique to allogeneic transplantation. It is an attack of the “new” bone marrow’s immune cells against the recipient’s tissues. This can occur even if the donor and recipient are HLA-identical because the immune system can still recognize other differences between their tissues. It is aptly named graft-versus-host disease because bone marrow transplantation is the only transplant procedure in which the transplanted cells must accept the body rather than the body accepting the new cells. *Acute graft-versus-host disease* typically occurs in the first 3 months after transplantation and may involve the skin, intestine, or the liver.

High-dose corticosteroids such as prednisone are a standard treatment; however this immuno-suppressive treatment often leads to deadly infections. *Chronic graft-versus-host disease* may also develop after allogeneic transplant. It is the major source of late treatment-related complications, although it less often results in death. In addition to inflammation, chronic graft-versus-host disease may lead to the development of fibrosis, or scar tissue, similar to scleroderma; it may cause functional disability and require prolonged immunosuppressive therapy. Graft-versus-host disease is usually mediated by T cells, which react to foreign peptides presented on the MHC of the host.

Graft-versus-tumor Effect

Graft-versus-tumor effect (GVT) or "graft versus leukemia" effect is the beneficial aspect of the Graft-versus-Host phenomenon. For example, HSCT patients with either acute and in particular chronic graft-versus-host disease after an allogeneic transplant tend to have a lower risk of cancer relapse. This is due to a therapeutic immune reaction of the grafted donor T lymphocytes against the diseased bone marrow of the recipient. This lower rate of relapse accounts for the increased success rate of allogeneic transplants, compared to transplants from identical twins, and indicates that allogeneic HSCT is a form of immunotherapy. GVT is the major benefit of transplants that do not employ the highest immuno-suppressive regimens.

Graft versus tumor is mainly beneficial in diseases with slow progress, e.g. chronic leukemia, low-grade lymphoma, and some cases multiple myeloma. However, it is less effective in rapidly growing acute leukemias.

If cancer relapses after HSCT, another transplant can be performed, infusing the patient with a greater quantity of donor white blood cells.

Oral Carcinoma

Patients after HSCT are at a higher risk for oral carcinoma. Post-HSCT oral cancer may have more aggressive behavior with poorer prognosis, when compared to oral cancer in non-HSCT patients.

Chapter 5: Veterinary Health: Prognosis and Treatments

Prognosis in HSCT varies widely dependent upon disease type, stage, stem cell source, HLA-matched status (for allogeneic HCST) and conditioning regimen. A transplant offers a chance for cure or long-term remission if the inherent complications of graft versus host disease, immuno-suppressive treatments and the spectrum of opportunistic infections can be survived. In recent years, survival rates have been gradually improving across almost all populations and sub-populations receiving transplants. Mortality for allogeneic stem cell transplantation can be estimated using the prediction model created by Sorror et al., using the Hematopoietic Cell Transplantation-Specific Comorbidity Index (HCT-CI). The HCT-CI was derived and validated by investigators at the Fred Hutchinson Cancer Research Center (Seattle, WA). The HCT-CI modifies and adds to a well-validated comorbidity index, the Charlson Comorbidity Index (CCI) (Charlson et al.) The CCI was previously applied to patients undergoing allogeneic HCT but appears to provide less survival prediction and discrimination than the HCT-CI scoring system.

Risks to Donor

The risks of a complication depend on patient characteristics, health care providers and the apheresis procedure, and the colony-stimulating factor used (G-CSF, GM-CSF). G-CSF drugs include Filgrastim (Neupogen, Neulasta), and lenograstim (Graslopin).

Drug Risks

Filgrastim is typically dosed in the 10 microgram/kg level for 4-5 days during the harvesting of stem cells. The documented adverse effects of filgrastim include splenic rupture (indicated by left upper abdominal or shoulder pain, risk 1 in 40000), Adult respiratory distress syndrome (ARDS), alveolar hemorrage, and allergic reactions (usually expressed in first 30 minutes, risk 1 in 300). In addition, platelet and hemoglobin levels dip post-procedure, not returning to normal until one month.

The question of whether patients over 65 react the same as patients under 65 has not been sufficiently examined. Coagulation issues and inflammation of atherosclerotic plaques are known to occur as a result of G-CSF injection.

G-CSF has also been described to induce genetic changes in mononuclear cells of normal donors. There is evidence that myelodysplasia (MDS) or acute myeloid leukaemia (AML) can be induced by GCSF in susceptible individuals.

Access Risks

Blood was drawn peripherally in a majority of patients, but a central line to jugular/subclavian/femoral veins may be used in 16% of women and 4% of men. Adverse reactions during apheresis were experienced in 20% of women and 8% of men, these adverse events primarily consisted of numbness/tingling, multiple line attempts, and nausea.

Clinical Observations

A study involving 2408 donors (18–60 years) indicated that bone pain (primarily back and hips) as a result of filgrastim treatment is observed in 80% of donors by day 4 post-injection. This pain responded to acetaminophen or ibuprofen in 65% of donors and was characterized as mild to moderate in 80% of donors and severe in 10%. Bone pain receded post-donation to 26% of patients 2 days post-donation, 6% of patients one week post-donation, and <2% 1 year post-donation. Donation is not recommended for those with a history of back pain. Other symptoms observed in more than 40% of donors include myalgia, headache, fatigue, and insomnia.

These symptoms all returned to baseline 1 month post-donation, except for some cases of persistent fatigue in 3% of donors. . In one metastudy that incorporated data from 377 donors, 44% of patients reported having adverse side effects after peripheral blood HSCT. Side effects included pain prior to the collection procedure as a result of GCSF injections, post-procedural generalized skeletal pain, fatigue and reduced energy.

Severe Reactions

A study that surveyed 2408 donors found that serious adverse events (requiring prolonged hospitalization) occurred in 15 donors (at a rate of 0.6%), although none of these events were fatal. Donors were not observed to have higher than normal rates of cancer with up to 4–8 years

of follow up. One study based on a survey of medical teams covered approximately 24,000 peripheral blood HSCT cases between 1993 and 2005, and found a serious cardiovascular adverse reaction rate of about 1 in 1500. This study reported a cardiovascular-related fatality risk within the first 30 days HSCT of about 2 in 10000. For this same group, severe cardiovascular events were observed with a rate of about 1 in 1500. The most common severe adverse reactions were pulmonary edema/deep vein thrombosis, splenic rupture, and myocardial infarction. Haematological malignancy induction was comparable to that observed in the general population, with only 15 reported cases within 4 years.

Donor Registration and Recruitment

At the end of 2010, 14.9 million people had registered their willingness to be a bone marrow donor with one of the 64 registries from 45 countries participating in Bone Marrow Donors Worldwide. 12.2 million of these registered donors had been ABDR typed, allowing easy matching. A further 453,000 cord blood units had been received by one of 44 cord blood units from 26 countries participating. The highest total number of bone marrow donors registered were those from the USA (6.4 million), and the highest number per capita were those from Cyprus (10.6% of the population).

Within the United States, racial minority groups are the least likely to be registered and therefore the least likely to find a potentially life-saving match. In 1990, only six African-Americans were able to find a bone marrow match, and all six had common European genetic signatures.

Africans are more genetically diverse than people of European descent, which means that more registrations are needed to find a match. Bone marrow and cord blood banks exist in South Africa, and a new program is beginning in Nigeria.

Experimental HIV Treatment

In 2007, a team of doctors in Berlin, Germany, including Gero Hütter, performed a stem cell transplant for leukemia patient Timothy Ray Brown, who was also HIV-positive. From 60 matching donors, they selected a [CCR5]-Ä32 homozygous individual with two genetic copies of a rare variant of a cell surface receptor.

This genetic trait confers resistance to HIV infection by blocking attachment of HIV to the cell. Roughly one in 1000 people of European ancestry have this inherited mutation, but it is rarer in other populations.

The transplant was repeated a year later after a relapse. Over three years after the initial transplant and despite discontinuing antiretroviral therapy, researchers cannot detect HIV in the transplant recipient's blood or in various biopsies. Levels of HIV-specific antibodies have also declined, leading to speculation that the patient may have been functionally cured of HIV.

However, scientists emphasise that this is an unusual case. Potentially fatal transplant complications (the "Berlin patient" suffered from graft-versus-host disease and leukoencephalopathy) mean that the procedure could not be performed in others with HIV, even if sufficient numbers of suitable donors were found.

Harvesting

The stem cells are typically harvested directly from the red marrow in the iliac crest, often under general anesthesia. The procedure is minimally invasive and does not require stitches afterwards. Depending on the donor's health and reaction to the procedure, the actual harvesting can be an outpatient procedure, or can require 1–2 days of recovery in the hospital.

Another option is to administer certain drugs that stimulate the release of stem cells from the bone marrow into circulating blood. An IV is inserted into the donor's arm, and the stem cells are filtered out of the blood.

This procedure is similar to donating blood or platelets. Bone marrow may also be taken from the sternum. The tibia may seem a good source, since it is very superficial, but adult tibia bone marrow does not contain any substantial amount of red marrow. In newborns, stem cells may be retrieved from the umbilical cord.

F ood

Many cultures have used bone marrow as food throughout history. Anthropologists believe that early humans were scavengers rather than hunters in some regions of the world. Marrow would have been a useful food source (largely due to its fat content) for tool-using hominids, who were able to crack open the bones of carcasses left by apex predators such as lions.

European diners in the 18th century often used a marrow scoop (or marrow spoon), often of silver and with a long, thin bowl, as a table implement for removing marrow from a bone. Bone marrow was also used in various preparations, such as pemmican. Bone marrow's popularity as a food is now relatively limited in the western world, but it remains in use in some gourmet restaurants, and is popular among food enthusiasts.

In Vietnam, beef bone marrow is used as the soup base for the national staple dish, *phß*, while in the Philippines, the soup *bulalo* is made primarily of beef stock and marrow bones, seasoned with vegetables and boiled meat; a similar soup in the Philippines is called *kansi*.

In Indonesia, bone marrow is called *sumsum* and can be found especially in Minangkabau cuisine. *Sumsum* is often cooked as soup or as *gulai* (a curry-like dish). In India and Pakistan, slow-cooked marrow is the core ingredient of the dish *nalli nihari*.

In Hungary, tibia is a main ingredient of beef soup; the bone is chopped into short (10–15 cm) pieces and the ends are covered with salt to prevent the marrow from leaking from the bone while cooking. Upon serving the soup, the marrow is usually spread on toast.

Beef bone marrow is also the main ingredient in Italian dish *ossobuco* (braised veal shanks), and beef marrow bones are often included in the French *pot-au-feu* broth, the cooked marrow being traditionally eaten on toasted bread with sprinkled coarse sea salt.

In Iranian cuisine, lamb shanks are usually broken before cooking to allow diners to suck out and eat the marrow when the dish is served. Similar practices are also common in Pakistani cuisine. Some Native Alaskans eat the bone marrow of caribou and moose.

Bone Marrow Examination

Bone marrow examination refers to the pathologic analysis of samples of bone marrow obtained by bone marrow biopsy (often called a trephine biopsy) and bone marrow aspiration. Bone marrow examination is used in the diagnosis of a number of conditions, including leukemia, multiple myeloma, lymphoma, anemia, and pancytopenia. The bone marrow produces the cellular elements of the blood, including platelets, red blood cells and white blood cells. While much information can be gleaned by testing the blood itself (drawn from a vein by phlebotomy), it

is sometimes necessary to examine the source of the blood cells in the bone marrow to obtain more information on hematopoiesis; this is the role of bone marrow aspiration and biopsy.

Components of the Procedure

Bone marrow samples can be obtained by aspiration and trephine biopsy. Sometimes, a bone marrow examination will include both an aspirate and a biopsy.

The aspirate yields semi-liquid bone marrow, which can be examined by a pathologist under a light microscope and analyzed by flow cytometry, chromosome analysis, or polymerase chain reaction (PCR).

Frequently, a trephine biopsy is also obtained, which yields a narrow, cylindrically shaped solid piece of bone marrow, 2mm wide and 2 cm long (80 iL), which is examined microscopically (sometimes with the aid of immunohistochemistry) for cellularity and infiltrative processes.

An aspiration, using a 20 mL syringe, yields approximately 300 iL of bone marrow. A volume greater than 300 iL is not recommended, since it may dilute the sample with peripheral blood

Comparison		
	Aspiration	***Biopsy***
Advantages	Fast Gives relative quantity of different cell types Gives material to further study, e.g. molecular genetics and flow cytometry	Gives cell and stroma constitution Represents all cells Explains cause of " dry tap" (aspiration gives no blood cells)
Drawbacks	Does not represent all cells	Slow processing

Aspiration does not always represent all cells since some such as lymphoma stick to the trabecula, and would thus be missed by a simple aspiration.

Site of Procedure

Bone marrow aspiration and trephine biopsy are usually performed on the back of the hipbone, or posterior iliac crest. However, an *aspirate* can also be obtained from the sternum (breastbone). A *trephine biopsy* should never be performed on the sternum, due to the risk of injury to blood vessels, lungs or the heart.

How the test is Performed?

Figure: *A needle used for bone marrow aspiration, with removable stylet.*

A bone marrow biopsy may be done in a health care provider's office or in a hospital. Informed consent for the procedure is typically required. The patient is asked to lie on his or her abdomen (prone position) or on his/her side (lateral decubitus position). The skin is cleansed, and a local anesthetic such as lidocaine is injected to numb the area. Patients may also be pretreated with analgesics and/or antianxiety medications, although this is not a routine practice.

Typically, the aspirate is performed first. An aspirate needle is inserted through the skin using manual pressure and force until it abuts the bone. Then, with a twisting motion of clinician's hand and wrist, the needle is advanced through the bony cortex (the hard outer layer of the bone) and into the marrow cavity. Once the needle is in the marrow cavity, a syringe is attached and used to aspirate ("suck out") liquid bone marrow. A twisting motion is performed during the aspiration to avoid

excess content of blood in the sample, which might be the case if an excessively large sample from one single point is taken.

Subsequently, the biopsy is performed if indicated. A different, larger trephine needle is inserted and anchored in the bony cortex. The needle is then advanced with a twisting motion and rotated to obtain a solid piece of bone marrow. This piece is then removed along with the needle. The entire procedure, once preparation is complete, typically takes 10–15 minutes.

If several samples are taken, the needle is removed between the samples to avoid blood coagulation.

In March 2010, Vidacare Corporation introduced a new technology to facilitate faster and easier insertion when compared to manual insertions with comparable or better core sample quality. The On Control™ Bone Marrow Biopsy and Aspiration System provides the first advance in bone marrow biopsy and aspiration procedures in over 50 years by combining a specially designed needle with a powered driver to obtain high-quality core samples. Validation testing completed prior to the product's launch on the On Control™ System showed the following results:

> *Mean length of core sample of 1.32 cm, Median time to core extraction of 81 seconds, Needle insertion success rate of 94%, Biopsy core acquisition success rate of 90%, Zero complications.*

In addition, an article dated April 19, 2010 published in the Journal of Clinical Pathology (Swords, Ronan, *et al.*) made the following conclusion about the System: "As clinicians, we feel that the speed and ease of use of this new powered system could change the way that bone marrow aspiration and biopsy is carried out in the future, particularly in large centres where this procedure is commonly performed."

After the Procedure

After the procedure is complete, the patient is typically asked to lie flat for 5–10 minutes to provide pressure over the procedure site. After that, assuming no bleeding is observed, the patient can get up and go about their normal activities. Paracetamol (acetaminophen) or other simple analgesics can be used to ease soreness, which is common for 2–3 days after the procedure. Any worsening pain, redness, fever, bleeding or swelling may suggest a complication. Patients are also advised to avoid

washing the procedure site for at least 24 hours after the procedure is completed.

Contraindications

There are few contraindications to bone marrow examination. The only absolute reason to avoid performing a bone marrow examination is the presence of a severe bleeding disorder which may lead to serious bleeding after the procedure. If there is a skin or soft tissue infection over the hip, a different site should be chosen for bone marrow examination. Bone marrow aspiration and biopsy can be safely performed even in the setting of extreme thrombocytopenia (low platelet count).

Complications

While mild soreness lasting 12–24 hours is common after a bone marrow examination, serious complications are extremely rare. In a large review, an estimated 55,000 bone marrow examinations were performed, with 26 serious adverse events (0.05%), including one fatality.

The same author collected data on over 19,000 bone marrow examinations performed in the United Kingdom in 2003, and found 16 adverse events (0.08% of total procedures), the most common of which was bleeding. In this report, complications, while rare, were serious in individual cases.

Bone Marrow Biopsy

A bone marrow biopsy is the removal of soft tissue, called marrow, from inside bone. Bone marrow is found in the hollow part of most bones. It helps form blood cells.

How the Test is Performed

A bone marrow biopsy may be done in the health care provider's office or in a hospital. The sample is usually taken from the hip bone. The health care provider will clean the skin and inject a numbing medicine into the area. Rarely, you may be given medicine to help you relax. The doctor inserts the biopsy needle into the bone. The center of the needle is removed and the hollowed needle is moved deeper into the bone. This captures a tiny sample, or core, of bone marrow within the needle. The sample and needle are removed. Pressure and a bandage is applied to the biopsy site.

A bone marrow aspirate may also be performed, usually before the biopsy is taken. After the skin is numbed, the needle is inserted into the bone, and a syringe is used to withdraw the liquid bone marrow. If this is done, the needle will be removed and either repositioned, or another needle may be used for the biopsy.

How to Prepare for the Test

Tell the health care provider:

- If you are allergic to any medications
- What medications you are taking
- If you have bleeding problems
- If you are pregnant

You must sign a consent form.

How the Test Will Feel

You will feel a sharp sting when the numbing medicine is injected. You may feel a brief, sharp pain when the liquid (aspirate) is removed.

The biopsy needle may also cause a brief, usually more dull, pain. Since the inside of the bone cannot be numbed, this test may cause some discomfort. However, not all patients have such pain.

Why the Test is Performed

Your doctor may order this test if you have abnormal types or numbers of red or white blood cells or platelets on a complete blood count (CBC).

This test is used to diagnose leukemia, infections, some types of anemia, and other blood disorders. It may also be used to help determine if a cancer has spread or responded to treatment.

Normal Results

A normal result means the bone marrow contains the proper number and types of blood-forming (hematopoietic) cells, fat cells, and connective tissues.

What Abnormal Results Mean

Abnormal results may be due to cancers of the bone marrow (leukemia or Hodgkin's disease).

The results may detect the cause of anemia (too few red blood cells), abnormal white blood cells, or thrombocytopenia (too few platelets).

Additional conditions under which the test may be performed:

- Disseminated coccidioidomycosis
- Hairy cell leukemia
- Hodgkin's lymphoma
- Idiopathic aplastic anemia
- Multiple myeloma
- Neuroblastoma
- Non-Hodgkin's lymphoma
- Polycythemia vera
- Primary amyloid
- Primary myelofibrosis
- Primary thrombocythemia
- Secondary aplastic anemia
- Secondary systemic amyloid

Risks

There may be some bleeding at the puncture site. More serious risks, such as serious bleeding or infection, are very rare.

Considerations

This test is often performed when there are problems with the various types of blood cells. The person may be at increased risk for bleeding, infection, or other problems.

Alternative Names

Biopsy - bone marrow

Bone Marrow Aspiration and Biopsy

How is it Used?

The bone marrow biopsy and aspiration procedure provides information about the status of and capability for blood cell production. It is not routinely ordered and in fact the majority of people will never have one done. A bone marrow aspiration and/or biopsy may be ordered to help evaluate blood cell production, to help diagnose leukemia, to help diagnose a bone marrow disorder, to help diagnose and stage a variety of other types of cancer that may have spread into the marrow, and to help

determine whether a severe anemia is due to decreased RBC production, increased loss, abnormal RBC production, and/or to a vitamin or mineral deficiency or excess. Conditions that affect the marrow can affect the number, mixture, and maturity of the cells, and can affect its fibrous structure.

A bone marrow sample may also be evaluated and cultured for the presence of microorganisms such as fungi, bacteria, or mycobacteria (such as that which causes tuberculosis) when the patient has a fever of unknown origin.

Additional marrow testing may be ordered when it is suspected that the patient has a chromosomal abnormality and/or a disorder associated with iron storage that may cause iron to accumulate in the marrow.

When a person is being treated for a cancer, a bone marrow aspiration and/or biopsy may be ordered to evaluate the response to therapy to determine whether suppressed marrow function is beginning to return to normal.

A CBC and reticulocyte count are frequently ordered along with the bone marrow aspiration/biopsy. The results are used to help evaluate cell production in the marrow and compare it to current cell populations in the blood.

When is it Ordered?

A bone marrow aspiration and/or biopsy may be ordered as a diagnostic procedure when, for example, one of the following is suspected:

- Aplastic Anemia
- Acute Leukemia
- Myelodysplastic Syndrome
- Chronic Myelogenous Leukemia
- Myelofibrosis and Essential Thrombocythemia
- Multiple Myeloma
- Severe thrombocytopenia and/or anemia and/or neutropenia

It may be ordered when staging certain cancers. Staging is a careful and thorough examination and classification of how far the cancer has spread and what body organs are involved. These may include:

- Hodgin's and Non-Hodgkins lymphomas
- Small Cell Carcinoma of the Lung (not frequently done anymore) It may be ordered for culturing in some cases, such as:

- When fever is present in HIV/AIDS or other immuno-compromised patients
- In patients suspected of having infectious diseases such as Brucellosis or Typhoid Fever

A bone marrow biopsy and aspiration may also be ordered at intervals when a person is being treated for a cancer to evaluate whether marrow function is being suppressed and, if it is, when its function begins to recover.

What does the Test Result Mean?

With a bone marrow biopsy and aspiration the doctor is evaluating what is in the marrow in order to determine whether the cells found are normal and present in typical quantities, to determine whether there are cells present that should not be there, and to determine what is missing.

The training and expertise of the pathologist or hematologist evaluating the marrow samples allow her to sort through the marrow clues and determine what is happening in the marrow. In most cases, this information can confirm or rule out a diagnosis and bone marrow involvement, but it can also point out the need for further investigation. For instance, if there are a decreased number of RBCs in the blood and an increased number of reticulocytes, and a marrow evaluation shows that RBC production appears normal but increased, then the patient's doctor knows that marrow production of RBCs has increased appropriately to meet a RBC demand. What she still doesn't know is the reason for the demand. It could be due to an acute or chronic loss of RBCs, such as may occur with gastrointestinal bleeding, or due to acute or chronic RBC destruction, such as sometimes occurs with an artificial heart valve.

A patient with few RBCs and no increase in reticulocytes may have aplastic anemia with suppressed RBC production in the marrow. An evaluation of the bone marrow may confirm this condition, but it does not necessarily tell the doctor whether it is due to a bone marrow disorder, radiation, exposure to certain chemicals, cancer, cancer treatment, or due to a tuberculosis infection.

The presence of some abnormal cells can be characteristic of specific cancers or disorders, such as the Gaucher cell found with Gaucher's disease or the foamy lipid-filled Niemann-Pick cell found with Niemann-Pick disease.

There may be masses of cells forming tumors in the marrow, such as can occur with multiple myeloma, and changes to or increases in the fibrous network that supports cellular production.

The doctor takes the information that she receives from the marrow evaluation and combines it with information from a clinical examination, blood tests, and a variety of other tests, such as imaging scans and X-rays, to reach a final diagnosis. It can be a straightforward process or it can be a complex diagnostic puzzle. Patients should stay involved in this process by talking to their doctor before and after a bone marrow biopsy and/or aspiration, asking her what her suspicions are, what kind of information she is hoping to obtain from the evaluation, and what follow-up tests might be indicated.

Is There Anything Else I Should Know?

Complications from the bone marrow aspiration and/or biopsy procedure are rare, but some patients may have excessive bleeding at the collection site or develop an infection.

Patients should tell the doctor about any allergies they have and about any medications or supplements they are taking prior to the procedure and should contact their doctor promptly if they experience persistent or spreading redness or bleeding at the site, a fever, or increasing pain.

Cerebrospinal Fluid

Cerebrospinal fluid (CSF), *Liquor cerebrospinalis*, is a clear, colorless, bodily fluid, that occupies the subarachnoid space and the ventricular system around and inside the brain and spinal cord.

The CSF occupies the space between the arachnoid mater (the middle layer of the brain cover, meninges) and the pia mater (the layer of the meninges closest to the brain). It constitutes the content of all intra-cerebral (inside the brain, cerebrum) ventricles, cisterns, and sulci (singular sulcus), as well as the central canal of the spinal cord.

It acts as a cushion or buffer for he cortex, providing a basic mechanical and immunological protection to the brain inside the skull and serves a vital function in cerebral autoregulation of cerebral blood flow.

It is produced in the choroid plexus.

Circulation

CSF is produced in the brain by modified ependymal cells in the choroid plexus (approx. 50-70%) and the remainder is formed around blood vessels and along ventricular walls. It circulates from the lateral ventricles to the foramen of Monro (Interventricular foramen), third ventricle, aqueduct of Sylvius (Cerebral aqueduct), fourth ventricle, foramen of Magendie (Median aperture) and foramina of Luschka (Lateral apertures), subarachnoid space over brain and spinal cord. It should be noted that the CSF moves in a pulsatile manner throughout the CSF system with nearly zero net flow. CSF is reabsorbed into venous sinus blood via arachnoid granulations.

It had been thought that CSF returns to the vascular system by entering the dural venous sinuses via the arachnoid granulations (or villi). However, some have suggested that CSF flow along the cranial nerves and spinal nerve roots allow it into the lymphatic channels; this flow may play a substantial role in CSF reabsorbtion, in particular in the neonate, in which arachnoid granulations are sparsely distributed.

The flow of CSF to the nasal submucosal lymphatic channels through the cribriform plate seems to be especially important.

Amount and Constitution

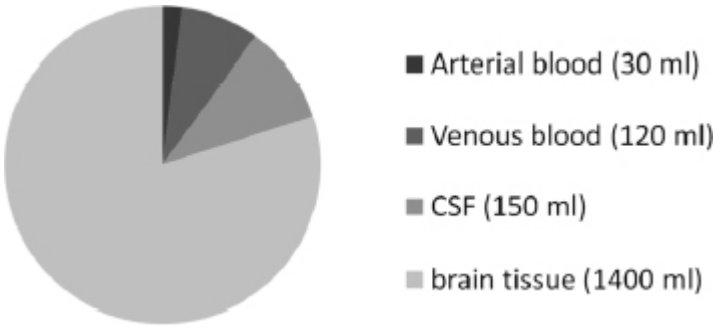


Figure: *Intracranial volumetric distribution of cerebrospinal fluid, blood, and brain parenchyma*

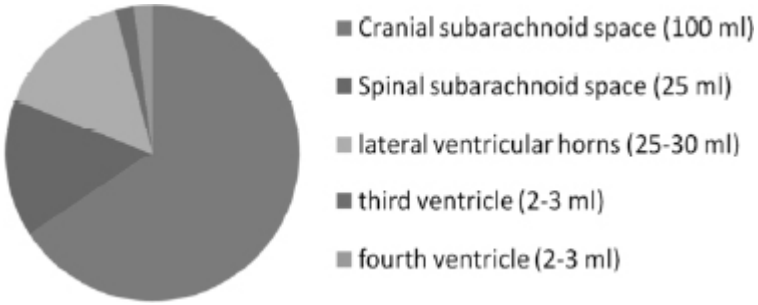


Figure: *Volumetric distribution of cerebrospinal fluid*

The CSF is produced at a rate of 500 ml/day. Since the subarachnoid space around the brain and spinal cord can contain only 135 to 150 ml, large amounts are drained primarily into the blood through arachnoid granulations in the superior sagittal sinus. Thus the CSF turns over about 3.7 times a day. This continuous flow into the venous system dilutes the concentration of larger, lipid-insoluble molecules penetrating the brain and CSF. The CSF contains approximately 0.3% plasma proteins, or approximately 15 to 40 mg/dL, depending on sampling site.

CSF pressure, as measured by lumbar puncture (LP), is 10-18 cmH_2O (8-15 mmHg or 1.1-2 kPa) with the patient lying on the side and 20-30cmH_2O (16-24 mmHg or 2.1-3.2 kPa) with the patient sitting up. In newborns, CSF pressure ranges from 8 to 10 cmH_2O (4.4– 7.3 mmHg or 0.78–0.98 kPa). Most variations are due to coughing or internal compression of jugular veins in the neck. When lying down, the cerebrospinal fluid as estimated by lumbar puncture is similar to the intracranial pressure.

There are quantitative differences in the distributions of a number of proteins in the CSF. In general, globular proteins and albumin are in lower concentration in ventricular CSF compared to lumbar or cisternal fluid.

The *IgG index* of cerebrospinal fluid is a measure of the immunoglobulin G content, and is elevated in multiple sclerosis. It is defined as:

$$IgG\ index = (IgG_{CSF}/IgG_{serum})/(albumin_{CSF}/albumin_{serum})$$

A cutoff value has been suggested to be 0.73, with a higher value indicating presence of multiple sclerosis.

Functions

CSF serves four primary purposes:

1. *Buoyancy:* The actual mass of the human brain is about 1400 grams; however, the net weight of the brain suspended in the CSF is equivalent to a mass of 25 grams. The brain therefore exists in neutral buoyancy, which allows the brain to maintain its density without being impaired by its own weight, which would cut off blood supply and kill neurons in the lower sections without CSF.

2. *Protection:* CSF protects the brain tissue from injury when jolted or hit. In certain situations such as auto accidents or sports injuries, the CSF cannot protect the brain from forced contact with the skull case, causing hemorrhaging, brain damage, and sometimes death.
3. *Chemical stability:* CSF flows throughout the inner ventricular system in the brain and is absorbed back into the bloodstream, rinsing the metabolic waste from the central nervous system through the blood–brain barrier. This allows for homeostatic regulation of the distribution of neuroendocrine factors, to which slight changes can cause problems or damage to the nervous system. For example, high glycine concentration disrupts temperature and blood pressure control, and high CSF pH causes dizziness and syncope.. To use Davson's term, the CSF has a "sink action" by which the various substances formed in the nervous tissue during its metabolic activity diffuse rapidly into the CSF and are thus removed into the bloodstream as CSF is absorbed.
4. *Prevention of brain ischemia:* The prevention of brain ischemia is made by decreasing the amount of CSF in the limited space inside the skull. This decreases total intracranial pressure and facilitates blood perfusion.

Pathology and Laboratory Diagnosis

When CSF pressure is elevated, cerebral blood flow may be constricted. When disorders of CSF flow occur, they may therefore affect not only CSF movement but also craniospinal compliance and the intracranial blood flow, with subsequent neuronal and glial vulnerabilities.

The venous system is also important in this equation. Infants and patients shunted as small children may have particularly unexpected relationships between pressure and ventricular size, possibly due in part to venous pressure dynamics. This may have significant treatment implications, but the underlying pathophysiology needs to be further explored.

CSF connections with the lymphatic system have been demonstrated in several mammalian systems. Preliminary data suggest that these CSF-lymph connections form around the time that the CSF secretory capacity of the choroid plexus is developing (in utero).

There may be some relationship between CSF disorders, including hydrocephalus and impaired CSF lymphatic transport.

CSF can be tested for the diagnosis of a variety of neurological diseases. It is usually obtained by a procedure called lumbar puncture. Removal of CSF during lumbar puncture can cause a severe headache after the fluid is removed, because the brain hangs on the vessels and nerve roots, and traction on them stimulates pain fibers.

The pain can be relieved by intrathecal injection of sterile isotonic saline. Lumbar puncture is performed in an attempt to count the cells in the fluid and to detect the levels of protein and glucose. These parameters alone may be extremely beneficial in the diagnosis of subarachnoid hemorrhage and central nervous system infections (such as meningitis). Moreover, a CSF culture examination may yield the microorganism that has caused the infection.

By using more sophisticated methods, such as the detection of the oligoclonal bands, an ongoing inflammatory condition (for example, multiple sclerosis) can be recognized. A beta-2 transferrin assay is highly specific and sensitive for the detection for, e.g., CSF leakage.

Lumbar Puncture

Lumbar puncture can also be performed to measure the intracranial pressure, which might be increased in certain types of hydrocephalus. However a lumbar puncture should never be performed if increased intracranial pressure is suspected because it could lead to brain herniation and ultimately death.

Baricity

This fluid has an importance in anesthesiology. Baricity refers to the density of a substance compared to the density of human cerebral spinal fluid. Baricity is used in anesthesia to determine the manner in which a particular drug will spread in the intrathecal space.

Alzheimer's Disease

A 2010 study showed analysis of CSF for three protein biomarkers can indicate the presence of Alzheimer's disease. The three biomarkers are CSF amyloid beta 1-42, total CSF tau protein and P-Tau_{181P}. In the study, the biomarker test showed good sensitivity, identifying 90% of persons with Alzheimer's disease, but poor specificity, as 36% of control subjects were positive for the biomarkers.

The researchers suggested the low specificity may be explained by developing but not yet symptomatic disease in controls.

Cerebral Spinal Fluid (CSF) Collection

Cerebrospinal fluid (CSF) collection is a test to look at the fluid that surrounds the brain and spinal cord.

CSF acts as a cushion, protecting the brain and spine from injury. The fluid is normally clear. The test is also used to measure pressure in the spinal fluid.

How the Test is Performed?

There are different ways to get a sample of CSF. Lumbar puncture, commonly called a spinal tap, is the most common method. The test is usually done like this:

- The patient lies on his or her side, with knees pulled up toward the chest, and chin tucked downward. Sometimes the test is done with the person sitting up, but bent forward.
- After the back is cleaned, the health care provider will inject a local numbing medicine (anesthetic) into the lower spine.
- A spinal needle is inserted, usually into the lower back area.
- Once the needle is properly positioned, CSF pressure is measured and a sample is collected.
- The needle is removed, the area is cleaned, and a bandage is placed over the needle site. The person is often asked to lie down for a short time after the test.

Occasionally, special x-rays are used to help guide the needle into the proper position. This is called fluoroscopy.

Lumbar puncture with fluid collection may also be part of other procedures, particularly a myelogram (x-ray or CT scan after dye has been inserted into the CSF).

Alternative methods of CSF collection are rarely used, but may be necessary if the person has a back deformity or an infection.

Cisternal puncture uses a needle placed below the occipital bone (back of the skull). It can be dangerous because it is so close to the brain stem. It is always done with fluoroscopy. Ventricular puncture is even more rare, but may be recommended in people with possible brain herniation. This test is usually done in the operating room. A hole is

drilled in the skull, and a needle is inserted directly into one of brain's ventricles.

CSF may also be collected from a tube that's already placed in the fluid, such as a shunt or a ventricular drain. These sorts of tubes are usually placed in the intensive care unit.

How to Prepare for the Test?

The patient (or guardian) must give the health care team permission to do the test.

Afterward, you should plan to rest for several hours, even if you feel fine. You won't be required to lie flat on your back the entire time, but rest is advised to prevent additional leakage of CSF around the site of the puncture.

How the Test Will Feel?

The test is usually done with you curled up on your side with knees pulled up and chin to chest. Sometimes, CSF is collected with the person seated and bent forward over a table or chair. Holding the position may be uncomfortable, but it is extremely important to stay in this bent position to avoid moving the needle and possibly injuring the spinal cord. The person doing the test may ask you to straighten out slightly after the needle is in place, in order to accurately measure the CSF pressure, called the "opening pressure."

The anesthetic will sting or burn when first injected. There will be a hard pressure sensation when the needle is inserted, and there is usually some brief pain when the needle goes through the tissue surrounding the spinal cord. This pain should stop in a few seconds.

Overall, discomfort is minimal to moderate. The entire procedure usually takes about 30 minutes, but it may take longer. The actual pressure measurements and CSF collection only take a few minutes.

Why the Test is Performed?

This test is done to measure pressures within the cerebrospinal fluid and to collect a sample of the fluid for further testing. CSF analysis can be used to diagnose certain neurologic disorders, particularly infections (such as meningitis) and brain or spinal cord damage.

Normal Results

Normal values typically range as follows:

- Pressure: 70 - 180 mm H20
- Appearance: clear, colorless
- CSF total protein: 15 - 60 mg/100 mL
- Gamma globulin: 3 - 12% of the total protein
- CSF glucose: 50 - 80 mg/100 mL (or greater than 2/3 of blood sugar level)
- CSF cell count: 0 - 5 white blood cells (all mononuclear), and no red blood cells
- Chloride: 110 - 125 mEq/L

Note: mg/mL = milligrams per milliliter; mEq/L = milliequivalents per liter

Note: Normal value ranges may vary slightly among different laboratories. Talk to your doctor about the meaning of your specific test results.

The examples above show the common measurements for results for these tests. Some laboratories use different measurements or may test different specimens.

What Abnormal Results Mean?

If the CSF looks cloudy, it could mean there is an infection or a build up of white blood cells or protein.

If the CSF looks bloody or red, it may be a sign of bleeding or spinal cord obstruction. If it is brown, orange, or yellow, it may be a sign of increased CSF protein or previous bleeding (more than 3 days ago). Occasionally, there may be blood in the sample that came fom the spinal tap itself. This makes it harder to interpret the test results.

CSF Pressure:

- Increased CSF pressure may be due to increased intracranial pressure (pressure within the skull).
- Decreased CSF pressure may be due to spinal cord tumor, shock, fainting, or diabetic coma.

CSF Protein”

- Increased CSF protein may be due to blood in the CSF, diabetes,polyneuritis, tumor, injury, or any inflammatory or infectiouscondition.
- Decreased protein is a sign of rapid CSF production.

CSF Glucose:

- Increased CSF glucose is a sign of high blood sugar.
- Decreased CSF glucose may be due to hypoglycemia (low bloodsugar), bacterial or fungal infection (such as meningitis),tuberculosis, or certain other types of meningitis.

Blood Cells in CSF:

- Increased white blood cells in the CSF may be a sign ofmeningitis, acute infection, beginning of a chronic illness, tumor, abscess, stroke, or demyelinating disease (such as multiple sclerosis).
- Red blood cells in the CSF sample may be a sign of bleedinginto the spinal fluid or the result of a traumatic lumbarpuncture.

Other CSF Results:

- Increased CSF gamma globulin levels may be due to diseasessuch as multiple sclerosis, neurosyphilis, or Guillain-Barresyndrome.

Additional conditions under which the test may be performed:

- Chronic inflammatory polyneuropathy
- Dementia due to metabolic causes
- Encephalitis
- Epilepsy
- Febrile seizure (children)
- Generalized tonic-clonic seizure
- Hydrocephalus
- Inhalation anthrax
- Normal pressure hydrocephalus (NPH)
- Pituitary tumor
- Reye syndrome

Risks

Risks of lumbar puncture include:

- Bleeding into the spinal canal
- Discomfort during the test
- Headache after the test
- Hypersensitivity (allergic) reaction to the anesthetic
- Infection introduced by the needle going through the skin

There is an increased risk of bleeding in people who take blood thinners. Brain herniation may occur if this test is done on a person with a mass in the brain (such as a tumor or abscess). This can result in brain damage or death.

This test is not done if an exam or test reveals signs of a brain mass. Damage to the nerves in the spinal cord may occur, particularly if the person moves during the test. Cisternal puncture or ventricular puncture carry additional risks of brain or spinal cord damage and bleeding within the brain.

Considerations

This test is particularly dangerous for people with:

- A tumor in the back of the brain that is pressing down on the brain stem
- Blood clotting problems
- Low platelet count (Thrombocytopenia)

Alternative Names

Spinal tap; Ventricular puncture; Lumbar puncture; Cisternal puncture; Cerebrospinal fluid culture

CSF Coccidioides Complement Fixation

CSF coccidioides complement fixation looks for antibodies to the fungus *Coccidioides immitis* in the cerebrospinal (CSF) fluid, the fluid surrounding the brain and spine.

How the Test is Performed?

Complement fixation is a specific laboratory technique that looks to see if the body has produced antibodies to a specific foreign substance (antigen) — in this case *Coccidioides immitis*.

If the antibodies are present, they stick, or "fix" themselves, to the antigen. That's why the test is called "fixation." The spinal fluid needed to perform this test is usually obtained by lumbar puncture (spinal tap).

How to Prepare for the Test?

A consent form must be signed. You will need to remain in the hospital for about 8 hours afterwards, and you should remain lying flat.

Considerations

In some cases, this test may be done as a blood test. In the initial stage of an illness, few antibodies may be detected. Antibody production increases during the course of an infection. For this reason, such tests are often repeated several weeks after the first test is done.

Note: While an abnormal result on the spinal fluid test specifically means that the central nervous system is infected, an abnormal result on the blood test does not pinpoint the exact area of infection. It only means that there is a coccidioides infection somewhere in the body.

CSF Oligoclonal Banding

CSF oligoclonal banding is a test to look for inflammation-related proteins in the cerebrospinal fluid (CSF), the clear fluid that flows in the space surrounding the spinal cord and brain.

Oligoclonal bands are proteins called immunoglobulins, which suggest inflammation of the central nervous system. The presence of oligoclonal bands may be a sign of multiple sclerosis.

How the Test is Performed?

A sample of CSF is needed. A lumbar puncture (spinal tap) is the most common way to collect this sample. For information on this procedure, see the article on lumbar puncture. Other methods for collecting CSF are rarely used, by may be recommended in some cases. They include:

- Cisternal puncture
- Ventricular puncture
- Removal of CSF from a tube that is already in the CSF, such as a shunt or ventricular drain.

After the sample is taken, it is sent to a laboratory for evaluation.

Why the Test is Performed?

This test helps support, but does not confirm, the diagnosis of multiple sclerosis (MS). The presence of oligoclonal bands in the CSF can also be seen in other illnesses.

Normal Results

Normally, one or no bands should be found in the CSF.

Note: Normal value ranges may vary slightly among different laboratories. Talk to your doctor about the meaning of your specific test

results.

The examples above show the common measurements for results for these tests. Some laboratories use different measurements or may test different specimens.

What Abnormal Results Mean

There are two or more bandings found in the CSF and not in the blood. This may be a sign of multiple sclerosis or other inflammatory processes.

Semen

Semen is an organic fluid, also known as *seminal fluid*, that may contain spermatozoa. It is secreted by the gonads (sexual glands) and other sexual organs of male or hermaphroditic animals and can fertilize female ova. In humans, seminal fluid contains several components besides spermatozoa: proteolytic and other enzymes as well as fructose are elements of seminal fluid which promote the survival of spermatozoa and provide a medium through which they can move or "swim".

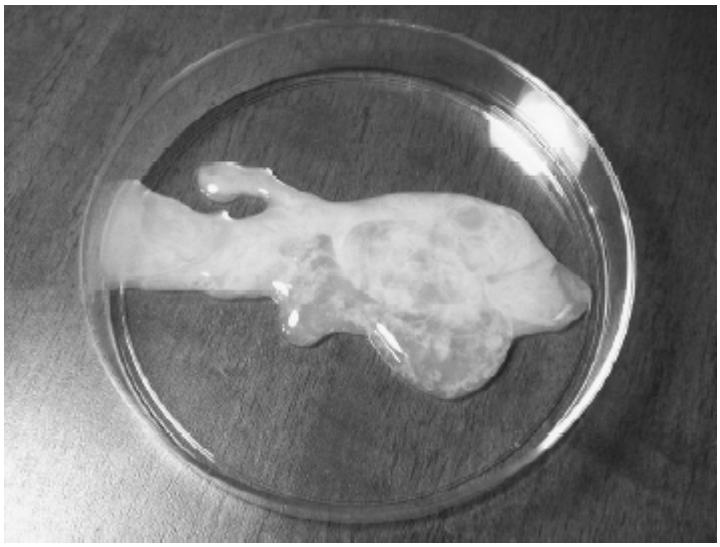


Figure: *Human semen in a petri dish.*

Semen is produced and originates from the seminal vesicle, which is located in the pelvis. The process that results in the discharge of semen is called *ejaculation*.

Chapter 6: Principles of Diagnosis in Animal Pathology

Internal and External Fertilization

Depending on the species, spermatozoa can fertilize ova externally or internally. In external fertilization, the spermatozoa fertilize the ova directly, outside of the female's sexual organs. Female fish, for example, spawn ova into their aquatic environment, where they are fertilized by the semen of the male fish. During internal fertilization, however, fertilization occurs inside the female's sexual organs. Internal fertilization takes place after insemination of a female by a male through copulation. In low vertebrates (amphibians, reptiles, birds and monotreme mammals), copulation is achieved through the physical mating of the cloaca of the male and female. In marsupial and placental mammals, copulation occurs through the vagina.

Composition of Human Semen

During the process of ejaculation, sperm passes through the ejaculatory ducts and mixes with fluids from the seminal vesicles, the prostate, and the bulbourethral glands to form the semen. The seminal vesicles produce a yellowish viscous fluid rich in fructose and other substances that makes up about 70% of human semen. The prostatic secretion, influenced by dihydrotestosterone, is a whitish (sometimes clear), thin fluid containing proteolytic enzymes, citric acid, acid phosphatase and lipids. The bulbourethral glands secrete a clear secretion into the lumen of the urethra to lubricate it.

Sertoli cells, which nurture and support developing spermatocytes, secrete a fluid into seminiferous tubules that helps transport sperm to the genital ducts. The ductuli efferentes possess cuboidal cells with microvilli and lysosomal granules that modify the semen by reabsorbing some fluid. Once the semen enters the ductus epididymis the principle cells, which contain pinocytotic vessels indicating fluid reabsorption, secrete glycerophosphocholine which most likely inhibits premature

capacitation. The accessory genital ducts, the seminal vesicle, prostate glands, and the bulbourethral glands, produce most of the seminal fluid.

Seminal plasma of humans contains a complex range of organic and inorganic constituents.

The seminal plasma provides a nutritive and protective medium for the spermatozoa during their journey through the female reproductive tract. The normal environment of the vagina is a hostile one for sperm cells, as it is very acidic (from the native microflora producing lactic acid), viscous, and patrolled by immune cells. The components in the seminal plasma attempt to compensate for this hostile environment. Basic amines such as putrescine, spermine, spermidine and cadaverine are responsible for the smell and flavor of semen. These alkaline bases counteract the acidic environment of the vaginal canal, and protect DNA inside the sperm from acidic denaturation.

Appearance and Consistency of Human Semen

Semen is typically translucent with white, grey or even yellowish tint. Blood in the semen can cause a pink or reddish colour, known as *hematospermia*, and may indicate a medical problem which should be evaluated by a doctor if the symptom persists.

After ejaculation, the latter part of the ejaculated semen coagulates immediately, forming globules, while the earlier part of the ejaculate typically does not. After a period typically ranging from 15 – 30 minutes, Prostate-specific antigen present in the semen causes the decoagulation of the seminal coagulum. It is postulated that the initial clotting helps keep the semen in the vagina, while liquefaction frees the sperm to make their journey to the ova.

Semen Quality

Semen quality is a measure of the ability of semen to accomplish fertilization. Thus, it is a measure of fertility in a man. It is the sperm in the semen that are of importance, and therefore semen quality involves both sperm quantity and quality. Decreased semen quality is a major factor of male infertility.

Semen Analysis

A semen analysis (plural: semen analyses) evaluates certain characteristics of a male's semen and the sperm contained in the semen. It is done to help evaluate male fertility, whether for those seeking pregnancy or verifying the success of vasectomy. Depending on the measurement method, just a few characteristics may be evaluated (such as with a home kit) or many characteristics may be evaluated (generally by a diagnostic laboratory). Collection techniques and precise measurement method may influence results.

Reasons for Testing

The most common reasons for laboratory semen analysis in humans are as part of a couple's infertility investigation and after a vasectomy to verify that the procedure was successful. It is also commonly used for testing human donors for sperm donation, and for animals semen analysis is commonly used in stud farming and farm animal breeding.

Occasionally a man will have a semen analysis done as part of routine pre-pregnancy testing. At the laboratory level this is rare, as most doctors will not test the semen and sperm unless specifically requested or there is a strong suspicion of a pathology in one of these areas discovered during the medical history or during the physical examination. Such testing is very expensive and time-consuming, and is unlikely to be covered by insurance.

Relation to Fertility

The characteristics measured by semen analysis are only some of the factors in semen quality. One source states that 30% of men with a normal semen analysis actually have abnormal sperm function. Conversely, men with poor semen analysis results may go on to father children.

Collection Methods

The most common way to collect a semen sample is through masturbation, directing the sample into a clean cup.

A sample may also be collected during intercourse in a special type of condom known as a collection condom. Collection condoms are made from silicone or polyurethane, as latex is somewhat harmful to sperm. Many men prefer collection condoms to masturbation, and some religions prohibit masturbation entirely. Adherents of religions that prohibit contraception may use collection condoms with holes pricked in them.

A third option for collecting a sample is through coitus interruptus (withdrawal). With this technique, the man removes his penis from his partner near the end of intercourse and ejaculates into a cup.

Finally, if a blockage in the vas deferens is suspected to impede fertility, semen can be taken directly from the epididymis. Such a collection is called per cutaneous epididymal sperm aspiration (PESA). Alternatively, the testicular tissue itself, instead of the sperm produced can be investigated. Then, the collecting method is called TESE.

Parameters

Examples of parameters measured in a semen analysis are: sperm count, motility, morphology, volume, fructose level and pH.

Sperm Count

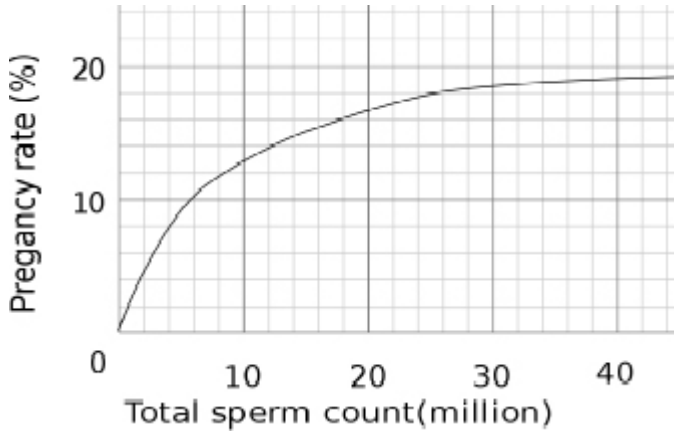


Figure: *Approximate pregnancy rate varies with amount of sperm used in an artificial insemination cycle. Values are for intrauterine insemination, with sperm number in* total sperm count*, which may be approximately twice the* total motile sperm count.

Sperm count, or *sperm concentration* to avoid mixup, measures the concentration of sperm in a man's ejaculate, distinguished from *total sperm count*, which is the sperm count multiplied with volume. Over 15 million sperm per milliliter is considered normal, according to the WHO in 2010. Older definitions state 20 million.

A lower sperm count is considered oligozoospermia. A vasectomy is considered successful if the sample is azoospermic. Some define success with rare non-motile sperm are observed (fewer than 100,000 per millilitre). Others advocate obtaining a second semen analysis to verify the counts are not increasing (as can happen with re-canalization) and others still may perform a repeat vasectomy for this situation. The average sperm count today is between 20 and 40 million per milliliter in

the Western world, having decreased by 1-2% per year from a substantially higher number decades ago.

Chips for home use are emerging that can give an accurate estimation of sperm count after three samples taken on different days. Such a chip may measure the concentration of sperm in a semen sample against a control liquid filled with polystyrene beads.

Total Sperm Count

Total sperm count, or *total sperm number*, is the total number of spermatozoa in the entire ejaculate. By WHO, lower reference limit (2.5th percentile) is 39 million per ejaculate.

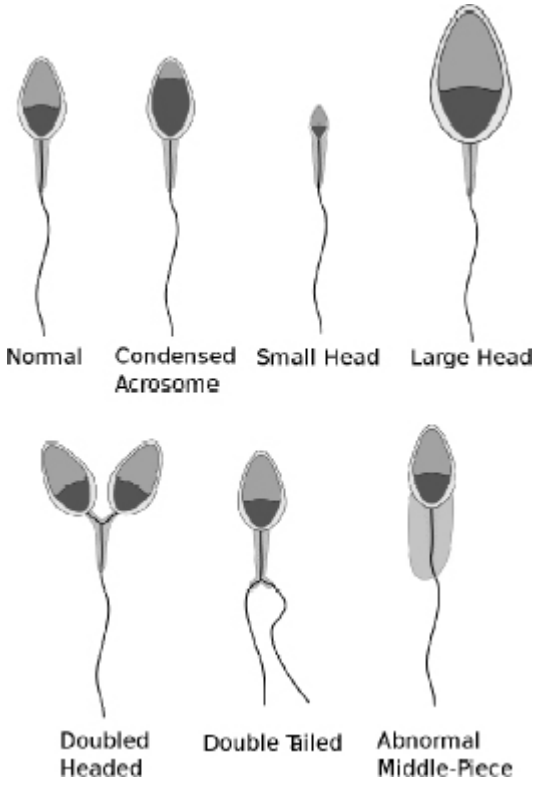
Motility

The World Health Organization has a value of 50% and this must be measured within 60 minutes of collection. WHO also has a parameter of *vitality*, with a lower reference limit of 60% live spermatozoa. A man can have a total number of sperm far over the limit of 20 million sperm cells per milliliter, but still have bad quality because too few of them are motile.

However, if the sperm count is very high, then a low motility (for example, less than 60%) might not matter, because the fraction might still be more than 8 million per millilitre. The other way around, a man can have a sperm count far less than 20 million sperm cells per millilitre and still have good motility, if more than 60% of those observed sperm cells show good forward movement.

A more specified measure is *motility grade*, where the motility of sperm are divided into four different grades:

- Grade a: Sperm with progressive motility. These are the strongest and swim fast in a straight line. Sometimes it is also denoted motility I.
- Grade b: (non-linear motility): These also move forward but tend to travel in a curved or crooked motion. Sometimes also denoted motility II.
- Grade c: These have non-progressive motility because they do not move forward despite the fact that they move their tails. Sometimes also denoted motility III.
- Grade d: These are immotile and fail to move at all. Sometimes also denoted motility IV.



Morphology

Regarding sperm morphology, the WHO criteria as described in 2010 state that a sample is normal (samples from men whose partners had a pregnancy in the last 12 months) if 4% (or 5th centile) or more of the observed sperm have normal morphology.

Morphology is a predictor of success in fertilizing oocytes during in vitro fertilization.

Up to 10% of all spermatozoa have observable defects and as such are disadvantaged in terms of fertilising an oocyte.

Also, sperm cells with tail-tip swelling patterns generally have lower frequency of aneuploidy.

Volume

WebMD advises that semen volumes between 1.0 mL and 6.5 mL are normal; WHO regards 1.5 ml as the lower reference limit. Low volume may indicate partial or complete blockage of the seminal vesicles, or that the man was born without seminal vesicles. In clinical practice, a volume of less than 2 mL in the setting of infertility and absent sperm should prompt an evaluation for obstructive azoospermia. A caveat to this is be sure it has been at least 48 hours since the last ejaculation to time of sample collection.

Fructose Level

Regarding the level of fructose in the semen, WebMD lists normal as at least 3 $^{mg}/_{mL}$. WHO specifies a normal level of 13 imol per sample. Absence of fructose may indicate a problem with the seminal vesicles.

pH

WebMD lists a normal pH range of 7.1-8.0; WHO criteria specify normal as 7.2-7.8. Acidic ejaculate (lower pH value) may indicate one or both of the seminal vesicles are blocked. A basic ejaculate (higher pH value) may indicate an infection. A pH value outside of the normal range is harmful to sperm.

Liquefaction

The liquefaction is the process when the gel formed by proteins from the seminal vesicles is broken up and the semen becomes more liquid. It normally takes less than 20 minutes for the sample to change from a thick gel into a liquid.

An abnormally long liquefaction (more than 30 minutes at 37 24°C) time may indicate an infection. In the NIC guidelines, a liquefaction time within 60 minutes is regarded as within normal ranges.

MOT

MOT is a measure of how many million sperm cells per ml are highly motile, that is, approximately of grade a (>25 micrometer per 5 sek. at room temperature) and grade b (>25 micrometer per 25 sek. at room temperature). Thus, it is a combination of sperm count and motility.

With a straw or a vial volume of 0.5 milliliter, the general guideline is that, for intracervical insemination (ICI), straws or vials making a total of 20 million motile spermatozoa in total is recommended. This is equal to 8 straws or vials 0.5 ml with MOT5, or 2 straws or vials of MOT20. For intrauterine insemination (IUI), 1-2 MOT5 straws or vials is regarded sufficient. In WHO terms, it is thus recommended to use approximately 20 million grade a+b sperm in ICI, and 2 million grade a+b in IUI.

Total Motile Spermatozoa

Total motile spermatozoa (TMS) or *total motile sperm count* (TMSC) is a combination of sperm count, motility and volume,

measuring how many million sperm cells in an entire ejaculate are motile.

Use of approximately 20 million sperm of motility grade c or d in ICI, and 5 million ones in IUI may be an approximate recommendation.

Others

The NICE guidelines also include testing *vitality*, with normal ranges defined as more than 75% of sperm cells alive.

The sample may also be tested for white blood cells. A high level of white blood cells (over 1 million per milliliter) may indicate an infection.

Abnormalities

- Aspermia: absence of semen
- Azoospermia: absence of sperm
- Hypospermia: low semen volume
- Oligozoospermia: low sperm count
- Asthenozoospermia: poor sperm motility
- Teratozoospermia: sperm carry more morphological defects than usual

Factors that Influence Results

Apart from the semen quality itself, there are various methodological factors that may influence the results, giving rise to inter-method variation.

Compared to samples obtained from masturbation, semen samples from collection condoms have higher total sperm counts, sperm motility, and percentage of sperm with normal morphology. For this reason, they are believed to give more accurate results when used for semen analysis.

If the results from a man's first sample are subfertile, they must be verified with at least two more analyses. At least 2 to 4 weeks must be allowed between each analysis. Results for a single man may have a large amount of natural variation over time, meaning a single sample may not be representative of a man's average semen characteristics. In addition, sperm physiologist Joanna Ellington believes that the stress of producing an ejaculate sample for examination, often in an unfamiliar setting and without any lubrication (most lubricants are somewhat

harmful to sperm), may explain why men's first samples often show poor results while later samples show normal results.

A man may prefer to produce his sample at home rather than at the clinic. The site of semen collection does *not* affect the results of a semen analysis.

Measurement Methods

Volume can be determined by measuring the weight of the sample container, knowing the mass of the empty container. Sperm count and morphology can be calculated by microscopy. Sperm count can also be estimated by kits that measure the amount of a sperm-associated protein, and are suitable for home use.

Computer Assisted Semen Analysis (CASA) is a catch-all phrase for automatic or semi-automatic semen analysis techniques. Most systems are based on image analysis, but alternative methods exist such as tracking cell movement on a digitizing tablet. Computer-assisted techniques are most-often used for the assessment of sperm concentration and mobility characteristics, such as velocity and linear velocity. Nowadays, there are CASA systems, based on image analysis and using new techniques, with near perfect results, and doing full analysis in a few seconds. With some techniques, sperm concentration and motility measurements are at least as reliable as current manual methods.

Hamster Zona-free Ovum Test

A man's sperm are mixed with hamster eggs that have had the zona pellucida (outer membranes) removed, and the number of sperm penetrations per egg is measured. The human sperm does not fertilize the hamster eggs. A negative result on the hamster test correlates with a lower probability of the man's partner becoming pregnant.

Sperm Chromatin Assay

Chromatin is the complex of DNA and protein that make up chromosomes. If a large percentage of a man's sperm (greater than 30%) have damaged chromatin, his chances of impregnating a partner are significantly reduced, and if he does impregnate his partner, she faces an increased risk of miscarriage. The portion of a man's sperm with damaged chromatin may be determined with a Sperm Chromatin Structure Assay (SCSA).

Cryopreservation

When performing cryopreservation of semen, it is the sperm quality after reviving the sample that is of importance, because many sperm cells die in the process.

To be of use in assisted reproductive technology, the sample should after thawing have more than 5 million motile sperm cells per ml with a good grade of mobility. If the grade of mobility is poor, 10 million motile cells per ml is required.

Home insemination of previously frozen sperm can be accomplished with the use of a cervical cap conception device as a delivery system for the sperm.

Bad Freezers

In 10–20% of all men, the semen doesn't endure cryopreservation. The cause is unknown. It does not necessarily mean an otherwise bad semen quality.

Sperm Washing

When a sperm sample is prepared for intrauterine insemination, it is washed at a facility such as a fertility clinic or a sperm bank. Some sperm does not survive the washing process, as is also the case when freezing the sperm.

Factors

There are many factors that influence the sperm quality. Exposure to any of the temporary factors can cause up to a three month delay before sperm quality returns to normal, due to spermiogenesis.

Age

Although it is possible for men to father children into old age, the genetic quality of sperm, as well as its volume and motility, all typically decrease with age. In other words, older sperm are less likely to result in a successful pregnancy and, moreover, the cumulative fragmentation of sperm DNA over time makes it more likely that an older father will pass on achondroplasia and possibly other genetic diseases. The onset of genetic deterioration of sperm is believed to have begun by the 24-28.

For example, the percentage of sperm with highly damaged DNA, comet extent, DNA break number, and other comet measures has been found to be significantly higher in men aged 31–57 years than in those

aged 20–30 years. Advancing paternal age has been implicated in a number of possible health effects. One particularly well-studied connection is the link between advancing age and autism. For example, one study of 943,664 children less than 10 years old, found that, with confounding variables controlled, those whose fathers were 32 years or older had a risk of autism of 15.39 compared to those whose fathers were 25–29 years old.

Prospective fathers should take up age-related fertility issues with a qualified fertility specialist such as a reproductive endocrinologist.

Masturbation vs Intercourse

Sperm samples obtained via sexual intercourse contain 70-120% more sperm, with sperm having a slightly higher motility and slightly more normal morphology, compared with sperm samples obtained via masturbation. Sexual intercourse also generates a 25–45% increase in ejaculate volume, mainly by increased prostate secretion.

This intercourse advantage is even greater for men with oligospermia.

However, the single factor or factors for the intercourse advantage have not yet been isolated. It cannot be explained by presence of visual perception of physical attractiveness alone during stimulation, although there may be a slight correlation. Neither does any substantial fluctuations in sex hormones explain the intercourse advantage. It is hypothesized that sexual intercourse subdues an inhibition from the central nervous system, but what, in turn, is the subduing factor is still not completely known.

Heat

Sperm are heat-sensitive, and cannot endure high temperatures. The body has compensatory mechanisms, like the cremaster muscle relaxing and letting the testicle hang further away from the warm body, sweating and a countercurrent exchange of blood cooling in flowing blood. However, despite these compensations, there are activities that should not be performed too often, in order of preventing infertility due to heat;

- sauna sessions
- bathing for a long time in hot water
- Long-time tanning bed sessions
- Placement of a laptop computer over the groin for extended use

Fever raises the body temperature, which can strike sperm quality. In the same way, sperm quality can be lower in the summer.

Contrary to widely held beliefs, no evidence supports that wearing constrictive underwear, or "briefs," decreases fertility. Even with an elevation in temperature of 0.8-1° caused by wearing constrictive underwear, no changes in sperm parameters, no decrease in spermatogenesis, and no changes in sperm function are observed

Physical Trauma

A blow from outside doesn't affect the sperm quality of already produced sperm cells. Furthermore, the testes are well protected in the scrotum, for example by the tunica vaginalis, making the testes slide away from external pressure rather than being malformed from it. However a hard enough hit can close or crush the capillaries that supply the sperm producing tissue, resulting in permanent or temporary partial or total inability to produce sperm in the affected testicle.

Chemicals

There is suspicion that many toxic substances, including several types of medication and hormones, and also constituents of the diet, influence sperm quality. While a few chemicals with known effects on fertility have been excluded from human consumption, we cannot know if others remain undiscovered.

Environmental Chemicals

There has been evidence for a general decline in sperm counts in Europe and the USA between 1938 and 1990. While these dates were critiqued, further analysis supported the findings. A 2008 report demonstrates further evidence of the effects of feminizing chemicals on male development in each class of vertebrate species as a worldwide phenomenon; these chemical are suspected of reducing the sex ratio and sperm counts in humans. Ninety-nine percent of over 100,000 recently introduced chemicals are poorly regulated.

At least three types of synthetic toxins have been found in the semen of student volunteers: polychlorinated biphenyls (PCBs), DDT, and hexachlorobenzene. Leaks of dibromochloropropane (DBCP) have caused sterility in men. Soldiers that were exposed to dioxin during the Vietnam war have given rise to children with an increased rate of birth defects. Plutonium, widely spread from nuclear weapon tests,

accumulates in the testes, where it disrupts zinc metabolism, in turn causing genetic damage.

Phthalates, a ubiquitous pollutant, may cause decreased sperm production when having been exposed to it during prenatal development.

Medication:

- Depo-Provera, Adjudin, and gossypol are examples of substances used as male contraceptives or in chemical castration. Recent studies have found that THC present in marijuana can confuse the movements of intact sperm, reducing their ability to achieve fertilization.
- Selective serotonin reuptake inhibitors (SSRI) may cause low sperm count.
- Many antibiotics, e.g. penicillin and tetracycline, suppress sperm production. It may indirectly reach humans through eating livestock given antibiotics as a growth promoter.

Hormones:

- Anabolic steroids use and use of other hormones can reducesperm quality. Changes in hormone homeostasis affect thespermatogenesis.

The body also has natural variations in hormone concentrations, giving sperm quality natural fluctuations as well.

Diet:

- Coffee and alcohol lower the quality of sperm. However, the influence is probably minor.
- Drinking over 1 liter of cola a day might decrease sperm quality by up to 30% (study claims there is correlation, but not causation)
- Soy products decrease sperm quality due to the high content of a type of phytoestrogen called isoflavones. Theoretically, this exposure to high levels of phytoestrogen in men may alter the hypothalamic-pituitary-gonadal axis. A few tudies on animals have shown that such a hormonal effect may be significant and decrease fertility. On the other hand, most studies have shown that isoflavone supplements have little to no effect on sperm concentration, count, or mobility, and cause no changes in testicular or ejaculate volume.
- A review in 2010 concluded that there is little evidence for a relationship with semen parameters and increased BMI.

- Folate (vitamin B_9) may protect sperm cells from aneuploidy.

Other chemicals:

- Gossypol present in crude cottonseed oil (and potentially theorgan meats from animals poisoned with it) has been associatedwith reduced sperm production. Misuse of anabolic steroidscan cause testicular atrophy and reduced fertility.

Last Ejaculation

How long the man has abstained prior to providing a semen sample correlates with the results of semen analysis and also with success rates in assisted reproductive technology (ART).

Both a too short period of time since last ejaculation and a too long one reduces semen quality.

- A period of time of less than one day reduces sperm count by at least 20%.
- Longer periods of abstinence correlate with poorer results – one study found that couples where the man had abstained for more than 10 days before an intrauterine insemination (IUI) had only a 3% pregnancy rate. An abstinence period of only 1 or 2 days produce the highest pregnancy rates per IUI cycle compared with longer intervals of ejaculatory abstinence. This increase in pregnancy rate occurs despite a lower value of total motile spermatozoa. Daily sexual activity increases sperm quality in men minimizing DNA damage in the sperm -because it is speculated to result in less storage time where damage may accumulate.

Environment

For semen that has been ejaculated, the quality deteriorates with time. However, this lifetime can be shortened or prolonged, depending on the environment.

Outside Body

Sperm outside of the body generally has a life expectancy which is considered to depend on pH, temperature, presence of air and other factors, and is unpredictable but smaller than the life expectancy inside the human body. For instance, sperm donors who collect the sample outside the clinic are advised to have handed in the sample before one

hour from collection, and to keep them in, if not at body temperature, then at least at room temperature.

In a non-harmful environment outside the body, such as in a sterile glass container the number of motile sperm decreases with approximately 5-10% per hour. In contrast, in a latex condom, the quality decreases with 60-80% per hour, rendering the sample unusable in not too long time.

In Female

The environment in the uterus and fallopian tubes are advantageous. A pregnancy resulting from sperm life of eight days has been documented.

Home or in Clinic

The sperm quality is better if the sample is collected at home than in the clinics. Collecting the sperm at home gives a higher sperm concentration, sperm count and motility particularly if the sperm is collected via sexual intercourse.

Mobile Phones

Having a mobile phone in talk mode in the pocket, like when using handsfree, has been suggested to be a risk if often used in the long-term.

In an *in vitro* study, sperm samples (in a petri dish) exposed to radio frequency electromagnetic waves (as in mobile phones) showed significantly decreased sperm motility and viability, increased ROS level, and decreased ROS-TAC score.

Others

Tobacco smoking lowers the sperm quality, perhaps by decreased ability to attach to hyaluronan on the egg cell. However, the influence is probably minor. Smoking marijuana can decrease sperm quantity.

Long-term Stress is also Suggested

Higher levels of intelligence are also correlated with higher levels of sperm quality in three key indicators: sperm concentration, sperm count and sperm motility. Men who scored high on a battery of intelligence tests tended to have higher counts of healthy sperm, while low scorers tended to have fewer and more sickly sperm. It is conceivable that intelligence might tip off a man's overall health to women looking for a mate with healthy genes, explained University of New Mexico

evolutionary psychologist Geoffrey Miller at a talk at Harvard University.

"Though the connections between brains and sperm were 'not awesome, they're there and highly significant,' Miller said. All things held equal, good sperm and good brains go together."

Regarding diet, malnutrition or an unhealthy diet can lead to e.g. Zinc deficiency, lowering sperm quality.

Sperm quality is better in the afternoon than in the morning. Adrenaline-levels are higher during awakening (~06.00 to noon), which may contribute similarly to general stress.

Lack of exercise, as well as excessive exercise, are minor factors. In professional sports, semen quality parameters tend to decrease as training requirements increase. The effect differs substantially between different professional sport types. For example, water polo appears substantially less harmful to semen quality than triathlon.

A longer duration of sexual stimulation before ejaculation slightly increases sperm quality.

During the three decades leading up to the early 1990s, several studies suggested a population-wide decline in the quality of semen over the past 50 years. Definitive evidence for decreasing semen quality was lacking until a 1996 study published in the New England Journal of Medicine concluded that from 1973 to 1992 "each successive calendar year of birth accounted for 2.6 percent of the yearly decline in the sperm concentration, for 0.3 percent of the yearly declines in the percentages of motile, and 0.7 percent of the yearly declines in the percentages of normal spermatozoa (all $P<0.001$)".

Males carrying Robertsonian translocations in their chromosomes have significantly higher degree of sperm cell apoptosis and lower concentration. Sperm cells also have decreased forward motility, but normal morphology.

Semen Storage

Semen can be stored in diluents such as the *Illini Variable Temperature* (IVT) diluent, which have been reported to be able to preserve high fertility of semen for over seven days. The IVT diluent is composed of several salts, sugars and antibacterial agents and gassed with CO_2.

Semen cryopreservation can be used for far longer storage durations. For human sperm, the longest reported successful storage with this method is 21 years.

Health Effects

In addition to its central role in reproduction, some studies have made claims that semen may have certain beneficial effects on human health:

- *Antidepressant:* One study suggested that vaginal absorption of semen could act as an antidepressant; the study compared two groups of women, one of which used condoms and the other did not.
- *Cancer prevention:* Studies suggested that seminal plasma might reduce breast cancer by "not less than 50 percent." This effect is attributed to its glycoprotein and selenium content, with apoptosis being induced by TGF-Beta. A related urban legend parodied these findings and claimed that performing fellatio at least three times a week reduced the risk of breast cancer.
- *Preeclampsia prevention:* It has been hypothesized that substances in semen condition a mother's immune system to accept the "foreign" proteins found in sperm as well as the resulting fetus and placenta, keeping blood pressure low and thereby reducing the risk of preeclampsia. A study shows that oral sex and swallowing sperm may help make a woman's pregnancy safer and more successful, because she is absorbing her partner's antigens.

Other studies claim adversarial effects:

- *Cancer worsening:* Seminal plasma has prostaglandin elementsthat could accelerate the development of an already existingcervical cancer.

Semen and Transmission of Disease

Semen can be the vehicle for many sexually transmitted diseases, including HIV, the virus that causes AIDS.

Further research, such as that by Mathur and Goust, demonstrated that non-preexisting antibodies were produced in humans in response to the sperm. These antibodies mistakenly recognized native T lymphocytes

as *foreign* antigens, and consequently T lymphocytes would fall under attack by the body's B lymphocytes.

Semen contains many proteins with potent antimicrobial activity against bacteria, fungi ad virus. But none of these proteins are not active against Neisseria gonorrhoeae, a common cause of sexually transmitted disease.

Blood in the Semen (hematospermia)

The presence of blood in semen or hematospermia may be undetectable (it only can be seen microscopically) or visible in the fluid. Its cause could be the result of inflammation, infection, blockage, or injury of the male reproductive tract or a problem within the urethra, testicles, epididymis or prostate.

It usually clears up without treatment, or with antibiotics, but if persistent further semen analysis and other urogenital system tests might be needed to find out the cause.

Semen Allergy

In rare cases, people have been known to experience allergic reactions to seminal fluids, known as human seminal plasma hypersensitivity. Symptoms can be either localized or systemic, and may include vaginal itching, redness, swelling, or blisters within 30 minutes of contact. They may also include generalized itching, hives, and even difficulty breathing.

One way to test for human seminal plasma sensitivity is to use a condom during intercourse. If symptoms dissipate with the use of a condom, it is possible that a sensitivity to semen is present. Mild cases of semen allergy can often be overcome by repeated exposure to seminal fluid. In more severe cases, it is important to seek the advice of a physician, particularly in the event that a couple is trying to conceive, in which case, artificial insemination may be indicated.

Scientists at Utrecht University studied the condition whereby some men "get flu-like symptoms such as feverishness, runny nose, extreme fatigue and burning eyes immediately after they ejaculate. Symptoms can last for up to week." This condition is termed post orgasmic illness syndrome or POIS, and it was discovered that this stemmed from an allergy to their own semen, its effects could be cured using hyposensitization therapy or allergen immunotherapy.

Psychological Aspects

A recent study has suggested that semen acts as an antidepressant in women, so that women physically exposed to semen are less likely to suffer from depression. It is thought that the psychological effects of semen are a result of its complex chemical make-up including several mood-altering hormones (testosterone, oestrogen, follicle-stimulating hormone, luteinizing hormone, prolactin and several different prostaglandins).

In a scientific survey of 293 college women it was also found that those who did not use condoms were most likely to initiate sex and to seek out new partners as soon as a relationship ended, suggesting that the chemical dependency to semen creates a "rebound effect". The effect of semen on a male sexual partner (as the receiver of semen) is not known.

Cultural Aspects

Qigong

Qigong and Chinese medicine place huge emphasis on a form of energy called ¾ – which one attempts to develop and accumulate. "Jing" is sexual energy and is considered to dissipate with ejaculation so masturbation is considered "energy suicide" amongst those who practice this art. According to Qigong theory, energy from many pathways/meridians becomes diverted and transfers itself to the sexual organs during sexual excitement. The ensuing orgasm and ejaculation will then finally expel the energy from the system completely. The Chinese proverb a drop of semen is equal to ten drops of blood) illustrates this point.

The scientific term for semen in Chinese is ¾|²m and the term for sperm is ¾|P two modern terms with classical referents.

Greek Philosophy

In Ancient Greece, Aristotle remarked on the importance of semen: "For Aristotle, semen is the residue derived from nourishment, that is of blood, that has been highly concocted to the optimum temperature and substance. This can only be emitted by the male as only the male, by nature of his very being, has the requisite heat to concoct blood into semen." According to Aristotle, there is a direct connection between

food and semen: “Sperms are the excretion of our food, or to put it more clearly, as the most perfect component of our food.”

The connection between food and physical growth, on the one hand, and semen, on the other, allows Aristotle to warn against “engaging in sexual activity at too early an age ... [since] this will affect the growth of their bodies. Nourishment that would otherwise make the body grow is diverted to the production of semen.... Aristotle is saying that at this stage the *body* is still growing; it is best for sexual activity to begin when its growth is ‘no longer abundant’, for when the body is more or less at full height, the transformation of nourishment into semen does not drain the body of needed material.”

Additionally, “Aristotle tells us that the region round the eyes was the region of the head most fruitful of seed (“most seedy”), pointing to generally recognised effects upon the eyes of sexual indulgence and to practices which imply that seed comes from liquid in the region of the eyes.” This may be explained by the belief of the Pythagoreans that “semen is a drop of the brain

Greek Stoic philosophy conceived of the *Logos spermatikos* (“seminal word”) as the principle of active reason that fecundated passive matter. The Jewish philosopher Philo similarly spoke in sexual terms of the Logos as the masculine principle of reason that sowed seeds of virtue in the feminine soul.

The Christian Platonist Clement of Alexandria likened the Logos to physical blood as the “substance of the soul,” and noted that some held “that the animal semen is substantially foam of its blood”. Clement reflected an early Christian view that “the seed ought not be wasted nor scattered thoughtlessly nor sown in a way it cannot grow.”

Sacred Semen

In some pre-industrial societies, semen and other body fluids were revered because they were believed to be magical. Blood is an example of such a fluid, but semen was also widely believed to be of supernatural origin and effect and was, as a result, considered holy or sacred.

Dew was once thought to be a sort of rain that fertilized the earth and, in time, became a metaphor for semen. The Bible employs the term “dew” in this sense in such verses as Song of Solomon 5:2 and Psalm 110:3, declaring, in the latter verse, for example, that the people should follow only a king who was virile enough to be full of the “dew” of youth.

It was widely believed, in ancient times, that gemstones were drops of divine semen which had coagulated after having fertilized the earth. There is an ancient Chinese belief that jade, in particular, was the dried semen of the celestial dragon.

Based upon the resemblance of dandelion juice to human semen, it was historically believed that the flower magically promoted the flow of sperm. (This belief probably derives from the doctrine of signatures.) The orchid's twin bulbs were thought to resemble the testicles, and there was an ancient Roman belief that the flower sprang from the spilled semen of copulating satyrs.

Barbara G. Walker recounts these examples of sacred semen in *The Woman's Dictionary of Symbols and Sacred Objects*, the thesis of which is that myth and folklore show a pre-patriarchic rule by women that was later supplanted by masculine culture.

In primitive mythology around the world, semen is very often considered analogous to breast milk in some way. In the traditions of Bali, it is considered to be the returning or refunding of the milk of the mother in an alimentary metaphor.

The wife feeds her husband who returns to her his semen, the milk of human kindness, as it were.

In some systems of medical philosophy, such as traditional Russian medicine and the Vital Force theory of Herbert Nowell, semen is regarded as the product of a complex physiological interaction between a man and a woman (rather than merely the product of the male reproductive system).

Semen in Espionage

When the British Secret Intelligence Service discovered that semen made a good invisible ink, Sir George Mansfield Smith-Cumming noted of his agents that "Every man (is) his own stylo".

Semen Ingestion

Some reasons for human ingestion of human or other semen are erotic gratification and physical and spiritual benefits.

Health Risks

There is no risk in ingesting the semen of a healthy man. Swallowing semen carries no additional risk other than those inherent in fellatio. Fellatio does carry some transmission risk for sexually

transmitted diseases such as HIV or herpes, especially for people with bleeding gums, gingivitis or open sores.

Even if semen is cold before the individual ingests it, viruses can stay active for a long period of time once outside the body.

Research has suggested that performing unprotected oral sex on a person infected with human papillomavirus (HPV) might increase the risk of oral or throat cancer.

The study found that 36% of the cancer patients had HPV compared to only 1% of the healthy control group. It is believed that this is due to the transmission of HPV because this virus has been implicated in the majority of cervical cancers.

Quantity

The volume of semen ejaculate varies. A review of 30 studies concluded that the average was around 3.4 milliliters (ml), with some studies finding amounts as high as 4.99 ml or as low as 2.3 ml. In a study with Swedish and Danish men, a prolonged interval between ejaculations caused an increase of the sperm count in the semen but not an increase of its amount.

Cultural Practices

In some cultures, semen is attributed with special properties of masculinity. Several tribes of Papua New Guinea, including the Sambia and the Etoro, believe that semen provides sexual maturation among the younger men of their tribe. To them, sperm possesses the manly nature of the tribal elders, and in order to pass down their authority and powers, younger men of their next generation must fellate their elders and ingest their semen.

This custom commences among prepubescent males and postpubescents. This act may also be attributed to the culturally active homosexuality throughout these and other tribes.

Spiritual Views

The church father Epiphanius records that the Borborites and other libertine Gnostic sects consumed semen as the Body of Christ. The *Pistis Sophia* and *Testimony of Truth* harshly condemn such practices.

In the modern St. Priapus Church, consumption of semen in the presence of others is a form of worship.

Sexual Practices

There are several sexual practices involving the ingestion of semen. They can be done with one or more partners, like snowballing, felching and creampie eating, or with multiple partners, like the practices of bukkake and gokkun, which originate from Japan.

Increasing Semen Volume

Some dietary supplements have been marketed with claims to increase seminal volume. Like other supplements, including so-called herbal viagra, these are not approved or regulated by the Food and Drug Administration (as licensed medications would be), and none of the claims have been scientifically verified. Similar claims are made about traditional aphrodisiac foods, with an equal lack of verification.

Euphemisms

A huge variety of euphemisms and dysphemisms have been invented to describe semen. For a complete list of terms, see *sexual slang*.

Hematospermia

Hematospermia (haematospermia), (or Hemospermia, haemospermia) or the presence of blood in semen, is most often a benign and idiopathic symptom, but can sometimes result from medical problems such as a urethral stricture, infection of the prostate, or a congenital bleeding disorder, and can occur transiently after surgical procedures such as a prostate biopsy. It is present in less than 2% of urology referrals, although prevalence in the overall population is unknown. Patients with hematospermia should be evaluated by a urologist to identify or rule out medical causes. Idiopathic hematospermia is sometimes treated with tetracycline and prostatic massage.

Serology

Serology is the scientific study of blood serum and other bodily fluids. In practice, the term usually refers to the diagnostic identification of antibodies in the serum. Such antibodies are typically formed in response to an infection (against a given microorganism), against other foreign proteins (in response, for example, to a mismatched blood

transfusion), or to one's own proteins (in instances of autoimmune disease).

Serological tests may be performed for diagnostic purposes when an infection is suspected, in rheumatic illnesses, and in many other situations, such as checking an individual's blood type. Serology blood tests help to diagnose patients with certain immune deficiencies associated with the lack of antibodies, such as X-linked agammaglobulinemia. In such cases, tests for antibodies will be consistently negative.

There are several serology techniques that can be used depending on the antibodies being studied. These include: ELISA, agglutination, precipitation, complement-fixation, and fluorescent antibodies. Some serological tests are not limited to blood serum, but can also be performed on other bodily fluids such as semen and saliva, which have (roughly) similar properties to serum. Serological tests may also be used forensically, specifically a piece of evidence (e.g., linking a rapist to a semen sample).

Serological Surveys

Serological surveys are often used by epidemiologists to determine the prevalence of a disease in a population. Such surveys are sometimes performed by random, anonymous sampling from samples taken for other sexual tests.

Serum (Blood)

In blood, the serum is the component that is neither a blood cell (serum does not contain white or red blood cells) nor a clotting factor; it is the blood plasma with the fibrinogens removed. Serum includes all proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances (e.g., drugs and microorganisms).

The study of serum is serology, and may also include proteomics. Serum is used in numerous diagnostic tests, as well as in blood typing.

Blood is centrifuged to remove cellular components. Anti-coagulated blood yields plasma containing fibrinogen and clotting factors. Coagulated blood (clotted blood) yields serum without fibrinogen, although some clotting factors remain.

Serum is an essential factor for the self-renewal of embryonic stem cells in combination with the cytokine leukemia inhibitory factor.

Chapter 7: Clinical Biochemistry and Complete Blood Counts

Blood plasma is the straw-colored liquid component of blood that normally holds the blood cells in whole blood in suspension. It makes up about 55% of total blood volume. It is the intravascular fluid part of extracellular fluid (all body fluid outside of cells). It is mostly water (93% by volume) and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide (plasma being the main medium for excretory product transportation). Plasma also serves as the protein reserve of the human body. It plays a vital role in intravascular osmotic effect that keeps electrolyte in balance form and protects the body from infection and other blood disorders.

Blood plasma is prepared by spinning a tube of fresh blood containing an anti-coagulant in a centrifuge until the blood cells fall to the bottom of the tube. The blood plasma is then poured or drawn off. Blood plasma has a density of approximately 1025 kg/m^3, or 1.025 kg/l. Blood serum is blood plasma without fibrinogen or the other clotting factors (i.e., whole blood minus both the cells *and* the clotting factors). Plasmapheresis is a medical therapy that involves blood plasma extraction, treatment, and reintegration.

Plasma Shift

Blood plasma volume may be expanded by or drained to extravascular fluid when there are changes in Starling forces across capillary walls. For example, when blood pressure drops in circulatory shock, Starling forces drive fluid into the interstitium, causing "third spacing."

If one stands still for a prolonged period, this causes an increase in transcapillary hydrostatic pressure. As a result, approximately 12% of blood plasma volume crosses into the extravascular compartment. This causes an increase in hematocrit, serum total protein, blood viscosity and, as a result of increased concentration

Medical History

"Dried plasmas" were developed and first used in World War II. Prior to the United States' involvement in the war, liquid plasma and whole blood were used. The "Blood for Britain" program during the early 1940s was quite successful (and popular in the United States) based on Dr. Charles Drew's contribution. A large project began in August 1940 to collect blood in New York City hospitals for the export of plasma to Britain.

Dr. Drew was appointed medical supervisor of the "Plasma for Britain" project. His notable contribution at this time was to transform the test tube methods of many blood researchers into the first successful mass production techniques.

Nonetheless, the decision was made to develop a dried plasma package for the armed forces as it would reduce breakage and make the transportation, packaging, and storage much simpler. The resulting dried plasma package came in two tin cans containing 400 cc bottles. One bottle contained enough distilled water to reconstitute the dried plasma contained within the other bottle. In about three minutes, the plasma would be ready to use and could stay fresh for around four hours.

Following the "Plasma for Britain" invention, Dr. Drew was named director of the Red Cross blood bank and assistant director of the National Research Council, in charge of blood collection for the United States Army and Navy. Dr. Drew argued against the armed forces directive that blood/plasma was to be separated by the race of the donor.

Dr. Drew argued that there was no racial difference in human blood and that the policy would lead to needless deaths as soldiers and sailors were required to wait for "same race" blood.

By the end of the war the American Red Cross had provided enough blood for over six million plasma packages. Most of the surplus plasma was returned to the United States for civilian use. Serum albumin replaced dried plasma for combat use during the Korean War.

Plasma Donation

Plasma is used in blood transfusions, typically as fresh frozen plasma (FFP) or Plasma Frozen Within 24 Hours After Phlebotomy (PF24). When donating whole blood or packed red blood cell (PRBC) transfusions, ABO blood type O- is the most desirable and is considered a "universal donor," since it has neither A nor B antigens and can be

safely transfused to most recipients. Type AB+ is the "universal recipient" type for whole blood or PRBC donations. However, for plasma the situation is somewhat reversed.

Blood donation centers will sometimes collect only plasma from AB donors through apheresis, as their plasma does not contain the antibodies that may cross react with recipient antigens. As such, AB is often considered the "universal donor" for plasma. Special programs exist just to cater to the male AB plasma donor, because of concerns about transfusion related acute lung injury (TRALI) and female donors who may have higher leukocyte antibodies. However, some studies do not show an increased risk of TRALI despite increased leukocyte antibodies in women who have been pregnant.

Safety and Compensation to Donors

In contrast to whole blood donation, plasma donation may be done much more often (2 times per week in some jurisdictions, compared to once per 8 weeks for whole blood donation), because the whole blood cells are returned to the body during the donation process, and most plasma is naturally replaced by the body within 48 hours. Also in contrast to whole blood donation, plasma donors are often compensated financially ($10-$100 per week for 5 +/- 2 hours of time, normally). It is usually a compensation for time and trouble, not a "purchase" or "sale" of a body part, for various legal, ethical, and cultural reasons. Professional donation centers use sterile and safe practices.

They process the plasma and sell it as a highly quality controlled medical product, selling the product for as high as $1200/week/donor. They set their donor compensation not as a percentage of the expected income, but rather according to how many donors they need, based upon demand for the product and supply of donations.

Blood (Serum) Chemistry

The serum chemistry profile is one of the most important initial tests that is commonly performed. A blood sample is collected from the patient. The blood is then separated into a cell layer and serum layer by spinning the sample at high speeds in a machine called a centrifuge. The serum layer is drawn off and a variety of compounds are then measured. These measurements aid in assessing the function of various organs and body systems.

Diagnostic Value: Very high. Sometimes a specific diagnosis may be made on the basis of a blood chemistry profile alone. More often than not, however, the profile provides information on a variety of body organs and systems, giving the doctor an indication of where a problem might be located. The profile can be extremely helpful in determining which of the many other diagnostic tests might be beneficial.

Risks to Patient: Virtually none, provided that the blood is collected under sterile conditions by a trained professional.

Relative Cost: Relatively low when a group of test are run as one panel.

Interpretation of Results:

A. *Glucose :* This is a measurement of the blood sugar level.

1. High glucose levels can occur just after a meal, during stressful situations, and with the use of certain drugs. The measurement of glucose is important in the diagnosis of diabetes mellitus.

2. Low glucose levels can occur when the patient does not eat, has liver or hormonal problems, or when there is a severe bacterial infection in the bloodstream. Too much insulin will also cause low levels of glucose.

B. *Blood Urea Nitrogen (BUN) :* Urea, which is normally excretedby the kidney, is a by-product of protein metabolism.

1. High levels of BUN may be the result of a high protein diet, dehydration, ulcers in the digestive tract, kidney disease, or blockage of the normal flow of urine (from a kidney or bladder stone, for example).

2. Low BUN levels can result from a low protein diet or liver disease.

C. *Calcium:* This mineral is normally found in the body, and isimportant for normal muscle and heart function.

1. High calcium levels occur in some types of cancer, bone disease, parathyroid problems, and kidney diseases. A variety of other conditions may also cause an elevated calcium level.

2. Low calcium levels can occur in a lactating women after giving birth. Low calcium levels are also associated with

dietary insufficiencies, parathyroid problems, and intestinal problems. Other causes also exist.

D. *Total Protein:* Several protein types circulate in the bloodstream.These protein types can be measured all together or may beseparated out and measured one at a time. On a routine bloodchemistry profile, total protein is measured as the total of allproteins together. Albumin, the most abundant protein type,is usually measured separately.

1. High protein levels may result from dehydration, inflammation, some cancers, and infections.
2. Low protein levels can occur in situations of malnutrition, intestine absorption problems, blood loss, and kidney or liver disease.

E. *Cholesterol*

1. High cholesterol levels can be associated with high-fat diets, hypothyroidism , diabetes mellitus, pancreatitis, Cushing's disease, liver disease, and kidney problems.
2. Low cholesterol levels may occur with low-fat diets, liver failure, digestive and absorption problems, pancreas disease, and with some types of seizure therapy.

F. *Creatine Phosphokinase (CPK) :* This is an enzyme found inmuscle cells.

1. High CK levels can occur in situations where muscles of the body are damaged, diseased, or nflamed. This can even occur with heart muscle problems.

G. *Alkaline Phosphatase (ALP):* This is an enzyme found in liverand bone cells.

1. High ALP levels may indicate a liver problem, some cancers, and increased bone growth or destruction. ALP levels can also be elevated in cases where steroids are administeredor in Cushing's disease where natural steroids are elevated. High levels of ALP are normal in growing children.

H. *Alanine Aminotransferase (ALT):* This is another enzyme found in liver cells.

1. High ALT levels occur when the liver is damaged. This damage can occur because of toxins, not enough oxygen,

inflammation, metabolic disorders, and other diseases.

I. *Aspartate Aminotransferase (AST):* This is another enzyme produced by a variety of tissues. Concentratios tend to be highest in muscle and liver cells.

1. High AST levels occur most often when the muscles and/ or te liver are damaged. This damage can occur because of toxins, lack of oxygen, inflammation, metabolic disorders, and other diseases.

J. *Chloride:* This is a negatively-charged electrolyte (dissolved salt).

1. High levels of chloride can occur with dehydration, fluid therapy, and acidosis (where the pH of the body is abnormally low). Some drugs such as phenobarbital can also cause elevated chloride levels.

2. Low levels may be the result of vomiting, especially right after eating; and treatment with certain drugs (diuretics such as furosemide).

K. *Potassium:* This is an electrolyte with a positive charge.

1. High levels are associated with acidosis, Addison's disease, during certain phases of severe kidney disease, rupture of the urinary bladder,, and with some treatments and syndromes.

2. Low levels are seen with chronic vomiting and diarrhea liver disease, Cushing's disease, certain phases of kidney disease, and administration of some drugs.

L. *Sodium:* This is also another electrolyte with a positive charge.

1. High sodium levels may accompany dehydration, a high salt diet, Cushing's disease, chronic kidney disease, and diabetes insipidus.

2. Low sodium occurs with vomiting, diarrhea, Addison's disease, fluid therapy, kidney problems, and hypothyroidism.

Blood Lipids

Blood lipids (or blood fats) are lipids in the blood, either free or bound to other molecules. They are mostly transported in a protein capsule, and the density of the lipids and type of protein determines the fate of the particle and its influence on metabolism. The concentration of

blood lipids depends on intake and excretion from the intestine, and uptake and secretion from cells. Blood lipids are mainly fatty acids and cholesterol. Hyperlipidemia is the presence of elevated or abnormal levels of lipids and/or lipoproteins in the blood, and is a major risk factor for cardiovascular disease.

Fatty Acids

Blood fatty acids are in different forms in different stages in the circulation. They are taken in through the intestine in chylomicrons, but also exist in very low density lipoproteins (VLDL) after processing in the liver. In addition, when released from adipocytes, fatty acids exist in the blood as free fatty acids

Intestine Intake

Short- and medium chain fatty acids are absorbed directly into the blood via intestine capillaries and travel through the portal vein. Long-chain fatty acids, on the other hand, are too large to be directly released into the tiny intestine capillaries. Instead they are coated with cholesterol and protein (protein coat of lipoproteins) into a compound called a chylomicron. The chylomicron enters a lymphatic capillary and enters into the bloodstream first at the left subclavian vein (having bypassed the liver).

In any case, the concentration of blood fatty acids increase temporarily after a meal.

Cell Uptake

After a meal, when the blood concentration of fatty acids rises, there is an increase in uptake of fatty acids in different cells of the body, mainly liver cells, adipocytes and muscle cells. This uptake is stimulated by insulin from the pancreas. As a result, the blood concentration of fatty acid stabilizes again after a meal.

Cell Secretion

After a meal, some of the fatty acids taken up by the liver is converted into very low density lipoproteins (VLDL) and again secreted into the blood.

In addition, when long time has passed since the last meal, the concentration of fatty acids in the blood decreases, which triggers adipocytes to release stored fatty acids into the blood as free fatty acids, in order to supply e.g. muscle cells with energy.

In any case, also the fatty acids secreted from cells are anew taken up by other cells in the body, until entering fatty acid metabolism.

Cholesterol

The fate of cholesterol in the blood is highly determined by its constitution of lipoproteins, where some types favour transport towards body tissues and others towards the liver for excretion into the intestines.

The 1987 report of National Cholesterol Education Program, Adult Treatment Panels suggest the total blood cholesterol level should be: <200 mg/dl normal blood cholesterol, 200–239 mg/dl borderline-high, >240 mg/dl high cholesterol.

The average amount of *blood cholesterol* varies with age, typically rising gradually until one is about 60 years old. There appear to be seasonal variations in cholesterol levels in humans, more, on average, in winter. These seasonal variations seem to be inversely linked to vitamin C intake.

Intestine Intake

In lipid digestion, cholesterol is packed into Chylomicrons in the small intestine, which are delivered to the Portal vein and Lymph. The chylomicrons are ultimately taken up by liver hepatocytes via interaction between apolipoprotein E and the LDL receptor or Lipoprotein receptor-related proteins.

In Lipoproteins

Cholesterol is minimally soluble in water; it cannot dissolve and travel in the water-based bloodstream. Instead, it is transported in the bloodstream by lipoproteins - protein “molecular-suitcases” that are water-soluble and carry cholesterol and triglycerides internally. The apolipoproteins forming the surface of the given lipoprotein particle determine from what cells cholesterol will be removed and to where it will be supplied.

The largest lipoproteins, which primarily transport fats from the intestinal mucosa to the liver, are called chylomicrons. They carry mostly fats in the form of triglycerides. In the liver, chylomicron particles release triglycerides and some cholesterol. The liver converts unburned food metabolites into very low density lipoproteins (VLDL) and secretes them into plasma where they are converted to intermediate

density lipoproteins (IDL), which thereafter are converted to low-density lipoprotein (LDL) particles and non-esterified fatty acids, which can affect other body cells. In healthy individuals, the relatively few LDL particles are large. In contrast, large numbers of small dense LDL (sdLDL) particles are strongly associated with the presence of atheromatous disease within the arteries. For this reason, LDL is referred to as “bad cholesterol”.

High-density lipoprotein (HDL) particles transport cholesterol back to the liver for excretion, but vary considerably in their effectiveness for doing this. Having large numbers of large HDL particles correlates with better health outcomes, and hence it is commonly called “good cholesterol”. In contrast, having small amounts of large HDL particles is independently associated with atheromatous disease progression within the arteries.

Intestine Excretion

After being transported to the liver by HDL, cholesterol is delivered to the intestines via bile production. However, 92-97% is reabsorbed in the intestines and recycled via enterohepatic circulation.

Cell Uptake

Cholesterol circulates in the blood in low-density lipoproteins and these are taken into the cell by LDL receptor-mediated endocytosis in clathrin-coated pits, and then hydrolysed in lysosomes.

Cell Secretion

In response to low blood cholesterol, different cells of the body, mainly in the liver and intestines, start to synthesize cholesterol from acetyl-CoA by the enzyme HMG-CoA reductase. This is then released into the blood.

Related Medical Conditions

Hyperlipidemia

Hyperlipidemia is the presence of elevated or abnormal levels of lipids and/or lipoproteins in the blood.

Lipid and lipoprotein abnormalities are extremely common in the general population, and are regarded as a highly modifiable risk factor for cardiovascular disease. In addition, some forms may predispose to

acute pancreatitis. One of the most clinically relevant lipid substances is cholesterol, especially on atherosclerosis and cardiovascular disease. The presence of high levels of cholesterol in the blood is called hypercholesterolemia. Hyperlipoproteinemia is elevated levels of lipoproteins.

Hypercholesterolemia

Hypercholesterolemia is the presence of high levels of cholesterol in the blood. It is not a disease but a metabolic derangement that can be secondary to many diseases and can contribute to many forms of disease, most notably cardiovascular disease. Familial hypercholesterolemia is a rare genetic disorder that can occur in families, where sufferers cannot properly metabolise cholesterol.

Hypocholesterolemia

Abnormally low levels of cholesterol are termed *hypocholesterolemia*. Research into the causes of this state is relatively limited, and while some studies suggest a link with depression, cancer and cerebral hemorrhage it is unclear whether the low cholesterol levels are a cause for these conditions or an epiphenomenon.

Serum-separating Tube

Serum-separating tubes, also known as serum separator tubes or SSTs, are used in medical clinical chemistry tests requiring blood serum.

SSTs are sometimes called “marble-top tubes” or “yellow topped tubes”, referring to the stoppers which are either gold or red-gray. Trademarked versions include Covidien “Corvac” tubes.

They contain a special gel that separates blood cells from serum, as well as particles to cause blood to clot quickly. The blood sample may then be centrifuged, allowing the clear serum to be removed for testing.

These tubes should be used with care when measuring therapeutic endocrine levels because the drug/hormone may diffuse from the serum into the gel, causing a reduction in measured drug level. The gel in SST II tubes (which appears slightly less opaque) is supposed to have less effect on drug levels in serum.

Chapter 8: Animal Dermatology and Skin Scraping Test

Skin problems and itchiness are common and frustrating disorders in animals. With so many underlying causes, finding the reason for the problem is important in order to find an appropriate treatment or even a cure. A skin scraping is a commonly performed test that can help diagnose certain skin inflammations, fungal infections, and skin cancer and is quite effective in determining the presence of mites. A skin scraping is a collection of a sample of skin cells that are evaluated under a microscope. A skin scraping is indicated in any skin disorder, especially those involving hair loss and itchiness. There are no real contraindications to performing this test.

What Does a Skin Scraping Reveal?

A skin scraping can reveal the presence of abnormal cells in the superficial layers of the skin. It can reveal certain fungi, bacteria, cancer cells and parasites. By determining the underlying cause of the skin disorder, an effective and appropriate treatment can begin.

How Is a Skin Scraping Done?

A skin scraping is performed by collecting a sample of skin cells with the use of a scalpel blade. The blade is used to gently scrape layers of the skin, usually until a small amount of blood is seen, so that your veterinarian can gather cells deep in the skin. This is important, especially if parasites are suspected, since they often live deep in the skin.

The skin cell sample is placed on a microscope slide, mixed with oil and evaluated under a microscope. Most often, results are available within 30 minutes. Sometimes, the veterinarian may need a second opinion and will submit the skin scraping sample to an outside laboratory. Results may take two to four days.

Is a Skin Scraping Painful?

Any pain involved is associated with the deep scraping of the skin. The level of pain varies from one animal to another and is more likely to cause discomfort rather than pain.

Is Sedation or Anesthesia Needed for a Skin Scraping?

Neither sedation nor anesthesia is needed in order to perform a skin scraping. Most animals tolerate this procedure quite well.

Cutaneous Candidiasis

Cutaneous candidiasis is infection of the skin with candida fungus.

Causes

The body normally hosts a variety of microorganisms, including bacteria and fungi. Some of these are useful to the body, some produce no harm or benefit, and some can cause harmful infections.

Some fungal infections are caused by fungi that live on the hair, nails, and outer skin layers. They include mold-like fungi (dermatophytes, which cause tinea infections) and yeast-like fungi (such as candida).

In cutaneous candidiasis, the skin is infected with candida fungi. It is fairly common. Infection can involve almost any skin on the body, but most often it occurs in warm, moist, creased areas such as the armpits and groin. The fungus that most often causes cutaneous candidiasis is *Candida albicans*.

Candida is the most common cause of diaper rash in infants. The fungi take advantage of the warm, moist conditions inside the diaper. Candida infection is particularly common in people with diabetes and in people who are obese. Antibiotics and oral contraceptives (birth control pills) increase the risk of cutaneous candidiasis. Candida can also cause infections of the nails (onychomycosis), at the edge of the nails (paronychia), and at the corners of the mouth (angular cheilitis).

Oral thrush, a form of candida infection of the moist lining (mucous membranes) of the mouth, is usually associated with taking antibiotics. It may also be a sign of HIV infection or other immunodeficiency disorders when it occurs in adults. Individuals with candida infections are not usually contagious, though in some settings immunocompromised people can catch the infection. Candida is also the most frequent cause of

vaginal yeast infections, which are extremely common and often associated with antibiotics use.

Symptoms

- Itching (may be intense)
- Skin lesion or rash
  - Growing red, inflammed area
  - Infection of hair follicles (folliculitis) may look like pimples
  - Located on the skin folds, genitals, trunk, buttocks, under the breasts, or on other skin areas
  - Macule or papule
  - May have satellite lesions (smaller lesions next to bigger ones)
  - Skin redness or inflammation

Exams and Tests

A diagnosis of cutaneous candidiasis is based mainly on the appearance of the skin, particularly if there are risk factors. Skin scrapings may reveal yeast forms, which usually indicates candida.

Treatment

General hygiene is vital to the treatment of cutaneous candidiasis. Keeping the skin dry and exposed to air is helpful. Weight loss may eliminate the problem in obese people. Proper blood sugar control may also be helpful to those with diabetes.

Antifungal skin creams or ointments may be used to treat infection of the skin, mouth, or vagina. Antifungal medications taken by mouth may be necessary for folliculitis, nail infection, or severe candida infections involvng the mouth, throat, or vagina.

Outlook (Prognosis)

Cutaneous candidiasis usually goes away with treatment. Repeat infections are common.

Possible Complications

- Infection of nails may cause nails to become oddly shaped and may cause infection around the nail
- Recurrence (repeat episodes) of candida skin infection

- Widespread (disseminated) candidiasis may occur in immunocompromised individuals

When to Contact a Medical Professional?

Call for an appointment with your health care provider if you develop symptoms of cutaneous candidiasis.

Prevention

Good general health and hygiene help prevent candida infections. Keep the skin clean and dry. Drying powders may help prevent fungal infections in people who are susceptible to them.

Alternative Names

Skin infection - fungal; Fungal infection - skin; Skin infection - yeast; Yeast infection - skin; Intertriginous candidiasis

Skin-abnormally Dark or Light

Skin that has turned darker or lighter than normal is usually not a sign of a serious medical condition.

Considerations

Normal skin contains cells called melanocytes. These cells produce melanin, the substance that gives skin its color.

Skin with too much melanin is called hyperpigmented skin.

Skin with too little melanin is called hypopigmented skin.

Pale skin areas are due to too little melanin or underactive melanocytes. Darker areas of skin (or an area that tans more easily) occurs when you have more melanin or overactive melanocytes.

Bronzing of the skin may sometimes be mistaken for a suntan. This skin discoloration often develops slowly, starting at the elbows, knuckles, and knees and spreading from there. Bronzing may also be seen on the soles of the feet and the palms of the hands. The bronze color can range from light to dark (in fair-skinned people) with the degree of darkness due to the underlying cause.

Causes

Causes of hyperpigmentation include:

- History of skin inflammation (post-inflammatory hyperpigmentation)
- Use of certain medications (such as minocycline)
- Endocrine diseases such as Addison's disease
- Hemochromatosis (iron overload)
- Sun exposure

Causes of hypopigmentation include:

- History of skin inflammation
- Certain fungal infections (such as tinea versicolor)
- Pityriasis alba
- Vitiligo

Home Care

Over-the-counter creams are available for lightening the skin. If you use these creams, follow instructions carefully and don't use one for more than 3 weeks at a time. Darker skin requires greater care when using these preparations. Cosmetics may also help cover a discoloration.

Avoid too much sun exposure. Always use sunscreen.

Abnormally dark skin may continue even after treatment. Experts recommend emotional support or counseling.

When to Contact a Medical Professional?

Call your doctor for an appointment if you have:

- Skin discoloration that causes significant concern
- Persistent, unexplained darkening or lightening of the skin
- Any skin sore or lesion that changes shape, size, or color — may be a sign of skin cancer

What to Expect at Your Office Visit?

Your doctor will perform a physical exam and ask questions about your symptoms, including:

- When did the discoloration develop?
- Did it develop suddenly?
- Is it getting worse? How fast?
- Has it spread to other parts of the body?
- What medicines do you take?
- Has anyone else in your family had a similar problem?

- How often are you in the sun? Do you use a sun lamp or go to tanning salons?
- What is your diet like?
- What other symptoms do you have? For example, are there any rashes or skin lesions?

Tests that may be done include:

- Adrenocorticotropin hormone stimulation test
- Skin biopsy
- Thyroid function studies
- Wood's lamp test

Your doctor may recommend creams, ointments, surgery, or phototherapy, depending on the type of skin condition you have. The following articles offer more detailed treatment information.

- Liver spots
- Mole
- Pityriasis versicolor
- Vitiligo Some skin color changes may return to normal without treatment.

Skin Abscess

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A skin abscess is a collection of pus and infected material in or on the skin.

Causes

Skin abscesses are fairly common. They occur when an infection causes pus and infected material to collect in the skin.

Skin abscesses may occur after:

- A bacterial infection (often staphylococcus)
- A minor wound or injury
- Boils
- Folliculitis

Skin abscesses may occur anywhere on the body. They affect people of all ages.

Symptoms

- Fever or chills, in some cases
- Local swelling, hardening of tissue (induration)
- Skin lesion
  - Open or closed sore, domed nodule
  - Red
  - May drain fluid
- Tender and warm affected area

Exams and Tests

Your doctor can diagnose the condition based on the appearance of the area. A culture or examination of any drainage from the lesion may help identify what organism is causing it.

Treatment

Moist heat (such as warm compresses) may speed healing and may help a skin abscess drain. DO NOT push and squeeze on the abscess.

The doctor may cut open and drain the abscess, after placing some numbing medicine in the area. Packing material may be left in wound to help it heal and not close over again..

Antibiotics are given by mouth to control the infection.

Outlook (Prognosis)

Most skin abscesses can be cured with proper treatment. More often now, the bacteria does not respond to regular antibiotics. This is called methicillin-resistant staph aureus (MRSA).

Possible Complications

- Prevention of the proper functioning of nearby tissues
- Spread of infection around the same area or throughout the body
- Spread of infection through the bloodstream, causing:
  - Abscess formation on the joints or other locations
  - Endocarditis
  - Many new abscesses (“seeding” of infection)
  - Osteomyelitis
- Tissue death (gangrene)

When to Contact a Medical Professional?

Call for an appointment with your health care provider if you have any signs of skin infection, including:

- Drainage of any kind
- Fever
- Pain
- Redness
- Swelling

Also call for an appointment if you develop new symptoms during or after treatment for a skin abscess.

Prevention

Prevent and watch for bacterial infections. Keep the skin around minor wounds clean and dry. Consult the health care provider if you develop signs of infection. Treat minor infections promptly.

Alternative Names

Abscess - skin; Cutaneous abscess; Subcutaneous abscess

Skin Blushing/flushing

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Skin blushing or flushing is a sudden reddening of the face, neck, or upper chest.

Considerations

Blushing is a normal body response that may occur when you are embarrassed, angry, excited, or experiencing some other strong emotion.

Flushing of the face may also be associated with certain medical conditions.

Causes

Common causes include:

- Extreme emotions
- Hot or spicy foods
- Rosacea

Other causes include:

- Alcohol use
- Carcinoid syndrome
- Certain medicines used to treat diabetes and high cholesterol
- High fever
- Menopause
- Rapid changes in temperature

Home Care

Try to avoid the things that cause your blushing. For example, you may need to avoid hot drinks, spicy foods, extreme temperatures, and bright sunlight.

When to Contact a Medical Professional?

Call your doctor if you have persistent flushing, particularly if you have other symptoms (such as diarrhea).

What to Expect at Your Office Visit?

Your doctor will perform a physical exam and may ask you questions about your medical history and symptoms, including:

- Does the flushing affect the whole body or just the face?
- Do you have hot flashes?
- How often do you have flushing or blushing?
- Are episodes getting worse or more frequent?
- Is it worse after you drink alcohol?
- What other symptoms do you have? For example, do you have diarrhea, wheezing, hives, or difficulty breathing?

Skin Color–patchy

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Patchy skin color is areas where the skin color is irregular. Mottling or mottled skin refers to blood vessel changes in the skin that cause a patchy appearance.

Considerations

Irregular or patchy discoloration of the skin can be caused by:

- Changes in melanin, a substance produced in the skin cells that gives skin its color
- Growth of bacteria or other organisms on the skin
- Blood vessel (vascular) changes

The following can increase or decrease melanin production:

- Your genes
- Heat
- Injury
- Exposure to radiation (such as from the sun)
- Exposure to heavy metals
- Changes in hormone levels

Exposure to sun or ultraviolet (UV) light, especially after taking a medicine called psoralens, may increase skin color (pigmentation). Increased pigment production is called hyperpigmentation.

Decreased pigment production is called hypopigmentation.

Skin color changes can be their own condition, or they may be caused by other medical conditions or disorders.

How much skin pigmentation you have can help determine which skin diseases you may be more likely to develop. For example, lighter-skinned people are more sensitive to sun exposure and damage, which raises the risk for skin cancers. However, too much sun exposure can lead to skin cancers even in darker-skinned people.

See also:

- Basal cell carcinoma
- Squamous cell carcinoma

Generally, skin color changes are cosmetic and do not affect physical health. However, mental stress can occur because of pigment changes. Some pigment changes may be a sign that you are at risk for other medical disorders.

Causes

- Cafe-au-lait spots
- Chloasma
- Cuts, scrapes, wounds, insect bites and minor skin infections
- Erythrasma
- Melasma

- Melanoma
- Moles (nevi), bathing trunk nevi, or giant nevi
- Mongolian blue spots
- Pityriasis alba
- Radiation therapy
- Rashes
- Sensitivity to the sun due to medication reactions or certain drugs
- Sunburn or suntan
- Tinea versicolor
- Unevenly applying sunscreen, leading to areas of burn, tan, and no tan
- Vitiligo

Home Care

Normal skin color may return on its own in some cases.

You may use lotions that bleach or lighten the skin to reduce discoloration or to even the skin tone where hypopigmented areas are large or very noticeable.

Selsun Blue, ketoconazole, or tolnaftate (Tinactin) lotion can help treat tinea versicolor. Apply as directed to the affected area daily until the discolored patches disappear. Tinea versicolor often returns, even with treatment.

You may use cosmetics or skin dyes to hide skin color changes. Makeup can also help hide mottled skin, but it will not cure the problem.

Avoid too much sun exposure and use sunblock. Hypopigmented skin sunburns easily, and hyperpigmented skin may get even darker. In darker-skinned people, skin damage may cause permanent hyperpigmentation.

When to Contact a Medical Professional?

Contact your doctor if:

- You have any lasting skin color changes that don’t have a known cause
- You notice a new mole or other growth
- An existing growth has changed color, size, or appearance

What to Expect at Your Office Visit?

The doctor will carefully examine the skin and ask questions about your medical history and symptoms, such as:

- When did the skin color change develop?
- Did it develop slowly or suddenly?
- Is it getting worse? How quickly?
- What is your normal skin color?
- Does the skin color change appear in more than one place?
- Have you had any injury to the skin (including sunburn or frequent suntans)?
- Are you pregnant?
- What medications do you take?
- What medical treatments have you had?
- What other symptoms do you have?

Tests that may be done include:

- Scrapings of skin lesions
- Skin biopsy
- Wood's lamp (ultraviolet light) examination of the skin

Skin Culture

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A skin or nail culture is a laboratory test to look for and identify germs that cause problems with the skin or nails.

It is called a mucosal culture if the sample involves the mucous membranes.

How the Test is Performed?

Your health care provider may use a cotton swab to collect a sample from an open skin rash or skin sore.

A sample of skin or mucous membrane is needed. For information on how this is done, see:

- Skin lesion biopsy
- Gum biopsy

A small sample of a fingernail or toenail may be taken. It may take up to three weeks to get results for this type of culture.

The sample is sent to a laboratory and checked at different time periods to see if bacteria, virus, or fungus has grown.

Further tests can be done to identify the specific germ that is causing your problem. This can help your doctor determine the best treatment.

How to Prepare for the Test?

There is no preparation needed for a culture. For information on how to prepare for a skin or mucosal sample, see:

- Skin lesion biopsy
- Gum biopsy

How the Test Will Feel?

The laboratory test does not involve the patient, so it is painless. For information on how it may feel to give a skin or mucosal sample, see:

- Skin lesion biopsy
- Gum biopsy

Why the Test is Performed?

This test may be done to diagnose the cause of:

- A fungus infection of the skin, finger or toenail
- A skin rash or sore that appears to be infected
- A skin ulcer that is not healing

Normal Results

A normal result means no disease-causing germs are seen in the test sample.

Some germs normally live on the skin. These are not a sign of infection and are considered a normal finding.

Normal value ranges may vary slightly among different laboratories. Some labs use different measurements or test different samples. Talk to your doctor about the meaning of your specific test results.

What Abnormal Results Mean?

An abnormal result means bacteria, fungus, or virus is present. This may be a sign of infection.

Common skin infections caused by bacteria include:

- Impetigo
- Diabetes foot ulcers

Common skin infections caused by fungus include:

- Athlete's foot
- Nail infections
- Scalp infections

Risks

A laboratory culture does not pose a risk to the patient. For information on risks related to removing a sample of skin or mucosal tissue, see:

- Skin lesion biopsy
- Gum biopsy

Alternative Names

Mucosal culture; Culture - skin; Culture - mucosal; Nail culture; Culture - fingernail; Fingernail culture

Skin Discoloration–Bluish

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Cyanosis is a bluish color to the skin or mucus membranes that is usually due to a lack of oxygen in the blood.

Considerations

Red blood cells provide oxygen to body tissues. Most of the time, almost all blood cells carry a full supply of oxygen. These blood cells are bright red, and the skin has a pinkish or red hue.

Blood that has lost its oxygen is dark bluish-red. People whose blood is low in oxygen tend to have a bluish color to their skin, called cyanosis.

Depending on the cause, cyanosis may develop suddenly, along with shortness of breath and other symptoms.

Cyanosis that is caused by long-term heart or lung problems may develop slowly. Symptoms may be present, but are often not severe.

When oxygen levels have dropped only a small amount, cyanosis may be hard to detect.

In dark-skinned people, cyanosis may be easier to see in the mucus membranes (lips, gums, around the eyes) and nails.

Causes

Cyanosis that is seen in only one part of the body may be due to:

- A blood clot that blocks the blood supply to a leg, foot, hand, or arm
- Raynaud's phenomenon

Lack of Oxygen in the Blood

Most cyanosis occurs because of a lack of oxygen in the blood. This can be caused by the following problems.

Problems with the lungs:

- Blood clot in the arteries of the lungs (pulmonary embolism)
- Infection in the smallest air passages in the lungs of children, called bronchiolitis
- Long-term lung problems that become more severe, such as COPD, asthma, and interstitial lung disease
- Drowning or near-drowning
- High altitudes
- Pneumonia (severe)

Problems with the airways leading to the lungs:

- Breath-holding
- Choking on something stuck in the airways
- Croup
- Epiglottitis
- Seizures that last a long time

Problems with the heart:

- Heart failure
- Heart defects that are present at birth (congenital)
- Heart stops working (cardiac arrest) Other problems:
- Drug overdoses (narcotics, benzodiazepines, sedatives)
- Exposure to cold air or water
- Toxins such as cyanide

Home Care

For cyanosis caused by exposure to cold or Raynaud's phenomenon, dress warmly when going outside or stay in a well-heated room.

When to Contact a Medical Professional?

Bluish skin (cyanosis) can be a sign of many serious medical problems and should be taken seriously. Call or visit your health care provider.

For adults, call your doctor or 911 if you have bluish skin and:

- Your breathing is getting harder, faster, or you cannot get a deep breath.
- You need to lean forward when sitting to breathe.
- You are using muscles around the ribs to get enough air.
- You have chest pain.
- You are having headaches more often than usual.
- You feel sleepy or confused.
- You have a fever.
- You are coughing up dark mucus.

For children, call the doctor or 911 if your child has bluish skin and:

- Is having a hard time breathing
- The child's chest muscles are pulling in with each breath
- Is breathing faster than 50 to 60 breaths per minute (when not crying)
- Is making a grunting noise
- Is sitting with shoulders hunched over
- Is very tired
- Is not moving around very much
- Has a limp or floppy body
- The nostrils are flaring out when the child breathes
- Loses his or her appetite
- Is irritable
- Has trouble sleeping

What to Expect at Your Office Visit?

Your health care provider will perform a physical examination, which includes listening to your breathing and heart sounds. In emergency situations (such as shock), you will be stabilized first.

Medical history questions may include:

- When did the bluish skin color develop?

- Did it develop suddenly?
- Has it been developing slowly?
- Are your lips blue?
- Are your nailbeds blue?
- Is your body blue all over?
- Have you been exposed to cold?
- Have you suddenly gone to a high altitude?
- Have you breathed in anything poisonous?
- What other symptoms do you have?
- Do you have difficulty breathing?
- Do you have ankle, foot, or leg swelling?
- Do you have a cough?
- Do you have chest pain?

Tests that may be performed include:

- Arterial blood gas analysis
- Blood oxygen saturation by pulse oximetry
- Complete blood count (CBC)
- Chest x-ray
- ECG For shortness of breath and cyanosis, you may receive oxygen.

Alternative Names

Lips - bluish; Fingernails - bluish; Cyanosis; Bluish lips and fingernails; Bluish skin

Skin Graft

A skin graft is a patch of skin that is removed by surgery from one area of the body and transplanted, or attached, to another area.

Description

Your surgery will probably be done while you are under general anesthesia (you will be unconscious and will not feel pain).

Healthy skin is taken from a place on your body called the donor site. Most people who are having a skin graft have a split-thickness skin graft. This takes the two top layers of skin from the donor site (the epidermis) and the layer under the epidermis (the dermis).

The donor site can be any area of the body. Most times, it is an area that is hidden by clothes, such as the buttock or inner thigh.

The graft is carefully spread on the bare area where it is being transplanted. It is held in place either by gentle pressure from a well-padded dressing that covers it, or by staples or a few small stitches. The donor-site area is covered with a sterile dressing for 3 to 5 days.

People with deeper tissue loss may need a full-thickness skin graft. This requires an entire thickness of skin from the donor site, not just the top two layers.

A full-thickness skin graft is a more complicated procedure. The flap of skin from the donor site includes the muscles and blood supply. It is transplanted to the area of the graft. Common donor sites for full-thickness skin grafts include the chest wall, back, or abdominal wall.

Why the Procedure is Performed?

Skin grafts may be recommended for:

- Areas where there has been infection that caused a large amount of skin loss
- Burns
- Cosmetic reasons or reconstructive surgeries where there has been skin damage or skin loss
- Skin cancer surgery
- Surgeries that need skin grafts to heal
- Venous ulcers, pressure ulcers, or diabetic ulcers that do not heal
- Very large wounds
- When the surgeon is unable to close a wound properly

Full-thickness grafts are done when a lot of tissue is lost. This can happen with open fractures of the lower leg, or after severe infections.

Risks

Risks for any anesthesia are:

- Reactions to medicines
- Problems with breathing

Risks for this surgery are:

- Bleeding
- Chronic pain (rarely)
- Infection

- Loss of grafted skin (the graft not healing, or the graft healing slowly)
- Reduced or lost skin sensation, or increased sensitivity
- Scarring
- Skin discoloration
- Uneven skin surface

Before the Procedure

Always tell your doctor or nurse:

- What drugs you are taking, even drugs or herbs you boughtwithout a prescription.
- If you have been drinking a lot of alcohol.

During the days before your surgery:

- You may be asked to stop taking aspirin, ibuprofen, warfarin (Coumadin), and any other drugs that make it hard for your blood to clot.
- Ask your doctor which drugs you should still take on the day of your surgery.
- If you smoke, try to stop.

If you have diabetes, follow your diet and take your medicines as usual.

On the day of the surgery:

- Usually you will be asked not to drink or eat anything for 8 to 12 hours before the surgery.
- Take the drugs your doctor told yu to take with a small sip of water.

Prepare your home. Plan to have the help you will need from your spouse, a friend, or a neighbor.

Make sure the bathroom and the rest of the house are set up safely so that you do not trip or fall. Make sure you can get in and out of your house easily.

After the Procedure

You should recover quickly after split-thickness skin grafting, except in cases of major burns.

The skin graft must be protected from trauma, such as being hit, or heavy stretching for at least 2 to 3 weeks.

Depending on the location of the graft, you may need to wear a dressing for 1 to 2 weeks. Avoid exercise that might stretch or injure the graft for 3 to 4 weeks. Some people need physical therapy after their skin graft.

Full-thickness grafts need a longer recovery period. Most people with these grafts need to stay in the hospital for 1 to 2 weeks.

Outlook (Prognosis)

New blood vessels begin growing within 36 hours. Most skin grafts are successful, but some do not heal well. You may need a second graft.

Alternative Names

Skin transplant; Skin autografting; FTSG; STSG; Split thickness skin graft; Full thickness skin graft

Skin Lesion Aspiration

Skin lesion aspiration is the withdrawal of fluid from a skin lesion (sore).

How the Test is Performed?

A needle is inserted into a skin lesion or skin abscess, which may contain fluid or pus. The fluid may be examined under the microscope or placed in a special laboratory dish (called a culture medium), where it will be watched for the growth of microorganisms.

How to Prepare for the Test?

There is no specialpreparation for this test.

How the Test Will Feel?

If the abscess is deep, the health care provider may inject a numbing medicine (anesthetic) into the skin before inserting the needle.

You may feel a pricking sensation as the needle enters the skin.

In many cases, the removal of fluid will decrease the pressure within the irritates skin sore, and relieve your pain.

Why the Test is Performed?

This test is used to help determine the cause of a fluid-filled skin lesion. It can be used to diagnose skin infections.

What Abnormal Results Mean?

Abnormal results may be a sign of a bacterial or fungal skin infection.

Risks

There is a slight risk of bleeding, mild pain, or infection.

Chapter 9: Purpose, Procedure and Risks of Skin Lesion Biopsy

A skin lesion biopsy is the removal of a piece of skin to diagnose or rule out an illness.

How the Test is Performed?

There are several ways to do a skin biopsy. Most procedures can be easily done in outpatient medical offices or your doctor's office.

Which procedure you have depends the location, size, and type of lump or sore. You will receive some type of numbing medicine (anesthetic) before any type of skin biopsy.

Types of skin biopsies include:

- Shave biopsy
- Punch biopsy
- Excisional biopsy
- Incisional biopsy

The shave biopsy is the least invasive of all three techniques. Your doctor will remove the outermost layers of skin. You will not need stitches. Punch biopsies are most often used for deeper skin spots or sores. Your doctor removes a small round piece of skin (usually the size of a pencil eraser) using a sharp, hollow instrument. If a large sample is taken, the area may be closed with stitches.

An excisional biopsy is done to remove the entire lesion. A numbing medicine is injected into the area. Then the entire lump, spot, or sore is removed, going as deep as needed to get the whole area. The area is closed with stitches. Pressure is applied to the area to stop any bleeding. If a large area is biopsied, a skin graft or flap of normal skin may be used to replace the skin that was removed.

An incisional biopsy takes a piece of a larger growth for examination. The area is injected with a numbing medicine. A piece of the growth is cut and sent to the lab for examination. You may have

stitches, if needed. The rest of the growth can be treated after the diagnosis is made.

Have stitches, if needed. The rest of the growth can be treated after the diagnosis is made.

How to Prepare for the Test?

Tell your health care provider:

- About the medications you are taking (including vitamins and supplements, herbal remedies, and over-the-counter preparations)
- Ifyou have any allergies
- If you have bleeding problems
- If you are pregnant

How the Test Will Feel?

There is a brief prick and sting as the anesthetic is injected. Afterward, the area may be tender.

Why the Test is Performed

Your doctor may order a skin biopsy if you have signs or symptoms of:

- Chronic skin infections
- Noncancerous (benign) growths
- Skin cancer

•Other skin conditions

Normal Results

Normal value ranges may vary slightly among different laboratories. Talk to your doctor about the meaning of your specific test results.

What Abnormal Results Mean?

The test may reveal skin cancers or noncancerous (benign) conditions. Bacteria and fungi can be identified. The test may also reveal some inflammatory diseases of the skin. Once the diagnosis is confirmed with the biopsy, a treatment plan is usually started.

Risks

Risks may include:

- Infection

- Scar (keloids)

You will bleed slightly during the procedure. Tell your doctor if you have a history of bleeding problems.

Considerations

Fluid-filled sores or growths may be examined by skin lesion aspiration instead of skin lesion biopsy.

Alternative Names

Punch biopsy; Shave biopsy; Skin biopsy; Biopsy - skin

Skin Lesion of Blastomycosis

A skin lesion of blastomycosis is a symptom of an infection with the fungus *Blastomyces dermatitidis*. The skin becomes infected as the fungus spreads throughout the body.

Causes

Blastomycosis is a rare fungal infection.It is most common in the central and southeastern United States, and in Canada, India, Israel, Saudi Arabia, and Africa. A person gets infected by inhaling fungal particles that are found in moist soil, particularly where there is rotting vegetation. People with immune system disorders are at highest risk for this infection.

The fungus enters the body through the lungs, infecting them. In some people, the fungus then spreads (disseminates) to other areas of the body. The infection may affect the skin, bones and joints, genitourinary tract, and other systems. Skin symptoms are a sign of widespread (disseminated) blastomycosis.

Symptoms

Skin symptoms occur in about 80% of people whose blastomycosis infection spreads beyond their lung.

Papules, pustules, or nodules are most frequently found on exposed body areas.

- They may look like warts or ulcers.
- They are usually painless.
- They may vary from gray to violet in color.

The pustules may:

- Form ulcers
- Bleed easily
- Occur in the nose or mouth

Over time, these skin lesions can lead to scarring and loss of skin color (pigment).

Exams and Tests

The infection is diagnosed by identifying the fungus in a culture taken from a skin lesion. This usually requires a skin biopsy.

Treatment

This infection is treated with antifungal drugs such as amphotericin B, itraconazole, ketoconazole, or fluconazole. Either oral or intravenous (directly in the vein) drugs are used, depending on the form and stage of the disease.

Outlook (Prognosis)

What happens depends on the form of blastomycosis and the individual's immune system. In immunouppressed individuals, long-term treatment may be required to prevent symptoms from coming back.

Possible Complications

- Abscesses (pockets of pus)
- Additional (secondary) skin infection caused by bacteria
- Complications related to medications (for instance, amphotericin B can have severely unpleasant side effects)
- Spontaneously draining nodules

When to Contact a Medical Professional?

Some of the skin problems caused by blastomycosis can be similar to skin problems caused by other illnesses. Tell your doctor if you develop any worrisome skin problems.

Skin Lesion of Coccidioidomycosis

Skin lesions of coccidioidomycosis are a symptom of infection with the *Coccidioides immitis* fungus.

This article discusses skin lesions due to coccidioidomycosis. For more general information about this disease, see: Coccidioidomycosis

Causes

Coccidioidomycosis is a fungal infection most commonly seen in the desert regions of the southwestern United States, and in Central and South America. You get it by breathing in fungal particles (called spores) found in infected soil. The infection starts in the lungs.

After the lungs are infected, the fungus may spread to other tissues including the skin. The skin rash, or skin lesions, include erythema nodosum or erythema multiforme. They are thought to be caused by an imune response to the infection, rather than by the fungus itself.

Skin lesions are a sign of widespread (disseminated) fungal disease. Those who are at higher risk f developing widespread infection include:

- People of Native American, African, or Philippine descent
- Those with weakened immune systems due to AIDS, diabetes, or medications that suppress the immune system

Symptoms

- Skin lesion of the initial (primar) infection
  - Erythema multiforme (target lesions)
  - Erythema nodosum
  - Papular rash
- Skin lesion of widespread (disseminated) disease: papule,pustule, nodule, or plaque
  - May form abscesses
  - May ulcerate
  - Most often on the face

Exams and Tests

The diagnosis depends on the stage of infection (primary or disseminated). If disseminated disease is suspected, the doctor may do a skin biopsy to look for the fungus in a skin lesion.

Treatment

This infection is treated with antifungal medications. Oral or intravenous (directly into a vein) drugs will be used, depending on the form and stage of the disease. Antifungal agents used include amphotericin B, itraconazole, ketoconazole, or fluconazole. People with disseminated disease and a suppressed immune system may need long-term treatment.

Outlook (Prognosis)

What happens depends on the stage and extent of the infection, as well as the person's immune system. The highest mortality rate is seen in immunosuppressed people with disseminated disease.

Possible Complications

- Additional skin infections caused by bacteria
- Complications related to medications (such as severe side effects)
- Skin abscess

When to Contact a Medical Professional?

There are many types of skin lesions, and they can be hard to tell apart. Notify your medical provider if you develop skin lesions and suspect this condition, because you will need to be tested.

Skin Lesion of Histoplasmosis

Skin lesions of histoplasmosis are a symptom of widespread infection with the fungus *Histoplasma capsulatum*.

This article talks about skin lesions due to histoplasmosis only. For more general information on the disease, see: Histoplasmosis

Causes

Histoplasmosis is a fungal infection. It occurs throughout the world. In the United States, it is most common in the southeastern, mid-Atlantic, and central states. Histoplasma fungus grows in soil. When particles become airborne, they can be breathed into the lungs, causing infection. Soil contaminated with bird or bat droppings may have a higher concentration of the fungus. After infecting the lungs, the fungus travels to distant areas of the body, including the skin. This is a sign of widespread (disseminated) infection. Skin lesions can be caused by an immune response to the infection (usually a rash called erythema nodosum or erythema multiforme), or by the fungus itself when it spreads to the skin.

Widespread infection is most common in immunosuppressed people, such as those with AIDS or cancer, or those who have had a transplant.

Symptoms

The symptoms may include:

- Mouth ulcer, usually painless
- Pustules or nodules all over the body
- Red spots on the skin (erythema nodosum)
- Red lumps on the skin (erythema multiforme), usually on the lower legs

Exams and Tests

In some cases, a biopsy of the skin lesion can identify the fungus. Rashes due to erythema nodosum and erythema multiforme, however, usually do not have fungus in them. Blood and urine tests may help detect the fungus in some cases.

Treatment

Antifungal drugs, such as amphotericin B, itraconazole, and ketoconazole, are the usual treatments. Antifungals may be given intravenously depending on the form or stage of disease. In some cases, long-term treatment with antifungal drugs may be needed.

Outlook (Prognosis)

The outlook depends on the form of histoplasmosis and the condition of the individual's immune system. Death can occur in some cases.

Possible Complications

- Bacterial skin infection
- Complications of medications (for example, amphotericin B can have severe, unpleasant side effects)

When to Contact a Medical Professional?

The skin lesions may be similar to skin problems from other infections or illnesses. Notify your health care provider if you develop any suspicious lesions on your skin, so that you may be tested correctly.

Prevention

Histoplasmsis may be prevented by reducing exposure to dust in chicken coops, bat caves, and other high-risk locations. Wear masks and other protective equipment if you work in these environments.

Skin Lumps

Skin lumps are any abnormal bump or swelling on the skin.

Considerations

Many people wonder if small bumps or lumps on the body are something to worry about. Most lumps and swellings are benign (not cancerous) and are harmless, especially the kind that feel soft and roll easily (lipomas).

A lump or swelling that appears suddenly (over 24 - 48 hours) and is painful is usually caused by an injury or an infection.

Causes

- Lipomas, fatty lumps under the skin
- Enlarged lymph glands, usually in the armpits, neck, and groin
- Cyst, a closed sac in or under the skin that is lined with skin tissue and contains fluid or semisolid material
- Benign skin growths such as seborrheic keratoses or neurofibromas
- Boils, painful, red bumps usually involving a hair follicle
- Corn or callus, caused by skin thickening in response to continued pressure (for example, from shoes) and usually occurring on a toe or foot
- Warts, a skin virus that develops a rough, hard bump, usually appearing on a hand or foot and often with tiny black dots in the bump
- Moles, skin-colored, tan, or brown bumps on the skin
- Abscess, infected fluid trapped in a closed space from which it cannot escape
- Cancer of the skin (colored or pigmented spot that bleeds easily, changes size or shape, or crusts and doesn't heal)

Home Care

Skin lumps from trauma can be treated with rest, ice, compression, and elevation. Most other lumps should be looked at by your health care provider before you try any home treatments.

When to Contact a Medical Professional?

Call your provider if there is any unexplained lump or swelling.

What to Expect at Your Office Visit?

Your doctor will perform a physical exam and ask you questions about your symptoms, including:

- Where is the lump?
- When did you first notice it?
- Is there more than one lump?
- Is it painful?
- What does the lump look like?
- What other symptoms do you have?

Your doctor may prescribe antibiotics if you have an infection. If cancer is suspected, a biopsy may be done.

Skin Self-exam

Skin self-exam means checking your own skin regularly for any abnormal growths or unusual changes. A skin self-exam helps find any suspicious skin problems early. The earlier skin cancer is diagnosed, the better chance you will have for a cure.

How the Test is Performed?

The National Cancer Institute (NCI) and the American Academy of Dermatology (AAD) recommend that people perform a skin self-exam once a month. The easiest time to do the exam may be after you take a bath or shower. Women may wish to perform their skin self-exam when they do their monthly breast self-exam. Men may want to do the skin self-exam when they perform their monthly testicular self-exam.

Ideally, the room should have a full-length mirror and bright lights so that you can see your entire body.

When you are performing the skin self-exam, look for:

- New skin markings (moles, blemishes, changes in color, bumps)
- Moles that have changed in size, texture, color, or shape
- Moles or sores that continue to bleed or won't heal
- Moles with uneven edges, differences in color, or lack of even sides (symmetry)
- Any mole or growth that looks very different from other skin growths

Experts recommend that you examne your skin in the following way:

- Look closely at your entire body, both front and bac, in the mirror.
- Check under your arms and on both sides of each arm.
- Examine your forearms after bending your arms at the elbows, and then look at the palms of your hands and underneath your upper arms.
- Look at the front and back of both legs.
- Look at your buttocks and between your buttocks.
- Examine your genital area.
- Look at your face, neck, back of the neck, and scalp. It is best to use both a hand mirror and full-length mirror, along with a comb, to see areas of your scalp.
- Look at your feet, including the soles and the space between your toes.
- Have a person you trust help by examining hard-to-see areas.

Considerations

Always tell your doctor if:

- You have any new or unusual sores or spots on your skin
- A mole or skin sore changes in size, color, or texture
- You have a sore that does not heal

Skin Turgor

Skin turgor is the skin's ability to change shape and return to normal (elasticity).

Considerations

Skin turgor is a sign commonly used by health care workers to assess the degree of fluid loss or dehydration. Fluid loss can occur from common conditions, such as diarrhea or vomiting. Infants and young children with vomiting, diarrhea, and decreased or no fluid intake can rapidly lose a significant amount of fluid. Fever speeds up this process.

To determine skin turgor, the health care provider grasps the skin on the back of the hand, lower arm, or abdomen between two fingers so that it is tented up. The skin is held for a few seconds then released. Skin with normal turgor snaps rapidly back to its normal position. Skin with

decreased turgor remains elevated and returns slowly to its normal position.

Decreased skin turgor is a late sign in dehydration. It occurs with moderate to severe dehydration. Fluid loss of 5% of the body weight is considered mild dehydration, 10% is moderate, and 15% or more is severe dehydration.

Note: Edema (a buildup of fluid in the tissues that causes swelling) causes the skin to be extremely difficult to pinch up.

Causes

- Decreased fluid intake
- Dehydration
- Diarrhea
- Diabetes
- Extreme weight loss
- Heat stroke (excessive sweating without enough fluid intake)
- Vomiting

Connective tissue disorders, such as scleroderma and Ehlers-Danlos syndrome, can also affect the elasticity of the skin. This does not have to do with fluids, but is a change in the elastic properties of the skin tissue.

Home Care

A quick check of skin turgor by pinching the skin over the back of the hand, on the abdomen, or over the front of the chest under the collarbone is a good way to check for dehydration at home.

Mild dehydration will cause the skin to be slightly slow in its return to normal. To rehydrate, drink more fluids — particularly water.

If turgor is severe, indicating moderate or severe dehydration, see your health care provider immediately.

When to Contact a Medical Professional?

- Poor skin turgor occurs with vomiting, diarrhea, or fever.
- During a check of skin turgor the skin is very slow to return to normal, or the skin “tents” up. This can indicate dehydration that is severe enough to require immediate treatment.
- You have reduced skin turgor and are unable to increase your intake of flids (for example, because of vomiting).

What to Expect at Your Office Visit?

The health care provider will perform a physical exam and ask questions about your medical history, including:

- How long have you had symptoms?
- What other symptoms came before the change in skin turgor (vomiting, diarrhea, others)?
- What have you done to try to treat the condition?
  - Did it make it better?
  - Is it getting worse?
- What other symptoms do you have (such as dry lips, decreasedurine output, and decreased tearing)?

Tests that may be performed:

- Blood chemistry (such as a chem-20)
- CBC
- Urinalysis

Intravenous fluids may be necessary for severe dehydration. You may need medications to treat other conditions that affect skin turgor and elasticity.

Alternative Names

Doughy skin; Poor skin turgor; Good skin turgor; Decreased skin turgor

Clinical Specimen Collection and Handling Unexplained Critical Illnesses and Deaths

Testing provided through the Unexplained Critical Illness and Deaths Project for unexplained deaths that appear likely to have infectious etiologies will be primarily pathology-directed. Therefore, it is important when possible to provide optimal pathology specimens.

These clinical specimens may be useful in determining an etiology for cases of unexplained illness or death that are likely to have infectious causes. These specimens should be saved for possible cases. While timing of collection and volume of some specimens may not be optimal, it may still be possible to use them for testing.

On this page:

- Types of specimens

- Specimen collection and handling
- Laboratory specimens
- Shipping

Types of Specimens

- Two types of specimens are needed in order to provide acomplete evaluation:
  - Fixed tissue
  - Fresh frozen tissue
- Body fluids are also helpful
- A nasopharyngeal swab can also be collected if a respiratory infection is suspected

Specimen Collection and Handling

Ideally, tissue for CDC would be collected separately at the time of autopsy and would include tissue from each organ and from several areas of each organ. Fixed tissue from multiple organs can be combined in one container.

If specimens are limited, priority should be given to providing fixed tissue, especially from the primary affected organ (for example, brain tissue for a meningoencephalitis case).

If a specifically affected area is noted by gross pathology or preliminary histopathology, it is helpful to submit tissue from this area.

Fixed tissue:

- Fixed tissue should either be in 10% buffered formalin or paraffin-embedded
- Fixed tissue is used for routine H&E stains and special stains as well as immunohistochemistry and in situ hybridazation.
- If fixed tissue is submitted to CDC more than 2 weeks after it is collected, paraffin-embedded tissue is preferred for submission.
- Over-fixation will form a strong formalin bond in tissue and make antigen retrieval more difficult.
- Blocks rather than slides are also preferred since epitopes and nucleic acids can degenerate more quickly once tissue is prepared for slides thus decreasing sensitivity of special assays.
- Fixed tissue from multiple organs can be combined in one container.

- Fixed tissue should be stored and shipped at room temperature.

Do not freeze fixed tissue.

Fresh frozen tissue :

- Frozen tissue is used for culture and molecular techniques including specific primer and consensus polymerase chain reaction (PCR).
- Specimens should be collected aseptically and as soon as possible after death.
- A separate sterile instrument should be used for each collection site and each specimen should be placed in a separate sterile container in small amounts of viral transport media or saline.
- Frozen tissue should be stored at -70° C and shipped on dry ice.

Body fluids:

- 5-10 cc of cerebrospinal fluid and 5 cc of heart blood (in a marbled red tube top) collected postmortem may also be used for testing.
- Body fluid specimens should be kept refrigerated and shipped with a cold pack (or dry ice if specimens are being sent directly to CDC).
- Please refer to the laboratory specimens chart for information on collection and handling of body fluid specimens obtained prior to death.

Nasopharyngeal swab:

- If a respiratory infection is suspected, a postmortem nasopharyngeal swab can be collected and sent to the Minnesota Department of Health (MDH) in a sterile container with viral transport media for viral culture.
- Laboratory Specimens: Collection and Handling of Clinical Specimens.

A chart listing clinical specimens, optimal timing of collection, optimal volume, container type, and temperature for storing and shipping.

Shipping

- Each specimen container should be labeled with a case identification number assigned by MDH (call 651-201-5414 for a case number).

- A pathology/autopsy report should accompany pathology specimens.
- Identifying information such as name, address, and medical record number may be removed. Preliminary reports are acceptable.
- A list of specimens being sent should also be included.
  - Fixed tissue should be shipped in a separate package from body fluids and/or frozen tissue.
  - Fixed tissue should be sent at room temperature while frozen tissue and body fluid should be sent on dry ice.
- Specimens may be sent directly to CDC or arrangements may be made for specimens to be picked up or delivered to MDH where they will be forwarded to CDC.
- Please call 651-201-5414 for more information on shipping.

Microbiology

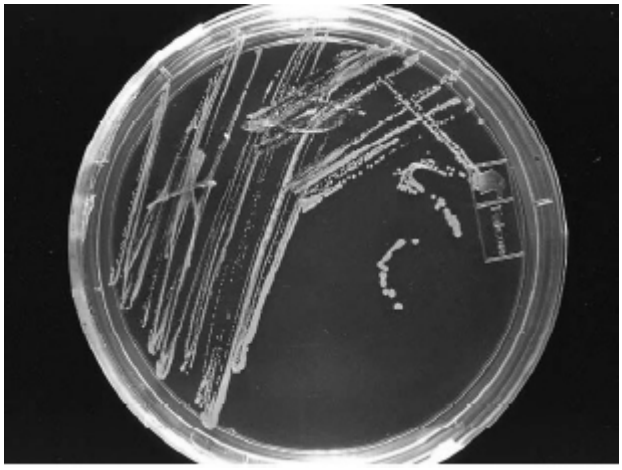

Figure: *An agar plate streaked with microorganisms*

Microbiology is the study of microscopic organisms, which are defined as any living organism that is either a single cell (unicellular), a cell cluster, or has no cells at all (acellular). This includes eukaryotes, such as fungi and protists, and prokaryotes. Viruses and prions, though not strictly classed as living organisms, are also studied. Microbiology typically includes the study of the immune system, or immunology. Generally, immune systems interact with pathogenic microbes; these two disciplines often intersect which is why many colleges offer a paired degree such as “Microbiology and Immunology”.

Microbiology is a broad term which includes virology, mycology, parasitology, bacteriology, immunology and other branches. A microbiologist is a specialist in microbiology and these related topics.

Microbiological procedures usually must be aseptic, and use a variety of tools such as light microscopes with a combination of stains and dyes. The most commonly used stains are called basic dyes, and are composed of positively charged molecules. Two types of basic dyes are simple stains and differential stains. Simple stains consist of one dye and identify the shape and multicell arrangement of bacteria. Methylene blue, carbolfuchsin, safranin, and crystal violet are some of the most commonly used stains. Differential stains on the other hand, use two or more dyes and help us to distinguish between two or more organisms or two or different parts of the organism.

Types of differential stains are gram, Ziehl-Neelsen acid fast, negative, flagella, and endospore. Specific constraints apply to particular fields of microbiology, such as parasitology, which heavily utilizes the light microscopy, whereas microscopy's utility in bacteriology is limited due to the similarity is many cells physiology. Indeed, most means of differentiating bacteria is based on growth or biochemical reactions. Virology has very little need for light microscopes, relying on almost entirely molecular means. Mycology relies on all technologies the most evenly, from macroscopy to molecular techniques.

Microbiology is actively researched, and the field is advancing continuously. It is estimated that only about one percent of the microorganisms present in a given environmental sample are culturable and the number of bacterial cells and species on Earth is still not possible to be determined, recent estimates indicate that it can be extremely high (5 Exp 30 clls on Earth, unknown number of species). Although microbes were directly observed over three hundred years ago, the precise determination, quantitation and description of its functions is far to be complete, given the overwhelming diversity detected by genetic and culture-independent means.

History

Ancient

The existence of microorganisms was hypothesized for many centuries before their actual discovery. The existence of unseen microbiological life was postulated by Jainism which is based on

Mahavira's teachings as early as 6th century BCE. Paul Dundas notes that Mahavira asserted existence of unseen microbiological creatures living in earth, water, air and fire.

Jain scriptures also describe nigodas which are sub-microscopic creatures living in large clusters and having a very short life and are said to pervade each and every part of the universe, even in tissues of plants and flesh of animals. The Roman Marcus Terentius Varro made references to microbes when he warned against locating a homestead in the vicinity of swamps "because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there by cause serious diseases."

In 1546 Girolamo Fracastoro proposed that epidemic diseases were caused by transferable seedlike entities that could transmit infection by direct or indirect contact, or vehicle transmission.

However, early claims about the existence of microorganisms were speculative, and not based on microscopic observation. Actual observation and discovery of microbes had to await the invention of the microscope in the 17th century.

Modern

In 1676, Anton van Leeuwenhoek observed bacteria and other microrganisms, using a single-lens microscope of his own design. Whil Van Leeuwenhok is often cited as the first to observe microbes, Robert Hooke made the first recorded microscopic observation, of the fruiting bodies of molds, in 1665. The first observation of microbes using a microscope is generally credited to the Dutch draper and haberdasher, Antonie van Leeuwenhoek, who lived for most of his life in Delft, Holland.

It has, however, been suggested that a Jesuit priest called Athanasius Kircher was the first to observe micro-organisms. He was among the first to design magic lanterns for projection purposes, so he must have been well acquainted with the properties of lenses. One of his books contains a chapter in Latin, which reads in translation – 'Concerning the wonderful structure of things in nature, investigated by Microscope.

Here, he wrote 'who would believe that vinegar and milk abound with an innumerable multitude of worms.' He also noted that putrid material is full of innumerable creeping animalcule. These observations antedate Robert Hooke's Micrographia by nearly 20 years an were published some 29 years before van Leeuwenhoek saw protozoa and 37

years before he described having seen bacteria. The field of bacteriology (later a subdiscipline of microbiology) was founded in the 19th century by Ferdinand Cohn, a botanist whose studies on algae and photosynthetic bacteria led him to describe several bacteria including *Bacillus* and *Beggiatoa*.

Cohn was also the first to formulate a scheme for the taxonomic classification of bacteria and discover spores. Louis Pasteur and Robert Koch were contemporaries of Cohn's and are often considered to be the father of microbiology and medical microbiology, respectively. Pasteur is most famous for his series of experiments designed to disprove the then widely held theory of spontaneous generation, thereby solidifying microbiology's identity as a biological science. Pasteur also designed methods for food preservation (pasteurization) and vaccines against several diseases such as anthrax, fowl cholera and rabies.

Koch is best known for his contributions to the germ theory of disease, proving that specific diseases were caused by specific pathogenic micro-organisms. He developed a series of criteria that have become known as the Koch's postulates. Koch was one of the first scientists to focus on the isolation of bacteria in pure culture resulting in his description of several novel bacteria including *Mycobacterium tuberculosis*, the causative agent of tuberculosis.

While Pasteur and Koch are often considered the founders of microbiology, their work did not accurately reflect the true diversity of the microbial world because of their exclusivefocus on microorganisms having direct medical relevance. It was not until the late 19th century and the work of Martinus Beijerinck and Sergei Winogradsky, the founders of general microbiology (an older term encompassing aspects of microbial physiology, diversity and ecology), that the true breadth of microbiology was revealed.

Beijerinck made two major contributions to microbiology: the discovery of viruses and the development of enrichment culture techniques. While his work on the Tobacco Mosaic Virus established the basic principles of virology, it was his development of enrichment culturing that had the most immediate impact on microbiology by allowing for the cultivation of a wide range of microbes with wildly different physiologies.

Winogradsky was the first to develop the concept of chemolithotrophy and to thereby reveal the essential role played by micro-organisms in geochemical processes. He was responsible for the

first isolation and description of both nitrifying and nitrogen-fixing bacteria.

Branches

The branches of microbiology can be classified into pure and applied sciences. Microbiology can be also classified based on taxonomy, in the cases of bacteriology, mycology, protozoology, and phycology. There is considerable overlap between the specific branches of microbiology with each other and with other disciplines.

Pure Microbiology

Taxonomic arrangement:

- Bacteriology: The study of bacteria.
- Mycology: The study of fungi.
- Protozoology: The study of protozoa.
- Phycology (or algology): The study of algae.
- Parasitology: The study of parasites.
- Immunology: The study of the immune system.
- Virology: The study of the viruses.
- Nematology: The study of the nematodes Integrative arrangement:
- Microbial cytology: The study of microscopic and submicroscopic details of microorganisms.
- Microbial physiology: The study of how the microbial cell functions biochemically. Includes the study of microbial growth, microbial metabolism and microbial cell structure.
- Microbial ecology: The relationship between microorganisms and their environment.
- Microbial genetics: The study of how genes are organized and regulated in microbes in relation to their cellular functions. Closely related to the field of molecular biology.
- Cellular microbiology: A discipline bridging microbiology and cell biology.
- Evolutionary microbiology: The study of the evolution of microbes. This field can be subdivided into:

- Microbial taxonomy: The naming and classification of microorganisms.
- Microbial systematics: The study of th diversity an genetic relationship of microorganisms.

- Generation microbiology: The study of those microorganisms that have the same characters as their parents.
- Systems microbiology: A discipline bridging systems biology and microbiology.
- Molecular microbiology: The study of the molecular principles of the physiological processes in microorganisms.

Other:

- Nano microbiology: The study of those microgasims
- Exo microbiology (or Astro microbiology): The study of microorganisms in outer space.

Applied Microbiology

- *Medical Microbiology:* The study of the pathogenic microbes and the role of microbes in human illness. Includes the study of microbial pathogenesis and epidemiology and is related to the study of disease pathology and immunology.
- *Pharmaceutical Microbiology*: The study of microorganisms that are related to the production of antibiotics, enzymes, vitamins,vaccines, and other pharmaceutical products and that cause pharmaceutical contamination and spoil.
- *Industrial Microbiology:* The exploitation of microbes for use in industrial processes. Examples include industrial fermentation and wastewater treatment. Closely linked to the biotechnology industry. This field also includes brewing, an important application of microbiology.
- *Microbial Biotechnology:* The manipulation of microorganisms at the genetic and molecular level to generate useful products.
- *Food Microbiology and Dairy Microbiology*: The study of microorganisms causing food spoilage and foodborne illness. Using microorganisms to produce foods, for example by fermentation.
- *Agricultural Microbiology:* The study of agriculturally relevant microorganisms. This field can be further classified into the following:

- *Plant Microbiology and Plant Pathology:* The study of the interactions between microorganisms and plants and plant pathogens.
- *Soil Microbiology:* The study of those microorganisms that are found in soil.

- *Veterinary Microbiology:* The study of the role in microbes in veterinary medicine or animal taxonomy.
- *Environmental Microbiology:* The study of the function and diversity of microbes in their natural environments. This involves the characterization of key bacterial habitats such as the rhizosphere and phyllosphere, soil and groundwater ecosystems, open oceans or extreme environments (extremophiles). This field includes other branches of microbiology such as:
  - Microbial ecology
  - Microbially-mediated nutrient cycling
  - Geomicrobiology
  - Microbial diversity
  - Bioremediation
- *Water microbiology (or Aquatic microbiology):* The study of those microorganisms that are found in water.
- *Aeromicrobiology (or Air microbiology):* The study of airborne microorganisms.
- *Epidemiology:* The study of the incidence, spread, and control of disease.

Benefits

Whilst there are undoubtedly some who fear all microbes due to the association of some microbes with various human illnesses, many microbes are also responsible for numerous beneficial processes such as industrial fermentation (e.g. the production of alcohol, vinegar and dairy products), antibiotic production and as vehicles for cloning in more complex organisms such as plants.

Scientists have also exploited their knowledge of microbe to produce biotechnologically important enzymes such as Taq polymerase, reporter genes for use in other genetic systems and novel molecular biology techniques such as the yast two-hybrid system.

Bacteria can be used for the industrial production of amino acids. *Corynebacterium glutamicum* is one of the most important bacterial

species with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine.

A variety of biopolymers, such as polysaccharides, polyesters, and polyamides, are produced by microorganisms. Microorganisms are used for the biotechnological production of biopolymers with tailored properties suitable for high-value medical application such as tissue engineering and drug delivery.

Microorganisms are used for the biosynthesis of xanthan, alginate, cellulose, cyanophycin, poly (gamma-glutamic acid), levan, hyaluronic acid, organic acids, oligosaccharides and polysaccharide, and polyhydroxyalkanoates.

Microorganisms are beneficial for microbial biodegradation or bioremediation of domestic, agricultural and industrial wastes and subsurface pollution in soils, sediments and marine environments. The ability of each microorganism to degrade toxic waste depends on the nature of each contaminant. Since sites typically have multiple pollutant types, the most effective approach to microbial biodegradation is to use a mixture of bacterial species and strains, each specific to the biodegradation of one or more types of contaminants.

There are also various claims concerning the contributions to human and animal health by consuming probiotics (bacteria potentially beneficial to the digestive system) and/or prebiotics (substances consumed to promote the growth of probiotic microorganisms).

Recent research has suggested that microorganisms could be useful in the treatment of cancer. Various strains of non-pathogenic clostridia can infiltrate and replicate within solid tumors. Clostridial vectors can be safely administered and their potential to deliver therapeutic proteins has been demonstrated in a variety of preclinical models.

Chapter 10: Essentials of Medical Laboratory Practice

A medical laboratory or clinical laboratory is a laboratory where tests are done on clinical specimens in order to get information about the health of a patient as pertaining to the diagnosis, treatment, and prevention of disease.

Figure: *Clinical laboratory in a Hospital setting showing several automated analysers.*

Departments

Laboratory medicine is generally divided into two sections, and each of which is further divided into a number of units. These two sections are:

- *Anatomic Pathology:* units included here are histopathology, cytopathology, and electron microscopy. Academically, each unit is studied alone in one course. Other courses pertaining to this section include anatomy, physiology, histology, pathology, and pathophysiology.
- Clinical pathology, including :
  - *Clinical Microbiology:* This encompasses five different sciences (units). These include bacteriology, virology, parasitology, immunology, and mycology.

- *Clinical Chemistry:* Units under this busy section include instrumental analysis of blood components, enzymology, toxicology and endocrinology.
- *Hematology:* This section consists of automated and manual analysis of blood cells. It includes two subunits, which are coagulation and blood bank.
- Genetics is also studied along with a subspecialty known as cytogenetics.
- *Reproduction biology :* Semen analysis, Sperm bank and assisted reproductive technology.

Distribution of clinical laboratories in health institutions varies greatly from one place to another. Take for example microbiology, some health facilities have a single laboratory for microbiology, while others have a separate lab for each unit, with nothing called a "microbiology" lab.

Here's a detailed breakdown of the responsibilities of each unit:

- Microbiology receives almost any clinical specimen, including swab, feces, urine, blood, sputum, cerebrospinal fluid, synovial fluid, as well as possible infected tissue. The work here is mainly concerned with cultures, to look for suspected pathogens which, if found, are further identified based on biochemical tests. Also, sensitivity testing is carried out to determine whether the pathogen is sensitive or resistant to a suggested medicine. Results are reported with the identified organism(s) and the type and amount of drug(s) that should be prescribed for the patient.
- Parasitology is a microbiology unit that investigates parasites. The most frequently encountered specimen here is faeces. However, blood, urine, sputum, and other samples may also contain parasites.
- Virology is concerned with identification of viruses in specimens such as blood, urine, and cerebrospinal fluid.
- Hematology works with whole blood to do full blood counts, and blood films as well as many other specialised tests.
- Coagulation requires citrated blood samples to analyze blood clotting times and coagulation factors.
- Clinical Biochemistry usally receives serum or pasma. They test the serum for chemicals present in blood. These include a wide

array of substances, such as lipids, blood sugar, enzymes, and hormones.

- Toxicology mainly tests for pharmaceuticl and recreational drugs. Urine and blood samples are submitted to this lab.
- Immunology/Serology uses the concept of antigen-antibody interaction as a diagnostic tool. Compatibility of transplanted organs is also determined.
- Immunohaematology, or Blood bank determines blood groups, and performs compatibility testing on donor blood and recipients. It also prepares blood components, derivatives, and products for transfusion. Regulated by the FDA since giving blood is considered a drug, this unit determines a patient's blood type and Rh status, checks for antibodies to common antigens found on red blood cells, and cross matches units that are negative for the antigen.
- Urinalysis tests urine for many analytes. Some health care providers have a urinalysis laboratory, while others don't. Instead, each component of the urinalysis is performed at the corresponding unit. If measuring urine chemicals is required, the specimen is processed in the clinical biochemistry lab, but if cell studies are indicated, the specimen should be submitted to the cytopathology lab, and so on.
- Histopathology processes solid tissue removed from the body (biopsies) for evaluation at the microscopic level.
- Cytopathology examines smears of cells from all over the body (such as from the cervix) for evidence of inflammation, cancer, and other conditions.
- Electron microscopy prepares specimens and takes micrographs of very fine details by means of TEM and SEM.
- Genetics mainly performs DNA analysis.
- Cytogenetics involves using blood and other cells to get a karyotype. This can be helpful in prenatal diagnosis (e.g. Down's syndrome) as well as in cancer (some cancers have abnormal chromosomes).
- Surgical pathology examines organs, limbs, tumors, fetuses,and other tissues biopsied in surgery such as breastmastectomys.

Medical Laboratory Staff

The staff of clinical laboratories may include:

- Pathologist,
- Clinical Biochemist
- Pathologists' assistant (PA)
- Biomedical Scientist (BMS) in the UK, also known as
- Medical Laboratory Scientist (MT, MLS or CLS) in the US, also known as
- Medical Laboratory Technologist (MLT) in Canada,
- Medical Laboratory Technician (MLT in US),
- Medical Laboratory Assistant (MLA),
- Phlebotomist (PBT),
- Transcriptionist,
- Specimen processor, Secretary.

The laboratory management often consists of:

- Laboratory Medical Director
- Laboratory Manager
- Department/Technical Supervisor
- Chief/Lead Technical Staff

Some of these titles may not exist in some countries or may have different names. In France, clinical biologists may also be Medical director and laboratory manager.

Types of Laboratory

In many countries, there are two main types of labs that process the majority of medical specimens. Hospital laboratories are attached to a hospital, and perform tests on patients. Private (or community) laboratories receive samples from general practitioners, insurance companies, clinical research sites and other health clinics for analysis. These can also be called reference laboratories where more unusual and obscure tests are performed.

For extremely specialised tests, samples may go to a research laboratory. A lot of samples are sent between different labs for uncommon tests. It is more cost effective if a particular laboratory specializes in a rare test, receiving specimens (and money) from other labs, while sending away tests it cannot do.

In many countries there are mainly three types of Medical Laboratories as per the types of investigations carried out. 1. Clinical

Pathology 2. Clinical Microbiology & 3. Clinical Biochemistry laboratories. 1. Clinical Pathology: Haematology, Histopathology, Cytology, Routine Pathology 2. Clinical Microbiology: Bacteriology, Mycobacteriology, Virology, Mycology, Parasitology, Immunology, Serology. 3. Clinical Biochemistry: Biochemical analysis, Hormonal assays etc. Blood Banks:- Blood bank is a separate body. Its laboratory need Microbiological analysis for infectious diseases that may be found in blood. Pathology to observe Blood grouping, Haematology & cross matching reactions. It also involves PRO department for the communication & contact for blood donations etc.. Molecular diagnostic lab or cyotgenetics and molecular biology lab is the latest addition to the three types of medical laboratories listed above in many countries.

Specimen Processing and Work Flow

Sample processing will usually start with a set of samples and a request form. Typically a set of vacutainer tubes containing blood, or any other specimen, will arrive to the laboratory in a small plastic bag, along with the form.

The form and the specimens are given a laboratory number. The specimens will usually all receive the same number, often as a sticker that can be placed on the tubes and form. This label has a barcode that can be scanned by automated analyzers and test requests uploaded from the LIS. Entry of requests onto a laboratory management system involves typing, or scanning (where barcodes are used) in the laboratory number, and entering the patient identification, as well as any tests requested. This allows laboratory machines, computers and staff to know what tests are pending, and also gives a place (such as a hospital department, doctor or other customer) for results to go.

For biochemistry samples, blood is usually centrifuged and serum is separated. If the serum needs to go on more than one machine, it can be divided into separate tubes. Many specimens end up in one or more sophisticated automated analysers, that process a fraction of the sample and return one or more “results”. Some laboratories use robotic sample handlers (Laboratory automation) to optimize the workflow and reduce contamination risk and sample handling of the staff. The work flow in a lab is usually heavy from 2:00 am to 10:00 am. Nurses and doctors generally have their patients tested at least once a day with general complete blood counts and chemistry profiles. These orders are then drawn during a morning run by phlebotomists for results to be available in the patient’s charts for the attending physicians to consult during their

morning rounds. Another busy time for the lab is after 3:00 pm when private practice physician offices are closing. Couriers will pick up specimens that have been drawn throughout the day and deliver them to the lab. Also, couriers will stop at outpatient drawing centers and pick up specimens. These specimens will be processed in the evening and overnight to ensure results will be available the following day.

Laboratory Informatics

Laboratories today are held together by a system of software programs and computers that exchange data about patients, test requests, and test results known as a Laboratory information system or LIS. The LIS is interfaced with the hospital information system.

This system enables hospitals and labs to order the correct test requests for each patient, keep track of individual patient or specimen histories, and help guarantee a better quality of results as well as printing hard copies of the results for patient charts and doctors to check.

Result Analysis, Validation and Interpretation

According to ISO 15189 norm, all pathological results must be verified by a competent professional. In some countries staff like clinical scientists do the majority of this work inside the laboratory with abnormal results referred to the relevant pathologist. In others, only medical staff (pathologist or clinical biologist) is concerned by this phase. It can be assisted by some software in order to validate normal or non modified results. Medical staff are sometimes also required in order to explain pathology results to physicians. For a simple result given by phone or for a technical problem it's a medical technologist or medical lab scientist explaining it to a registered nurse.

Departments in some countries are exclusively directed by a specialized Pathologist, in others a consultant, medical or non-medical, may be the Head of Department. Clinical Scientists have the right to interpret and discuss pathology results in their discipline in many countries, in Europe they are qualified to at least Masters level, may have a PhD and can have an exit qualification equivalent to medical staff e.g. FRCPath in the UK. In France only medical staff (Pharm.D. and M.D. specialized in Anatomical pathology or Clinical biology) can discuss pathological results, clinical scientists are not considered as a part of medical staff.

Medical Laboratory Accreditation

Credibility of medical laboratories is paramount to the health and safety of the patients relying on the testing services provided by these labs. The international standard in use today for the accreditation of medical laboratories is ISO 15189 - Medical laboratories - particular requirements for quality and competence.

Accreditation is done by the Joint Commission, College of American Pathologists, AAB (American Association of Bioanalysts), and other state and federal agencies. CLIA 88 or the Clinical Laboratory Improvement Amendments also dictate testing and personnel. The accrediting body in Australia is NATA, all laboratories must be NATA accredited to receive payment from Medicare. In France, where accrediting body is COFRAC, in 2010, modification of legislation established ISO 15189 accreditation as an obligation for all clinical laboratories.

Parasitology

Parasitology is the study of parasites, their hosts, and the relationship between them. As a biological discipline, the scope of parasitology is not determined by the organism or environment in question, but by their way of life. This means it forms a synthesis of other disciplines, and draws on techniques from fields such as cell biology, bioinformatics, biochemistry, molecular biology, immunology, genetics, evolution and ecology.

Fields

The study of these diverse organisms means that the subject is often broken up into simpler, more focused units, which use common techniques, even if they are not studying the same organisms or diseases. Much research in parasitology falls somewhere between two or more of these definitions. In general, the study of prokaryotes falls under the field of bacteriology rather than parasitology.

Medical Parasitology

One of the largest fields in parasitology, medical parasitology is the subject which deals with the parasites that infect humans, the diseases caused by them, clinical picture and the response generated by humans against them. It is also concerned with the various methods of their diagnosis, treatment and finally their prevention & control. A parasite is

an organism that live on or within another organism called the host. These include organisms such as:

- *Plasmodium* spp., the protozoan parasite which causes malaria. The four species of malaria parasites infective to humans are *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* & *Plasmodium ovale*.
- *Leishmania donovani*, the unicellular organism which causes leishmaniasis
- *Entamoeba* and *Giardia*, which cause intestinal infections (dysentery and diarrhoea)
- Multicellular organisms and worms such as *Schistosoma* spp., *Wuchereria bancrofti*, *Necator americanus* (hookworm) and *Taenia* spp. (tapeworm)
- Ectoparasites such as ticks, scabies and lice

Medical parasitology can involve drug development, epidemiological studies and study of zoonoses.

Veterinary Parasitology

The study of parasites that cause economic losses in agriculture or aquaculture operations, or which infect companion animals. Examples of species studied are:

- *Lucilia sericata*, a blowfly, which lays eggs on the skins of farm animals. The maggots hatch and burrow into the flesh, distressing the animal and causing economic loss to the farmer
- *Otodectes cynotis*, the cat ear mite, responsible for Canker.
- *Gyrodactylus salaris*, a monogenean parasite of salmon, which can wipe out populations which are not resistant.

Structural Parasitology

This is the study of structures of proteins from parasites. Determination of parasitic protein structures may help to better understand how these proteins function differently from homologous proteins in humans. In addition, protein structures may inform the process of drug discovery.

Quantitative Parasitology

Parasites exhibit an aggregated distribution among host individuals, thus the majority of parasites live in the minority of hosts. This feature forces parasitologists to use advanced biostatistical methodologies.

Parasite Ecology

Parasites can provide information about host population ecology. In fisheries biology, for example, parasite communities can be used to distinguish distinct populations of the same fish species co-inhabiting a region. Additionally, parasites possess a variety of specialized traits and life-history strategies that enable them to colonize hosts. Understanding these aspects of parasite ecology, of interest in their own right, can illuminate parasite-avoidance strategies employed by hosts

Conservation Biology of Parasites

Conservation biology is concerned with the protection and preservation of vulnerable species, including parasites. A large proportion of parasite species are threatened by extinction, partly due to efforts to eradicate parasites which infect humans or domestic animals, or damage human economy, but also caused by the decline or fragmentation of host populations and the extinction of host species.

Taxonomy and Phylogenetics

The huge diversity between parasitic organisms creates a challenge for biologists who wish to describe and catalogue them. Recent developments in using DNA to identify separate species and to investigate the relationship between groups at various taxonomic scales has been enormously useful to parasitologists, as many parasites are highly degenerate, disguising relationships between species.

Sputum

Sputum is mucus that is coughed up from the lower airways. It is usually used for microbiological investigations of respiratory infections.

The best sputum samples contain very little saliva, as this contaminates the sample with oral bacteria. This event is assessed by the clinical microbiologist by examining a Gram stain of the sputum. More than 25 squamous epithelial cells at low enlargement indicates salivary contamination.

When a sputum specimen is plated out, it is best to get the portion of the sample that most looks like pus onto the swab. If there is any blood in the sputum, this should also be on the swab.

Microbiological sputum samples are usually used to look for infections by *Moraxella catarrhalis*, *Mycobacterium tuberculosis*,

Streptococcus pneumoniae and *Haemophilus influenzae*. Other pathogens can also be found.

Purulent Sputum contains a lot of pus, composed of white blood cells, cellular debris, dead tissue, serous fluid and viscous liquid (mucus).

Mostly, it's yellow in color, as well as green. That is always seen in cases of bronchiectasis, lung abscess, or advanced stage of bronchitis, acute upper respiratory tract infection (cold, laryngitis).

Sputum can be:

1. Bloody (Hemoptysis)
    1. blood-streaked sputum - inflammation of throat, bronchi; lung cancer;
    2. Pink sputum - sputum evenly mixed with blood, from alveoli, small bronchi;
    3. massive blood - cavitary tuberculosis of lung, lung abscess, bronchiectasis, infarction, embolism.
2. Rusty colored - usually caused by pneumococcal bacteria (in pneumonia)
3. Purulent - containing pus. The color can provide hints as to effective treatment in Chronic Bronchitis Patients:
    1. a yellow-greenish (mucopurulent) color suggests that treatment with antibiotics can reduce symptoms. Green color is caused by Neutrophil Myeloperoxidase.
    2. a white, milky, or opaque (mucoid) appearance often means that antibiotics will be ineffective in treating symptoms. (This information may correlate with the presence of bacterial or viral infections, though current research does not support that generalization.)
4. Foamy white - may come from obstruction or even edema.
5. Frothy pink - pulmonary edema

Laboratory Specimen

In medicine, a laboratory specimen is a biological specimen taken by sampling, that is, gathered matter of a medical patient's tissue, fluid, or other material derived from the patient used for laboratory analysis to assist differential diagnosis or staging of a disease process. Common

examples include throat swabs, sputum, urine, blood, surgical drain fluids, tissue biopsies, *etc.*

In zoology, a laboratory specimen is a sample of a species which is preserved and made available to researchers for elucidating the structure, general appearance, various organs, and details related to the specimen's body.

A specimen can also refer to a sample of any other substance taken by sampling, for example - soil, air, chemicals, entire living creatures, or anything else which can be studied to represent a whole entity.

Laboratory Techniques in Immunology

Various laboratory techniques exist that rely on the use of antibodies to visualize components of microorganisms or other cell types and to distinguish one cell or organism type from another.

Electrophoresis is a technique whereby the protein or carbohydrate components of microorganisms can be separated based upon their migration through a gel support under the driving influence of electricity. Depending upon the composition of the gel, separation can be based on the net charge of the components or on their size. Once the components are separated, they can be distinguished immunologically. This application is termed immunoelectrophoresis.

Immunoelectrophoresis relies upon the exposure of the separated components in the gel to a solution that contains an antibody that has been produced to one of the separated proteins. Typically, the antibody is generated by the injection of the purified protein into an animal such as a rabbit. For example, the protein that comprises the flagellar appendage of a certain bacteria can be purified and injected into the rabbit, so as to produce rabbit anti-flagellar protein.

Immunoelectrophoresis can be used in a clinical immunology laboratory in order to diagnose illness, especially those that alter the immunoglobulin composition of body fluids. Research immunology laboratories also employ immunoelectrophoresis to analyze the components of organisms, including microorganisms.

One example of an immunoelectrophoretic technique used with microorganisms is known as the Western Blot. Proteins that have been separated on a certain type of gel support can be electrically transferred to a special membrane. Application of the antibody will produce binding between the antibody and the corresponding antigen. Then, an antibody

generated to the primary antibody (for example, goat anti-rabbit antibody) is added. The secondary antibody will bind to the primary antibody. Finally, the secondary antibody can be constructed so that a probe binds to the antibody's free end. A chemical reaction produces a color change in the probe.

Thus, bound primary antibody is visualized by the development of a dark band on the support membrane containing the electrophoretically separated proteins. Various controls can be invoked to ensure that this reaction is real and not the result of an experimental anomaly.

A similar reaction can be used to detect antigen in sections of biological material. This application is known as immuno-histo-chemistry. The sections can be examined using either an electron microscope or a light microscope.

The preparation techniques differ for the two applications, but both are similar in that they ensure that the antigen is free to bind the added antibody. Preservation of the antigen binding capacity is a delicate operation, and one that requires a skilled technician. The binding is visualized as a color reaction under light microscopic illumination or as an increased electron dense area under the electron beam of the electron microscope.

The binding between antigen and antibody can be enhanced in light microscopic immunohistochemistry by the exposure of the specimen to heat. Typically a microwave is used. The heat energy changes the configuration of the antigen slightly, to ease the fit of the antigen with the antibody. However, the shape change must not be too great or the antibody will not recognize the altered antigen molecule.

Another well-establish laboratory immunological technique is known as enzyme-linked immunosorbent assay. The technique is typically shortened to ELISA. In the ELISA technique, antigen is added to a solid support. Antibody is flooded over the support. Where an antibody recognizes a corresponding antigen, binding of the two will occur. Next an antibody raised against the primary antibody is applied, and binding of the secondary antibody to the primary molecule occurs. Finally, a substrate is bound to a free portion of the secondary antibody, and the binding can be subsequently visualized as a color reaction. Typically, the ELISA test is

done using a plastic plate containing many small wells. This allows up to 100 samples to be tested in a single experiment. ELISA can reveal the presence of antigen in fluids such as a patient's serum, for example.

The nature of the antibody can be important in laboratory immunological techniques. Antibodies such as those raised in a rabbit or a goat are described as being polyclonal in nature. That is, they do recognize a certain antigenic region. But if that region is present on different molecules, the antibody will react with all the molecules. The process of monoclonal antibody production can make antigenic identification much more specific, and has revolutionized immunological analysis.

Monoclonal antibodies are targeted against a single antigenic site. Furthermore, large amounts of the antibody can be made. This is achieved by fusing the antibody-producing cell obtained from an immunized mouse with a tumor cell. The resulting hybrid is known as a hybridoma. A particular hybridoma will mass-produce the antibody and will express the antibody on the surface of the cell. Because hybridoma cells are immortal, they grow and divide indefinitely. Hence the production of antibody can be ceaseless.

Monoclonal antibodies are very useful in a clinical immunology laboratory, as an aid to diagnose diseases and to detect the presence of foreign or abnormal components in the blood. In the research immunology laboratory, monoclonal technology enables the specific detection of an antigenic target and makes possible the development of highly specific vaccines.

One example of the utility of monoclonal antibodies in an immunology laboratory is their use in the technique of flow cytometry. This technique separates sample as individual sample molecules pass by a detector. Sample can be treated with monoclonal antibody followed by a second treatment with an antibody to the monoclonal to which is attached a molecule that will fluoresce when exposed to a certain wavelength of light. When the labeled sample passes by the detector and is illuminated (typically by laser light of the pre-determined wavelength), the labeled sample molecules will fluoresce. These can be detected and will be shunted off to a special collection receptacle. Many sorts of analyses are possible using flow cytometry, from the distinguishing of one type of bacteria from another to the level of the genetic material comprising such samples.

Serology for Brucellosis

Serology for brucellosis is a blood test to look for antibodies against *Brucella*, the bacteria that causes the disease brucellosis.

Brucellosis is an infection that occurs from coming into contact with animals carrying *Brucella* bacteria.

How the Test is Performed?

A blood sample is needed. For information on how this is done, see: Venipuncture

The blood is then tested in a laboratory to look for antibodies. For *Brucella*, the serum agglutination test (SAT) is the simplest and most widely used testing method.

How to Prepare for the Test?

When the needle is inserted to draw blood, some people feel moderate pain, while others feel only a prick or stinging sensation. Afterward, there may be some throbbing.

Why the Test is Performed?

Your doctor may order this test if you have signs or symptoms of brucellosis. People working in jobs where they often come in contact with animals or meat, such as slaughterhouse workers, farmers, and veterinarians, are most likely to get this disease.

Normal Results

A normal (negative) result usually means you have not come in contact with the bacteria that causes brucellosis. However, this test may not detect the disease at an early stage. Your doctor may have you come back for another test in 10 days to 3 weeks.

Infection with other bacteria, such as *Yersinia*, *Francisella*, and *Vibrio*, and certain immunizations can cause false-positive results.

Normal value ranges may vary slightly among different laboratories. Some labs use different measurements or test different samples. Talk to your doctor about the meaning of your specific test results.

What Abnormal Results Mean?

An abnormal (positive) results usually means you have come in contact with the bacteria that causes brucellosis.

However, this does not mean that you have an active infection. Your doctor will repeat the test after a few weeks to see if the test result

increases. This is more likely to be a sign of a current infection.

Risks

Veins and arteries vary in size from one patient to another and from one side of the body to the other. Obtaining a blood sample from some people may be more difficult than from others.

Other risks associated with having blood drawn are slight but may include:

- Excessive bleeding
- Fainting or feeling light-headed
- Hematoma (blood accumulating under the skin)
- Infection (a slight risk any time the skin is broken)

Alternative Names

Brucella serology; Brucella antibody test or titer

Serology for Tularemia

This blood test looks for antibodies against *Francisella tularensis,* the bacteria that cause the disease tularemia.

How the Test is Performed?

Antibodies defend the body against some bacteria, viruses, fungi, or other foreign substances called antigens. Certain cells of the immune system cause the body to produce antibodies during an active infection.

When you first get sick, few antibodies may be detected. Antibody production increases during the course of an infection.

For this reason, antibody tests are often repeated several weeks after the first test is done, so the results can be compared. A rising level of antibodies tells the health care provider that there is an infection.

This test looks for antibodies to *F. tularensis* in the clear liquid portion of the blood, which is called the serum. (This technique is called serology.) The presence of these antibodies means you have a current or past infection with *F. tularensis*. In some cases, a single

How the Test Will Feel?

When the needle is inserted to draw blood, you may feel moderate pain, or only a prick or stinging sensation. Afterward, there may be some throbbing.

Normal Results

Antibodies are absent. However, during the first few days to weeks of exposure to an antigen, there may be slight antibody production. As the disease progresses, more antibodies will be present. If a disease is suspected, the test may need to be repeated several weeks after the first test.

Note: Normal value ranges may vary slightly among different laboratories. Talk to your doctor about the meaning of your specific test results

What Abnormal Results Mean?

If antibodies are detected, there has been exposure to *Francisella tularensis* (possible tularemia).

Risks

Veins and arteries vary in size from one patient to another and from one side of the body to the other. Obtaining a blood sample from some people may be more difficult than from others.

Other risks associated with having blood drawn are slight but may include:

- Excessive bleeding
- Fainting or feeling light-headed
- Hematoma (blood accumulating under the skin)
- Infection (a slight risk any time the skin is broken)

Considerations

A serology test can determine whether you have ever been exposed to a certain antigen, but this does not mean that you have a current infection. Increasing antibody levels are more likely to indicate a current infection.

Alternative Names

Tularemia test; Serology for Francisella tularensis

Serosanguineous

Serosanguineous means containing or relating to both blood and the liquid part of blood (serum). It usually refers to fluids collected from or

leaving the body. For example, fluid exiting a wound is serosanguineous, and usually is yellowish with small amounts of blood.

Immunopathology

Immunopathology is a branch of medicine that deals with immune responses associated with disease. It includes the study of the pathology of an organism, organ system, or disease with respect to the immune system, immunity, and immune responses.

It is a subspecialty of Clinical Pathology which consists in analysis of body fluids for detection of immune system diseases. In biology, it refers to damage caused to an organism by its own immune response, as a result of an infection.

Molecular Biology

Molecular biology is the branch of biology that deals with the molecular basis of biological activity. This field overlaps with other areas of biology and chemistry, particularly genetics and biochemistry.

Molecular biology chiefly concerns itself with understanding the interactions between the various systems of a cell, including the interactions between the different types of DNA, RNA and protein biosynthesis as well as learning how these interactions are regulated.

Writing in *Nature* in 1961, William Astbury described molecular biology as

not so much a technique as an approach, an approach from the viewpoint of the so-called basic sciences with the leading idea of searching below the large-scale manifestations of classical biology for the corresponding molecular plan.

It is concerned particularly with the *forms* of biological molecules and [...] is predominantly three-dimensional and structural—which does not mean, however, that it is merely a refinement of morphology. It must at the same time inquire into genesis and function.

Relationship to Other Biological Sciences

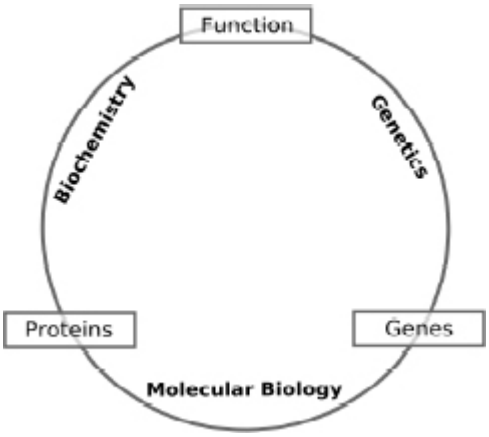

Figure: *Schematic relationship between biochemistry, genetics, and molecular biology*

Researchers in molecular biology use specific techniques native to molecular biology but increasingly combine these with techniques and ideas from genetics and biochemistry. There is not a defined line between these disciplines. The figure above is a schematic that depicts one possible view of the relationship between the fields:

- *Biochemistry* is the study of the chemical substances and vital processes occurring in living organisms. Biochemists focus heavily on the role, function, and structure of biomolecules. The study of the chemistry behind biological processes and the synthesis of biologically active molecules are examples of biochemistry.
- *Genetics* is the study of the effect of genetic differences on organisms. Often this can be inferred by the absence of a normal component (e.g. one gene). The study of "mutants" – organisms which lack one or more functional components with respect to the so-called "wild type" or normal phenotype. Genetic interactions (epistasis) can often confound simple interpretations of such "knock-out" studies.
- *Molecular biology* is the study of molecular underpinnings of the processes of replication, transcription, translation, and cell function. The central dogma of molecular biology where genetic material is transcribed into RNA and then translated into protein, despite being an oversimplified picture of molecular biology, still provides a good starting point for understanding the field. This picture, however, is undergoing revision in light of emerging novel roles for RNA.

Much of the work in molecular biology is quantitative, and recently much work has been done at the interface of molecular biology and computer science in bioinformatics and computational biology. As of the early 2000s, the study of gene structure and function, molecular genetics, has been among the most prominent sub-field of molecular biology.

Increasingly many other loops of biology focus on molecules, either directly studying their interactions in their own right such as in cell biology and developmental biology, or indirectly, where the techniques of molecular biology are used to infer historical attributes of populations or species, as in fields in evolutionary biology such as population genetics and phylogenetics. There is also a long tradition of studying biomolecules "from the ground up" in biophysics.

Techniques of Molecular Biology

Since the late 1950s and early 1960s, molecular biologists have learned to characterize, isolate, and manipulate the molecular components of cells and organisms. These components include DNA, the repository of genetic information; RNA, a close relative of DNA whose functions range from serving as a temporary working copy of DNA to actual structural and enzymatic functions as well as a functional and structural part of the translational apparatus; and proteins, the major structural and enzymatic type of molecule in cells. For more extensive list on protein methods, see protein methods. For more extensive list on nucleic acid methods, see nucleic acid methods.

Expression Cloning

Expression cloning is a technique in DNA cloning that uses expression vectors to generate a library of clones, with each clone expressing one protein. This *expression library* is then screened for the property of interest and clones of interest recovered for further analysis. An example would be using an expression library to isolate genes that could confer antibiotic resistance.

Expression Vectors

Expression vectors are a specialized type of cloning vector in which the transcriptional and translational signals needed for the regulation of the gene of interest are included in the cloning vector. The transcriptional and translational signals may be synthetically created to make the expression of the gene of interest easier to regulate.

Purpose

Usually the ultimate aim of expression cloning is to produce large quantities of specific proteins. To this end, a bacterial expression clone may include a ribosome binding site (Shine-Dalgarno sequence) to enhance translation of the gene of interest's mRNA, a transcription termination sequence, or, in eukaryotes, specific sequences to promote the post-translational modification of the protein product.

Chapter 11: Veterinary PCR Diagnostics in Veterinary Laboratory

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis.

The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of

temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting.

At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

PCR Principles and Procedure

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

A basic PCR set up requires several components and reagents. These components include:

- *DNA template* that contains the DNA region (target) to be amplified.
- Two *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70 °C.
- *Deoxynucleoside triphosphates* (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- *Divalent cations*, magnesium or manganese ions; generally Mg is used, but Mn can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis
- *Monovalent cation* potassium ions.

The PCR is commonly carried out in a reaction volume of 10– 200 il in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction (see below).

Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid theral equilibration.

Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of il on top of the reaction mixture or a ball of wax inside the tube.

Procedure

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Fig. 2). The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

- *Initialization step*: This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.
- *Denaturation step*: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- *Annealing step*: The reaction temperature is lowered to 50– 65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation .

- *Extension/elongation step*: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.
- *Final elongation*: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- *Final hold*: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products (see Fig. 3).

PCR Stages

The PCR process can be divided into three stages:

***Exponential Amplification*:** At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA need to be present.

***Leveling off Stage*:** The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

***Plateau*:** No more product accumulates due to exhaustion of reagents and enzyme.

PCR Optimization

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups.

Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.

Applications of PCR

The Polymerase Chain Reaction (PCR) has found widespread application in many areas of genetic analysis. This is a list of some of these applications:

Medical Applications

PCR has been applied to a large number of medical procedures:

- The first application of PCR was for genetic testing, where a sample of DNA is analyzed for the presence of genetic disease mutations. Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease. DNA samples for Prenatal testing can be obtained by amniocentesis, chorionic villus sampling, or even by the analysis of rare fetal cells circulating in the mother's bloodstream. PCR analysis is also essential to Preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

- PCR can also be used as part of a sensitive test for tissue typing, vital to organ transplantation. As of 2008, there is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.
- Many forms of cancer involve alterations to oncogenes. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient.

Infectious Disease Applications

Characterization and detection of infectious disease organisms have been revolutionized by PCR:

- The Human Immunodeficiency Virus (or HIV), responsible for AIDS, is a difficult target to find and eradicate. The earliest tests for infection relied on the presence of antibodies to the virus circulating in the bloodstream. However, antibodies don't appear until many weeks after infection, maternal antibodies mask the infection of a newborn, and therapeutic agents to fight the infection don't affect the antibodies.
- PCR tests have been developed that can detect as little as one viral genome among the DNA of over 50,000 host cells. Infections can be detected earlier, donated blood can be screened directly for the virus, newborns can be immediately tested for infection, and the effects of antiviral treatments can be quantified.
- Some disease organisms, such as that for Tuberculosis, are difficult to sample from patients and slow to be grown in the laboratory. PCR-based tests have allowed detection of small numbers of disease organisms (both live or dead), in convenient samples. Detailed genetic analysis can also be used to detect antibiotic resistance, allowing immediate and effective therapy. The effects of therapy can also be immediately evaluated.
- The spread of a disease organism through populations of domestic or wild animals can be monitored by PCR testing. In many cases, the appearance of new virulent sub-types can be detected and monitored. The sub-types of an organism that were responsible for earlier epidemics can also be determined by PCR analysis.

Forensic Applications

The development of PCR-based genetic (or DNA) fingerprinting protocols has seen widespread application in forensics:

- In its most discriminating form, Genetic fingerprinting can uniquely discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts. Simpler versions of these tests are often used to rapidly rule out suspects during a criinal investigation. Evidence from decades-old crimes can be tested, confirming or exonerating the people originally convicted.
- Less discriminating forms of DNA fingerprinting can help in Parental testing, where an individual is matched with their close relatives. DNA from unidentified human remains can be tested, and compared with that from possible parents, siblings, or children. Similar testing can be used to confirm the biological parents of an adopted (or kidnapped) child. The actual biological father of a newborn can also be confirmed (or ruled out).

Research Applications

PCR has been applied to many areas of research in molecular genetics:

- PCR allows rapid production of short pieces of DNA, even when nothing more than the sequence of the two primers is known. This ability of PCR augments many methods, such as generating hybridization probes for Southern or northern blot hybridization. PCR supplies these techniques with large amounts of pure DNA, sometimes as a single strand, enabling analysis even from very small amounts of starting material.
- The task of DNA sequencing can also be assisted by PCR. Known segments of DNA can easily be produced from a patient with a genetic disease mutation. Modifications to the amplification technique can extract segments from a completely unknown genome, or can generate just a single strand of an area of interest.
- PCR has numerous applications to the more traditional process of DNA cloning. It can extract segments for insertion into a vector from a larger genome, which may be only available in small quantities. Using a single set of ‘vector primers’, it can

also analyze or extract fragments that have already been inserted into vectors. Some alterations to the PCR protocol can generate mutations (general or site-directed) of an inserted fragment.

- Sequence-tagged sites is a process where PCR is used as an indicator that a particular segment of a genome is present in a particular clone. The Human Genome Project found this application vital to mapping the cosmid clones they were sequencing, and to coordinating the results from different laboratories.
- An exciting application of PCR is the phylogenic analysis of DNA from ancient sources, such as that found in the recovered bones of Neanderthals, or from frozen tissues of Mammoths. In some cases the highly degraded DNA from these sources might be reassembled during the early stages of amplification.
- A common application of PCR is the study of patterns of gene expression. Tissues (or even individual cells) can be analyzed at different stages to see which genes have become active, or which have been switched off. This application can also use Q-PCR to quantitate the actual levels of expression
- The ability of PCR to simultaneously amplify several loci from individual sperm has greatly enhanced the more traditional task of genetic mapping by studying chromosomal crossovers after meiosis. Rare crossover events between very close loci have been directly observed by analyzing thousands of individual sperms. Similarly, unusual deletions, insertions, translocations, or inversions can be analyzed, all without having to wait (or pay for) the long and laborious processes of fertilization, embryogenesis, etc.

Others

PCR is also important in answering basic scientific questions. In the field of evolutionary biology, PCR has been used to establish relationships among species. In anthropology, it has been used to understand ancient human migration patterns.

In archaeology, it has been used to help identify ancient human remains. Paleontologists have used PCR to amplify DNA from extinct insects preserved in amber for 20 million years. The Human Genome Project, which had a goal of determining the sequence of the 3 billion base pairs in the human genome, relied heavily on PCR. The genes

responsible for a variety of human diseases have been identified using PCR. For example, a PCR technique called multiplex PCR identifies a mutation in a gene in boys suffering from Duchenne muscular dystrophy. PCR can also be used to search for DNA from foreign organisms such as viruses or bacteria.

Selective DNA Isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism. Bacterial colonies (E. coli) can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing. This technique may also be used to determine evolutionary relationships among organisms.

Amplification and Quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old.

These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar.

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

PCR in Diagnosis of Diseases

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000-fold higher than that of other methods.

PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

Viral DNA can likewise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in treatment. The amount of virus ("viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques (see below).

Variations on the Basic PCR Technique

- *Allele-specific PCR*: a diagnostic or cloning technique based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful

amplification with an SNP-specific primer signals presence of the specific SNP in a sequence. See SNP genotyping for more information.

- *Assembly PCR* or *Polymerase Cycling Assembly (PCA)*: artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.
- *Asymmetric PCR*: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as *L*inear-*A*fter-*T*he-*E*xponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.
- *Helicase-dependent amplification*: similar to traditional PCR, but usesa constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.
- *Hot start PCR*: a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

- *Intersequence-specific PCR* (ISSR): a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.
- *Inverse PCR*: is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.
- *Ligation-mediated PCR*: uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA foot printing.
- *Methylation-specific PCR* (MSP): developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.
- *Miniprimer PCR*: uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to ampify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.
- *Multiplex Ligation-dependent Probe Amplification* (*MLPA*): permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR (see below).
- *Multiplex-PCR*: consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and

more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

- *Nested PCR*: increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.
- *Overlap-extension PCR* or *Splicing by overlap extension (SOE)* : a genetic engineering technique that is used to splice together two or more DNA fragments that contain complementary sequences. It is used to join DNA pieces containing genes, regulatory sequences, or mutations; the technique enables creation of specific and long DNA constructs.
- *Quantitative PCR (Q-PCR)*: used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. *Quantitative real-time PCR* has a very high degree of precision. QRT-PCR (or QF-PCR) methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (*R*eal *T*ime PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR (see below), often used in conjunction with Q-PCR.
- *R*everse *T*ranscription PCR (RT-PCR): for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA,

which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (*Rapid Amplification of cDNA Ends*).

- *Solid Phase PCR*: encompasses multiple meanings, including Polony Amplification (where PCR colonies are derived in a gel matrix, for example), Bridge PCR (primers are covalently linked to a solid-support surface), conventional Solid Phase PCR (where Asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced Solid Phase PCR (where conventional Solid Phase PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal 'step' to favour solid support priming).
- *Thermal asymmetric interlaced PCR (TAIL-PCR)*: for isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.
- *Touchdown PCR* (*Step-down PCR*): a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the T_m of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer T_m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.
- *PAN-AC*: uses isothermal conditions for amplification, and may be used in living cells.
- *Universal Fast Walking*: for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (using only one gene-specific primer and one general primer — which can lead to artefactual 'noise') by virtue of a mechanism involving lariat

structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe.

- *In silico PCR* (digital PCR, virtual PCR, electronic PCR, e-PCR) refers to computational tools used to calculate theoretical polymerase chain reaction results using a given set of primers (probes) to amplify DNA sequences from a sequenced genome or transcriptome.

History

A 1971 paper in the Journal of Molecular Biology by Kleppe and co-workers first described a method using an enzymatic assay to replicate a short DNA template with primers *in vitro*. However, this early manifestation of the basic PCR principle did not receive much attention, and the invention of the polymerase chain reaction in 1983 is generally credited to Kary Mullis.

When Mullis developed the PCR in 1983, he was working in Emeryville, California for Cetus Corporation, one of the first biotechnology companies. There, he was responsible for synthesizing short chains of DNA. Mullis has written that he conceived of PCR while cruising along the Pacific Coast Highway one night in his car. He was playing in his mind with a new way of analyzing changes (mutations) in DNA when he realized that he had instead invented a method of amplifying any DNA region through repeated cycles of duplication driven by DNA polymerase.

In *Scientific American*, Mullis summarized the procedure: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute.

It requires no more than a test tube, a few simple reagents, and a source of heat." He was awarded the Nobel Prize in Chemistry in 1993 for his invention, seven years after he and his colleagues at Cetus first put his proposal to practice. However, some controversies have remained about the intellectual and practical contributions of other scientists to Mullis' work, and whether he had been the sole inventor of the PCR principle (see below).

At the core of the PCR method is the use of a suitable DNA polymerase able to withstand the high temperatures of >90 °C (194 °F) required for separation of the two DNA strands in the DNA double helix

after each replication cycle. The DNA polymerases initially employed for in vitro experiments presaging PCR were unable to withstand these high temperatures. So the early procedures for DNA replication were very inefficient and time consuming, and required large amounts of DNA polymerase and continuous handling throughout the process.

The discovery in 1976 of Taq polymerase — a DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus*, which naturally lives in hot (50 to 80 °C (122 to 176 °F)) environments such as hot springs — paved the way for dramatic improvements of the PCR method. The DNA polymerase isolated from *T. aquaticus* is stable at high temperatures remaining active even after DNA denaturation, thus obviating the need to add new DNA polymerase after each cycle. This allowed an automated thermocycler-based process for DNA amplification.

Patent Wars

The PCR technique was patented by Kary Mullis and assigned to Cetus Corporation, where Mullis worked when he invented the technique in 1983. The *Taq* polymerase enzyme was also covered by patents. There have been several high-profile lawsuits related to the technique, including an unsuccessful lawsuit brought by DuPont. The pharmaceutical company Hoffmann-La Roche purchased the rights to the patents in 1992 and currently holds those that are still protected.

A related patent battle over the Taq polymerase enzyme is still ongoing in several jurisdictions around the world between Roche and Promega. The legal arguments have extended beyond the lives of the original PCR and Taq polymerase patents, which expired on March 28, 2005.

Gel Electrophoresis

Gel electrophoresis is a method used in clinical chemistry to separate proteins by charge and or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the

gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.

Gel electrophoresis uses a gel as an anticonvective medium and or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

Separation

In simple terms: Electrophoresis is a procedure which enables the sorting of molecules based on size and charge. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agar or polyacrylamide. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel.

The term “gel” in this instance refers to the matrix used to contain, then separate the target molecules. In most cases, the gel is a crosslinked polymer whose composition and porosity is chosen based on the specific weight and composition of the target to be analyzed.

When separating proteins or small nucleic acids (DNA, RNA, or oligonucleotides) the gel is usually composed of different concentrations of acrylamide and a cross-linker, producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified agarose. In both cases, the gel forms a solid, yet porous matrix. Acrylamide, in contrast to polyacrylamide, is a neurotoxin and must be handled using appropriate safety precautions to avoid poisoning. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores

allowing for the separation of macromolecules and macromolecular complexes.

"Electrophoresis" refers to the electromotive force (EMF) that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass when the charge to mass ratio (Z) of all species is uniform, toward the (negatively charged) cathode if positively charged or toward the (positively charged) anode if negatively charged.

If several samples have been loaded into adjacent wells in the gel, they will run parallel in individual lanes. Depending on the number of different molecules, each lane shows separation of the components from the original mixture as one or more distinct bands, one band per component. Incomplete separation of the components can lead to overlapping bands, or to indistinguishable smears representing multiple unresolved components. Bands in different lanes that end up at the same distance from the top contain molecules that passed through the gel with the same speed, which usually means they are approximately the same size. There are molecular weight size markers available that contain a mixture of molecules of known sizes. If such a marker was run on one lane in the gel parallel to the unknown samples, the bands observed can be compared to those of the unknown in order to determine their size. The distance a band travels is approximately inversely proportional to the logarithm of the size of the molecule.

There are limits to electrophoretic techniques. Since passing current through a gel causes heating, gels may melt during electrophoresis. Electrophoresis is performed in buffer solutions to reduce pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can exhaust the buffering capacity of the solution. Further, different preparations of genetic material may not migrate consistently with each other, for morphological or other reasons.

Types of Gel

Agarose

Agarose gels are easily cast and handled compared to other matrices, because the gel setting is a physical rather than chemical change.

Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases) using specialized apparatus. The distance between DNA bands of a given length is determined by the percent agarose in the gel. The disadvantage of higher concentrations is the long run times (sometimes days). Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis.

"Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications."

Agarose gels do not have a uniform pore size, but are optimal for electrophoresis of proteins that are larger than 200 kDa.

Polyacrylamid

Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by controlling the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered form.

Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read. Most modern DNA separation methods now use agarose gels, except for particularly small DNA fragments. It is currently most often used in the field of immunology and protein analysis, often used to separate different proteins or isoforms of the same protein into separate bands. These can be transferred onto a nitrocellulose or PVDF membrane to be probed with antibodies and corresponding markers, such as in a western blot.

Typically resolving gels are made in 6%, 8%, 10%, 12% or 15%. Stacking gel (5%) is poured on top of the resolving gel and a gel comb

(which forms the wells and defines the lanes where proteins, sample buffer and ladders will be placed) is inserted. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight, the higher the percentage that should be used. Changes on the buffer system of the gel can help to further resolve proteins of very small sizes.

Starch

Partially hydrolysed potato starch makes for another non-toxic medium for protein electrophoresis. The gels are slightly more opaque than acrylamide or agarose. Non-denatured proteins can be separated according to charge and size. They are visualised using Napthal Black or Amido Black staining. Typical starch gel concentrations are 5% to 10%.

Gel Conditions

Denaturing

A denaturing gel is a type of electrophoresis in which the native structure of macromolecules that are run within the gel is not maintained. For instance, gels used in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) will unfold and denature the native structure of a protein. In contrast to native gel electrophoresis, quaternary structure cannot be investigated using this method.

Native

Native gel electrophoresis is an electrophoretic separation method typically used in proteomics and metallomics.

Native PAGE separations are run in non-denaturing conditions. Detergents are used only to the extent that they are necessary to lyse lipid membranes in the cell. Complexes remain—for the most part—associated and folded as they would be in the cell. One downside, however, is that complexes may not separate cleanly or predictably, since they cannot move through the polyacrylamide gel as quickly as individual, denatured proteins.

Unlike denaturing methods, such as SDS-PAGE, native gel electrophoresis does not use a charged denaturing agent. The molecules being separated (usually proteins or nucleic acids) therefore differ not only in molecular mass and intrinsic charge, but also the cross-sectional area, and thus experience different electrophoretic forces dependent on

the shape of the overall structure. Since the proteins remain in the native state they may be visualised not only by general protein staining reagents but also by specific enzyme-linked staining.

Buffers

There are a number of buffers used for electrophoresis. The most common being, for nucleic acids Tris/Acetate/EDTA (TAE), Tris/Borate/EDTA (TBE). Many other buffers have been proposed, e.g. lithium borate, which is almost never used, based on Pubmed citations (LB), iso electric histidine, pK matched goods buffers, etc.; in most cases the purported rationale is lower current (less heat) and or matched ion mobilities, which leads to longer buffer life. Borate is problematic; Borate can polymerize, and/or interact with cis diols such as those found in RNA. TAE has the lowest buffering capacity but provides the best resolution for larger DNA.

This means a lower voltage and more time, but a better product. LB is relatively new and is ineffective in resolving fragments larger than 5 kbp; However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm), which means a shorter analysis time for routine electrophoresis. As low as one base pair size difference could be resolved in 3 % agarose gel with an extremely low conductivity medium (1 mM Lithium borate).

Visualization

After the electrophoresis is complete, the molecules in the gel can be stained to make them visible. DNA may be visualized using ethidium bromide which, when intercalated into DNA, fluoresce under ultraviolet light, while protein may be visualised using silver stain or Coomassie Brilliant Blue dye. Other methods may also be used to visualize the separation of the mixture's components on the gel. If the molecules to be separated contain radioactivity, for example in DNA sequencing gel, an autoradiogram can be recorded of the gel. Photographs can be taken of gels, often using Gel Doc.

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into the major groove of DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20 ng DNA becomes distinctly visible. EtBr is a known mutagen, and safer

alternatives are available, such as GelRed, which binds to the minor groove.

SYBR Green I is another dsDNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans.

SYBR Safe is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste under U.S. Federal regulations. It has similar sensitivity levels to EtBr, but, like SYBR Green, is significantly more expensive. In countries where safe disposal of hazardous waste is mandatory, the costs of EtBr disposal can easily outstrip the initial price difference, however.

Since EtBr stained DNA is not visible in natural light, scientists mix DNA with negatively charged loading buffers before adding the mixture to the gel. Loading buffers are useful because they are visible in natural light (as opposed to UV light for EtBr stained DNA), and they co-sediment with DNA (meaning they move at the same speed as DNA of a certain length).

Xylene cyanol and Bromophenol blue are common dyes found in loading buffers; they run about the same speed as DNA fragments that are 5000 bp and 300 bp in length respectively, but the precise position varies with percentage of the gel. Other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp, respectively.

Visualization can also be achieved by transferring DNA to a nitrocellulose membrane followed by exposure to a hybridization probe. This process is termed Southern blotting.

Analysis

After electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation). The illuminator apparatus mostly also contains imaging apparatus that takes an image of the gel, after illumination with UV radiation.

The ethidium bromide fluoresces reddish-orange in the presence of DNA, since it has intercalated with the DNA. The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA. The gel can then be photographed usually with a digital or

polaroid camera. Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white (see figures).

Even short exposure of nucleic acids to UV light causes significant damage to the sample. UV damage to the sample will reduce the efficiency of subsequent manipulation of the sample, such as ligation and cloning. If the DNA is to be used after separation on the agarose gel, it is best to avoid exposure to UV light by using a blue light excitation source such as the XcitaBlue UV to blue light conversion screen from Bio-Rad or Dark Reader from Clare Chemicals.

A blue excitable stain is required, such as one of the SYBR Green or GelGreen stains. Blue light is also better for visualization since it is safer than UV (eye-protection is not such a critical requirement) and passes through transparent plastic and glass. This means that the staining will be brighter even if the excitation light goes through glass or plastic gel platforms.

Downstream Processing

After separation, an additional separation method may then be used, such as isoelectric focusing or SDS-PAGE. The gel will then be physically cut, and the protein complexes extracted from each portion separately. Each extract may then be analysed, such as by peptide mass fingerprinting or de novo sequencing after in-gel digestion. This can provide a great deal of information about the identities of the proteins in a complex.

Applications

- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

Gel electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software. Depending on

the type of analysis being performed, other techniques are often implemented in conjunction with the results of gel electrophoresis, providing a wide range of field-specific applications.

Nucleic Acids

In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally-occurring negative charge carried by their sugar-phosphate backbone.

Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size or, for cyclic fragments, their radius of gyration. Circular DNA such as plasmids, however, may show multiple bands, the speed of migration may depend on whether it is relaxed or supercoiled. Single-stranded DNA or RNA tend to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as sodium hydroxide or formamide, are used to denature the nucleic acids and cause them to behave as long rods again.

Gel electrophoresis of large DNA or RNA is usually done by agarose gel electrophoresis. See the “Chain termination method” page for an example of a polyacrylamide DNA sequencing gel. Characterization through ligand interaction of nucleic acids or fragments may be performed by mobility shift affinity electrophoresis. Electrophoresis of RNA samples can be used to check for genomic DNA contamination and also for RNA degradation. RNA from eukaryotic organisms shows distinct bands of 28s and 18s rRNA, the 28s band being approximately twice as intense as the 18s band. Degraded RNA has less sharpely defined bands, has a smeared appearance, and intensity ratio is less than 2:1.

Proteins

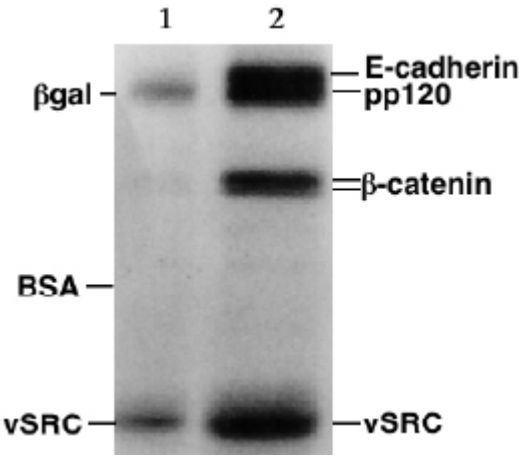

Figure: *SDS-PAGE autoradiography – The indicated proteins are present in different concentrations in the two samples.*

Proteins, unlike nucleic acids, can have varying charges and complex shapes, therefore they may not migrate into the polyacrylamide gel at similar rates, or at all, when placing a negative to positive EMF on the sample. Proteins therefore, are usually denatured in the presence of a detergent such as sodium dodecyl sulfate (SDS) that coats the proteins with a negative charge. Generally, the amount of SDS bound is relative to the size of the protein (usually 1.4g SDS per gram of protein), so that the resulting denatured proteins have an overall negative charge, and all the proteins have a similar charge to mass ratio. Since denatured proteins act like long rods instead of having a complex tertiary shape, the rate at which the resulting SDS coated proteins migrate in the gel is relative only to its size and not its charge or shape.

Proteins are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), by native gel electrophoresis, by quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE), or by 2-D electrophoresis.

Characterization through ligand interaction may be performed by electroblotting or by affinity electrophoresis in agarose or by capillary electrophoresis as for estimation of binding constants and determination of structural features like glycan content through lectin binding.

History

- 1930s – first reports of the use of sucrose for gel electrophoresis
- 1955 – introduction of starch gels, mediocre separation
- 1959 – introduction of acrylamide gels; disc electrophoresis (Ornstein and Davis); accurate control of parameters such as

pore size and stability; and (Raymond and Weintraub)

- 1969 – introduction of denaturing agents especially SDS separation of protein subunit (Weber and Osborn)
- 1970 – Laemmli separated 28 components of T4 phage using a stacking gel and SDS
- 1975 – 2-dimensional gels (O'Farrell); isoelectric focusing then SDS gel electrophoresis
- 1977 – sequencing gels
- late 1970s – agarose gels
- 1983 – pulsed field gel electrophoresis enables separation of large DNA molecules
- 1983 – introduction of capillary electrophoresis

A 1959 book on electrophoresis by Milan Bier cites references from the 1800s. However, Oliver Smithies made significant contributions. Bier states: "The method of Smithies ... is finding wide application because of its unique separatory power." Taken in context, Bier clearly implies that Smithies' method is an improvement.

Southern Blot

A Southern blot is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern. Other blotting methods (i.e., western blot, northern blot, eastern blot, southwestern blot) that employ similar principles, but using RNA or protein, have later been named in reference to Edwin Southern's name. As the technique was eponymously named, Southern blot is capitalized as is conventional for proper nouns. The names for other blotting methods may follow this convention, by analogy. Method:

1. Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments.
2. The DNA fragments are then electrophoresed on an agarose gel to separate them by size.
3. If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute

HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane.

4. If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (typically containing sodium hydroxide) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe (see below), and destroys any residual RNA that may still be present in the DNA. The choice of alkaline over neutral transfer methods, however, is often empirical and may result in equivalent results.
5. A sheet of nitrocellulose (or, alternatively, nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. If transferring by suction 20X SSC buffer is used to ensure a seal and prevent drying of the gel. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
6. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours (standard conditions; nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.
7. The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, the hybridization probe may be made from RNA, rather than DNA. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon or herring sperm DNA for

blocking of the membrane surface and target DNA, deionized formamide, and detergents such as SDS to reduce non-specific binding of the probe.

8. After hybridization, excess probe is washed from the membrane (typically using SSC buffer), and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of color on the membrane if a chromogenic detection method is used.

Result

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. It also allows for the fixation of the target-probe hybrids, required for analysis by autoradiography or other detection methods.

Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome.

A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (for example, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

Applications

Southern transfer may be used for homology-based cloning on the basis of amino acid sequence of the protein product of the target gene. Oligonucleotides are designed that are similar to the target sequence. The oligonucleotides are chemically synthesised, radiolabeled, and used to screen a DNA library, or other collections of cloned DNA fragments. Sequences that hybridise with the hybridisation probe are further analysed, for example, to obtain the full length sequence of the targeted gene. Second, Southern blotting can also be used to identify methylated sites in particular genes. Particulary useful are the restriction nucleases

MspI and *HpaII*, both of which recognize and cleave within the same sequence. However, *HpaII* requires that a C within that site be methylated, whereas *MspI* cleaves only DNA methylated at that site. Therefore, any methylated sites within a sequence analyzed with a particular probe will be cleaved by the former, but not the latter, enzyme.

Western Blot

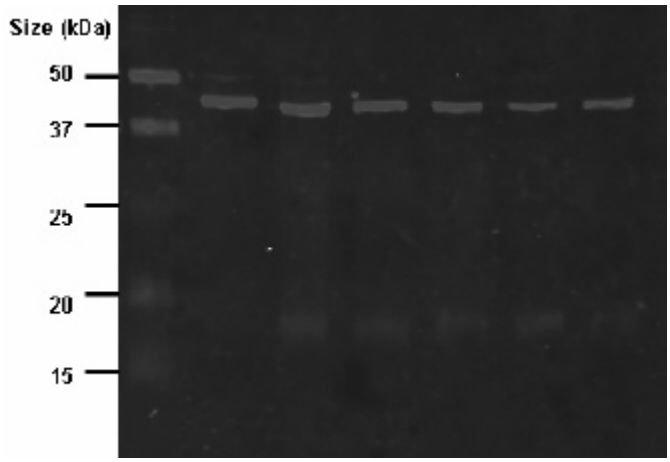

Figure: *Western blot using an antibody that recognizes proteins modified with lipoic acid.*

The western blot (sometimes called the protein immunoblot) is a widely used analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins. Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. Other related techniques include using antibodies to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

The method originated in the laboratory of George Stark at Stanford. The name *Western blot* was given to the technique by W. Neal Burnette and Sushant Bhat and is a play on the name Southern blot, a technique

for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blot.

Steps in a Western Blot

Tissue Preparation

Samples cannot be taken from whole tissue or from cell culture. Solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. However, virus or environmental samples can be the source of protein and thus western blotting is not restricted to cellular studies only.

Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing and degradation. A combination of biochemical and mechanical techniques – comprising various types of filtration and centrifugation – can be used to separate different cell compartments and organelles.

Gel Electrophoresis

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to identify a protein.

By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa).

The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into *wells* in the gel. One lane is usually reserved for a *marker* or *ladder*, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different *electrophoretic mobilities*) separate into *bands* within each *lane*. It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

Transfer

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of *nitrocellulose or polyvinylidene difluoride (PVDF*). The primary method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. An older method of transfer involves placing a membrane on top of the gel, and a stack of filter papers on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. In practice this method is not used as it takes too much time; electroblotting is preferred. As a result of either “blotting” process, the proteins are exposed on a thin surface layer for detection (see below). Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.

The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with

Coomassie Brilliant Blue or Ponceau S dyes. Ponceau S is the more common of the two, due to its higher sensitivity and water solubility, the latter making it easier to subsequently destain and probe the membrane, as described below.

Blocking

Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the western blot, leading to clearer results, and eliminates false positives.

Detection

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate this enzyme drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

Two steps:

- *Primary Antibody:* The primary antibodies are generated when a host species or immune cell culture is exposed to protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly. After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also

be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

- *Secondary Antibody:* After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to almost any mouse-sourced primary antibody, which allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark purple stain that can be photographed without using specialized photographic film. As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane (see the figure below with blue bands).

Another method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as

compared to chemiluminescence, in which light is measured in a dynamic state.

A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like *Staphylococcus* Protein A or Streptavidin with a radioactive isotope of iodine. Since other methods are safer, quicker, and cheaper, this method is now rarely used; however, an advantage of this approach is the sensitivity of auto-radiography based imaging, which enables highly accurate protein quantification when combined with optical software (e.g. Optiquant).

One Step

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.

Analysis

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis.

The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is normalized to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

Colorimetric Detection

The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescent Detection

Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which capture a digital image of the western blot.

The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.

Radioactive Detection

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image to the right).

The importance of radioactive detections methods is declining due to its hazardous radiation, because it is very expensive, health and safety risks are high, and ECL (enhanced chemiluminescence) provides a useful alternative.

Fluorescent Detection

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the western blot and allows further data analysis such as molecular weight analysis and a quantitative western blot analysis. Fluorescence is considered to be one of the best methods for quantification, but is less sensitive than chemiluminescence.

Secondary Probing

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

2-D Gel Electrophoresis

2-dimensional SDS-PAGE uses the principles and techniques outlined above. 2-D SDS-PAGE, as the name suggests, involves the migration of polypeptides in 2 dimensions. For example, in the first dimension polypeptides are separated according to isoelectric point, while in the second dimension polypeptides are separated according to their molecular weight. The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine and arginine) and negatively (e.g. glutamate and aspartate) charged amino acids, with negatively charged amino acids contributing to a high isoelectric point and positively charged amino acids contributing to a low isoelectric point. Samples could also be separated first under nonreducing conditions using SDS-PAGE and under reducing conditions in the second dimension, which breaks apart disulfide bonds that hold subunits together. SDS-PAGE might also be coupled with urea-PAGE for a 2-dimensional gel.

In principle, this method allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different isoforms of a particular protein - e.g. a protein that has been phosphorylated (by addition of a negatively charged group). Proteins that have been separated can be cut out of the gel and then analysed by mass spectrometry, which identifies the protein.

Medical Diagnostic Applications

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is

added. The stained bands then indicate the proteins to which the patient's serum contains antibody.

- A western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, western blot is sometimes used to confirm FIV+ status in cats.

Chapter 12: Diagnosis of Viral and Bacterial Diseases

The northern blot is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample.

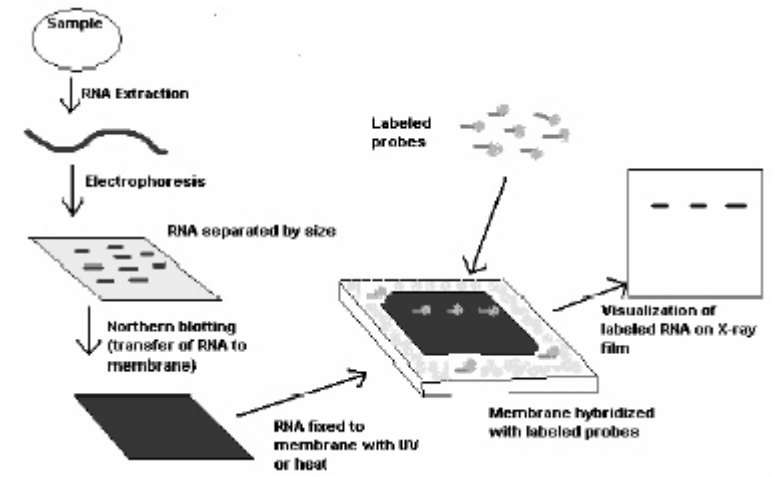

Figure: *Flow diagram outlining the general procedure for RNA detection by northern blotting.*

With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern. The major difference is that RNA, rather than DNA, is analyzed in the northern blot.

Procedure

A general blotting procedure starts with extraction of total RNA from a homogenized tissue sample or from cells. Eukaryotic mRNA can then be isolated through the use of oligo (dT) cellulose chromatography to isolate only those RNAs with a poly(A) tail. RNA samples are then separated by gel electrophoresis. Since the gels are fragile and the probes are unable to enter the matrix, the RNA samples, now separated by size, are transferred to a nylon membrane through a capillary or vacuum blotting system.

A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition. The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film and can be quantified by densitometry. To create controls for comparison in a northern blot, samples not displaying the gene product of interest can be used after determination by microarrays or RT-PCR.

Gels

The RNA samples are most commonly separated on agarose gels containing formaldehyde as a denaturing agent for the RNA to limit secondary structure. The gels can be stained with ethidium bromide (EtBr) and viewed under UV light to observe the quality and quantity of RNA before blotting. Polyacrylamide gel electrophoeresis with urea can also be used in RNA separation but it is most commonly used for fragmented RNA or microRNAs. An RNA ladder is often run alongside the samples on an electrophoresis gel to observe the size of fragments obtained but in total RNA samples the ribosomal subunits can act as size markers. Since the large ribosomal subunit is 28S (approximately 5kb) and the small ribosomal subunit is 18S (approximately 2kb) two

prominent bands will appear on the gel, the larger at close to twice the intensity of the smaller.

Probes

Probes for northern blotting are composed of nucleic acids with a complementary sequence to all or part of the RNA of interest, they can be DNA, RNA, or oligonucleotides with a minimum of 25 complementary bases to the target sequence. RNA probes (riboprobes) that are transcribed in vitro are able to withstand more rigorous washing steps preventing some of the background noise. Commonly cDNA is created with labelled primers for the RNA sequence of interest to act as the probe in the northern blot. The probes need to be labelled either with radioactive isotopes (P) or with chemiluminescence in which alkaline phosphatase or horseradish peroxidase breakdown chemiluminescent substrates producing a detectable emission of light.

The chemiluminescent labelling can occur in two ways: either the probe is attached to the enzyme, or the probe is labelled with a ligand (e.g. biotin) for which the antibody (e.g. avidin or streptavidin) is attached to the enzyme. X-ray film can detect both the radioactive and chemiluminescent signals and many researchers prefer the chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels. The same membrane can be probed up to five times without a significant loss of the target RNA.

Applications

Northern blotting allows one to observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment. The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs. If an upregulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding. The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or

repetitive sequence motifs. The variance in size of a gene product can also indicate deletions or errors in transcript processing, by altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.

BlotBase is an online database publishing northern blots. BlotBase has over 700 published northern blots of human and mouse samples, in over 650 genes across more than 25 different tissue types. Northern blots can be searched by a blot ID, paper reference, gene identifier, or by tissue. The results of a search provide the blot ID, species, tissue, gene, expression level, blot image (if available), and links to the publication that the work originated from. This new database provides sharing of information between members of the science community that was not previously seen in northern blotting as it was in sequence analysis, genome determination, protein structure, etc.

Advantages and Disadvantages

Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, serial analysis of gene expression (SAGE), as well as northern blotting. Microarrays are quite commonly used and are usually consistent with data obtained from northern blots; however, at times northern blotting is able to detect small changes in gene expression that microarrays cannot. The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time, while northern blotting is usually looking at one or a small number of genes.

A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate). The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity which is important to reduce false positive results.

The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured

on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.

For years after blotting.

Reverse Northern Blot

A variant of the procedure known as the reverse northern blot is occasionally used. In this procedure, the substrate nucleic acid (that is affixed to the membrane) is a collection of isolated DNA fragments, and the probe is RNA extracted from a tissue and radioactively labelled.

The use of DNA microarrays that have come into widespread use in the late 1990s and early 2000s is more akin to the reverse procedure, in that they involve the use of isolated DNA fragments affixed to a substrate, and hybridization with a probe made from cellular RNA. Thus the reverse procedure, though originally uncommon, enabled northern analysis to evolve into gene expression profiling, in which many (possibly all) of the genes in an organism may have their expression monitored.

Eastern Blotting

Eastern blotting is a biochemical technique used to analyze protein post translational modifications (PTM) such as lipids and glycoconjugates. It is most often used to detect carbohydrate epitopes. Thus, Eastern blotting can be considered an extension of the biochemical technique of Western blotting. Multiple techniques have been described by the term Eastern blotting, most use proteins or lipids blotted from SDS-PAGE gel on to a PVDF or nitrocellulose membrane.

Transferred proteins are analyzed for post-translational modifications using probes that may detect lipids, carbohydrate, phosphorylation or any other protein modification. Eastern blotting should be used to refer to methods that detect their targets through specific interaction of the PTM and the probe, distinguishing them from a standard Far-western blot. In principle, Eastern blotting is similar to lectin blotting (i.e. detection of carbohydrate epitopes on proteins or lipids); however, the term *lectin blotting* is more prevalent in the literature.

History and Multiple Definitions

Definition of the term *Eastern blotting* is somewhat confused due to multiple sets of authors dubbing a new method as *Eastern blotting*, or a derivative thereof. All of the definitions are a derivative of the technique of Western blotting developed by Towbin in 1979. The current definitions are summarized below in order of the first use of the name; however, all are based on some earlier works. In some cases, the technique had been in practice for some time before the introduction of the term.

- (1982) The term *Eastern blotting* was specifically rejected by two separate groups: Reinhart and Malamud referred to a protein blot of a native gel as a *native blot*; Peferoen et al., opted to refer to their method of drawing SDS-gel separated proteins onto nitr oce llulose using a vacuum as *Vacuum blotting*.
- (1984) *Middle Eastern blotting* has been described as a blot of polyA RNA (resolved by agarose) which is then immobilized. The immobilized RNA is then probed using DNA.
- (1996) *Eastern-Western blot* was first used by Bogdanov et al. The method involved blotting of phospholipids on PVDF or nitrocellulose membrane prior to transfer of proteins onto the same nitrocellulose membrane by conventional Western blotting and probing with conformation specific antibodies. This method is based on earlier work by Taki et al. in 1994, which they originally dubbed *TLC blotting*, and was based on a similar method introduced by Towbin in 1984.
- (2000) *Far-Eastern blotting* seems to have been first named in 2000 by Ishikawa & Taki. The method is described more fully in the article on Far-Eastern blotting, but is based on antibody or lectin staining of lipids transferred to PVDF membranes.
- (2001) *Eastern blotting* was described as a technique for detecting glycoconjugates generated by blotting BSA onto PVDF membranes, followed by periodate treatment. The oxidized protein is then treated with a complex mixture, generating a new conjugate on the membrane. The membrane is then probed with antibodies for epitopes of interest. This method has also been discussed in later work by the same group. The method is essentially Far-Eastern blotting.
- (2002) *Eastern blot* has also been used to describe an immunoblot performed on proteins blotted to a PVDF membrane from a PAGE gel run with opposite polarity. Since this is

essentially a Western blot, the charge reversal was used to dub this method an *Eastern blot*.

- (2005) *Eastern blot* has been used to describe a blot of proteins on PVDF membrane where the probe is an aptamer rather than an antibody. This could be seen as similar to a Southern blot, however the interaction is between a DNA molecule (the aptamer) and a protein, rather than two DNA molecules.
- (2006) *Eastern blotting* has been used to refer to the detection of fusion proteins through complementation. The name is based on the use of an enzyme activator (EA) as part of the detection.
- (2009) *Eastern blotting* has most recently been re-dubbed by Thomas et al. as a technique which probes proteins blotted to PVDF membrane with lectins, cholera toxin and chemical stains to detect glycosylated, lipoylated or phosphorylated proteins. These authors distinguish the method from the *Far-eastern blot* named by Taki et al. in that they use lectin probes and other staining reagents.

There is clearly no single accepted definition of the term. A recent highlight article has interviewed Ed Southern, originator of the Southern blot, regarding a re-christening of *Eastern blotting* from Tanaka et al. The article likens the *Eastern blot* to "fairies, unicorns, and a free lunch" and states that Eastern blots "don't exist." The *Eastern blot* is mentioned in an Immunology textbook which compares the common blotting methods (Southern, Northern, and Western), and states that "the Eastern blot, however, exists only in test questions."

The principles used for Eastern blotting to detect glycans can be traced back to the use of lectins to detect protein glycosylation. The earliest example for this mode of detection is Tanner and Anstee in 1976, where lectins were used to detect glycosylated proteins isolated from human erythrocytes. The specific detection of glycosylation through blotting is usually referred to as *lectin blotting*. A summary of more recent improvements of the protocol has been provided by H. Freeze.

Applications

One application of the technique includes detection of protein modifications in two bacterial species *Ehrlichia- E. muris* and IOE. Cholera toxin B subunit (which binds to gangliosides), Concanavalin A (which detects mannose-containing glycans) and nitrophospho molybdate-methyl green (which detects phosphoproteins) were used to

detect protein modifications. The technique showed that the antigenic proteins of the non-virulent *E.muris* is more post-translationally modified than the highly virulent IOE.

Significance

Most proteins that are translated from mRNA undergo modifications before becoming functional in cells. These modifications are collectively known as post-translational modifications (PTMs). The nascent or folded proteins, which are stable under physiological conditions, are then subjected to a battery of specific enzyme-catalyzed modifications on the side chains or backbones.

Post-translational protein modifications can include: acetylation, acylation (myristoylation, palmitoylation), alkylation, arginylation, ADP-Ribosylation, biotinylation, formylation, geranylgeranylation, glutamylation, glycosylation, glycylation, hydroxylation, isoprenylation, lipoylation, methylation, nitroalkylation, phosphopantetheinylation, phosphorylation, prenylation, selenation, S-nitrosylation, succinylation, sulfation, transglutamination and ubiquitination (sumoylation, neddylation).

Post-translational modifications occurring at the N-terminus of the amino acid chain play an important role in translocation across biological membranes. These include secretory proteins in prokaryotes and eukaryotes and also proteins that are intended to be incorporated in various cellular and organelle membranes such as lysosomes, chloroplast, mitochondria and plasma membrane. Expression of post-translated proteins is important in several diseases.

DNA Microarray

A DNA microarray (also commonly known as gene chip, DNA chip, or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10 moles) of a specific DNA sequence, known as *probes* (or *reporters or oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

The Basic Microarray

Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation. In standard microarrays, the probes are synthesized and then attached via surface engineering to a solid surface by a covalent bond to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can be glass or a silicon chip, in which case they are colloquially known as an *Affy chip* when an Affymetrix chip is used. Other microarray platforms, such as Illumina, use microscopic beads, instead of the large solid support. Alternatively, microarrays can be constructed by the direct synthesis of oligonucleotide probes on solid surfaces. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or targeted resequencing (*see uses and types section*). Microarrays also differ in fabrication, workings, accuracy, efficiency, and cost (*see fabrication section*). Additional factors for microarray experiments are the experimental design and the methods of analyzing the data (*see Bioinformatics section*) and the methods of analyzing the data (*see Bioinformatics section*).

History

Microarray technology evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known DNA sequence. The first reported use of this approach was the analysis of 378 arrayed lysed bacterial colonies each harboring a different sequence which were assayed in multiple replicas for expression of the genes in multiple normal and tumor tissue. This was expanded to analysis of more than 4000 human sequences with computer driven scanning and image processing for quantitative analysis of the sequences in human colonic tumors and normal tissue and then to comparison of colonic tissues at different genetic risk. The use of a collection of distinct DNAs in arrays for expression profiling was also described in 1987, and the arrayed DNAs were used to identify genes whose expression is modulated by interferon. These early gene arrays were made by spotting cDNAs onto filter paper with a pin-spotting device. The use of

miniaturized microarrays for gene expression profiling was first reported in 1995, and a complete eukaryotic genome (*Saccharomyces cerevisiae*) on a microarray was published in 1997.

Nucleic Acid Thermodynamics

Nucleic acid thermodynamics is the study of how temperature affects the nucleic acid structure of double-stranded DNA (dsDNA). The melting temperature (T_m) is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the random coil state.

T_m depends on the length of the DNA molecule and its specific nucleotide sequence. DNA, when in a state where its two strands are dissociated (i.e., the dsDNA molecule exists as two independent strands), is referred to as having been denatured by the high temperature.

Concepts

Hybridization

Hybridization is the process of establishing a non-covalent, sequence-specific interaction between two or more complementary strands of nucleic acids into a single complex, which in the case of two strands is referred to as a duplex. Oligonucleotides, DNA, or RNA will bind to their complement under normal conditions, so two perfectly complementary strands will bind to each other readily.

In order to reduce the diversity and obtain the most energetically preferred complexes, a technique called annealing is used in laboratory practice. However, due to the different molecular geometries of the nucleotides, a single inconsistency between the two strands will make binding between them less energetically favorable. Measuring the effects of base incompatibility by quantifying the temperature at which two strands anneal can provide information as to the similarity in base sequence between the two strands being annealed.

The complexes may be dissociated by thermal denaturation, also referred to as melting. Here, the solution of complexes is heated to break the hydrogen bonds between nucleic bases, after which the two strands separate.

In the absence of external negative factors, the processes of hybridization and melting may be repeated in succession indefinitely, which lays the ground for polymerase chain reaction. Most commonly, the pairs of nucleic bases A=T and Ga”C are formed, of which the latter is more stable.

Denaturation

DNA denaturation, also called DNA melting, is the process by which double-stranded deoxyribonucleic acid unwinds and separates into single-stranded strands through the breaking of hydrogen bonding between the bases.

Both terms are used to refer to the process as it occurs when a mixture is heated, although “denaturation” can also refer to the separation of DNA strands induced by chemicals like urea.

The process of DNA denaturation can be used to analyze some aspects of DNA. Because cytosine / guanine base-pairing is generally stronger than adenosine / thymine base-pairing, the amount of cytosine and guanine in a genome (called the “GC content”) can be estimated by measuring the temperature at which the genomic DNA melts. Higher temperatures are associated with high GC content.

DNA denaturation can also be used to detect sequence differences between two different DNA sequences. DNA is heated and denatured into single-stranded state, and the mixture is cooled to allow strands to rehybridize. Hybrid molecules are formed between similar sequences and any differences between those sequences will result in a disruption of the base-pairing.

On a genomic scale, the method has been used by researchers to estimate the genetic distance between two species, a process known as DNA-DNA hybridization. In the context of a single isolated region of DNA, denaturing gradient gels and temperature gradient gels can be used to detect the presence of small mismatches between two sequences, a process known as temperature gradient gel electrophoresis.

Methods of DNA analysis based on melting temperature have the disadvantage of being proxies for studying the underlying sequence; DNA sequencing is generally considered a more accurate method.

The process of DNA melting is also used in molecular biology techniques, notably in the polymerase chain reaction (PCR). Although the temperature of DNA melting is not diagnostic in the technique,

methods for estimating T_m are important for determining the appropriate temperatures to use in a protocol.

DNA melting temperatures can also be used as a proxy for equalizing the hybridization strengths of a set of molecules, e.g. the oligonucleotide probes of DNA microarrays.

Annealing

Annealing, in genetics, means for DNA or RNA to pair by hydrogen bonds to a complementary sequence, forming a double-stranded polynucleotide. The term is often used to describe the binding of a DNA probe, or the binding of a primer to a DNA strand during a polymerase chain reaction (PCR). The term is also often used to describe the reformation (renaturation) of complementary strands that were separated by heat (thermally denatured). Proteins such as RAD52 can help DNA anneal.

Uses and Types

Many types of arrays exist and the broadest distinction is whether they are spatially arranged on a surface or on coded beads:

- The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with a thousands of identicaland specific probes attached to a solid surface, such as glass, plastic or silicon biochip (commonly known as a *genome chip*, *DNA chip* or *gene array*). Thousands of these features can be placed in known locations on a single DNA microarray.
- The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence.

DNA microarrays can be used to detect DNA (as in comparative genomic hybridization), or detect RNA (most commonly as cDNA after reverse transcription) that may or may not be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling.

Applications Include: Application or technology Synopsis Gene expression profiling In an mRNA or gene expression profiling experiment the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression. For example,

microarray-based gene expression profiling can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing gene expression in infected to that in uninfected cells or tissues. Comparative genomic hybridization Assessing genome content in different cells or closely related organisms. Gene ID Small microarrays to check IDs of organisms in food and feed (like GMO), mycoplasms in cell culture, or pathogens for disease detection, mostly combining PCR and microarray technology.

Chromatin Immunoprecipitation on Chip: DNA sequences bound to a particular protein can be isolated by immunoprecipitating that protein (ChIP), these fragments can be then hybridized to a microarray (such as a tiling array) allowing the determination of protein binding site occupancy throughout the genome.

Example protein to immunoprecipitate are histone modifications (H3K27me3, H3K4me2, H3K9me3, etc.), Polycomb-group protein (PRC2:Suz12, PRC1:YY1) and trithorax-group protein (Ash1) to study the epigenetic landscape or RNA Polymerase II to study the transcription landscape.

DamID: Analogously to ChIP, genomic regions bound by a protein of interest can be isolated and used to probe a microarray to determine binding site occupancy. Unlike ChIP, DamID does not require antibodies but makes use of adenine methylation near the protein's binding sites to selectively amplify those regions, introduced by expressing minute amounts of protein of interest fused to bacterial DNA adenine methyltransferase.

SNP Detection: Identifying single nucleotide polymorphism among alleles within or between populations. Several applications of microarrays make use of SNP detection, including Genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating germline mutations in individuals or somatic mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.

Alternative Splicing Detection: An '*exon junction array* design uses probes specific to the expected or potential splice sites of predicted exons for a gene. It is of intermediate density, or coverage, to a typical gene expression array (with 1-3 probes per gene) and a genomic tiling array (with hundreds or thousands of probes per gene). It is used to assay the expression of alternative splice forms of a gene. Exon arrays have a different design, employing probes designed to detect each individual

exon for known or predicted genes, and can be used for detecting different splicing isoforms.

Fusion Genes Microarray: A Fusion gene microarray can detect fusion transcripts, *e.g.* from cancer specimens. The principle behind this is building on the alternative splicing microarrays. The oligo design strategy enables combined measurements of chimeric transcript junctions with exon-wise measurements of individual fusion partners.

Tiling Array: Genome tiling arrays consist of overlapping probes designed to densely represent a genomic region of interest, sometimes as large as an entire human chromosome. The purpose is to empirically detect expression of transcripts or alternatively splice forms which may not have been previously known or predicted.

Fabrication

Microarrays can be manufactured in different ways, depending on the number of probes under examination, costs, customization requirements, and the type of scientific question being asked. Arrays may have as few as 10 probes or up to 2.1 million micrometre-scale probes from commercial vendors.

Spotted vs. in Situ Synthesised Arrays

Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micromirror devices, ink-jet printing, or electrochemistry on microelectrode arrays.

In *spotted microarrays*, the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs. The probes are synthesized prior to deposition on the array surface and are then "spotted" onto glass. A common approach utilizes an array of fine pins or needles controlled by a robotic arm that is dipped into wells containing DNA probes and then depositing each probe at designated locations on the array surface.

The resulting "grid" of probes represents the nucleic acid profiles of the prepared robes and is ready to receive complementary cDNA or cRNA "targets" derived from experimental or clinical samples. This technique is used by research scientists around the world to produce "in-house" printed microarrays from their own labs. These arrays may be easily customized for each experiment, because researchers can choose

the probes and printing locations on th arrays, synthesize the probes in their own lab (or collaborating facility), and spot the arrays.

They can then generate their own labeled samples for hybridization, hybridize the samples to the array, and finally scan the arrays with their own equipment. This provides a relatively low-cost microarray that may be customized for each study, and avoids the costs of purchasing often more expensive commercial arrays that may represent vast numbers of genes that are not of interest to the investigator. Publications exist which indicate in-house spotted microarrays may not provide the same level of sensitivity compared to commercial oligonucleotide arrays, possibly owing to the small batch sizes and reduced printing efficiencies when compared to industrial manufactures of oligo arrays.

In *oligonucleotide microarrays*, the probes are short sequences designed to match parts of the sequence of known or predicted open reading frames. Although oligonucleotide probes are often used in "spotted" microarrays, the term "oligonucleotide array" most often refers to a specific technique of manufacturing.

Oligonucleotide arrays are produced by printing short oligonucleotide sequences designed to represent a single gene or family of gene splice-variants by synthesizing this sequence directly onto the array surface instead of depositing intact sequences. Sequences may be longer (60-mer probes such as the Agilent design) or shorter (25-mer probes produced by Affymetrix) depending on the desired purpose; longer probes are more specific to individual target genes, shorter probes may be spotted in higher density across the array and are cheaper to manufacture.

One technique used to produce oligonucleotide arrays include photolithographic synthesis (Affymetrix) on a silica substrate where light and light-sensitive masking agents are used to "build" a sequence one nucleotide at a time across the entire array. Each applicable probe is selectively "unmasked" prior to bathing the array in a solution of a single nucleotide, then a masking reaction takes place and the next set of probes are unmasked in preparation for a different nucleotide exposure. After many repetitions, the sequences of every probe become fully constructed. More recently, Maskless Array Synthesis from NimbleGen Systems has combined flexibility with large numbers of probes.

Two-channel vs. One-channel Detection

Two-color microarrays or *two-channel microarrays* are typically hybridized with cDNA prepared from two samples to be compared (e.g. diseased tissue versus healthy tissue) and that are labeled with two different fluorophores. Fluorescent dyes commonly used for cDNA labeling include Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum). The two Cy-labeled cDNA samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes.

Oligonucleotide microarrays often carry control probes designed to hybridize with RNA spike-ins. The degree of hybridization between the spike-ins and the control probes is used to normalize the hybridization measurements for the target probes.

Although absolute levels of gene expression may be determined in the two-color array in rare instances, the relative differences in expression among different spots within a sample and between samples is the preferred method of data analysis for the two-color system. Examples of providers for such microarrays includes Agilent with their Dual-Mode platform, Eppendorf with their DualChip platform for colorimetric Silverquant labeling, and TeleChem International with Arrayit.

In *single-channel microarrays* or *one-color microarrays*, the arrays provide intensity data for each probe or probe set indicating a relative level of hybridization with the labeled target. However, they do not truly indicate abundance levels of a gene but rather relative abundance when compared to other samples or conditions when processed in the same experiment. Each RNA molecule encounters protocol and batch-specific bias during amplification, labeling, and hybridization phases of the experiment making comparisons between genes for the same microarray uninformative.

The comparison of two conditions for the same gene requires two separate single-dye hybridizations. Several popular single-channel systems are the Affymetrix “Gene Chip”, Illumina “Bead Chip”, Agilent single-channel arrays, the Applied Microarrays “CodeLink” arrays, and the Eppendorf “DualChip & Silverquant”.

One strength of the single-dye system lies in the fact that an aberrant sample cannot affect the raw data derived from other samples, because each array chip is exposed to only one sample (as opposed to a two-color system in which a single low-quality sample may drastically impinge on overall data precision even if the other sample was of high quality).

Another benefit is that data are more easily compared to arrays from different experiments so long as batch effects have been accounted for. A drawback to the one-color system is that, when compared to the two-color system, twice as many microarrays are needed to compare samples within an experiment.

Microarrays and Bioinformatics

The advent of inexpensive microarray experiments created several specific bioinformatics challenges:

- the multiple levels of replication in experimental design (Experimental design)
- the number of platforms and independent groups and data format (Standardization)
- the treatment of the data (Statistical analysis)
- accuracy and precision (Relation between probe and gene)
- the sheer volume of data and the ability to share it (Data warehousing)

Experimental Design

Due to the biological complexity of gene expression, the considerations of experimental design that are discussed in the expression profiling article are of critical importance if statistically and biologically valid conclusions are to be drawn from the data.

There are three main elements to consider when designing a microarray experiment. First, replication of the biological samples is essential for drawing conclusions from the experiment. Second, technical replicates (two RNA samples obtained from each experimental unit) help to ensure precision and allow for testing differences within treatment groups. The biological replicates include independent RNA extractions and technical replicates may be two aliquots of the same extraction. Third, spots of each cDNA clone or oligonucleotide are present as replicates (at least duplicates) on the microarray slide, to provide a measure of technical precision in each hybridization. It is critical that information about the sample preparation and handling is discussed, in

order to help identify the independent units in the experiment and to avoid inflated estimates of statistical significance.

Standardization

Microarray data is difficult to exchange due to the lack of standardization in platform fabrication, assay protocols, and analysis methods. This presents an interoperability problem in bioinformatics. Various grass-roots open-source projects are trying to ease the exchange and analysis of data produced with non-proprietary chips:

- For example, the "Minimum Information About a MicroarrayExperiment" (MIAME) checklist helps define the level of detailthat should exist and is being adopted by many journals as a requirement for the submission of papers incorporating microarray results. But MIAME does not describe the format for the information, so while many formats can support the MIAME requirements, as of 2007 no format permits verification of complete semantic compliance.
- The "MicroArray Quality Control (MAQC) Project" is being conducted by the US Food and Drug Administration (FDA) to develop standards and quality control metrics which will eventually allow the use of MicroArray data in drug discovery, clinical practice and regulatory decision-making.
- The MGED Society has developed standards for the representation of gene expression experiment results and relevant annotations.

Statistical Analysis

Microarray data sets are commonly very large, and analytical precision is influenced by a number of variables. Statistical challenges include taking into account effects of background noise and appropriate normalization of the data. Normalization methods may be suited to specific platforms and, in the case of commercial platforms, the analysis may be proprietary. Algorithms that affect statistical analysis include:

- *Image analysis:* gridding, spot recognition of the scanned image (segmentation algorithm), removal or marking of poor-quality and low-intensity features (called *flagging*).
- *Data processing:* background subtraction (based on global or local background), determination of spot intensities and intensity ratios, visualisation of data (e.g. see MA plot), and log-

transformation of ratios, global or local normalization of intensity ratios, and segmentation into different copy number regions using step detection algorithms.

- *Identification of statistically significant changes:* t-t est, ANO VA, Bayesian method Mann–Whitney test methods tailored to microarray data sets, which take into account multiple comparisons or cluster analysis. These methods assess statistical power based on the variation present in the data and the number of experimental replicates, and can help minimize Type I and type II errors in the analyses.
- *Network-based methods:* Statistical methods that take the underlying structure of gene networks into account, representing either associative or causative interactions or dependencies among gene products.

Microarray data may require further processing aimed at reducing the dimensionality of the data to aid comprehension and more focused analysis. Other methods permit analysis of data consisting of a low number of biological or technical replicates; for example, the Local Pooled Error (LPE) test pools standard deviations of genes with similar expression levels in an effort to compensate for insufficient replication.

Relation Between Probe and Gene

The relation between a probe and the mRNA that it is expected to detect is not trivial. Some mRNAs may cross-hybridize probes in the array that are supposed to detect another mRNA. In addition, mRNAs may experience amplification bias that is sequence or molecule-specific. Thirdly, probes that are designed to detect the mRNA of a particular gene may be relying on genomic EST information that is incorrectly associated with that gene.

Data Warehousing

Microarray data was found to be more useful when compared to other similar datasets. The sheer volume of data, specialized formats (such as MIAME), and curation efforts associated with the datasets require specialized databases to store the data.

Allele-specific Oligonucleotide

An allele-specific oligonucleotide (ASO) is a short piece of synthetic DNA complementary to the sequence of a variable target DNA. It acts as a probe for the presence of the target in a Southern blot assay or, more commonly, in the simpler Dot blot assay. It is a common tool used in genetic testing, forensics, and Molecular Biology research. An ASO is typically an oligonucleotide of 15–21 nucleotide bases in length. It is designed (and used) in a way that makes it specific for only one version, or allele, of the DNA being tested. The length of the ASO, which strand it is chosen from, and the conditions by which it is bound to (and washed from) the target DNA all play a role in its specificity. These probes can usually be designed to detect a difference of as little as 1 base in the target's genetic sequence, a basic ability in the assay of single-nucleotide polymorphisms (SNPs), important in genotype analysis and the Human Genome Project.

To be detected after it has bound to its target, the ASO must be labeled with a radioactive, enzymatic, or fluorescent tag. The Illumina Methylation Assay technology takes advantage of ASO to detect one base pair difference (cytosine versus thymine) to measure methylation at a specific CpG site.

Alternatives

ASO analysis is only one of the methods used to detect genetic polymorphisms. Direct DNA sequencing is used to initially characterize the mutation, but is too laborious for routine screening. An earlier method, Restriction Fragment Length Polymorphism (RFLP) didn't need to know the sequence change beforehand, but required that the mutation affect the cleavage site of a Restriction Enzyme. The RFLP assay was briefly adapted to the use of oligonucleotide probes, but this technique was quickly supplanted by ASO analysis of polymerase chain reaction (PCR) amplified DNA. The PCR technique itself has been adapted to detect polymorphisms, as allele-specific PCR. However, the simplicity and versatility of the combined PCR/ASO method has led to its continued use, including with non-radioactive labels, and in a "reverse dot blot" format where the ASO probes are bound to the membrane and the amplified sample DNA is used for hybridization.

History

The use of synthetic oligonucleotides as specific probes for genetic sequence variations was pioneered by R. Bruce Wallace, working at the City of Hope National Medical Center in Duarte, California. In 1979

Wallace and his coworkers reported the use of ASO probes to detect variations in a single-stranded bacterial virus, and later applied the technique to cloned human genes. In 1983 and 1985 Wallace's lab reported the detection of the mutation for sickle cell anemia in samples of whole genomic DNA, although this application was hampered by the small amount of label that could be carried by the ASO.

Fortunately PCR, a method to greatly amplify a specific segment of DNA, was also reported in 1985. In less than a year PCR had been paired with ASO analysis. This combination solved the problem of ASO labeling, since the amount of target DNA could be amplified over a million-fold. Also, the specificity of the PCR process itself could be added to that of the ASO probes, greatly reducing the problem of spurious binding of the ASO to non-target sequences. The combination was specific enough that it could be used in a simple Dot blot, avoiding the laborious and inefficient Southern blot method.

Autopsy

An autopsy—also known as a post-mortem examination, necropsy (particularly as to non-human bodies), autopsia cadaverum, or obduction—is a highly specialized surgical procedure that consists of a thorough examination of a corpse to determine the cause and manner of death and to evaluate any disease or injury that may be present. It is usually performed by a specialized medical doctor called a pathologist.

Autopsies are performed for either legal or medical purposes. For example, a forensic autopsy is carried out when the cause of death may be a criminal matter, while a clinical or academic autopsy is performed to find the medical cause of death and is used in cases of unknown or uncertain death, or for research purposes. Autopsies can be further classified into cases where external examination suffices, and those where the body is dissected and internal examination is conducted. Permission from next of kin may be required for internal autopsy in some cases. Once an internal autopsy is complete the body is reconstituted by sewing it back together.

History

The term "autopsy" derives from the Ancient Greek *autopsia*, "to see for oneself", derived from áõôïò (*autos*, "oneself") and üøeò (*opsis*, "eye"). Around 3000 BC ancient Egyptians were one of the first

civilizations to practice the removal and examination of the internal organs of humans in the religious practice of mummification.

Autopsies that opened the body to determine the cause of death were attested at least in the early third millennium BC, although they were opposed in many ancient societies where it was believed that the outward disfigurement of dead persons prevented them from entering the afterlife (as with the Egyptians, who removed the organs through tiny slits in the body). Notable Greek autopsists were Erasistratus and Herophilus of Chalcedon, who lived in 3rd century BC Alexandria, but in general, autopsies were rare in ancient Greece. In 44 BC, Julius Caesar was the subject of an official autopsy after his murder by rival senators, the physician's report noting that the second stab wound Caesar received was the fatal one. By around 150 BC, ancient Roman legal practice had established clear parameters for autopsies.

The dissection of human remains for medical reasons continued to be practiced irregularly after the Romans, for instance by the Arab physicians Avenzoar and Ibn al-Nafis, but the modern autopsy process derives from the anatomists of the Renaissance. Giovanni Morgagni (1682–1771), celebrated as the father of anatomical pathology, wrote the first exhaustive work on pathology, *De Sedibus et Causis Morborum per Anatomen Indagatis* (The Seats and Causes of Diseases Investigated by Anatomy, 1769).

The great 19th-century medical researcher Rudolf Virchow, in response to a lack of standardization of autopsy procedures, established and published specific autopsy protocols (one such protocol still bears his name).

Purpose

The principal aim of an autopsy is to determine the cause of death, the state of health of the person before he or she died, and whether any medical diagnosis and treatment before death was appropriate.

In most Western countries the number of autopsies performed in hospitals has been decreasing every year since 1955. Critics, including pathologist and former *JAMA* editor George Lundberg, have charged that the reduction in autopsies is negatively affecting the care delivered in hospitals, because when mistakes result in death, they are often not investigated and lessons therefore remain unlearned.

When a person has given permission in advance of their death, autopsies may also be carried out for the purposes of teaching or medical

research.

An autopsy is frequently performed in cases of sudden death, where a doctor is not able to write a death certificate, or when death is believed to result from an unnatural cause. These examinations are performed under a legal authority (Medical Examiner or Coroner or Procurator Fiscal) and do not require the consent of relatives of the deceased.

The most extreme example is the examination of murder victims, especially when medical examiners are looking for signs of death or the murder method, such as bullet wounds and exit points, signs of strangulation, or traces of poison. Many religions such as Judaism and Islam usually discourage the performing of autopsies on their adherents. Organizations such as Zaka in Israel and Misaskim in the USA generally guide families how to ensure that an unnecessary autopsy is not made.

In Medicine

Autopsies are important in clinical medicine as they can identify medical error and assist continuous improvement.

A study that focused on myocardial infarction (heart attack) as a cause of death found significant errors of omission and commission, i.e. a sizable number cases ascribed to myocardial infarctions (MIs) were not MIs and a significant number of non-MIs were actually MIs.

A systematic review of studies of the autopsy calculated that in about 25% of autopsies a major diagnostic error will be revealed. However, this rate has decreased over time and the study projects that in a contemporary US institution, 8.4% to 24.4% of autopsies will detect major diagnostic errors.

A large meta-analysis suggested that approximately one-third of death certificates are incorrect and that half of the autopsies performed produced findings that were not suspected before the person died. Also, it is thought that over one fifth of unexpected findings can only be diagnosed histologically, i.e. by biopsy or autopsy, and that approximately one quarter of unexpected findings, or 5% of all findings, are major and can similarly only be diagnosed from tissue.

One study found that “Autopsies revealed 171 missed diagnoses, including 21 cancers, 12 strokes, 11 myocardial infarctions, 10 pulmonary emboli, and 9 endocarditis, among others”.

Focusing on intubated patients, one study found “abdominal pathologic conditions—abscesses, bowel perforations, or infarction—were as frequent as pulmonary emboli as a cause of class I errors. While

patients with abdominal pathologic conditions generally complained of abdominal pain, results of examination of the abdomen were considered unremarkable in most patients, and the symptom was not pursued".

In Veterinary Medicine

Post-mortem examination is far more common in veterinary medicine than in human medicine. For many species that exhibit few external symptoms (sheep), or that are not suited to detailed clinical examination (poultry, cage birds, zoo animals), it is a common method used by veterinarians to come to a diagnosis.

Types

There are four main types of autopsies:

- *Medico-Legal Autopsy or Forensic* or *coroner's autopsies* seek to find the cause and manner of death and to identify the decedent. They are generally performed, as prescribed by applicable law, in cases of violent, suspicious or sudden deaths, deaths without medical assistance or during surgical procedures.
- *Clinical* or *Pathological autopsies* are performed to diagnose a particular disease or for research purposes. They aim to determine, clarify, or confirm medical diagnoses that remained unknown or unclear prior to the patient's death.
- *Anatomical* or *academic autopsies* are performed by students of anatomy for study purpose only.
- *Virtual* or *medical imaging autopsies* are performed utilizing imaging technology only, primarily magnetic resonance imaging (MRI) and computed tomography (CT).

Forensic Autopsy

A forensic autopsy is used to determine the cause of death. Forensic science involves the application of the sciences to answer questions of interest to the legal system. In United States law, deaths are placed in one of five manners:

- Natural
- Accident
- Homicide
- Suicide
- Undetermined

In some jurisdictions, the Undetermined category may include deaths in absentia, such as deaths at sea and missing persons declared dead in a court of law; in others, such deaths are classified under "Other". But, medical examiners also attempt to determine the time of death, the exact cause of death, and what, if anything, preceded the death, such as a struggle. A forensic autopsy may include obtaining biological specimens from the deceased for toxicological testing, including stomach contents.

Toxicology tests may reveal the presence of one or more chemical "poisons" (all chemicals, in sufficient quantities, can be classified as a poison) and, the quantity of those chemicals. Because post-mortem deterioration of the body, together with the gravitational pooling of bodily fluids, will necessarily alter the bodily environment, toxicology tests may overestimate, rather than underestimate, the quantity of the suspected chemical.

Most states require the State medical examiner to complete an autopsy report and many mandate that the autopsy be videotaped.

Following an in-depth examination of all the evidence, a medical examiner or coroner will assign a manner of death as one of the five listed above, and detail the evidence on the mechanism of the death.

Clinical Autopsy

Clinical autopsies serve two major purposes. They are performed to gain more insight into pathological processes and determine what factors contributed to a patient's death. Autopsies are also performed to ensure the standard of care at hospitals. Autopsies can yield insight into how patient deaths can be prevented in the future.

Within the United Kingdom, clinical autopsies can be carried out only with the consent of the family of the deceased person as opposed to a medico-legal autopsy instructed by a Coroner (England & Wales) or Procurator Fiscal (Scotland) to which the family cannot object.

Prevalence

In 2004 in England and Wales, there were 514,000 deaths of which 225,500 were referred to the coroner. Of those, 115,800 (22.5%) resulted in post-mortem examinations and there were 28,300 inquests, 570 with a jury.

In the United States, autopsy rates fell from 17% in 1980 to 14% in 1985 and 11.5% in 1989, although the figures vary notably from county

to county.

Process

The body is received at a medical examiner's office or hospital in a body bag or evidence sheet. A new body bag is used for each body to ensure that only evidence from that body is contained within the bag. Evidence sheets are an alternate way to transport the body. An evidence sheet is a sterile sheet that the body is covered in when it is moved. If it is believed there may be any significant residue on the hands, for instance gunpowder, a separate paper sack is put around each hand and taped shut around the wrist.

There are two parts to the physical examination of the body: the external and internal examination. Toxicology, biochemical tests and/ or genetic testing often supplement these and frequently assist the pathologist in assigning the cause or causes of death.

External Examination

At many institutions the person responsible for handling, cleaning, and moving the body is often called a diener, the German word for *servant*. In the UK this role is performed by an Anatomical Pathology Technologist who will also assist the pathologist in eviscerating the body and reconstruction after the autopsy. After the body is received, it is first photographed. The examiner then notes the kind of clothes and their position on the body before they are removed. Next, any evidence such as residue, flakes of paint or other material is collected from the external surfaces of the body. Ultraviolet light may also be used to search body surfaces for any evidence not easily visible to the naked eye. Samples of hair, nails and the like are taken, and the body may also be radiographically imaged.

Once the external evidence is collected, the body is removed from the bag, undressed, and any wounds present are examined. The body is then cleaned, weighed, and measured in preparation for the internal examination. The scale used to weigh the body is often designed to accommodate the cart that the body is transported on; its weight is then deducted from the total weight shown to give the weight of the body.

If not already within an autopsy room, the body is transported to one and placed on a table. A general description of the body as regards ethnicity, sex, age, hair color and length, eye color and other distinguishing features (birthmarks, old scar tissue, moles, tattoos, etc.) is then made. A handheld voice recorder or a standard examination form is

normally used to record this information. In some countries e.g. France, Germany, and Canada, an autopsy may comprise an external examination only.

This concept is sometimes termed a "view and grant". The principles behind this being that the medical records, history of the deceased and circumstances of death have all indicated as to the cause and manner of death without the need for an internal examination.

Internal Examination

If not already in place, a plastic or rubber brick called a "body block" is placed under the back of the body, causing the arms and neck to fall backward whilst stretching and pushing the chest upward to make it easier to cut open. This gives the prosector, a pathologist or assistant, maximum exposure to the trunk. After this is done, the internal examination begins. The internal examination consists of inspecting the internal organs of the body for evidence of trauma or other indications of the cause of death. For the internal examination there are a number of different approaches available:

- A large and deep Y-shaped incision can be made starting at the top of each shoulder and running down the front of the chest, meeting at the lower point of the sternum. This is the approach most often used.
- A T-shaped incision made from the tips of both shoulder, in a horizontal line across the region of the collar bones to meet at the sternum (breastbone) in the middle.
- A single vertical cut is made from the middle of the neck (in the region of the 'adam's apple' on a male body)

In all of the above cases the cut then extends all the way down to the pubic bone (making a deviation to the left side of the navel).

Bleeding from the cuts is minimal, or non-existent, because the pull of gravity is producing the only blood pressure at this point, related directly to the complete lack of cardiac functionality. However, in certain cases there is anecdotal evidence to prove that bleeding can be quite profuse, especially in cases of drowning.

At this point, shears are used to open the chest cavity. It is also possible to utilise a simple scalpel blade. The prosector uses the tool to saw through the ribs on the lateral sides of the chest cavity to allow the sternum and attached ribs to be lifted as one chest plate; this is done so that the heart and lungs can be seen in situ and that the heart, in

particular the pericardial sac is not damaged or disturbed from opening. A scalpel is used to remove any soft tissue that is still attached to the posterior side of the chest plate. Now the lungs and the heart are exposed. The chest plate is set aside and will be eventually replaced at the end of the autopsy.

At this stage the organs are exposed. Usually, the organs are removed in a systematic fashion. Making a decision as to what order the organs are to be removed will depend highly on the case in question. Organs can be removed in several ways: The first is the en masse technique of letulle whereby all the organs are removed as one large mass. The second is the en bloc method of Ghon. The most popular in the UK is a modified version of this method which is divided into four groups of organs. Although these are the two predominant evisceration techniques in the UK variations on these are widespread.

One method is described here: The pericardial sac is opened to view the heart. Blood for chemical analysis may be removed from the inferior vena cava or the pulmonary veins. Before removing the heart, the pulmonary artery is opened in order to search for a blood clot. The heart can then be removed by cutting the inferior vena cava, the pulmonary veins, the aorta and pulmonary artery, and the superior vena cava. This method leaves the aortic arch intact, which will make things easier for the embalmer. The left lung is then easily accessible and can be removed by cutting the bronchus, artery, and vein at the hilum. The right lung can then be similarly removed. The abdominal organs can be removed one by one after first examining their relationships and vessels.

Some pathologists, however, prefer to remove the organs all in one “block”. Then a series of cuts, along the vertebral column, are made so that the organs can be detached and pulled out in one piece for further inspection and sampling. During autopsies of infants, this method is used almost all of the time. The various organs are examined, weighed and tissue samples in the form of slices are taken. Even major blood vessels are cut open and inspected at this stage. Next the stomach and intestinal contents are examined and weighed. This could be useful to find the cause and time of death, due to the natural passage of food through the bowel during digestion. The more area empty, the longer the deceased had gone without a meal before death.

The body block that was used earlier to elevate the chest cavity is now used to elevate the head. To examine the brain, an incision is made from behind one ear, over the crown of the head, to a point behind the

other ear. When the autopsy is completed, the incision can be neatly sewn up and is not noticed when the head is resting on a pillow in an open casket funeral. The scalp is pulled away from the skull in two flaps with the front flap going over the face and the rear flap over the back of the neck. The skull is then cut with what is called a Stryker saw, named for its manufacture, to create a "cap" that can be pulled off, exposing the brain. The brain is then observed in situ. Then the brain's connection to the cranial nerves and spinal cord are severed, and the brain is then lifted out of the skull for further examination. If the brain needs to be preserved before being inspected, it is contained in a large container of formalin (15 percent solution of formaldehyde gas in buffered water) for at least two but preferably four weeks. This not only preserves the brain, but also makes it firmer allowing easier handling without corrupting the tissue.

Reconstitution of the Body

An important component of the autopsy is the reconstitution of the body such that it can be viewed, if desired, by relatives of the deceased following the procedure. After the examination, the body has an open and empty chest cavity with chest flaps open on both sides, the top of the skull is missing, and the skull flaps are pulled over the face and neck. It is unusual to examine the face, arms, hands or legs internally.

In the UK, following the Human Tissue Act 2004 all organs and tissue must be returned to the body unless permission is given by the family to retain any tissue for further investigation. Normally the internal body cavity is lined with cotton wool or an appropriate material, the organs are then placed into a plastic bag to prevent leakage and returned to the body cavity.

The chest flaps are then closed and sewn back together and the skull cap is sewed back in place. Then the body may be wrapped in a shroud and it is common for relatives to not be able to tell the procedure has been done when the body is viewed in a funeral parlor after embalming.

Histology

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. It is commonly performed by examining cells and tissues by sectioning and staining, followed by examination under a light microscope or electron microscope. Histological studies may be conducted via tissue culture, where live cells can be isolated and

maintained in a proper environment outside the body for various research projects. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine.

Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of cancer and other diseases usually requires histopathological examination of samples. Trained medical doctors, frequently board-certified as pathologists, are the personnel who perform histopathological examination and provide diagnostic information based on their observations.

The trained scientists who perform the preparation of histological sections are *histotechnicians,* histology technicians (HT), histology technologists (HTL), medical scientists, medical laboratory technicians, or biomedical scientists. Their field of study is called *histotechnology.*

Fixation (Histology)

In the fields of histology, pathology, and cell biology, fixation is a chemical process by which biological tissues are preserved from decay, thereby preventing autolysis or putrefaction. Fixation terminates any ongoing biochemical reactions, and may also increase the mechanical strength or stability of the treated tissues.

Purposes of Fixation

Fixation of tissue is done for several reasons. One reason is to kill the tissue so that postmortem decay (autolysis and putrefaction) is prevented. Fixation preserves a sample of biological material (tissue or cells) as close to its natural state as possible in the process of preparing tissue for examination. To achieve this, several conditions usually must be met.

First, a fixative usually acts to disable intrinsic biomolecules—particularly proteolytic enzymes—which otherwise digests or damages the sample.

Second, a fixative typically protects a sample from extrinsic damage. Fixatives are toxic to most common microorganisms (bacteria in particular) that might exist in a tissue sample or which might otherwise colonise the fixed tissue. In addition, many fixatives

chemically alter the fixed material to make it less palatable (either indigestible or toxic) to opportunistic microorganisms.

Finally, fixatives often alter the cells or tissues on a molecular level to increase their mechanical strength or stability. This increased strength and rigidity can help preserve the morphology (shape and structure) of the sample as it is processed for further analysis.

Even the most careful fixation does alter the sample and introduce artifacts that can interfere with interpretation of cellular ultrastructure. A prominent example is the bacterial *mesosome*, which was thought to be an organelle in gram-positive bacteria in the 1970s, but was later shown by new techniques developed for electron microscopy to be simply an artifact of chemical fixation. Standardization of fixation and other tissue processing procedures takes this introduction of artifacts into account, by establishing what procedures introduce which kinds of artifacts. Researchers who know what types of artifacts to expect with each tissue type and processing technique can accurately interpret sections with artifacts, or choose techniques that minimize artifacts in areas of interest.

Fixation Process

Fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis. Therefore, the choice of fixative and fixation protocol may depend on the additional processing steps and final analyses that are planned. For example, immunohistochemistry uses antibodies that bind to a specific protein target. Prolonged fixation can chemically mask these targets and prevent antibody binding. In these cases, a ‘quick fix’ method using cold formalin for around 24 hours is typically used.

Types of Fixation

There are Generally Three Types of Fixation Process. Heat fixation: After a smear has dried at room temperature, the slide is gripped by tongs or a clothespin and passed through the flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide. Routinely used with bacteria and archaea. Heat fixation generally preserves overall morphology but not internal structures. Heat denatures the proteolytic enzyme and prevent autolysis. Heat fixation cannot be used in the capsular stain method as heat fixation will shrink or destroy the capsule (glycocalyx) and cannot be seen in stains.

Perfusion: Fixation via blood flow. The fixative is injected into the heart with the injection volume matching cardiac output. The fixative

spreads through the entire body, and the tissue doesn't die until it is fixed. This has the advantage of preserving perfect morphology, but the disadvantages that the subject dies and the cost is high (because of the volume of fixative needed for larger organisms)

Immersion: The sample of tissue is immersed in fixative of volume at a minimum of 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative must be considered. Using a larger sample means it takes longer for the fixative to reach the deeper tissue. Best in a slight vacuum.

Chemical Fixation

In this process, structures are preserved in a state (both chemically and structurally) as close to living tissue as possible. This requires a chemical fixative that can stabilise the proteins, nucleic acids and mucosubstances of the tissue by making them insoluble.

Types of Chemical Fixatives

Crosslinking Fixatives–Aldehydes

Crosslinking fixatives act by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton, and lends additional rigidity to the tissue.

By far the most commonly used fixative in histology is formaldehyde. It is usually used as a 10% Neutral Buffered Formalin (NBF), that is approx. 3.7%-4.0% formaldehyde in phosphate buffered saline. Because formaldehyde is a gas at room temperature, formalin-formaldehyde gas dissolved in water (~37% w/v)-is used when making the former fixative.

Paraformaldehyde is a polymerised form of formaldehyde, usually obtained as a fine white powder, which depolymerises back to formalin when heated. Formaldehyde fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine. Its effects are reversible by excess water and it avoids formalin pigmentation. Other benefits include: Long term storage and good tissue penetration. It is particularly good for immunohistochemistry techniques. Also the formaldehyde vapour can be used as a fixatives for cell smears.

Another popular aldehyde for fixation is glutaraldehyde. It operates in a similar way to formaldehyde by causing deformation of the alpha-

helix structures in proteins.

However glutaraldehyde is a larger molecule, and so its rate of diffusion across membranes is slower than formaldehyde. Consequently glutaraldehyde fixation on thicker tissue samples may be hampered, but this problem can be overcome by reducing the size of the tissue sample.

One of the advantages of glutaraldehyde fixation is that it may offer a more rigid or tightly linked fixed product—its greater length and two aldehyde groups allow it to 'bridge' and link more distant pairs of protein molecules. It causes rapid and irreversible changes, fixes quickly, is well suited for electron microscopy, fixes well at 4C, and gives best overall cytoplasmic and nuclear detail. However it is not ideal for immunohistochemistry staining.

Some fixation protocols call for a combination of formaldehyde and glutaraldehyde so that their respective strengths complement one another.

These crosslinking fixatives–especially formaldehyde–tend to preserve the secondary structure of proteins and may protect significant amounts of tertiary structure as well.

Precipitating Fixatives–Alcohols

Precipitating (or *denaturing*) fixatives act by reducing the solubility of protein molecules and (often) by disrupting the hydrophobic interactions that give many proteins their tertiary structure. The precipitation and aggregation of proteins is a very different process from the crosslinking that occurs with the aldehyde fixatives.

The most common precipitating fixatives are ethanol and methanol. They are commonly used to fix frozen sections and smears. Acetone is also used and has been shown to produce better histological preservation than frozen sections when employed in the Acetone Methylbenzoate Xylene (AMEX) technique.

The protein denaturants - methanol, ethanol and acetone - are rarely used alone for fixing blocks unless studying nucleic acids.

Acetic acid is a denaturant that is sometimes used in combination with the other precipitating fixatives. The alcohols, by themselves, are known to cause considerable shrinkage and hardening of tissue during fixation while acetic acid alone is associated with tissue swelling; combining the two may result in better preservation of tissue morphology.

Oxidising Agents

The oxidising fixatives can react with various side chains of proteins and other biomolecules, allowing formation of crosslinks that stabilize tissue structure. However they cause extensive denaturation despite preserving fine cell structure and are used mainly as secondary fixatives. Osmium tetroxide is often used as a secondary fixative when samples are prepared for electron microscopy. (It is not used for light microscopy as it penetrates thick sections of tissue very poorly.)

Potassium dichromate, chromic acid, and potassium permanganate all find use in certain specific histological preparations.

Mercurials

Mercurials such as B-5 and Zenker's fixative hav an unknown mechanism that increases staining brightness and give excellent nuclear detail. Despite being fast, mercurials penetrate poorly and produce tissue shrinkage. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Also note that since they contain mercury care must be taken with disposal.

Picrates

Picrates penetrate tissue well to react with histones and basic proteins to form crystalline picrates with amino acids and precipitate all proteins. It is a good fixative for connective tissue, preserves glycogen well, and extracts lipids to give superior results to formaldehyde in immunostaining of biogenic and polypeptide hormones However, it causes a loss of basophilia unless the specimen is thoroughly washed following fixation.

HOPE Fixative

Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) gives formalin-like morphology, excellent preservation of protein antigens for immunohistochemistry and enzyme histochemistry, good RNA and DNA yields and absence of crosslinking proteins.

Frozen Sections

Small pieces of tissue (5×5×3mm) are placed in a cryoprotective embedding medium—OCT, TBS, or Cryogel—then snap frozen in isopentane cooled by liquid nitrogen. Tissue is then sectioned in a freezing microtome or cryostat.

Sections are then fixed in one of the following fixatives: Absolute acetone for 10–15 minutes, 95% ethanol for 10–15 minutes or Absolute acetone 10 minutes followed by 95% ethanol 10 minutes

Advantages

- Give better preservation of antigenicity
- Minimal exposure to fixative
- Not exposed to the organic solvents
- Much faster than other forms of fixations.

Disadvantages

- Lack morphological detail
- Present a potential biohazard

Factors Affecting Fixation

pH

Should be kept in the physiological range, between pH 4-9. The pH for the ultrastructure preservation should be buffered between 7.2 to 7.4

Osmolarity

Hypertonic solutions give rise to cell shrinkage. Hypotonic solutions result in cell swelling and poor fixation. 10% neutral buffer formalin is 4% formaldehyde (1.33 osmolar) in PBS buffer (0.3 osmolar) sums to 1.63 osmolar.

This is a very hypertonic solution yet it has worked well as a general tissue fixation condition for many years in pathology labs.

Time from Removal to Fixation

Fixation is a chemical process, and time must be allowed for the process to complete. Although “over fixation” can be detrimental, under-fixation has recently been appreciated as a significant problem and may be responsible for inappropriate results for some assays.

Frozen Section Fixation

Frozen section is a rapid way to fix and mount histology sections. It is used in surgical removal of tumors, and allow rapid determination of margin (that the tumor has been completely removed). It is done using a refrigeration device called a cryostat. The frozen tissue is sliced using a

microtome, and the frozen slices are mounted on a glass slide and stained the same way as other methods.

It is a necessary way to fix tissue for certain stain such as antibody linked immunofluorescence staining. It can also be used to determine if a tumour is malignant when it is found incidentally during surgery on a patient.

Processing - dehydration, Clearing and Infiltration

The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 im (micrometres; 1000 micrometres = 1 mm) thick for light microscopy and 80-100 nm (nanometre; 1,000,000 nanometres = 1 mm) thick for electron microscopy. For light microscopy, paraffin wax is most frequently used.

Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene.

Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohisto-chemistry is required. Thicker sections (0.35im to 5im) of resin-embedded tissue can also be cut for light microscopy. Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

Embedding

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine, or wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. The acrylic resins are polymerised by heat, ultraviolet light, or chemical catalysts. The

hardened blocks containing the tissue samples are then ready to be sectioned.

Because Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, FFPE tissues are an important resource for historical studies in medicine.

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, OCT, TBS, Cryogel, or resin, which is then frozen to form hardened blocks.

Sectioning

Sectioning can be done in limited ways. Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of the hair follicles and pilosebaceous units. Tangential to horizontal sectioning is done in Mohs surgery and in methods of CCPDMA.

For light microscopy, a steel knife mounted in a microtome is used to cut 10-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain.

Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat.

Staining

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used. Hematoxylin and eosin (H&E stain) is the most commonly used light microscopical stain in histology and histopathology. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Uranyl acetate and lead citrate are commonly used to impart contrast to tissue in the electron microscope.

Special staining: There are hundreds of various other techniques that have been used to selectively stain cells and cellular components. Other

compounds used to color tissue sections include safranin, oil red o, Congo red, fast green FCF, silver salts, and numerous natural and artificial dyes that were usually originated from the development dyes for the textile industry.

Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the Perls Prussian blue reaction, used to demonstrate iron deposits in diseases like hemochromatosis.

Histology samples have often been examined by radioactive techniques. In historadiography, a slide (sometimes stained histochemically) is X-rayed. More commonly, autoradiography is used to visualize the locations to which a radioactive substance has been transported within the body, such as cells in S phase (undergoing DNA replication) which incorporate tritiated thymidine, or sites to which radiolabeled nucleic acid probes bind in situ hybridization. For autoradiography on a microscopic level, the slide is typically dipped into liquid nuclear tract emulsion, which dries to form the exposure film. Individual silver grains in the film are visualized with dark field microscopy.

Recently, antibodies have been used to specifically visualize proteins, carbohydrates, and lipids. This process is called immunohistochemistry, or when the stain is a fluorescent molecule, immunofluorescence. This technique has greatly increased the ability to identify categories of cells under a microscope. Other advanced techniques, such as nonradioactive *in situ* hybridization, can be combined with immunochemistry to identify specific DNA or RNA molecules with fluorescent probes or tags that can be used for immunofluorescence and enzyme-linked fluorescence amplification (especially alkaline phosphatase and tyramide signal amplification).

Fluorescence microscopy and confocal microscopy are used to detect fluorescent signals with good intracellular detail. Digital cameras are increasingly used to capture histological and histopathological image

Alternative Techniques

Alternative techniques include cryosection. The tissue is frozen using a cryostat, and cut. Tissue staining methods are similar to those of wax sections. Plastic embedding is commonly used in the preparation of material for electron microscopy. Tissues are embedded in epoxy resin.

Very thin sections (less than 0.1 micrometer) are cut using diamond or glass knives. The sections are stained with electron dense stains (uranium and lead) so that they can possibly be seen with the electron microscope.

History

In the 19th century, histology was an academic discipline in its own right. The 1906 Nobel Prize in Physiology or Medicine was awarded to histologists Camillo Golgi and Santiago Ramon y Cajal. They had dueling interpretations of the neural structure of the brain based in differing interpretations of the same images. Cajal won the prize for his correct theory and Golgi for the staining technique he invented to make it possible.

Histological Classification of Animal Tissues

There are four basic types of tissues: muscle tissue, nervous tissue, connective tissue, and epithelial tissue. All tissue types are subtypes of these four basic tissue types (for example, blood cells are classified as connective tissue, since they generally originate inside bone marrow).

- *Epithelium:* the lining of glands, bowel, skin, and some organs like the liver, lung, and kidney
- *Endothelium:* the lining of blood and lymphatic vessels
- *Mesothelium:* the lining of pleural and pericardial spaces
- *Mesenchyme:* the cells filling the spaces between the organs, including fat, muscle, bone, cartilage, and tendon cells
- *Blood cells:* the red and white blood cells, including those found in lymph nodes and spleen
- *Neurons:* any of the conducting cells of the nervous system
- *Germ cells:* reproductive cells (spermatozoa in men, oocytes in women)
- *Placenta:* an organ characteristic of true mammals during pregnancy, joining mother and offspring, providing endocrine secretion and selective exchange of soluble, but not particulate, blood-borne substances through an apposition of uterine and trophoblastic vascularised parts
- *Stem cells:* cells with the ability to develop into different cell types

Note that tissues from plants, fungi, and microorganisms can also be examined histologically. Their structure is very different from animal tissues.

Related Sciences

- Cell biology is the study of living cells, their DNA and RNA and the proteins they express.
- Anatomy is the study of organs visible by the naked eye.
- Morphology studies entire organisms.

Artifacts

Artifacts are structures or features in tissue that interfere with normal histological examination. These are not always present in normal tissue and can come from outside sources. Artifacts interfere with histology by changing the tissues appearance and hiding structures. These can be divided into two categories:

Pre-histology

These are features and structures that have been introduced prior to the collection of the tissues. A common example of these include: ink from tattoos and freckles (melanin) in skin samples.

Post-histology

Artifacts can result from tissue processing. Processing commonly leads to changes like shrinkage, washing out of particular cellular components, color changes in different tissues types and alterations of the structures in the tissue. Because these are caused in a laboratory the majority of post histology artifacts can be avoided or removed after being discovered. A common example is mercury pigment left behind after using Zenker's fixative to fix a section.

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