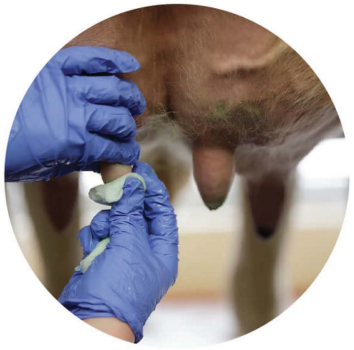

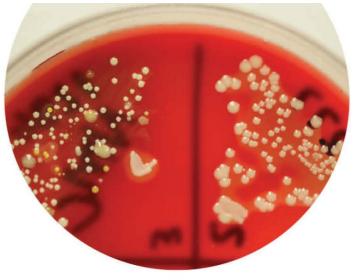

Laboratory Handbook on Bovine Mastitis

THIRD EDITION

A Global Organization for Mastitis Control and Milk Quality

Laboratory Handbook on Bovine Mastitis

THIRD EDITION

National Mastitis Council, Inc.
605 Columbus Avenue South
New Prague, Minnesota 56071 USA

Foreword

The *Laboratory and Field Handbook on Bovine Mastitis* was first published in 1987, with its first revision being published in 1999 as the *Laboratory Handbook on Bovine Mastitis.* The contributors to the most recent revision of the *Laboratory Handbook on Bovine Mastitis* would like to acknowledge the excellent work of the Writing Committee of the previous editions. Previous editions were comprehensive and organized in an excellent manner. Much of the material that was presented in the first revision remains very relevant and presentable today. Thus, readers of the first revision will be familiar with the current handbook. All readers will benefit from the hard work of the previous and current contributors to this practical publication.

The format of the current book is very similar to the previous edition. Microbial culture is still the backbone of mastitis diagnosis. The microbial culture methods have been updated when appropriate. Many of the procedures used to culture and identify mastitis pathogens have been used for many decades and are still very relevant today. New procedures are always being developed and the last several years have seen several molecular methods introduced that can be applied to more accurately identify causative organisms by genus and species, as well as strain type. Because of this changing landscape, it is difficult to provide detail on all new and developing methods. A new chapter (Chapter 4) has been included that discusses molecular techniques in general terms and is intended to provide an overview, rather than a discussion of the specifics of each individual test. Hence, the discussion is focused on the advantages and disadvantages of these newer techniques in terms of accuracy, specificity, and sensitivity of detection, cost, and turnaround time. Many of the other chapters reference some of these new techniques. Mycoplasmas are now discussed in their own chapter (Chapter 8) instead of with miscellaneous organisms; and a chapter (Chapter 12) has been added for on-farm culture.

What was written in the first revision foreword still applies. "The popularity of the first and second editions has been a testament to the acceptance of the Laboratory Handbook on Bovine Mastitis as an international reference for diagnosis of bovine mastitis by veterinarians, researchers, and diagnostic laboratories. The National Mastitis Council Research Committee anticipates that this revised edition will continue to be used by students, veterinarians, technical specialists, diagnostic laboratories, and others in the dairy industry involved with mastitis control."

Mention of specific equipment, products, or supplies does not imply endorsement by National Mastitis Council, Inc.

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Sample Collection and Handling

Introduction

Aseptic technique in sample collection is an absolute necessity. The quality of samples is extremely important in any diagnostic procedure; however, the quality of samples for mastitis diagnosis can be more difficult to obtain than other diseases. Organisms that have the potential to cause mastitis are common and prevalent throughout any dairy, and therefore samples can easily become contaminated if proper technique is not followed in collecting the sample. Contaminated samples lead to misdiagnosis, increased work, confusion, and frustration.

Sample storage

Storage and handling of samples are as important as the collection. Most mastitis-causing organisms survive refrigeration for several days or freezing for several weeks. Improper cooling, chemicals, and contaminating organisms in the sample can alter diagnostic results by altering pathogen growth or, in the case of contaminating organisms, overgrow the pathogen and obscure the diagnostic results. Some organisms, such as *Escherichia coli, Nocardia* spp., and *Mycoplasma* spp., may not survive extended periods of refrigeration or freezing.

Contaminated samples

The user of this handbook must thoroughly understand, practice, and insist upon aseptic sampling techniques and proper handling procedures in order to obtain highly reliable results. Much time, energy, and money can be wasted if shortcuts are employed in the very early stages of diagnosis. Some environmental conditions may render aseptic collection of samples impossible. Diagnosticians must learn to recognize contaminated samples. The most prudent decision following detection of a contaminated sample is to make no diagnosis of the infection status and to resample the cow.

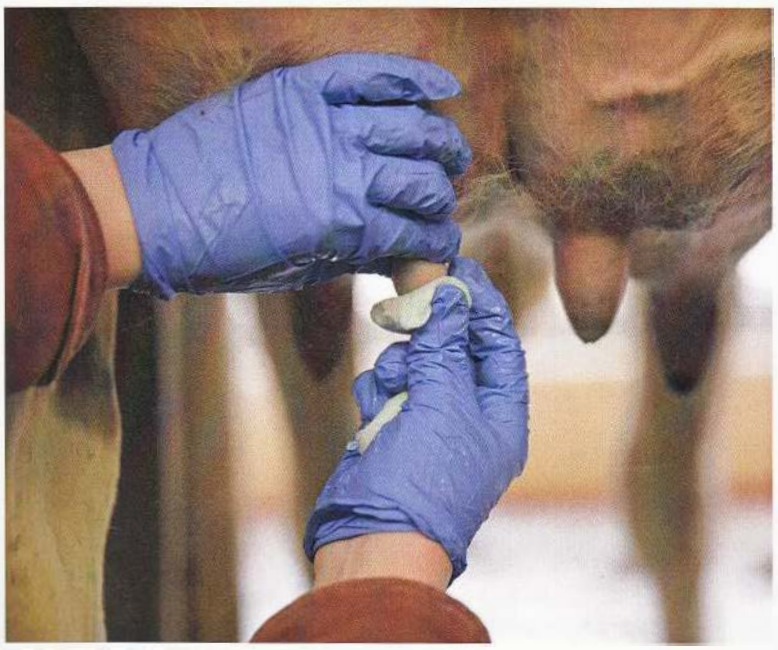

Figure 1.1
Aseptic technique for sample collection is an absolute necessity for accurate diagnosis.

Materials for Sampling

Sterile tubes	Sterile vials or tubes, 5- to 15-ml capacities
Alcohol	70% alcohol (ethyl or isopropyl)
Cotton or gauze wipes	Cotton balls, gauze squares, or pledgets soaked in 70% alcohol, or commercially prepared, individually packaged alcohol swabs
Gloves (latex or nitrile)	To prevent bacteria on hands from contaminating sample
Cooler with ice packs	Cooler with ice or freezer packs for storing samples
Markers	Means of identifying samples: permanent ink pen (with ink that is stable in both water and alcohol) or typed labels
Plastic wrap or sealable bag	To either wrap or place samples in, respectively, and provide secondary containment during transport
Racks	Racks for holding sample tubes or vials while sampling cows and for storage in the cooler
Teat dip	Disinfectant for cleaning teats (germicidal products used for premilking teat dipping are recommended)
Towels	Paper towels or individual cloth towels

Figure 1.2
Materials for aseptic collection of milk samples.

Sampling Technique

1. Label tubes	Label tubes prior to sampling (date, farm, cow, and quarter, as applicable).
2. Gloves	Put on clean latex or nitrile gloves prior to starting sampling. Gloves should be kept clean and replaced if they become contaminated or ripped.
3. Clean teats	Using a hand or a dry paper towel, brush loose dirt, bedding, and hair from the gland and teats. Grossly dirty teats and udders should be washed and dried thoroughly before proceeding with sample collection. Udders should be washed as a last resort.
4. Forestrip	Discard a few streams of milk from the teat (strict foremilk) and observe milk and gland for signs of clinical mastitis. Record all observations of clinical signs.
5. Pre-dip	Pre-dip all quarters in an effective predip product and allow 30 seconds contact time.
6. Dry teats	Dry teats thoroughly with a paper towel or clean individual cloth towel.
7. Alcohol scrub	Beginning with teats on the far side of the udder, scrub teat ends vigorously (10 to 15 seconds) with cotton balls, gauze squares or wipes, moist (not dripping wet) with 70% alcohol. When cotton balls are saturated with alcohol, simply squeeze out excess alcohol prior to use. Use as many cotton balls, gauze squares or wipes, as necessary, to clean the teat ends. Teat ends should be scrubbed until no more dirt appears on the swab or is visible on the teat end. A single cotton ball, gauze square, or wipe should not be used on more than one teat. Care should be taken not to touch clean teat ends. Also, care should be taken to avoid clean teats coming into contact with dirty tail switches, feet, and legs. In herds where cows are not cooperative, begin by scrubbing the nearest teat until clean, obtain the sample, and move to the next teat.

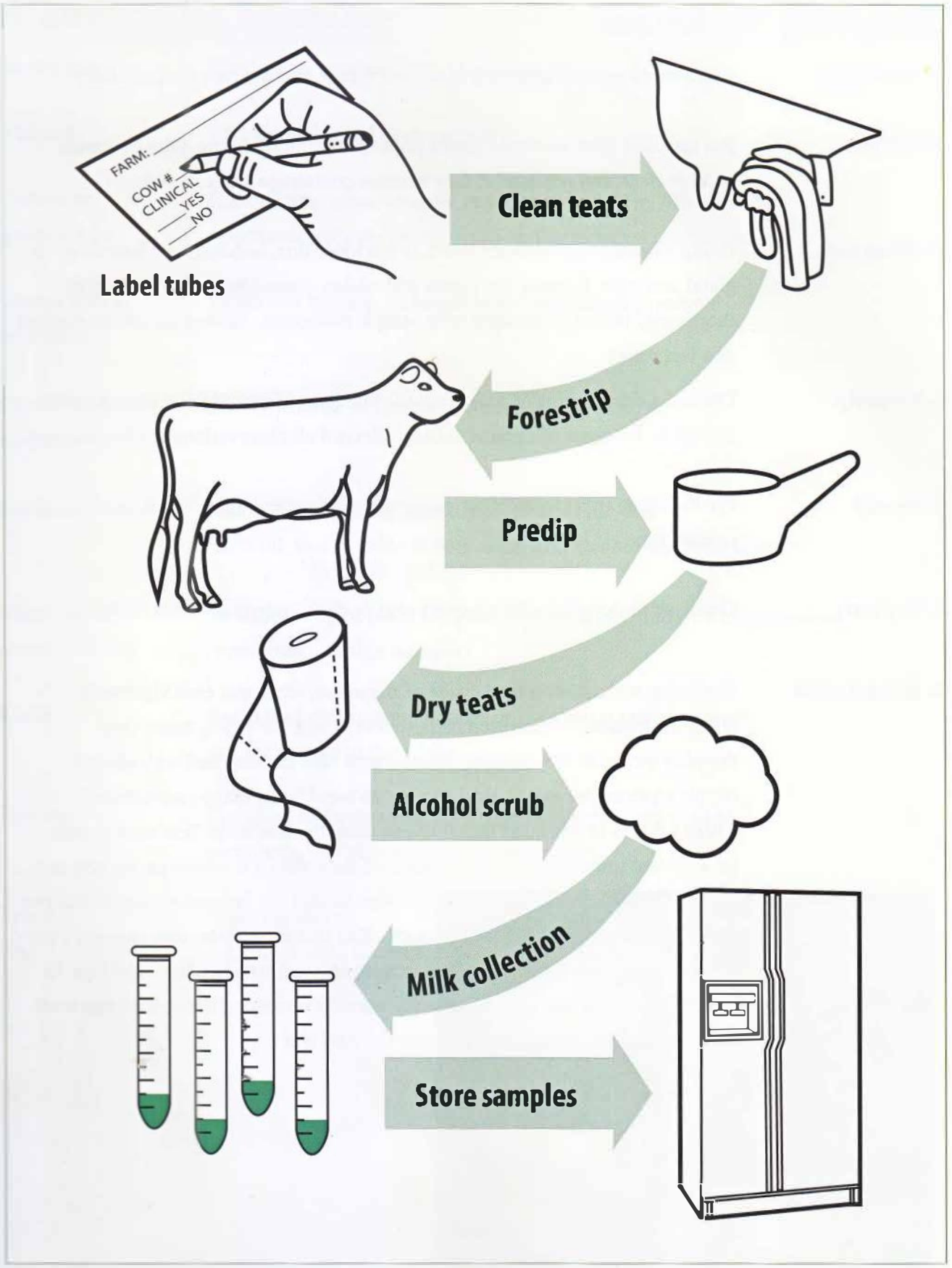

Figure 1.3
Flow diagram of sampling procedures.

8. Sample

To collect individual quarter milk samples, begin sample collection from the closest teat and move to teats on the far side of the udder – the reverse order from cleaning – to collect the sample, remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap. Always keep the open end of the cap facing downward. Maintain the tube or vial at approximately a 45° angle while taking the sample. Do not allow the lip of the sample tube to touch the teat end. Strip one stream of milk outside the tube, then collect one to three streams of milk and immediately replace and tightly secure the cap. Make sure milk entering the tube does not touch fingers or hands. Two to 3 ml of milk is generally a sufficient sample size, and there is seldom need to collect >5 ml. Sample vials should never be filled more than three-fourths full. Large volume samples are not required and increase the risk of contamination.

To collect a composite sample (milk from all four quarters in the same tube), begin sample collection with the nearest teats and progress to teats on the far side of the udder. A representative sample (1 to 2 ml) should be collected from each quarter of the udder. There is greater risk of contamination of composite samples because tubes are open for a longer period of time.

9. Teat dip

When samples are taken at the end of milking or between milkings, teats should be dipped in an effective germicidal teat dip following sample collection.

10. Store samples

Store samples immediately on ice or refrigerate. Samples to be cultured at a later date (after 24 to 48 hours) should be immediately frozen (-20°C).

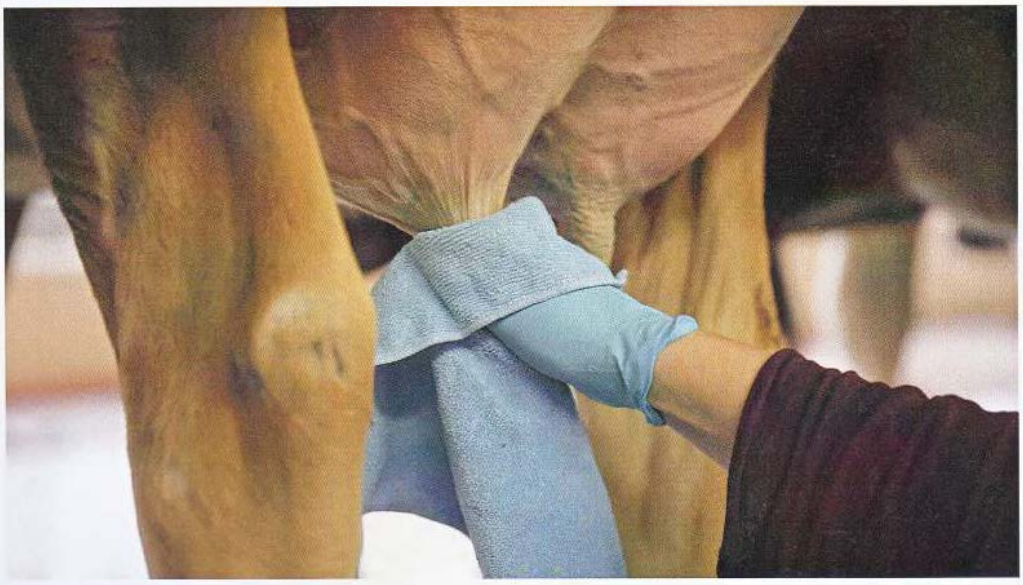

Figure 1.4
Teats must be clean and dry prior to sample collection.

Sample Storage and Shipping

Keep cold — Samples should be properly packaged and kept cold when they are transported any distance. Samples should be placed in a plastic bag or surrounded in plastic wrap to provide secondary containment during transport. If samples are in a rack, samples can remain in the rack. Samples should be surrounded with ice/cold packs or dry ice to keep samples at refrigeration temperature or frozen, respectively.

Ship overnight — Use only a next-day delivery service when shipping samples any significant distance. Do not use first class mail service.

Refrigerate or freeze — Samples must be maintained at refrigeration temperature or must remain frozen during transport.

Avoid weekends — Avoid shipping samples that may arrive at the laboratory on weekends or holidays.

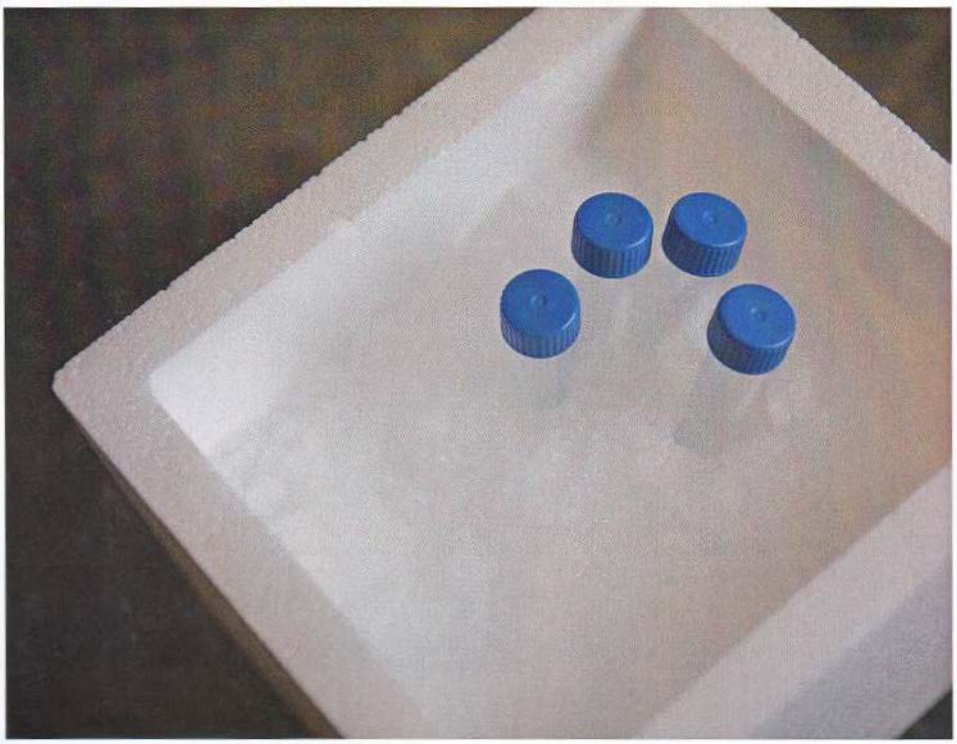

Figure 1.5
Samples should be properly packaged and kept cold when transported.

Chapter 2
Diagnostic Equipment and Materials

Equipment necessary for identification and differentiation of mastitis pathogens varies considerably, depending on need and level of diagnostic capabilities. The majority of diagnostic procedures continue to be based on microbial culture. Thus, elaborate equipment and materials are not required for routine bovine mastitis diagnostic work. In addition, there are several suppliers of commercially prepared microbiological media. Preparing media in-house may become more economical as sample volume increases. The most common mastitis pathogens, staphylococci, streptococci, and Gram-negative bacteria, grow readily on standard media. Additional equipment and materials may be necessary as diagnostic capabilities and services increase beyond the very basics. Additional and more specific descriptions of pathogen identification, in addition to a general description of molecular-based diagnostic techniques, will follow in subsequent chapters.

Essential equipment

The following items are necessary for routine diagnostic work-up of mastitis pathogens:

1) incubator set 35 to 37°C
2) refrigerator
3) microscope with low power and oil immersion objectives

Optional equipment

The following items are not absolutely necessary; however, they are useful and facilitate mastitis diagnostic work:

1) autoclave
2) balance
3) vortex,
4) water distillation unit
5) CO_2 incubator
6) anaerobic system
7) water bath
8) centrifuge
9) calibrated pipettes

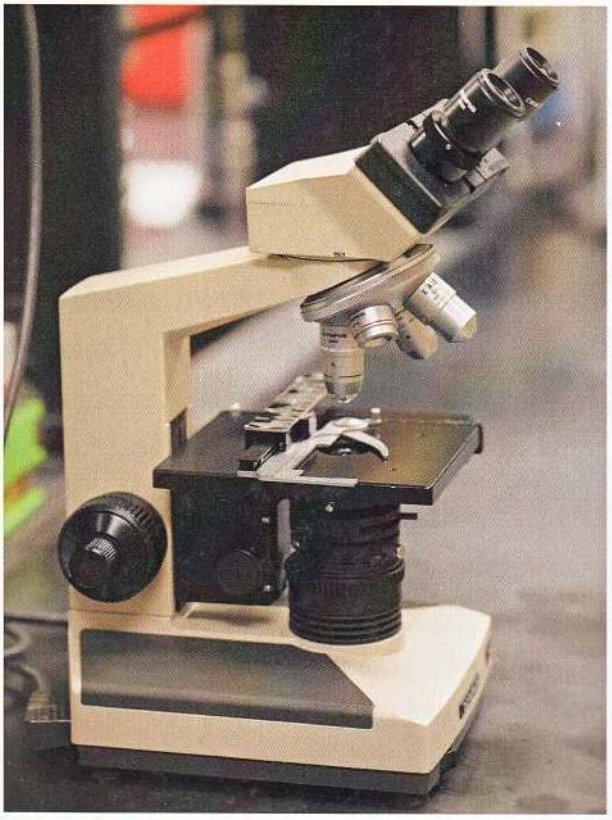

Figure 2.1
A microscope is essential diagnostic equipment.

Additional materials

Loops or swabs; media; Petri plates; tubes for slants and coagulase test; microscope slides; stains; reagents, such as potassium hydroxide, hydrogen peroxide, and oxidase discs; and an incandescent or fluorescent light source (60 to 100 watt).

Media for general isolation of mastitis-causing pathogens

The most common media used for identifying mastitis pathogens include blood agar, blood-esculin agar, or blood agar with hemolysin.

Blood supply for media preparation

Agar containing washed bovine red blood cells or whole bovine blood is well suited for bovine mastitis diagnostic work. Ovine blood may be substituted if necessary. Blood must be collected aseptically from healthy animals. Whole blood (use citrate or EDTA, not heparin, as anticoagulant) must be checked prior to use for presence of staphylococcal antihemolysin, which may inhibit hemolysins produced by *Staphylococcus aureus.* A CAMP test should be run on a test plate from each bottle of blood to verify that the blood is sensitive to the beta-hemolysin of *S. aureus* and will yield a positive CAMP reaction. Always use a known CAMP-positive streptococcal species.

Washed RBC

Use of washed red blood cells eliminates the need to test the "whole blood" donor. Citrate or EDTA may be used for collection of blood when washed red blood cells are used. Washing is done by centrifuging whole blood 30 minutes at 900 to 1,000 x *g* to pack the red blood cells. Remove serum and buffy coat aseptically by aspiration and resuspend cells in sterile saline (0.85%). Repeat the process twice to wash erythrocytes thoroughly. Cells are then resuspended in sterile saline to the original volume and added to the agar in the same percentage as in the original whole blood.

Commercially prepared media

Commercially available sheep blood or unwashed cow blood may be used if control strains of *Streptococcus agalactiae* and *S. aureus* produce typical zones of hemolysis on these media. Commercially prepared blood agar plates are also acceptable if tested similarly.

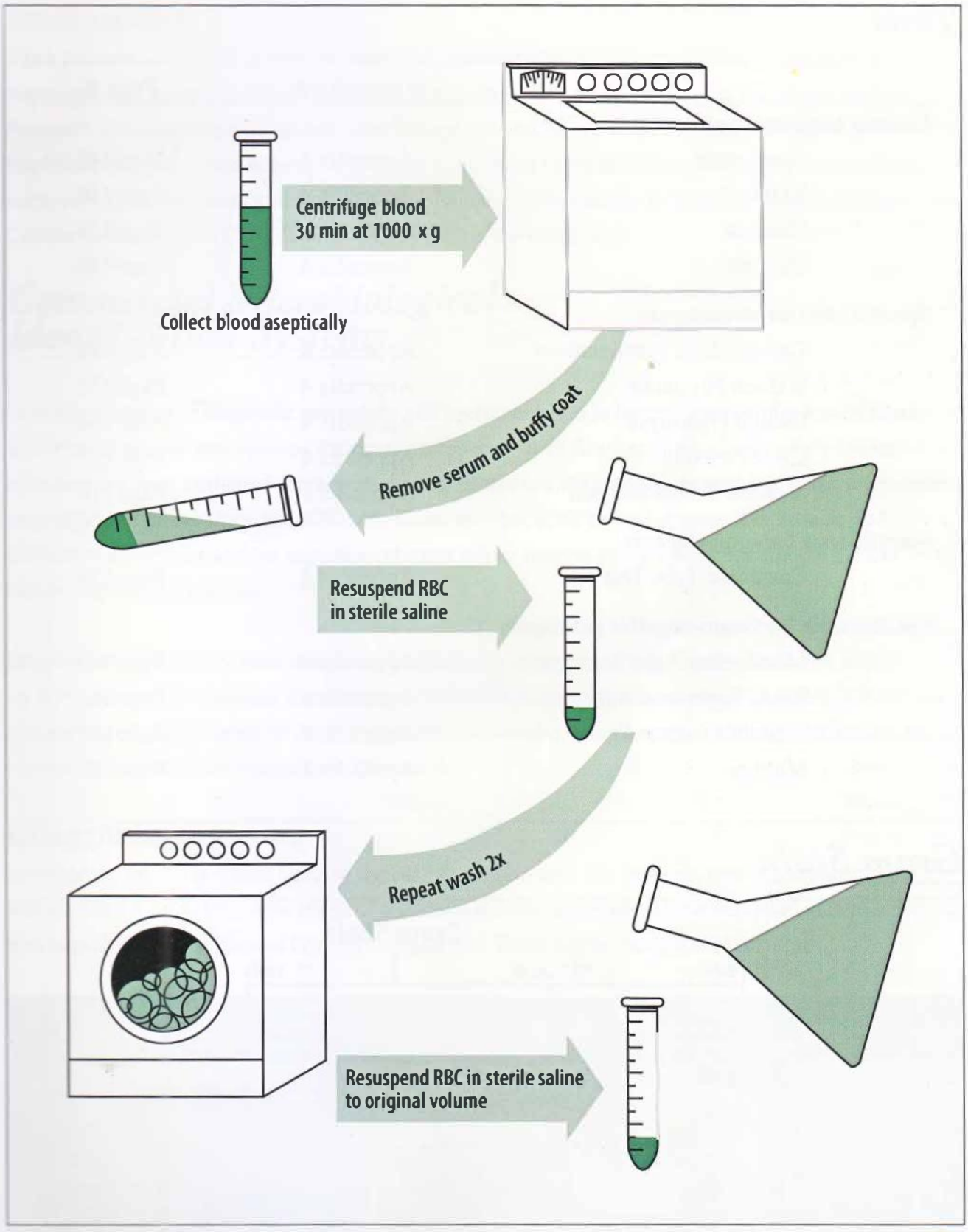

Figure 2.2
Flow chart for washing red blood cells.

Tests

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Gram Stain

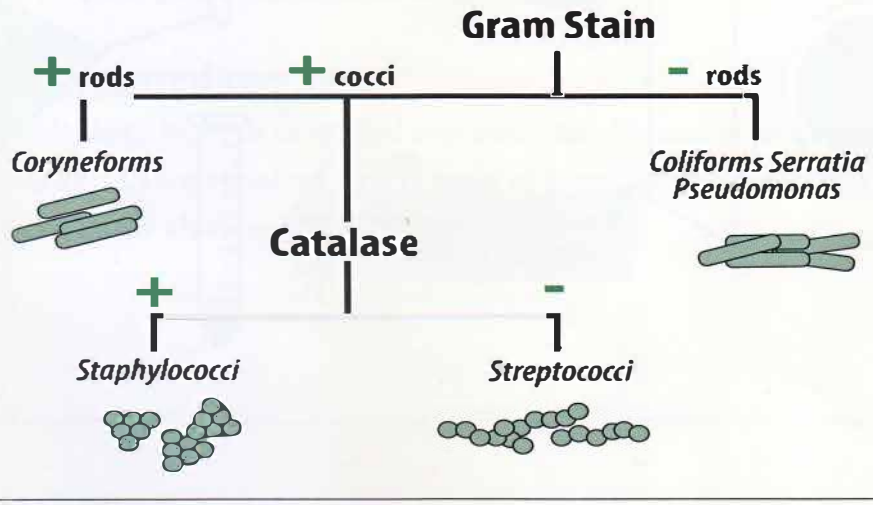


Figure 2.3
Gram stain reaction is the pivotal test for identifying mastitis pathogens.

Stock cultures

Stock cultures are excellent tools for observing macroscopic and microscopic morphologies of organisms that cause mastitis and are used also as controls for test procedures and media quality. Repeated sub-culturing may slightly alter colony morphology of stock cultures. Streptococci, staphylococci, and Gram-negative organisms retain most characteristics of the stock isolate. Stock cultures may be obtained from some mastitis diagnostic laboratories or from the American Type Culture Collection (ATCC), Rockville, MD (http://www.atcc.org).

Commercial Microbiological Identification Systems

Scientific advances in diagnostic technology and instrumentation in the past few decades have resulted in a wide variety of new systems for genus and species identification of bacteria. Some of these systems have been evaluated for their usefulness in mastitis diagnostic laboratories. These procedures vary in degree of accuracy and difficulty. Some are very accurate and require very little or no additional equipment and/or expertise, whereas others require costly laboratory equipment and higher degrees of expertise.

Diagnostic systems vary from simple agglutination or tube tests to colorimetric test strips based on fermentation of substrates to tests centered on molecular methods based on detection and/or sequencing of nucleic acids or detecting proteins or metabolites. For more detailed information on molecular diagnostic techniques, see Chapter 4.

Coagglutination tests

Investigators must stay within limits of the coagglutination tests. For example, beta-hemolytic systems will not work for non-hemolytic streptococci. These systems are excellent for differentiation between beta-hemolytic *S. agalactiae* and Group G streptococci. Broth culture renders the best results.

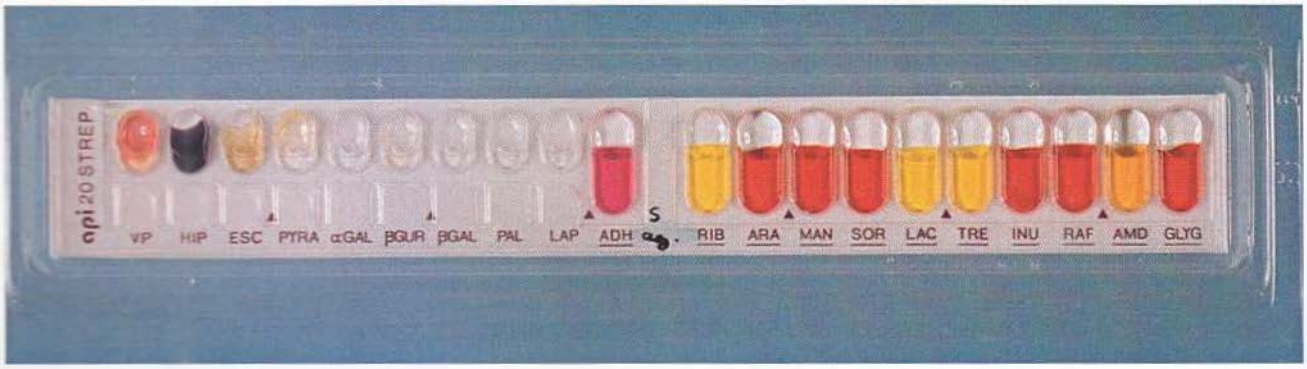


Figure 2.4
Example of a commercial miniaturized biochemical identification system.

Latex agglutination tests

This is a good system for identification of Lancefield groups B, C, D, and G.

Coagulase tests

The ability to clot plasma is the most widely used method to distinguish *S. aureus* from coagulase-negative *Staphylococcus* spp. The tube coagulase test for free coagulase is considered to be more definitive than the slide test for bound coagulase. See Appendix 4.

Rapid microbial identification systems

Methods for rapid identification of most bacteria that cause mastitis in dairy cows have been evaluated (e.g., Figure 2.4). These systems vary in accuracy, depending on the genus and species of bacteria being evaluated. For example, the accuracy of commercial biochemical test strips for identifying the coagulase-negative staphylococci has been shown to be inferior to genotypic methods. These systems are generally rapid and can identify mastitis pathogens within 4 to 24 hours of inoculation. Minimal training is necessary to use most of these systems; however, some difficulty in interpretation of color reactions can occur.

DNA-based diagnosis, speciation, and strain-typing

Real-time polymerase chain reaction- (PCR) based methods for detecting DNA of common mastitis pathogens are commercially available and are being utilized in the diagnosis of intramammary infection. DNA-based methods can also be used to better define the specific genus and species of bacteria isolated from milk and for strain-typing isolates to understand their epidemiology on the farm. Special equipment and training are needed to perform diagnostics based on these methods. These so-called molecular diagnostic methods are discussed in greater detail in Chapter 4.

Antimicrobial Susceptibility Testing

In clinical practice, bacterial culture followed by antimicrobial susceptibility testing are often performed to help determine the best course of treatment for an infection. While antimicrobial susceptibility testing can be applied to bacterial mastitis isolates, the consensus is that susceptibility testing is not very useful in determining the outcome of treating an intramammary infection with an antimicrobial agent. Therefore, empirical evidence of what works for a given organism on a given farm is the usual approach to selecting an antimicrobial therapy.

If antimicrobial susceptibility testing is performed, the laboratory should follow the testing and diagnostic threshold guidelines published by the Clinical & Laboratory Standards Institute (CLSI; http://clsi.org).

Chapter 3
Diagnostic Procedures

Procedures used in the diagnosis of intramammary infections are critical to developing control measures for the microorganisms that are isolated. Microbiological procedures used in the diagnosis of intramammary infections must be accurate; an approximate 90% correlation between samples collected consecutively from the same quarter and among interpretations of the same culture by different diagnosticians is expected. Thus, specific guidelines are presented for each microorganism to improve repeatability of final results among diagnosticians. Clearly for an accurate diagnosis of the inciting pathogen, milk samples should be collected before any antimicrobial therapy is administered to the cow. Additionally, post-treatment samples should ideally be collected when a sufficient withdrawal time has passed.

Microbiology

An understanding of basic microbiology is necessary for conducting and interpreting the diagnostic procedures outlined in this handbook. Such procedures are neither expensive nor difficult, as long as they are carried out following proper techniques. Implementation of imprecise techniques and use of shortcuts will only lead to improper diagnoses, wasted materials, and increased expenditures.

Herd problem

In the following sections, particular microbial genera, species, or groups of microorganisms are discussed. The comments on control and eradication are derived from the concept that mastitis is a herd problem that can be characterized by information gained from sampling individual cows and quarters.

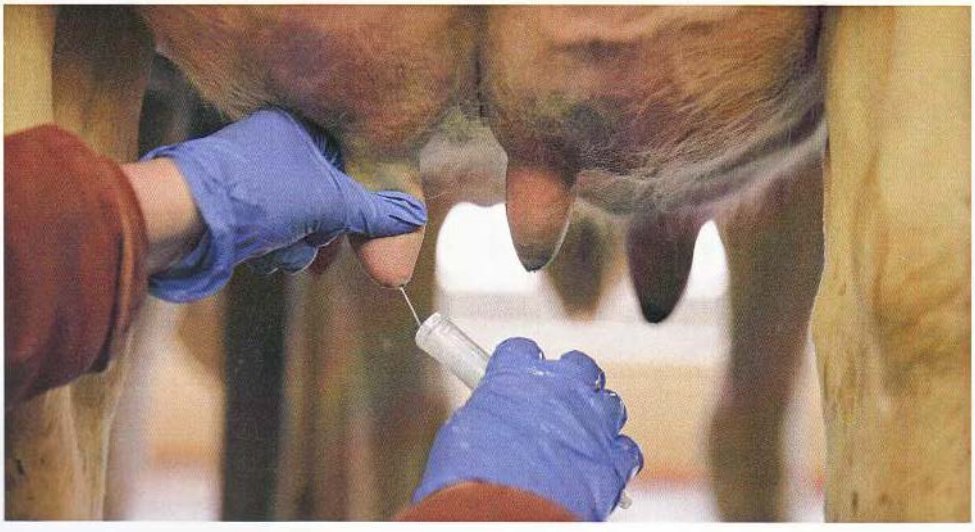


Figure 3.1
Mastitis is a herd problem characterized by information gained from sampling individual cows.

Sources of pathogens

Identification of microorganisms is carried out only to the extent that is useful in identifying likely sources of infection and effective measures of control. Thus, identification may be carried to the species level (e.g., *Staphylococcus aureus, Streptococcus agalactiae*), genus level (e.g., *Pseudomonas* spp.), or to groups of microorganisms (e.g., coliforms). For further species level identification, other identification systems will need to be employed.

Primary Isolation

Inoculum volume

1. For the routine culturing of milk on blood agar or esculin-blood agar plates, 0.01-ml platinum or disposable plastic inoculating loops are generally used.
2. Larger volumes may be applied to plates using loops, swabs, or pipettes.

Procedure

1. Using an aliquot of 0.01 ml, streak across one quadrant (quarter) of a blood agar plate (or one-half plate for composite samples) in a fashion to permit growth of isolated colonies. Other media should be used where indicated.

Figure 3.2a
Method for streaking milk on one quadrant of a plate.

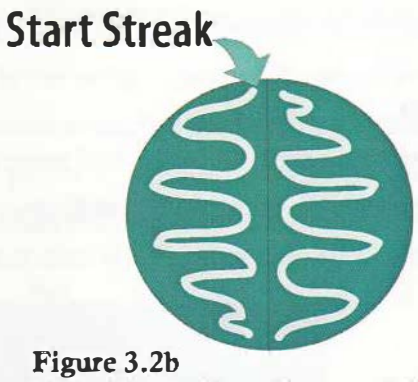


Figure 3.2b
Method for streaking milk on one half of a plate.

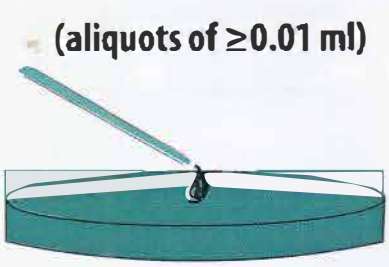


Figure 3.2c
Apply milk using a sterile pipette.

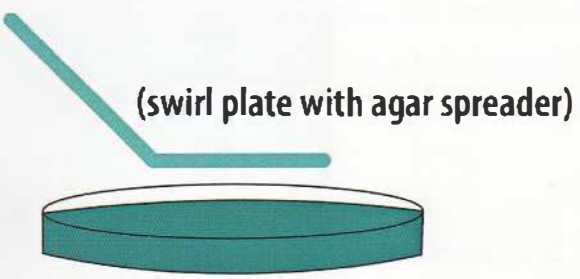


Figure 3.2d
Use an agar spreader to inoculate milk over entire plate surface.

2. Incubate plates in an inverted position at 37°C for 24 to 48 hours. At 24 hours, observe plates for microbial growth and check for purity.
3. All isolates should be treated as potential human pathogens and all materials should be autoclaved before discarding in an approved manner.
4. Perform a Gram-stain or potassium hydroxide test (KOH) to differentiate Gram-positive and Gram-negative microorganisms.

Microbial growth

Growth of isolated microbial colonies is essential for an accurate interpretation. If the sample is streaked onto too small of an area, certain factors in milk may inhibit the growth of microorganisms. In addition, contaminants in milk may overgrow the microorganism that is causing the infection. Incubation times and temperatures vary by microorganisms and groups of microorganisms (Table 3.1).

Table 3.1

Incubation times and temperatures vary by microorganism and groups of microorganisms. Culture techniques for each pathogen are described in greater detail in the chapters that follow and should be consulted for specifics.

Microorganism	Time*	Temperature (°C)
Streptococci	1 to 2 days	35 to 37
Staphylococci	1 to 2 days	35 to 37
Gram-negative bacteria	1 to 2 days	35 to 37
Yeast, mold, other fungi	1 to 3 days	23 to 37
Nocardia spp.	2 to 5 days	35 to 37
Prototheca spp.	2 to 3 days	23 to 37
Corynebacterium bovis	2 to 3 days	35 to 37
Trueperella pyogenes	2 to 4 days	35 to 37
Corynebacterium spp.	1 to 4 days	35 to 37
Gram-positive bacilli	1 to 2 days	35 to 37
Mycobacterium spp.	3 to 5 days	23 to 37
Mycoplasma spp.	2 to 10 days**	35 to 37

*1 day = 24 hours

**Special culture conditions are needed; Chapter 8 and Appendix 2

Interpretation of Results

The infection status of mammary quarters is determined by microbiological culture of aseptically obtained milk samples and interpretation of the culture results. As in all biological data, the diagnosis of intramammary infection is subject to error. Culture of milk samples generally results in one of three events: 1) no bacterial growth; 2) growth of a pure culture; or 3) growth of multiple colony types. Any of the three outcomes may not represent the true infection status of the quarter. Therefore, strict adherence to aseptic sampling technique and proper storage and handling of milk samples are absolutely essential. In addition, diagnosis of intramammary infection status based on multiple samples may be more reliable than diagnosis based on a single sample. When results of a second sample collected within 72 hours of a first sample do not agree with results of the first sample, then a third sample should be collected to arrive at a best diagnosis.

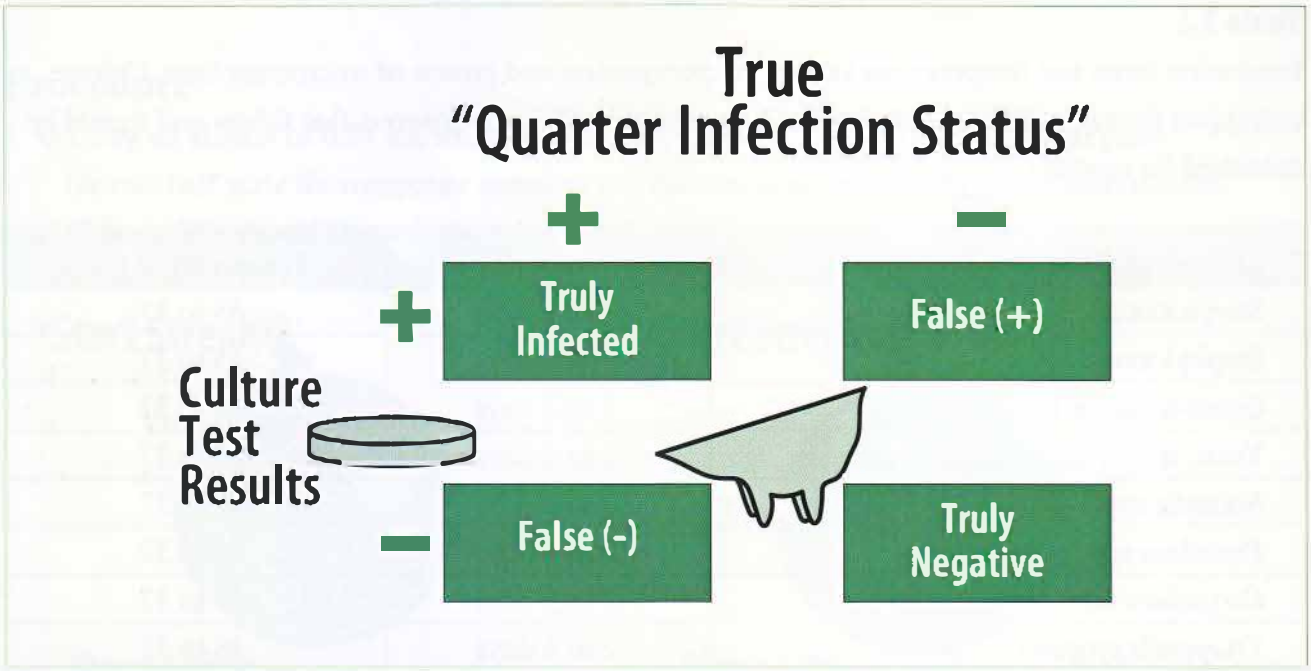


Figure 3.3
Possible outcome of test results.

False-positive samples

False-positive samples [F (+)] result when a pathogen is isolated in pure culture, but the quarter is truly not infected. Such samples occur as a result of contamination at some point during sample collection and/or processing. When intramammary infection status is based on culture of a single sample, F (+) samples get interpreted as an infected quarter.

A frequent assumption is that the recovery of the contagious pathogens *S. aureus, Mycoplasma* spp., or *S. agalactiae* from a single milk sample is evidence of intramammary infection. However, F (+) samples can occur with all pathogens, including *S. aureus* and *S. agalactiae.* False-positive samples associated with the environmental pathogens likely will increase as environmental contamination increases.

False-negative samples

False-negative samples [F (-)] result when no microbial growth is detected following microbiological culture, but the mammary quarter is truly infected. Reasons for such samples: 1) the colony-forming units of the organism in the milk are less than the detection limit of the assay; 2) special media or growth conditions are required; 3) inhibitors in the milk sample, such as antibiotics, have interfered with the growth of the pathogen; or 4) the pathogen died after the sample was collected because the sample was stored incorrectly or for too long. There is evidence to suggest that false-negative samples are more likely to occur with coliform, *Mycoplasma* spp., and *S. aureus* infections than infections caused by other pathogens. *Staphylococcus aureus* can be shed in low numbers in milk from an infected gland and their population may be less than the level of detection of routine culture, <100 colony-forming units per ml of milk when 0.01 ml of milk is cultured. The same can be true for the *Mycoplasma* spp. Coliform organisms may be quickly eliminated by the immune system; however, given the toxic nature of their infectious process, the inflammatory condition may persist for several days after clearance of the coliform organism.

Attempts to reduce the number of F (-) samples by using enrichment techniques or a period of preliminary incubation should be done judiciously. Such procedures can yield F (+) results when a very small number of contaminants are encouraged to grow with the enrichment procedures. Plating larger volumes of milk (0.1 ml per one-half plate) will help reduce the number of F (-) samples but may increase the number of F (+) samples if aseptic sampling technique is poor and contaminants are misclassified as pathogens. Clinical quarters are generally assumed to be infected. A common finding is that 20 to 30% of samples from clinical quarters will result in no microbial growth. Clinical signs may be present, but the pathogen has likely been eliminated by the cow's immune system, which may not be uncommon with coliform infections as discussed above.

Contaminated Samples

When a quarter milk sample results in the culture of three or more dissimilar colony types, the milk sample is most likely contaminated and the sample should be recorded as such. All mastitis pathogens present in milk samples can be the result of contamination, including *S. aureus*, *Mycoplasma* spp., and *S. agalactiae*. Two levels of contamination are generally recognized.

Low-level contamination

The clearest indication of infection status is growth of numerous colonies of a single morphology of a known mastitis pathogen (positive infected quarter) or no growth of any visible pathogens after proper incubation and growth conditions (negative or non-infected quarter). However, it is not unusual that a few errant colonies of one or more different morphologies may appear in addition to the predominant colony type. In such cases, the diagnostician must make a decision whether the mammary quarter is

infected with one pathogen or more than one pathogen. In these instances, it would be wise to have a second sample collected to confirm the diagnosis and determine if the quarter is truly infected with more than one pathogen or if the pathogen type that has appeared in low numbers is a contaminant. In cases where collection of a second sample is not easily achieved, then the diagnostician should record the result as infection with the predominant pathogen and either low level contamination microbe or coinfection with a second microbe.

Gross contamination

The second type of contamination is "gross contamination." Three or more colony types are present on the milk streak, often in relatively heavy growth. Such samples should be declared contaminated and no attempt should be made to identify potential pathogens within the mix of microbial growth. When gross contamination is observed, the quarter should be resampled.

Sources of contamination

Common sources of contaminants include: dirty teat ends, milk touches hands or fingers before entering the tube, non-sterile tubes or inoculating needles, streaking milk samples on contaminated media, excess alcohol on teat ends or hands, contamination of the container containing the cotton swabs, and the container lid not being sealed tightly resulting in alcohol evaporation from the cotton swabs.

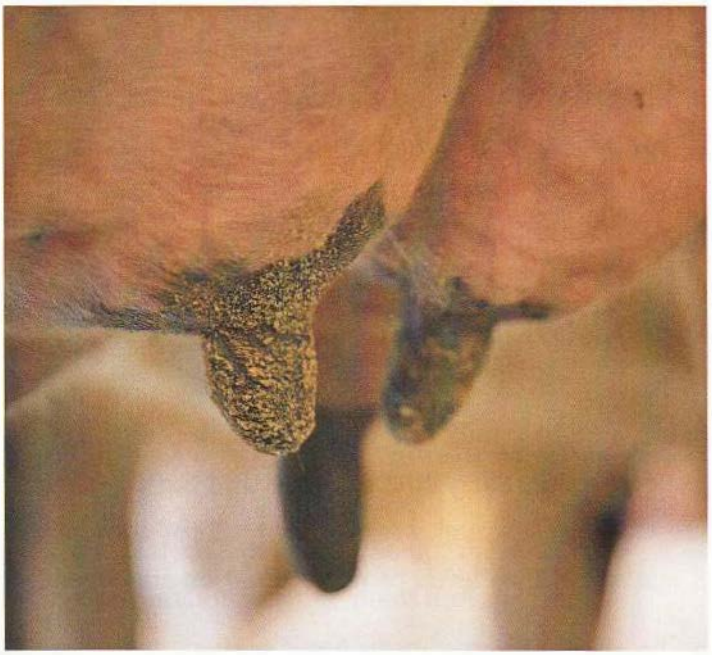


Figure 3.4
Dirty teat ends are a common source of contaminants in milk samples.

Chapter 4
Molecular Diagnostics

During the last decade, diagnostic tests based on molecular techniques have become increasingly more commonplace in the study and diagnosis of infectious disease. By definition, molecular diagnostic tests detect deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, or other metabolites associated with either the host or an inciting pathogen to help us differentiate health from disease. In the case of mastitis, these methods are currently employed to define whether or not a cow or mammary quarter has an intramammary infection (IMI), or to study the epidemiology of mastitis-causing bacteria. Note that, while IMI and mastitis are often used synonymously, each has a different definition. An IMI is defined as an infection occurring in the secretory tissue and/or the ducts and tubules of the mammary gland, which is generally diagnosed based on the detection of a microbial pathogen, whereas mastitis is defined as inflammation of one or more quarters of the mammary gland, which is almost always caused by an IMI but is quantified by measures of inflammation, such as somatic cell count or visible changes in milk quality.

For many years, conventional microbiological methods, based on morphology and phenotype expression on non-specific or specific growth media, have been considered the "gold standard" for detection of an IMI. These methods are still in widespread use in veterinary practice and are outlined elsewhere in this text. In contrast, molecular methods, based on detection of microbial DNA, RNA, proteins, or other metabolites, have historically been used in research laboratories to understand pathogen epidemiology. However, there are now commercially available DNA-based detection systems marketed for the diagnosis of IMI. Understanding how these techniques work and their limitations is key to making a diagnosis and subsequently implementing management decisions.

Goals of Diagnostic Testing

Broadly speaking, diagnostic tests are performed to decide whether there is an infection present and if there is an infection present, what, specifically, is causing it. In the case of mastitis, the goal of milk microbiology is to define broad groups of pathogens to facilitate the implementation of mastitis control and/or treatment measures. Mastitis pathogens can be classified based on their reservoir of infection as contagious or environmental, or based on disease manifestations as clinical or subclinical. In clinical disease, broad categorization into Gram-positive and Gram-negative pathogens can be useful in defining treatment, as Gram-positive organisms more often respond to antimicrobial therapy. With subclinical mastitis, defining pathogens as contagious or environmental can be useful in making decisions on where and how control measures need to be implemented. This has historically been

done by identifying specific genera and species of bacteria and lumping them into contagious or environmental categories, based primarily on phenotype and empirical knowledge (Table 4.1). For example, if we identified *Staphylococcus aureus* on a blood agar plate from a cow with subclinical mastitis, it was considered contagious and control measures would have been focused on milking time hygiene, segregation and milking cows last, and culling of chronically infected cows, whereas if we identified *Escherichia coli* from a cow with clinical mastitis, the cow would be treated with symptomatic and supportive care and prevention measures would be focused on the cow's environment.

While the same principles can be applied when using molecular diagnostic methods, molecular methods can also be employed to fine-tune our understanding of mastitis pathogen epidemiology. For example, strain-typing methods may be used to document whether a specific organism is found in multiple cows, potentially indicating a contagious reservoir. Or, conversely, if multiple strains are shared by very few cows, this might indicate an environmental reservoir of infection. Using molecular methods has demonstrated that some pathogens classically defined as contagious or environmental may exhibit one or both characteristics, and, hence, mastitis pathogens may be better referred to as host adapted or non-host adapted.

Finally, the goals of the research scientist may differ from those of the clinician. In clinical medicine, as discussed above, our diagnostic goal is usually to facilitate the implementation of treatment or control measures. In the research laboratory, there may be additional goals, including definitive speciation of pathogens, understanding pathogen specific virulence, and an in-depth understanding of host-pathogen interactions.

Sample Collection

Milk is the usual medium being examined for the presence of microbes when diagnosing the cause of mastitis. The key to making a definitive diagnosis at the cow or mammary quarter level, regardless of whether conventional or molecular diagnostic methods will be applied, is aseptic collection of milk samples (see Chapter 1). In contrast, if the goal is herd-level monitoring, bulk milk samples or milk collected using an in-line sampling device may be used. In the latter case, the goal is to get a picture of what might be causing mastitis in a herd. But with such a strategy, management practices cannot be implemented at the cow level as the affected cows cannot be definitively identified. Furthermore, interpretation of the results from bulk milk or in-line samplers needs to take into account possible sources for bacteria in a non-aseptically collected, comingled milk sample. For pathogens such as *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma* spp., it can likely be assumed that there are infected cattle in the herd, as the primary reservoir for these pathogens is the cow's mammary gland. However, the source of pathogens, such as *E. coli* in non-aseptically, commingled milk samples, is open to question as these pathogens can be on the udder or teat skin surface (dirty cows), in the mammary gland, or enter milk via post-harvest contamination.

Molecular Diagnostic Methods

A variety of molecular methods have been applied to the study of mastitis epidemiology in research laboratories for several years. Two main approaches have been used: 1) detection of microbial DNA using polymerase chain reaction (PCR) to diagnose IMI; and 2) using molecular methods for genus and species identification, or to strain-type bacteria within a specific genus and species. In the first case, PCR-based methods have been applied directly to milk samples, negating the need for time-consuming conventional culture. Thus, these techniques have particular appeal because, in many cases, they are more rapid than conventional bacterial culture methods. In addition, these methods may be used to detect DNA from organisms that are difficult to grow under routine culture conditions, e.g., *Mycoplasma* spp. or anaerobic bacterial genera. In the second case, bacterial culture is still needed to harvest an isolate for further manipulation. A variety of methods, including PCR, DNA sequencing, or proteomic methods [e.g., matrix-assisted laser desorption ionization time-of-flight analysis (MALDI-TOF)], have been employed for genus and species identification. Grouping of pathogens within a given genus and species, according to strain, has been performed using techniques such as pulsed-field gel electrophoresis, randomly amplified polymorphic DNA typing (RAPD), amplified fragment length polymorphism (AFLP), and multi-locus sequence typing (MLST). With the advent of high throughput inexpensive DNA sequence analysis, sequence-based methods are becoming quite commonplace, especially for bacterial genus and species identification, and even whole genomes are being studied to identify factors associated with pathogen virulence.

Today, molecular methods are no longer only in the realm of the research laboratory. There are now commercial test kits marketed for diagnosis of IMI that utilize real-time PCR methods to detect bacterial DNA in milk. One such test available through Thermo-Fisher (Pathoproof™) can be applied directly to milk without routine culture. This test detects DNA from up to 16 different commonly occurring mastitis pathogens and boasts results in 4 hours with "unparalleled sensitivity and specificity," and applicability to various samples, including mammary quarter milk samples, Dairy Herd Information Association (DHIA) samples, and bulk tank samples. While these claims can be substantiated, as with conventional culture, one must put the results in the context of where and how the milk sample was collected. As discussed above, non-aseptically collected samples from in-line samplers (DHIA samples) or bulk milk have their limitations. Furthermore, as with conventional culture, detection of multiple genera and species (≥3 types) in a mammary quarter or four-quarter composite, aseptically collected milk samples still indicates a contaminated sample. Also, bear in mind that this test will only detect the 16 pathogens for which PCR primers are included. So, unlike conventional culture where, depending on the culture media used, there is the potential to detect an infinite number of genera and species, PCR has its diagnostic limitations. Finally, the test only detects DNA and therefore there is no "proof of life" in the udder, calling into question whether an active IMI is present.

In addition to PCR-based mastitis diagnostics, proteomics-based (MALDI-TOF), bacterial identification systems are finding their way into veterinary diagnostic labs for genus and species identification of bacteria, and these high throughput methods may ultimately replace classical phenotypic identification systems based on biochemical profiling (e.g., Sensititre AP90™). However, few validation studies regarding the diagnostic accuracy of MALDI-TOF are currently available and hence one has to be cautious when interpreting results reported using this methodology.

Advantages and Disadvantages of Molecular Diagnostics

When evaluating a "new test," its sensitivity (Se), specificity (Sp), positive predictive value, negative predictive value, and accuracy are usually calculated by comparing the results of the new test to a "gold standard." One of the difficulties in diagnosing bacterial IMI is we do not have a perfect "gold standard" test. Milk culture is often considered the "gold standard" test, but manipulation of the milk inoculum size and growth media will impact Se and Sp. The National Mastitis Council recommendation for routine bacterial culture is to inoculate solid media (usually a blood-based agar) with 10 µl of milk. Therefore, the limit of detection of this test is 100 colony-forming units (CFU)/ml. Hence, if an individual mammary quarter is shedding <100 CFU/ml of bacteria, it will be declared culture negative. Similarly, if the organism has specific growth conditions not used in routine screening, e.g., *Mycoplasma* spp. or anaerobes, the sample will likewise be declared negative. Inhibitors in milk, such as antimicrobial treatments, can also lead to a negative test result. These are examples of false-negative test results that will impact Se. The opposite is true if, for example, the teat is not aseptically prepared prior to sample collection and bacteria from the outside of the teat are washed into the sample during collection. In this instance, we may get a false-positive result impacting test Sp.

Similar issues arise when diagnosing an IMI using PCR-based methods. Because the PCR assay detects only those bacteria for which there are DNA primers in the assay profile, organisms not included in the assay profile will be missed, leading to false-negative test results. An example of such a situation was recently documented in Europe using PCR detection of methicillin-resistant *S. aureus* (MRSA). A series of isolates were found to have an oxacillin-resistant phenotype based on conventional antimicrobial susceptibility testing, but were negative for the mecA gene, the gene encoding methicillin resistance, during PCR screening. Further investigation of the bacterial genome revealed that these isolates were a *mec*A variant, now termed *mec*C, which were missed because the original PCR primers did not detect this variant. False positives will occur with contamination or when the assay detects bacterial DNA from organisms that the cow's immune system or antimicrobial therapy has already killed. The issues associated with bulk milk and in-line sampler collected milk samples are discussed in the section above and are also potential examples of false-positive results. Recent work has shown that while carryover of bacterial organism (DNA) between cows when collecting milk from an in-line sampler (DHIA sample) is apparently low, carryover nonetheless

occurs and may lead to a cow being falsely identified as having an IMI. The false-positive and false-negative rates can also be influenced with the cycle threshold (Ct-values) used to make a diagnosis. Similar to manipulating the inoculum size in conventional culture, moving the Ct-value threshold up (more PCR amplification cycles needed to detect bacterial DNA, indicating fewer bacterial DNA present in the sample) or conversely down will impact the Sp and Se, respectively.

When using molecular methods to make a definitive genus and species diagnosis, one must consider whether the techniques have been appropriately validated against bovine reference strains. By example, recent work has clearly demonstrated the superiority of DNA-sequence-based methods over phenotypic methods (commercial biochemical test strips) for speciation of coagulase-negative staphylococcal isolates. That said, laboratories are now beginning to use bacterial proteomics (MALDI-TOF) to speciate bacteria. While new technologies have appeal from a speed and per sample expense standpoint, it is critical that the results be validated before being placed in widespread use.

With regard to strain-typing methods, it is important to understand the discriminatory power of the tests being used. Does the test lump isolates into one group or split them into many groups? How will the results be used to manage mastitis cases? If we are trying to determine contagiousness of *S. aureus* isolates in a group of cows, for example, a discriminatory test will be needed so that we do not needlessly cull cows falsely identified as possessing the contagious strain because the strain-typing test failed to adequately differentiate the strains.

Future Developments

The discussion so far has focused primarily on DNA-based (genomic) methods. There are other modalities that can be applied to the study of infectious disease, including metagenomics (study of microbiomes), transcriptomics, proteomics, and metabolomics. These techniques are being used by researchers to understand bovine mastitis and some have promise as future diagnostic methods. For example, recent studies evaluating the milk microbiome have revealed some interesting results to further our understanding of mastitis epidemiology. The milk microbiome consists of all the microorganisms in the cow's mammary gland environment. Using DNA extraction, PCR amplification, and subsequent DNA sequencing of a fragment of the 16S rRNA gene, multiple genera and species of bacteria are identified using bioinformatics analysis. By studying the microbiome of healthy versus mastitic mammary glands, it can be determined where differences in the microbial population lie to better understand what may be causing inflammation in the udder.

The development of new techniques to diagnose intramammary infections is likely to continue to occur at a relatively rapid pace. New techniques may be specific to a single pathogen type, group of pathogens, or all common mastitis pathogens. These techniques may have the advantage of being

rapid and/or have application at the cowside. Several of the new molecular type tests have undoubtedly improved mastitis diagnostics with precise speciation, which has not been easily achieved with traditional phenotypic tests. The traditional milk culture, at least to the genus or group level, is still the backbone of mastitis diagnosis. Standard culture techniques have been well described in this handbook, whereas newer methodologies have been described in general terms.

As with any test, new or traditional, it is critical that the interpreter know the limitations of the test and view the results in the context of how and from where the sample was collected. While new technologies add to our toolbox for mastitis diagnosis, and in some cases improve the Se and Sp of diagnosis, it is important that the clinician, researcher, or advisor have a clear understanding of the techniques and how they were validated. Field test comparisons with milk culture are highly recommended in the validation process. It is incumbent upon the diagnostician and/or researcher to have a full understanding of the advantages and disadvantages of each test used.

Table 4.1

Comparison of conventional bacterial culture and polymerase chain reaction - (PCR) based approaches to diagnosing intramammary infection

Parameter	Bacteriologic Culture	PCR
Detects	Bacterial colonies	Bacterial DNA
Diagnostic threshold	Colony forming units (CFU)/ml	Cycle threshold (Ct)
Live organism	Yes	Not necessarily
Virulence factor detection	Limited (e.g., hemolysins)	If PCR primers are included
Factors influencing sensitivity (Se)	Growth media & conditions; incubation time; CFU/ml detection threshold/inoculum volume; interpreter	Included primers; primer specificity; Ct threshold
Factors influencing specificity (Sp)	Contaminated sample; CFU/ml threshold/inoculum volume; interpreter	Contaminated sample; Ct threshold; detection of DNA from non-viable bacteria; primer specificity; carryover when using in-line sampler
Time to result	24 h to 10 d	4 h
Cost	Low	Currently, 4 to 5 x conventional culture

Chapter 5
Streptococci and Related Genera

Many streptococcal and streptococcal-like organisms have been isolated from milk of cows with mastitis. Streptococci can be among the most prevalent organisms isolated from lactating and non-lactating dairy cow mammary glands. Cows infected with streptococci may have subclinical mastitis or episodes of mild to moderate clinical mastitis.

The genus *Streptococcus* was separated into three distinct genera several decades ago: *Streptococcus*, *Enterococcus*, and *Lactococcus*. Examination of colony morphology and simple biochemical testing may not adequately distinguish between these genera and additional biochemical testing will be needed to make an accurate diagnosis. The predominant *Streptococcus* spp. involved in bovine mastitis are *S. agalactiae, S. dysgalactiae,* and *S. uberis*. *Streptococcus agalactiae* is classified as contagious pathogens because their reservoir is the cow's udder and they are spread from cow to cow, primarily at milking. Control measures centered on milking time hygiene and dry cow therapy have substantially reduced the prevalence of this pathogen in many regions. *Streptococcus uberis* and *S. dysgalactiae* have generally been considered environmental mastitis pathogens because they primarily reside in the dairy environment. However, there is evidence to suggest that both pathogens can establish reservoirs in the udder and spread cow to cow during milking. Other *Streptococcus* spp. isolated less frequently include *S. acidominimus, S. alactolyticus, S. canis, S. equi, S. equinus* (formerly called *S. bovis*), and *S. parauberis*. *Enterococcus* spp. involved in bovine mastitis include *E. durans, E. faecalis* (formerly *S. faecalis*), *E. faecium* (formerly called *S. faecium*), and *E. saccharolyticus* (formerly called *S. saccharolyticus*). Recently, *Lactococcus lactis* ssp. *lactis* and *Lactococcus garvieae* have been reported as causes of intramammary infection.

Identification of streptococci and streptococcal-like organisms can be accomplished both through phenotypic methods and molecular methods, e.g., genotypic (PCR amplification and sequencing or DNA-based strain-typing methods) or proteomic methods (MALDI-TOF). The rapid biochemical test-strip identification methods that have been used in the past to identify bacterial genus and species differ in their diagnostic accuracy by the genus and species being identified, and while they may provide an accurate diagnosis for one particular genus and species, accuracy may be lacking for other genera and species. Hence, many diagnostic laboratories are starting to rely on molecular typing methods to classify these organisms. With the advent of molecular methods for identification, it has been recognized that some of the organisms that were historically being classified as environmental streptococci were likely enterococci or even lactococci. Hence, it is unknown whether enterococci and lactococci are emerging mastitis pathogens, or whether the historical lack of incidence was due to misclassification.

The following information on streptococci and related genera is intended to provide the reader with a broad overview of these mastitis pathogens and present tests that can be used to identify and distinguish between these pathogens.

Colony morphology and hemolysis

Colonies of streptococci and related genera on blood agar are small (1 to 3 mm diameter), smooth, translucent, and convex. In comparison to streptococci, enterococci often have a creamier gray colony rather than the traditional gray translucent colonies of streptococci. Colonies may be surrounded by a zone of greenish, discolored erythrocytes (referred to as alpha-hemolysis), surrounded by a clear zone of lysed erythrocytes (referred to as beta-hemolysis), or be non-hemolytic (also referred to as gamma-hemolysis). Most typically, enterococci are accompanied by alpha-hemolysis or are non-hemolytic (gamma).

Gram-positive, catalase-negative, cocci

Other mastitis bacteria may have similar colony morphology on blood agar. Colonies should be Gram-stained and tested for catalase production. Catalase-negative, Gram-positive cocci should be selected for subsequent testing to distinguish streptococcal species from each other and related genera (Table 5.1). Note that, while enterococci are categorized as catalase-negative cocci, they may cause weak effervescence when mixed with hydrogen peroxide. Additional tests for differentiating streptococci and related genera include CAMP reaction, hydrolysis of esculin, sodium hippurate, and pyroglutamic acid, acid production in broth containing various carbohydrates (e.g., inulin and raffinose), growth in broth containing 6.5% NaCl (Figure 5.8), growth on bile-esculin-azide medium, reduction of 0.1% methylene blue milk, and molecular methods for speciation and strain-typing (Table 5.1).

Table 5.1
Differentiation of streptococci and streptococcal-like organisms

Organism	Lancefield Serogroup	CAMP	Inulin	Hippurate	Esculin	Bile-esculin	NaCl	Pyro-glutamic acid	Trehalose
Frequently Isolated									
S. agalactiae	B	+	-/+	+	-		-		
S. dysgalactiae	C	-	-/+	-	-/+		-		+
S. uberis	NG	-/+	+	+	+	-	-	+	
Less Frequently Isolated									
S. equi	C	-	-	-/+	-		-		-
Enterococcus faecalis	D	-	+/-	+	+	+	+	+	
E. faecium	D	-	-	+/-	+		+		
S. alactolyticus	D	-	-	-	-	-	-	+	
S. equinus	D	-	-/+	-/+	+	+	-	-	
S. canis	G	-*	-	-	+/-		-		
Aerococcus viridans	NG			+	+	+	+	+	
E. saccharolyticus	NG	-	-	-	+	+	-/+	-/+	
S. acidominimus	NG	-	-	+/-	-	-	-	-/+	

*May produce a club-shaped CAMP reaction.

+ Means majority of strains are positive.

- Means majority of strains are negative.

+/- Means more strains positive than negative.

-/+ Means more strains negative than positive.

NG = not serogrouped.

Streptococcus agalactiae

Source

The primary source of *S. agalactiae* is the udder of infected cows. This organism is considered an obligate parasite of the udder in dairy cows. However, *S. agalactiae* has been isolated from human genitalia and gastrointestinal tracts.

Means of spread

Streptococcus agalactiae is considered contagious mastitis pathogens and are readily spread from cow to cow, primarily during milking.

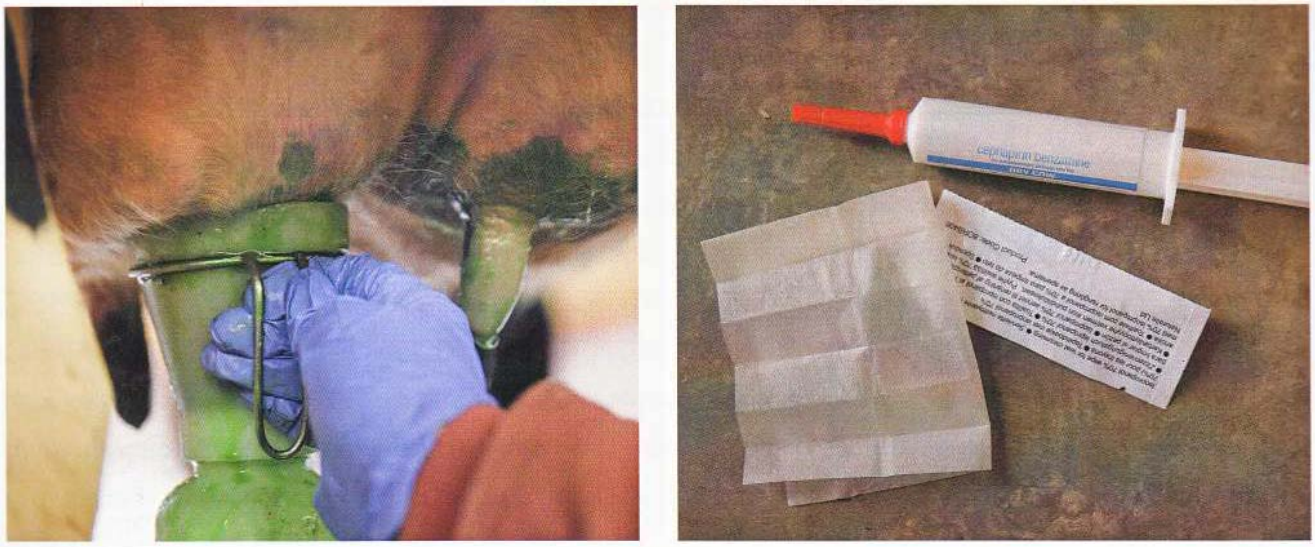


Figure 5.1
Postmilking teat disinfection and dry cow therapy are essential for controlling *Streptococcus agalactiae*.

Prevention and control measures

Eradication of *S. agalactiae* from dairy herds is possible. However, *S. agalactiae* remain a problem in individual herds and in some countries lacking either organized mastitis control programs or where the use of blanket dry cow therapy is not practiced. *Streptococcus agalactiae* is controlled effectively by procedures that prevent the spread of bacteria at milking time, which include pre-milking teat disinfection and drying teats with individual towels, post-milking teat disinfection with an effective teat disinfectant, and antibiotic dry cow therapy. It is also recommended to test new additions to the milking herd for *S. agalactiae* and to milk these cows last until they are shown to be culture negative for *S. agalactiae*.

Laboratory identification procedures

Appearance on blood agar – Small (1 to 2 mm diameter) colonies on blood agar; colonies are moist, convex, often lacking color and translucent. Most are beta-hemolytic (clear zone) on blood agar, can be non-hemolytic (gamma), and a few are alpha-hemolytic (green).

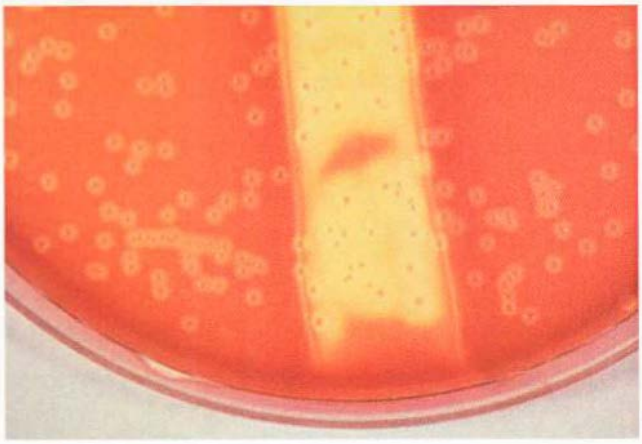


Figure 5.2a
Streptococcus agalactiae on blood agar with *S. aureus* beta-hemolysin streaked down center. CAMP reaction is clearing in area of hemolysin streak.

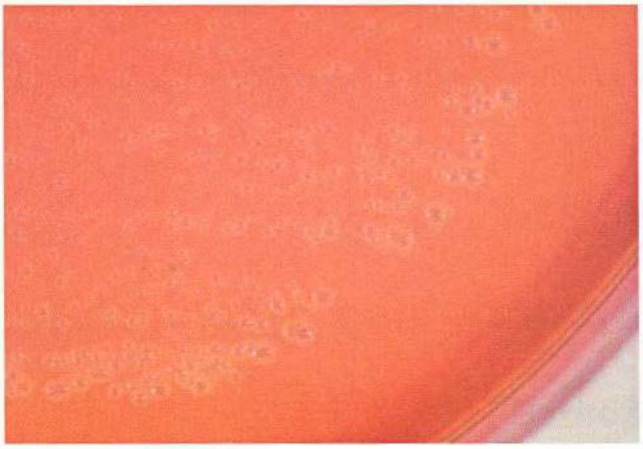


Figure 5.2b
Beta-hemolytic *Streptococcus agalactiae* on blood agar containing esculin; no esculin hydrolysis, therefore no change in media color.

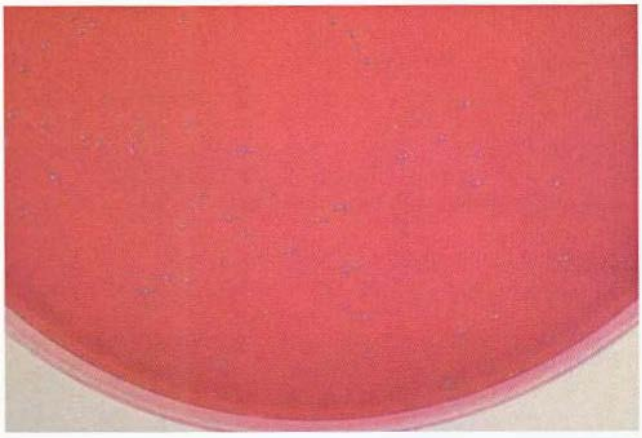


Figure 5.2c
Alpha-hemolytic *Streptococcus agalactiae* on blood agar containing esculin.

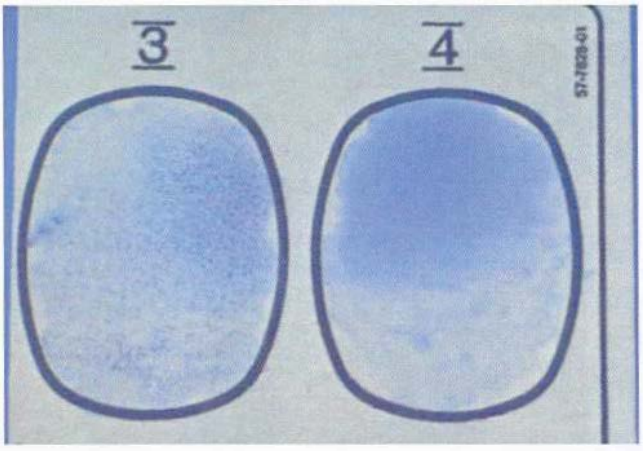


Figure 5.2d
Lancefield group B coagulation test.

Gram stain – Gram-positive cocci occurring in chains

Catalase – Negative

CAMP-esculin agar – CAMP positive, esculin negative

Lancefield group – Lancefield serological group B. *Streptococcus agalactiae* can be identified solely by serotyping.

Sodium hippurate – Positive

Streptococcus dysgalactiae

Source

Streptococcus dysgalactiae is rather unique mastitis pathogens in that they have characteristics of both contagious and environmental pathogens. Sources of the organism can be infected udders or the environment.

Means of spread

Streptococcus dysgalactiae may be spread from cow to cow during milking and cows can become infected from organisms in the environment.

Prevention and control measures

Pre-milking teat disinfection and drying teats with individual towels are effective milking time hygiene procedures for the control of *S. dysgalactiae*. Other recommended control procedures include post-milking teat disinfection, antibiotic dry cow therapy, and maintaining a clean, dry environment.

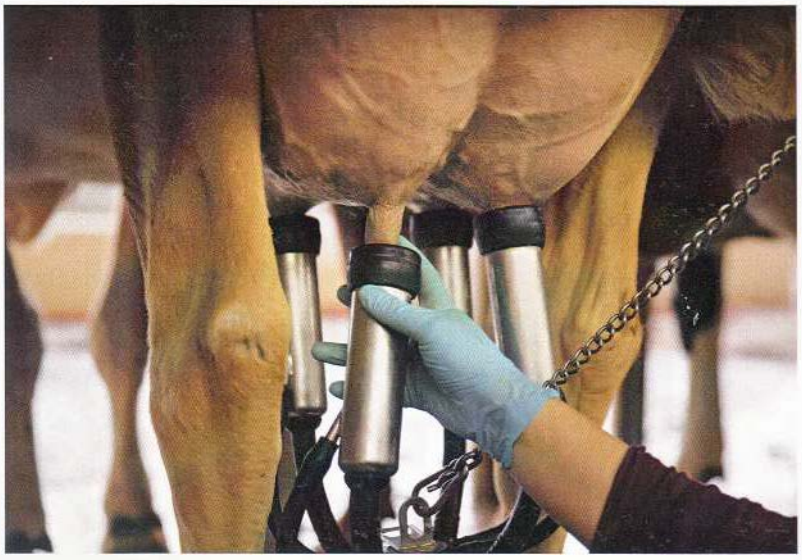


Figure 5.3
Streptococcus dysgalactiae may be spread from cow to cow during milking and cows can become infected from organisms in the environment.

Laboratory identification procedures

Appearance on blood agar – Small (1 to 2 mm diameter) colonies on blood agar; colonies are moist, convex, and translucent. Most display alpha-hemolysis (green) or are non-hemolytic (gamma) on blood agar.

Gram stain – Gram-positive cocci occurring in chains

Catalase – Negative

CAMP-esculin agar – CAMP negative, primarily esculin negative

Lancefield group – Lancefield serological group C. *Streptococcus dysgalactiae* can be identified by serotyping and trehalose utilization.

Sodium hippurate – Negative

Trehalose – Positive

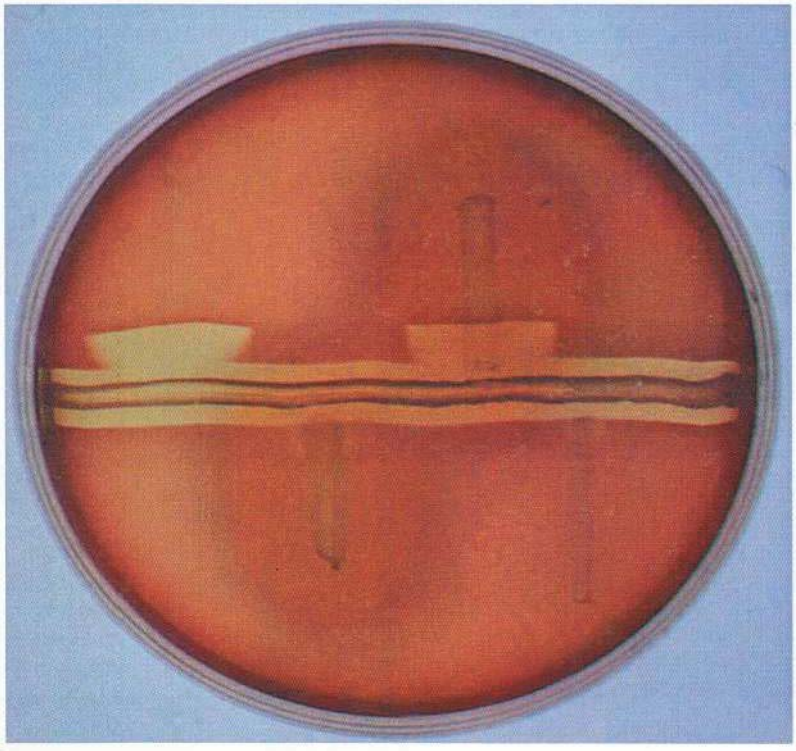


Figure 5.4
CAMP plate with (from left to right)
Top:
1) *S. agalactiae* - CAMP positive, esculin negative
2) *S. uberis* - CAMP positive, esculin positive
Bottom:
1) *S. uberis* - CAMP negative, esculin positive
2) *S. dysgalactiae* - CAMP negative, esculin negative

Streptococcus uberis

Source

Streptococcus uberis are one of the most frequently isolated streptococcal species from bovine mammary glands. The organism has been found in the dairy environment and has also been isolated from the udder, skin, lips, and genital area of dairy cows.

Means of spread

Cows apparently become infected with *S. uberis* from environmental sources, but recent data suggest that they may also behave like contagious pathogens in that multiple cows can be found infected with the same strain. *Streptococcus uberis* are a common cause of new intramammary infections in cows during the dry period and can be frequently isolated from cows with clinical mastitis during early lactation.

Prevention and control measures

Effective milking time hygiene procedures and pre-milking teat disinfection are useful for the control of *S. uberis*. Other recommended control procedures include post-milking teat disinfection, antibiotic dry cow therapy, and maintaining a clean, dry environment. Antibiotic therapy at drying off is effective in preventing *S. uberis* infections during the early dry period, but ineffective near calving when udders are highly susceptible to new *S. uberis* infections.

Figure 5.5a
Straw bedding often contains large populations of *Streptococcus uberis*.

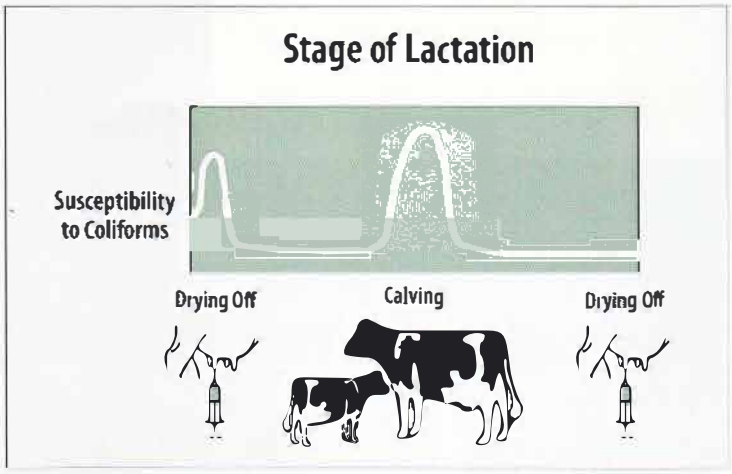


Figure 5.5b
Dry period is an important focus point for controlling *Streptococcus uberis*.

Laboratory identification procedures

Appearance on blood agar – Small (1 to 3 mm diameter) colonies on blood agar; colonies are moist, convex, with dense centers and translucent. Most are alpha-hemolytic (green) on blood agar, but can be non-hemolytic (gamma).

Gram stain – Gram-positive cocci occurring in chains

Catalase – Negative

CAMP-esculin agar – CAMP variable (primarily CAMP negative), esculin positive

Sodium hippurate – Positive

Inulin – Primarily positive

Lancefield group – Not-typable, some reported to belong to Lancefield group E, G, P, or U.

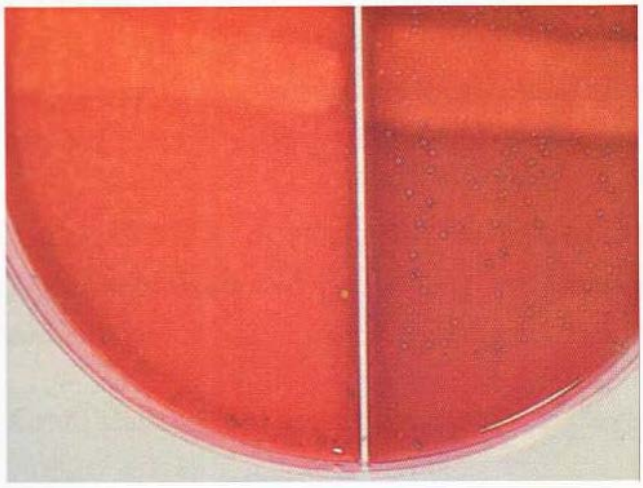


Figure 5.6a
Split plate of *Streptococcus uberis* on blood agar (left) and esculin-blood agar (right) with *Staphylococcus aureus* beta-hemolysin streak. Note pigment around colony when esculin is hydrolized. This *S. uberis* is CAMP negative.

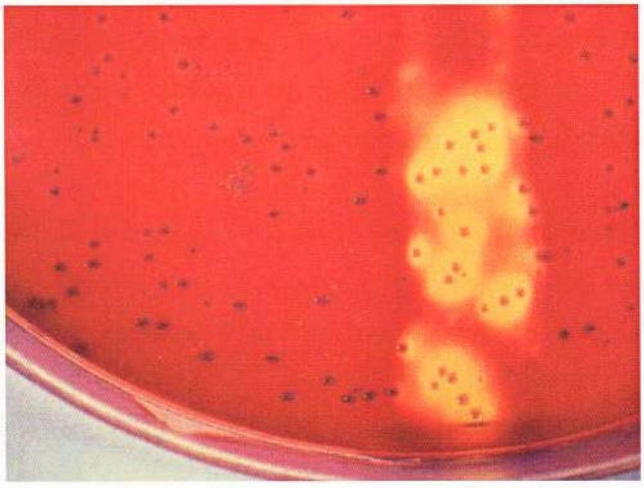


Figure 5.6b
CAMP-positive *S. uberis* on blood agar with esculin, streaked with *S. aureus* beta-helolysin.

Other Streptococcus spp.

Source

Other *Streptococcus* spp. less frequently isolated from cows with mastitis include *S. acidominimus*, *S. alactolyticus*, *S. canis*, *S. zooepidemicus* (Lancefield group G), *S. equi*, *S. equinus* (formerly *S. bovis*), and *S. parauberis* (formerly *S. uberis* genotype 2). These organisms have been isolated from soil, skin, infected udders, and in the general dairy environment.

Means of spread

Cows apparently become infected with *Streptococcus* spp. from environmental sources (Figure 5.7).

Prevention and control measures

Effective milking time hygiene procedures and pre-milking teat disinfection are useful for the control of streptococcal species. Other recommended control procedures include post-milking teat disinfection, antibiotic dry cow therapy, and maintaining a clean, dry environment.

Laboratory identification procedures

Appearance on blood agar – Small (1 to 3 mm diameter) colonies on blood agar; colonies are moist, convex, with dense centers and translucent. Colonies can be non-hemolytic (gamma) or alpha-hemolytic (green).

Gram stain – Gram-positive cocci occurring in chains

Catalase – Negative

CAMP-esculin agar – CAMP variable (primarily CAMP negative), primarily esculin positive

Lancefield group – Lancefield serological groups C, D, G, and many are not typable

Sodium hippurate – Primarily positive

Note:

Streptococcus uberis genotype 1 and *S. parauberis* (formerly *S. uberis* genotype 2) are phenotypically indistinguishable. *Streptococcus parauberis* can be definitively identified by DNA-based methods and does not appear to be a frequent cause of mastitis in dairy cows in the United States. *Streptococcus bovis* are now referred to as *S. equinus*. Two genotypes of *S. equinus* have been reported and these can be differentiated by commercial identification systems and by DNA-based methods.

Enterococcus spp.

Source

Enterococcus spp. involved in bovine mastitis include *E. casseliflavus, E. durans, E. faecalis* (formerly *S. faecalis*), *E. faecium* (formerly called *S. faecium*), *E. gallinarum*, and *E. saccharolyticus* (formerly called *S. saccharolyticus*). These organisms have been isolated from the intestinal tract, manure, infected udders, feed, bedding, and in the general dairy environment. Reports suggest that clinical and subclinical mastitis due to *Enterococcus* spp. may account for 8 to 13% of all bovine intramammary infections. It is further suggested that due to misclassification of enterococci as streptococci, that an additional 24 to 30% of intramammary infections classified as being caused by environmental streptococci are actually enterococcal infections. *Enterococcus faecalis* and *E. faecium* have been reported to occasionally cause herd problems.

Means of spread

Cows apparently become infected with *Enterococcus* spp. from environmental sources.

Prevention and control measures

Effective milking time hygiene procedures and pre-milking teat disinfection are useful procedures for the control of enterococcal species. Other recommended control procedures include post-milking teat disinfection, antibiotic dry cow therapy, and maintaining a clean, dry environment.

Laboratory identification procedures

Appearance on blood agar – Small (1 to 3 mm diameter) colonies on blood agar; colonies are moist, convex, and semi-translucent. Colonies can be non-hemolytic (gamma) or alpha-hemolytic (green).

Gram stain – Gram-positive cocci (occurring in chains when grown in broth)

Catalase – Negative (some may cause weak effervescence)

CAMP-esculin agar – CAMP negative, esculin positive

Lancefield group – Lancefield serological groups D and some may not be typable

Sodium hippurate – Positive

Growth in 6.5% NaCl broth or agar – Positive

Bile-esculin – Positive

Lactococcus spp.

Source

Lactococcus spp. involved in bovine mastitis include *Lactococcus lactis* ssp. *lactis* and *Lactococcus garvieae.* The source of this pathogen, although not intensively studied, appears to be the environment, particularly associated with surface water. A recent survey in New York State indicated that nearly two-thirds of all streptococcus-like isolates associated with mastitis were either *S. dysgalactiae* or *S. uberis*, and more than one-fourth were *Lactococcus* spp.

Means of spread

Cows apparently become infected with *Lactococcus* spp. from environmental sources.

Prevention and control measures

Effective milking time hygiene procedures and pre-milking teat disinfection are useful procedures for the control of lactococcal species. Other recommended control procedures include post-milking teat disinfection, antibiotic dry cow therapy, and maintaining a clean, dry environment.

Laboratory identification procedures

For many years, streptococcus-like mastitis pathogens were classified as simply either *S. agalactiae, S. uberis*, or *S. dysgalactiae.* As diagnostic techniques have become more refined and transitioned from phenotypic to genotypic methods of speciation, it has been possible to more accurately distinguish between pathogen types. Thus, there is accumulating evidence that many of the isolates that would have been previously classified as *Streptococcus* spp. are now being classified as *Lactococcus* spp. Genotypic methods, such as PCR and proteomic methods, such as MALDI-TOF, can be used to distinguish between genera and species of these streptococcus-like organisms, such as the lactococci.

Figure 5.7
Cows may become infected with *Streptococcus* spp. from environmental sources.

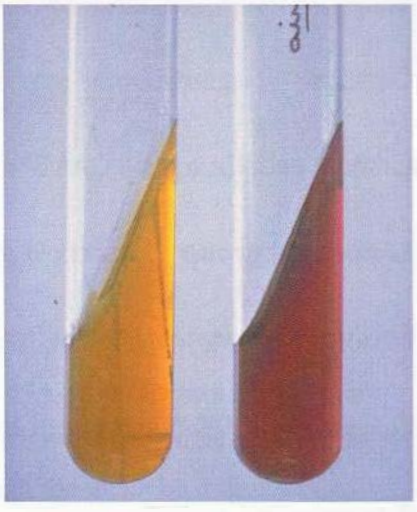


Figure 5.8
Growth on 6.5% NaCl slants is a characteristic of enterococci.

Chapter 6
Staphylococci

Staphylococcus species are the most common microorganisms isolated from aseptically collected milk samples.

Staphylococcus aureus can be a significant cause of mastitis in some dairy herds. However, they are isolated infrequently from milk samples from most well-managed dairy farms that have fully implemented a regime of post-milking teat dipping, dry cow therapy, and effective milking management. *Staphylococcus aureus* often cause chronic subclinical infections with intermittent clinical flare-ups, but in some regions can be a significant cause of clinical mastitis. A severe form of clinical disease, gangrenous mastitis, may occur in some cows. Subclinical disease is associated with elevations in milk SCC and decreased milk production.

In contrast, coagulase-negative staphylococci (CNS) are the microorganisms most commonly isolated from infected mammary glands in most herds. Coagulase-negative staphylococcal infections are usually associated with subclinical to mild clinical infections that result in low to moderate increases in milk SCC. The impact of CNS infections on milk production appears to differ among CNS species. Some species are associated with chronic infections and mean SCC >200,000 cells/ml.

Practical classification

The suggested and most practical classification of *Staphylococcus* spp. for mastitis diagnostic purposes and for reporting to veterinary practitioners and dairy farmers is:

***Staphylococcus aureus*:** distinct (sharp-edged), broad zones of incomplete and/or complete hemolysis; catalase positive; tube coagulase positive; pigmented colonies (i.e., color other than white);

OR

Coagulase-negative staphylococci: species producing no hemolysis or narrow, diffuse zones of complete hemolysis; catalase positive; tube coagulase negative; white or pigmented colonies.

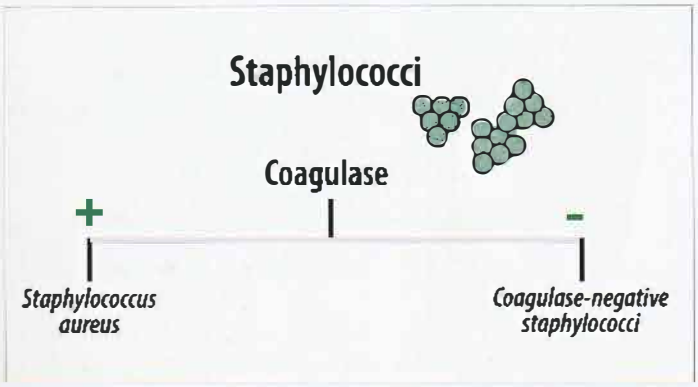


Figure 6.1
Practical classification of *Staphylococcus* spp. for mastitis diagnostics.

Staphylococcus aureus

Source

The primary reservoir is infected udders. *Staphylococcus aureus* readily colonize the teat orifice, teat canal, and damaged or roughened skin (e.g., chapping, frostbite, cuts, scabs, herpes lesions, or warts). *Staphylococcus aureus* can be isolated from the cow's environment but likely do not survive for long periods in the environment (i.e., not free living in the environment).

Means of spread

Staphylococcus aureus is generally considered to be contagious pathogens and are primarily transmitted from infected to uninfected glands or teats during the milking process.

Basic prevention and control measures

Teat dipping and dry cow therapy are key. Reduction of *S. aureus* to <1% of the quarters in a herd may be possible through a regimen of identification, segregation, strict milking time hygiene (including single-use udder cloths and gloves worn by milkers), efficacious treatment, and culling. Post-milking teat disinfection with an efficacious germicide and treatment of all cows with a commercial, efficacious dry cow product after the last milking of the lactation are the primary management factors for controlling contagious pathogens. Poor teat skin health may compromise the effectiveness of teat dipping. Identification and segregation of *S. aureus* infected cows and milking them last may reduce exposure of uninfected cows. Treatment response of clinical and subclinical infections during lactation will be variable and chronic infections are typically unresponsive to antibiotic therapy. Culling of chronically infected cows is an important measure in reducing the prevalence of *S. aureus* infections.

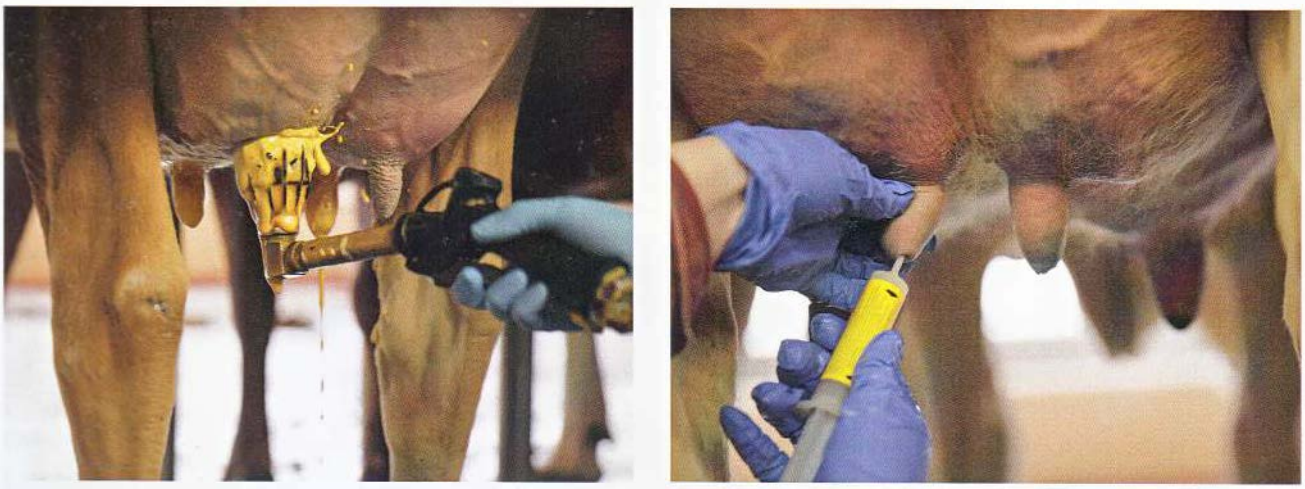


Figure 6.2
Post-milking teat dipping with an efficacious germicide and treating all cows with a commercial dry cow antibiotic product are the primary management factors for controlling *Staphylococcus aureus*.

Vaccination

Vaccination against *S. aureus* mastitis has been extensively studied and there are two commercially marketed vaccines (one in Europe and one in the United States). Given that *S. aureus* is a contagious mastitis pathogen, the goal of an *S. aureus* vaccine should be to either prevent or facilitate clearance of an *S. aureus* intramammary infection (IMI), thus eliminating the contagious reservoir of infection. None of the vaccines studied to date adequately prevent IMI. Current vaccines have been shown to decrease the clinical severity of mastitis and may improve spontaneous cure rates. In addition, some studies have shown decreased new IMI rates in dairy heifers at calving when they receive multiple vaccinations starting in calfhood. Hence, vaccines may be useful in herds with a high incidence of clinical mastitis or when used to decrease the incidence of *S. aureus* IMI in first-calf heifers. Additionally, recent work in Europe has shown that vaccination may decrease duration of intramammary infection and cow-to-cow transmission, but more well-controlled large field trials are needed before universal recommendations on the utility of vaccination can be made.

Backflushing and pre-dipping

Automated backflushing or pasteurizing of milking units between cows can significantly reduce contamination. However, the impact of pasteurizing teat cups on new infections is small compared with that of post-milking teat disinfection and dry cow therapy. Pre-dipping generally has a small effect on the control of *S. aureus* infections.

Additional information

Staphylococcus aureus may be carried at other body sites, such as the nares, vagina, and hock skin. Although *S. aureus* prevalence may be reduced to a low level in the lactating herd, this pathogen has

Figure 6.3
Heifers can be a source of introduction of new *Staphylococcus aureus* infection in a herd.

been isolated from the glands of heifers of all ages, which may represent an important source of infection at milking for uninfected lactating cows. Some researchers have suggested transmission to heifers may occur via flies, especially if fly infestation results in teat end sores. Geographical differences in the prevalence of *S. aureus* in heifers have been reported; ranges of 0 to 20% of glands have been observed.

Laboratory identification procedures

Appearance on blood agar – *Staphylococcus aureus* produce large (2 to 5 mm, 24 hours; 3 to 8 mm, 48 hours), smooth, pigmented colonies on blood agar. Pigmentation is creamy, grayish-white, tan, or golden-yellow. Hemolysis at 24 hours is characterized by a broad, distinct zone of incomplete hemolysis, which may be accompanied by a narrower clear zone of complete hemolysis extending 2 mm or more from the edge of the colony. Other strains of *S. aureus* may produce a well-defined zone of complete hemolysis. A minority of isolates express no hemolysis. Hemolytic patterns can vary depending on the blood-based culture media being used (see below). The presence of incomplete and/or complete hemolysis are strong indicators for *S. aureus*. Hemolytic zones typically have sharp, distinct outer edges (i.e., not diffuse).

Mastitis literature may incorrectly use the terms alpha-, beta-, or alpha-beta-hemolysis to describe hemolytic patterns rather than applying the terms to the hemolysins that produce the hemolysis. Alpha-hemolysin produces complete or clear hemolysis, and beta-hemolysin produces partial or incomplete hemolysis.

Blood used in media preparation is critical (see Appendix 1). All fresh blood agar should be tested with a stock *S. aureus* strain to ensure growth characteristics. A new donor should be used for media preparation or erythrocytes should be washed when <95% of *S. aureus* isolates are hemolytic on blood agar.

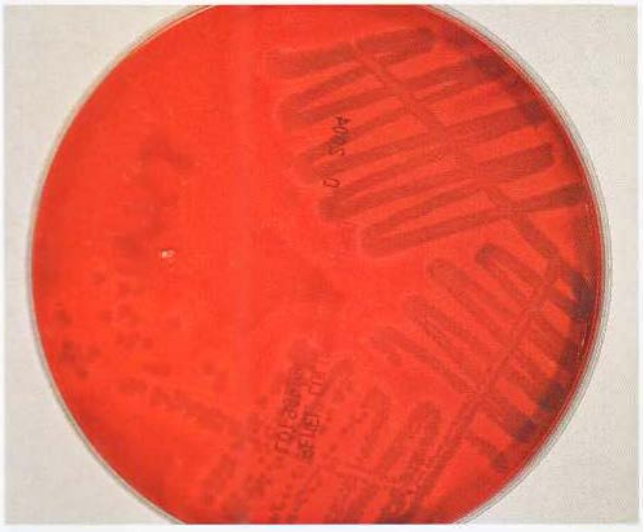


Figure 6.4a
Staphylococcus aureus producing zones of both complete and incomplete hemolysis on blood agar.

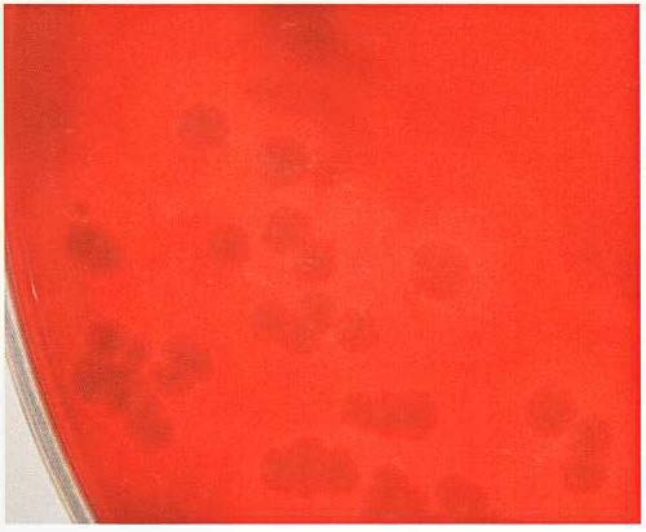


Figure 6.4b
Close-up of *Staphylococcus aureus* colonies.

Gram stain – Gram-positive cocci in pairs or irregular clusters

Catalase – Positive

Tube coagulase – Positive. The tube test for free coagulase is generally considered more definitive than the slide test for bound coagulase or "clumping factor." Although human clinical manuals recommend the slide test as a rapid screening test for *S. aureus*, the tube coagulase test is strongly recommended to avoid erroneous results with bovine isolates. On rare occasion, isolates that have been confirmed to be *S. aureus* by conventional or rapid identification systems display a negative tube coagulase reaction. Such atypical strains should be suspected of being *S. aureus* if characteristic hemolytic patterns and pigmentation are displayed and Gram stain are typical of a *Staphylococcus* spp.

Methods for species identification – The use of coagulase reaction alone will not adequately differentiate *S. aureus* from the other coagulase-positive species (CPS), *Staphylococcus pseudintermedius,* or the coagulase-variable species, *Staphylococcus agnetis* and *Staphylococcus hyicus.* However, based on current speciation methods, these other species are not commonly isolated from milk and generally have phenotypic characteristics that differ from *S. aureus.* Also, non-aureus CPS species have non-pigmented (white) to light gray colonies compared with the pigmented colonies of *S. aureus. Staphylococcus agnetis* and *Staphylococcus hyicus* do not produce hemolysis. The diagnostic criteria of hemolysin production and coagulase reactions are considered adequate for identifying *S. aureus* in prevention and control programs.

The use of commercially available, miniaturized biochemical test systems were once recommended to identify staphylococcal species. However, recent work suggests that the currently available miniaturized biochemical test strips lack diagnostic accuracy and DNA-based or proteomic methods are now being used for species identification.

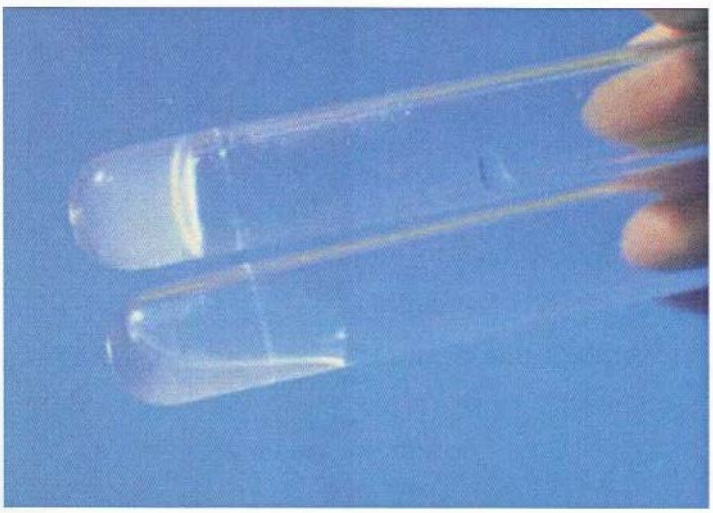


Figure 6.5
Coagulase test: positive upper tube; negative lower tube.

Staphylococci can be positively identified as *S. aureus* by PCR amplification of several genes, including the *nuc* gene or the *spa* gene. Furthermore, sequencing of housekeeping genes, including the *rpoB* gene or the *tuf* gene, can be done to speciate staphylococcal species. Commercially available PCR kits for the identification of major mastitis pathogens, including *S. aureus*, from bovine milk are currently available and are being used by some commercial laboratories, including some DHIA labs. Strain-typing *S. aureus* isolates may be beneficial to understand contagious spread in the face of a herd outbreak. Many strain-typing methods are available and are well described for *S. aureus*, including pulsed-field gel electrophoresis (PFGE), spa-typing, and multilocus sequence typing (MLST).

Acetoin production – Testing for acetoin production (Voges-Proskauer test) from glucose or pyruvate is a useful alternative characteristic to distinguish *S. aureus* from the other coagulase-positive staphylococci. *Staphylococcus pseudintermedius* and *S. hyicus* are acetoin negative, but *S. aureus* is acetoin positive.

Latex agglutination – Latex agglutination tests developed for rapid screening of *S. aureus* isolates based on the presence of clumping factor and protein A may be unreliable when performed on bovine strains of *S. aureus*, with some reports showing up to 54% of bovine isolates exhibiting a latex agglutination-negative phenotype. Never pick colonies for testing from media containing high salt concentrations (e.g., mannitol-salt agar; 7.5% NaCl), as this may lead to auto-agglutination and false-positive results.

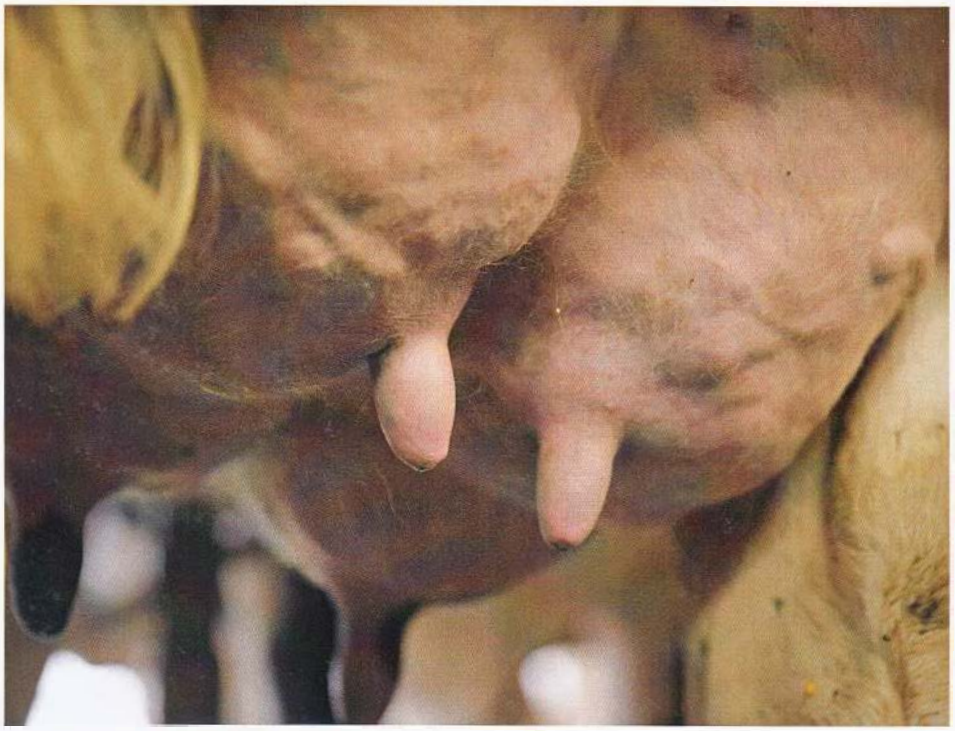


Figure 6.6
The commonly isolated CNS are part of the normal skin flora.

Coagulase-negative Staphylococci

Source

More than 50 species of coagulasenegative staphylococci (CNS) have been identified, many of which can be isolated from the cow's mammary gland. However, five species are most commonly found, including *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis*. For some species, including *S. chromogenes* and *S. epidermidis*, the udder is thought to be the main reservoir. For other species, including *S. haemolyticus* and *S. simulans*, the environment appears to the main reservoir and these species seem to act as environmental opportunists. *Staphylococcus hyicus* were reported as being quite prevalent in some herds in early studies. However, recent work using molecular methods suggests that *S. hyicus* is not as prevalent as once thought. This discrepancy may be due to misclassification of other CNS species as *S. hyicus* when using biochemical test methodologies. Furthermore, recent evidence suggests that bovine *S. hyicus* strains are actually a newly identified staphylococcal species, *S. agnetis*.

Means of spread

Coagulase-negative staphylococci appear to be opportunists and infect the teat canal and gland from skin sources or the environment. *Staphylococcus chromogenes* appear to readily colonize the teat canal and may persist for longer periods of time than the other CNS species. Many CNS infections are transient. Cow-to-cow spread is thought to be a low risk for intramammary infection.

Basic prevention and control measures

Post-milking teat dipping with an effective germicide and treatment of all quarters with a commercial dry cow antibiotic product after the last milking of lactation are the primary means of controlling CNS infections. Milking clean, dry teats will help reduce the contamination of bulk tank milk with bacteria from the teat surface. Dry cow antibiotic therapy should eliminate the majority of CNS infections. However, some infections have been shown to span the dry period and new infections may occur during the dry period because of the continuous presence of teat skin flora.

Miscellaneous information

Coagulase-negative staphylococci are the most common bacteria isolated from milk samples obtained from well-managed herds. A prevalence of 10 to 15% or greater of mammary quarters is common. Some of these isolations probably reflect teat canal colonization, rather than true intramammary infections. When an unusually high prevalence of CNS infections is observed, improper application of teat dip or the use of an ineffective germicide after milking should be investigated.

Intramammary infection

Infections are typically subclinical and generally result in moderate elevations in milk somatic cell count. Infected mammary quarters tend to have a higher milk somatic cell count than uninfected mammary quarters and some species may be more inflammatory than others. There is also evidence that some species, e.g., some strains of *S. chromogenes*, may protect the mammary gland from infection with more pathogenic staphylococci, such as *S. aureus*. Coagulase-negative staphylococci also may be associated with mild clinical mastitis.

Parity and stage of lactation

Cows in the first lactation may have a higher prevalence of CNS infection than older cows. Cattle at calving generally have a higher prevalence of CNS infection, which tends to decline rapidly over the first few weeks of lactation. This decline in CNS prevalence early in lactation is presumed to be an elimination of teat canal colonization/infection.

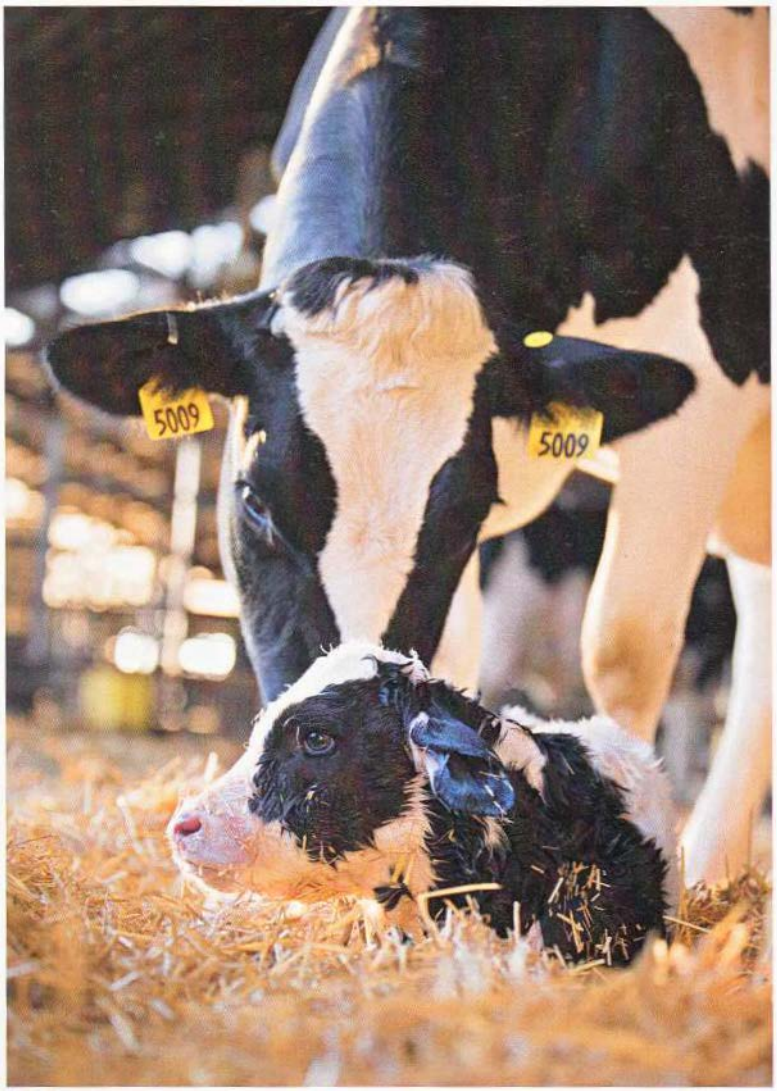


Figure 6.7
The highest prevalence of CNS during lactation occurs at calving. Photo provided by Accelerated Genetics.

Laboratory identification procedures

Note: Few *Micrococcus* spp. are isolated from milk. For diagnostic purposes, all Gram-positive, catalase positive, and coagulase-negative cocci are presumptively considered CNS.

Appearance on blood agar – Coagulase-negative staphylococci produce large (2 to 5 mm, 24 hours; 3 to 8 mm, 48 hours), smooth, colonies that are white (non-pigmented), cream, grayish-white, tan, or golden-yellow. The colonies of some species may have a rough, irregular appearance. The colonies generally have no hemolysis or may produce a narrow (<2 mm), diffuse zone of complete hemolysis at 24 hours. A few species will produce a larger, diffuse zone of incomplete hemolysis. These hemolytic patterns are not to be confused with the sharp-bordered zones of hemolysis displayed by *S. aureus*. Coagulase testing is recommended to confirm colonies as CNS.

Gram stain – Gram-positive cocci in pairs, tetrads, or irregular clusters

Catalase – Positive

Tube coagulase – Negative

Methods for species identification – The broad classification of these organisms as CNS based on phenotype should be adequate for most diagnostic and case management purposes. If identification of individual species of CNS is desired, DNA-based methods of speciation are recommended at this time. There are several described methods of DNA-based speciation, including sequencing of housekeeping genes (e.g., *rpo*B, *hsp*60, or *tuf* gene), PCR-restriction fragment length polymorphism of the gap gene, and transfer RNA-intergenic spacer PCR.

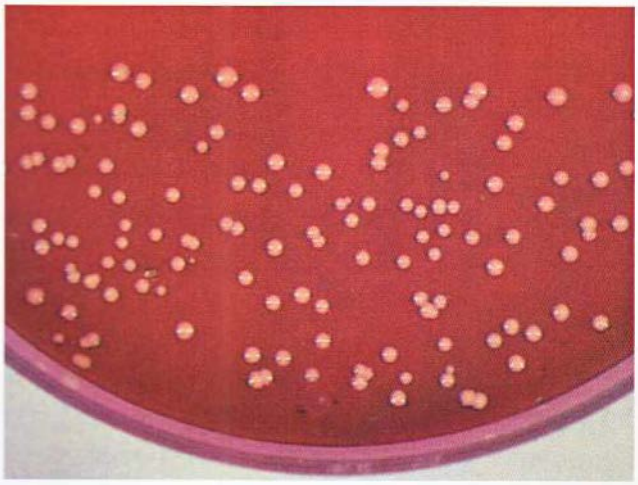


Figure 6.8a
Staphylococcus spp. on blood agar. No hemolysis.

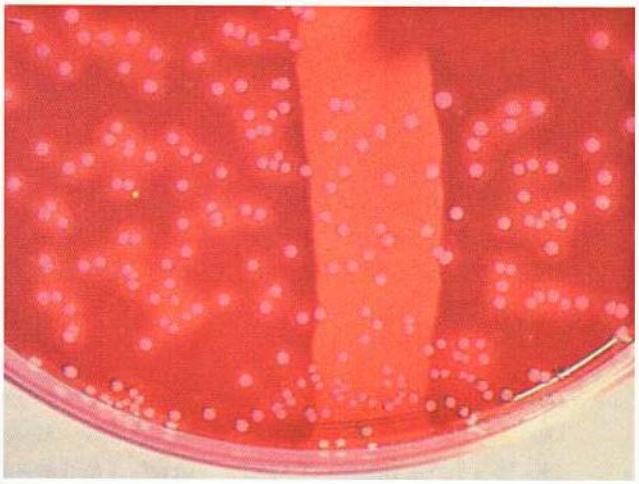


Figure 6.8b
Staphylococcus spp. showing narrow zones of complete hemolysis on blood agar streaked with *S. aureus* beta-hemolysin. The hemolysis within the area of the beta-hemolysin streak has been enhanced.

Since CNS species are so commonly isolated from the mammary gland and dairy environment, strain-typing may be necessary to determine if CNS species are associated with chronic infections or to determine epidemiologic relatedness of strains within a herd. Several strain-typing methods have been previously used for CNS species, including amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE).

Latex agglutination – Latex agglutination tests designed to detect clumping factor and protein A may give erroneous results with coagulase-negative staphylococci.

Chapter 7
Gram-negative Bacteria

Gram-negative bacteria are environmental mastitis pathogens commonly found in bedding, water, soil, and plant material. A high proportion of new infections caused by Gram-negative bacteria occur during the two weeks after drying off and the two weeks before calving. During lactation, susceptibility is highest at calving and decreases progressively as lactation advances. Infection risk tends to increase during hot and/or humid weather.

Clinical mastitis

Gram-negative bacterial infections may account for more than half of clinical mastitis cases in herds with bulk tank milk somatic cell counts consistently <200,000 cells/ml. Most cases of Gram-negative bacterial clinical cases will be limited to visibly abnormal milk and mild to moderate swelling of the infected quarter. However, 10% of Gram-negative intramammary infections present during lactation will result in severe clinical mastitis, requiring intensive therapy and veterinary services.

Antimicrobial therapy

Most antibiotics approved for intramammary use in lactating dairy cows are of marginal benefit for treating Gram-negative bacterial intramammary infections. In the United States, only one product has a label claim for a Gram-negative pathogen, being specifically labelled for the intramammary treatment of *Escherichia coli* mastitis. Cows with severe clinical mastitis that are exhibiting clinical "shock" may require systemic support, including fluid and electrolyte therapy and possibly anti-inflammatories. In addition, approximately 50% of severe clinical cases in one study have been shown to be bacteremic and hence systemic antimicrobial therapy may be warranted.

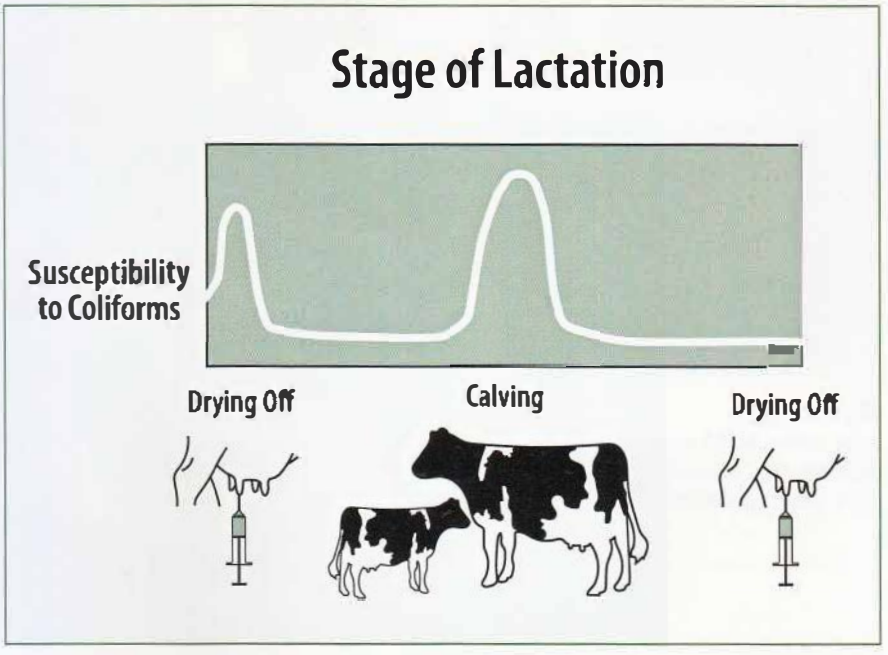


Figure 7.1
Incidence of new infection is greater during the dry period than during lactation.

Escherichia coli

Source

The primary sources of *E. coli* are bedding, manure, and soil.

Means of spread

Exposure of teats to *E. coli* occurs primarily between milkings when teats contact contaminated environmental sources.

Basic prevention and control measures

The most effective means of reducing the rate of intramammary infections is by keeping cows clean and dry to decrease teat end exposure to *E. coli*. Effective environmental sanitation includes the use of inorganic bedding, avoiding overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicidal pre-dip and thoroughly drying teats prior to milking may reduce new infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce the incidence and severity of clinical cases caused by many Gram-negative, mastitis-causing bacteria.

Additional information

The majority of *E. coli* infections last less than 10 days and respond poorly to antibiotic therapy. Infection rates are highest during the periparturient period and decrease as lactation progresses.

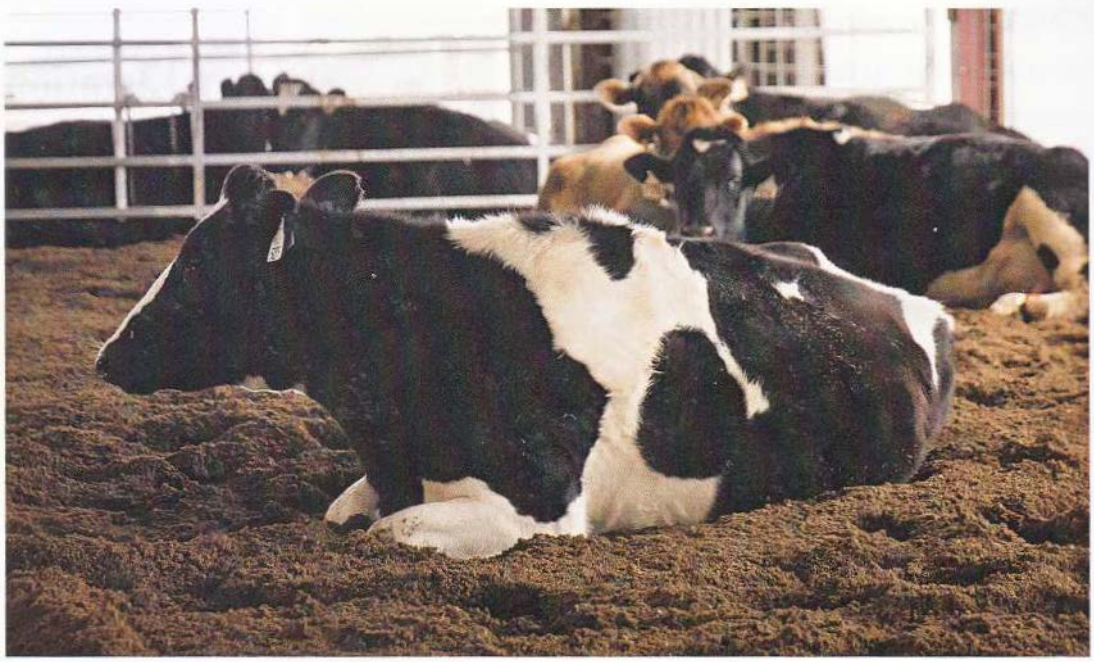


Figure 7.2
Escherichia coli infection rates are highest during the periparturient period.

Laboratory identification procedures

Appearance on blood agar – Colonies are 3 to 5 mm in diameter, gray, moist, and typically have a fecal odor. Colonies are generally not hemolytic; <15% of isolates are hemolytic.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Negative

Lactose fermentation – Positive within 18 hours at 37°C

Appearance on MacConkey agar – *Escherichia coli* form pink to red, dry, flat colonies (2 to 4 mm diameter), surrounded by a pink zone of precipitated bile salts (Figure 7.3b)

Triple Sugar Iron reaction – Acid slant, acid butt, with production of gas

Growth on Simmons Citrate agar – Negative

Motility – >90% are motile

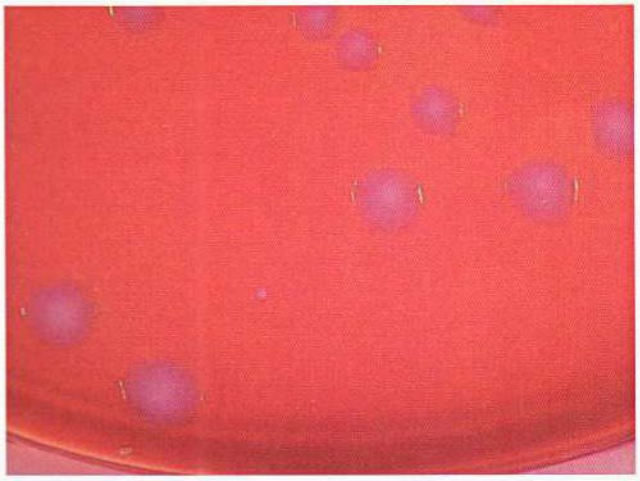


Figure 7.3a
Escherichia coli on blood sugar.

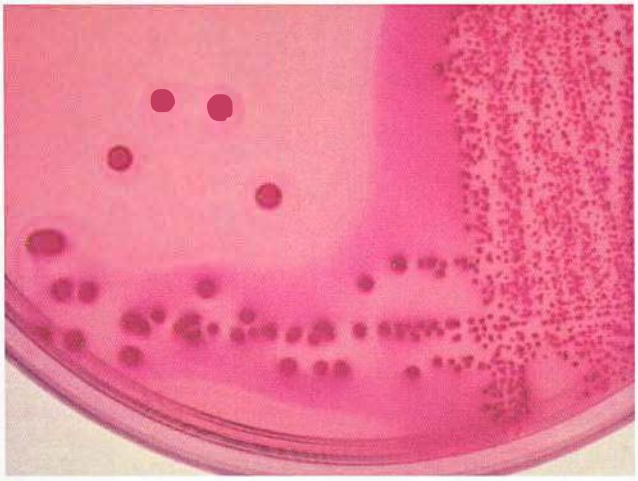


Figure 7.3b
Escherichia coli on MacConkey agar.
Pink coloy pigment is due to lactose fermentation.
Note the precipitation of bile salts.

Klebsiella spp.

Source

The primary source of *Klebsiella* spp. in the cow's environment is organic bedding, such as sawdust, shavings, and recycled manure solids.

Means of spread

Klebsiella spp. typically gain access to teats as a result of contact with organic bedding.

Basic prevention and control measures

A key control procedure for reducing *Klebsiella* spp. infections is to avoid using either sawdust or recycled manure bedding. In situations where sawdust bedding must be used, the use of a kiln-dried product will reduce the bacterial load in the unused product. Effective environmental sanitation includes the use of inorganic bedding, avoiding the overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicidal pre-dip and thoroughly drying teats prior to milking may reduce new infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce the incidence and severity of clinical cases caused by many Gram-negative, mastitis-causing bacteria.

Additional information

Klebsiella spp. are reported as a leading cause of severe Gram-negative clinical mastitis in dairy cows. Outbreaks of *Klebsiella* spp. mastitis are frequently associated with sawdust bedding. Rates of infection are higher in the summer than other seasons. *Klebsiella* spp. infections often persist for several weeks to months and respond poorly to antibiotic therapy. Infection rates are highest during the periparturient period and decrease as lactation progresses. Recent work suggests that fecal shedding of *Klebsiella* spp. likely contributes to pathogen loads in the cow's environment. Additionally, evidence shows that, in some herds, multiple cows can become infected with a common strain that either spreads by contact with milking equipment or a point source in the environment.

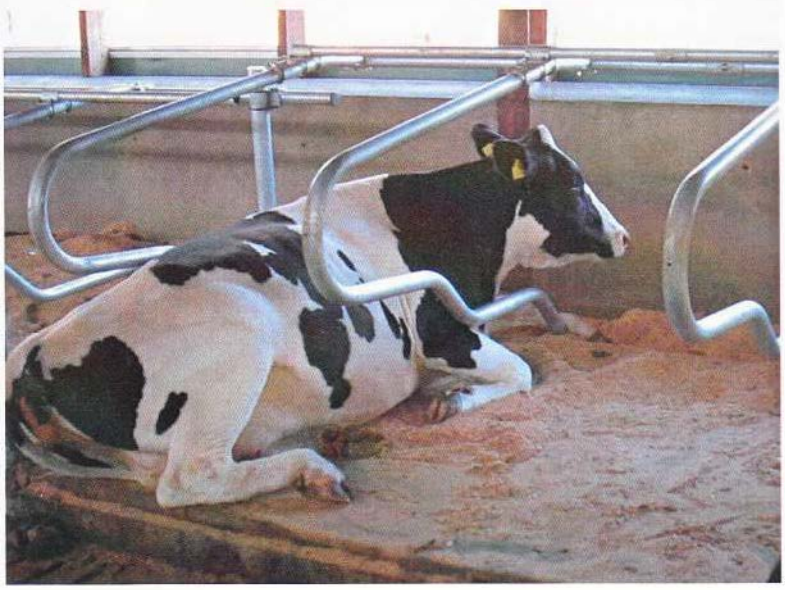


Figure 7.4
Outbreaks of *Klebsiella* spp. mastitis are commonly associated with sawdust bedding.

Laboratory identification procedures

Appearance on blood agar – Colonies are 3 to 5 mm in diameter, gray, moist, and often mucoid. *Klebsiella* spp. are not hemolytic.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Negative

Lactose fermentation – Positive within 18 hours at 37°C

Appearance on MacConkey agar – Pink-yellow mucoid colonies

Triple Sugar Iron reaction – Acid slant, acid butt, with gas production

Growth on Simmons Citrate agar – Positive

Motility – Negative

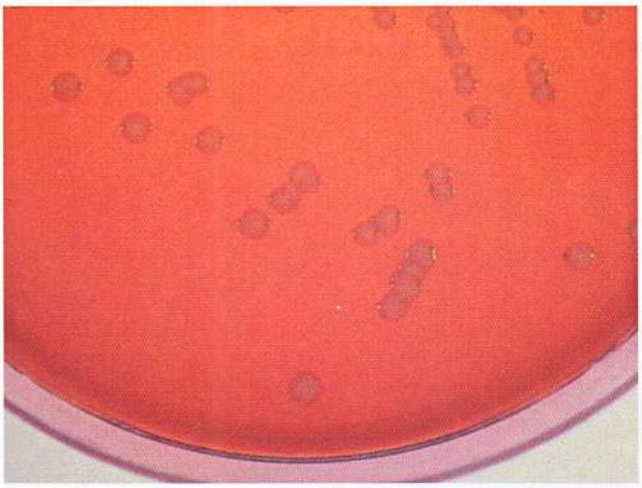


Figure 7.5a
Klebsiella spp. on blood agar.

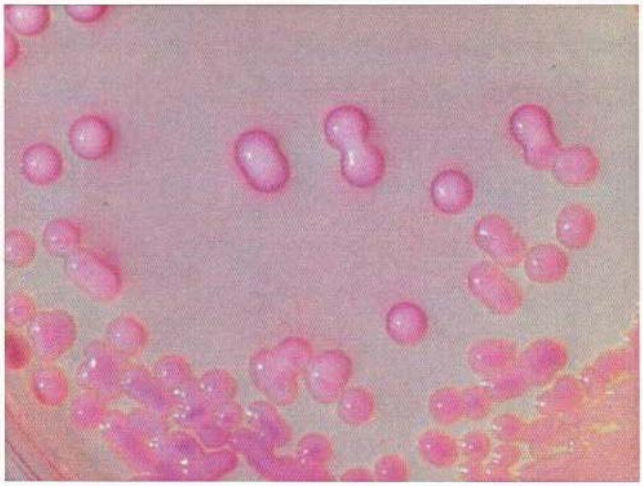


Figure 7.5b
Klebsiella spp. on MacConkey agar. Wet, mucoid colonies are due to capsule.

Enterobacter spp.

Source

The primary sources of *Enterobacter* spp. include bedding, manure, and soil.

Means of spread

Exposure of teats to *Enterobacter* spp. occurs primarily between milkings when teats contact the environmental sources.

Basic prevention and control measures

The most effective means of reducing the rate of intramammary infections is by keeping cows clean and dry to decrease teat end exposure to *Enterobacter* spp. Effective environmental sanitation practices include the use of inorganic bedding, avoiding overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicide to pre-dip and thoroughly drying teats prior to milking may reduce new infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce incidence and severity of clinical cases caused by many Gram-negative, mastitis-causing bacteria.

Additional information

Enterobacter spp. isolates are often misidentified as *Klebsiella* spp. *Enterobacter* spp. infections respond poorly to antibiotic therapy.

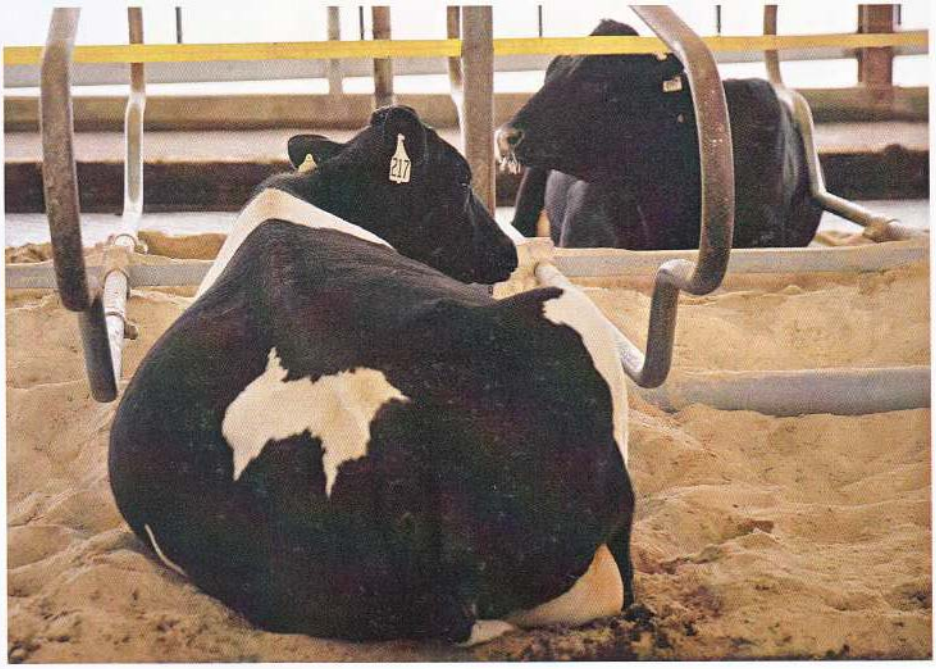


Figure 7.6
Exposure of teats to *Enterobacter* spp. occurs primarily between milkings.

Laboratory identification procedures

Appearance on blood agar – *Enterobacter* spp. colonies are 3 to 5 mm in diameter, gray, moist, and usually have a fecal odor. *Enterobacter* spp. are not hemolytic.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Negative

Lactose fermentation – Positive within 18 hours at 37°C

Appearance on MacConkey agar – Pink, dry colonies

Triple Sugar Iron reaction – Acid slant, acid butt, gas

Growth on Simmons Citrate agar – Positive

Motility – Positive

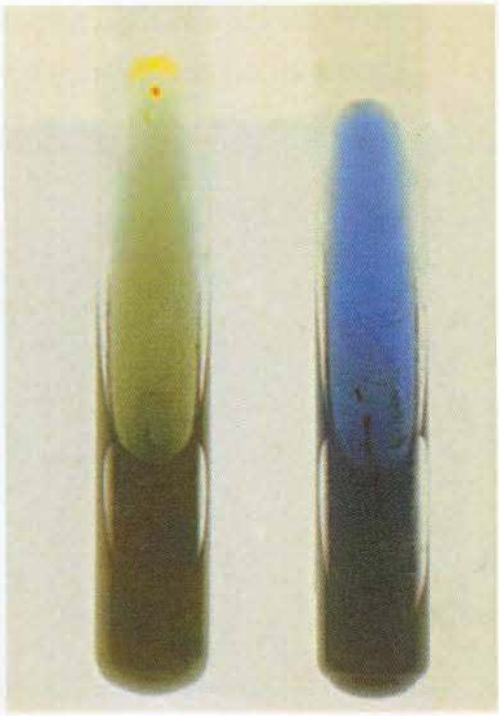


Figure 7.7a
Simmons citrate - left tube - negative (green); right tube - positive (blue).

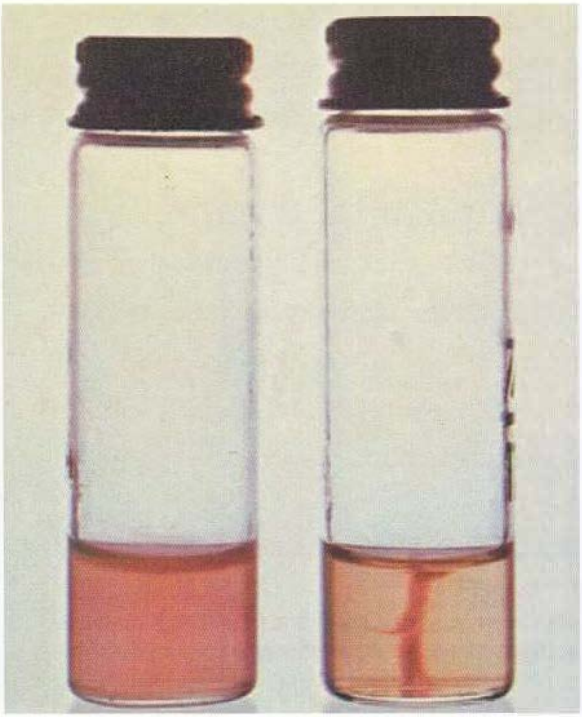


Figure 7.7b
Motility test - left tube - positive (turbid); right tube - negative (clear).

Serratia spp.

Source

Serratia spp. are environmental pathogens that commonly inhabit soil and plants.

Means of spread

Exposure of teats to *Serratia* spp. occurs primarily between milkings as teats contact the environmental sources.

Basic prevention and control measures

The most effective means of reducing the rate of intramammary infections is by keeping cows clean and dry to decrease teat end exposure. Effective environmental sanitation practices include the use of inorganic bedding, avoiding overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicidal pre-dip and thoroughly drying of teats prior to milking may reduce new infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce incidence and severity of clinical cases caused by many Gram-negative, mastitis-causing bacteria.

Germicidal resistance

Some strains of *Serratia* spp. are resistant to germicides containing chlorhexidine gluconate. Avoid teat dips containing chlorhexidine gluconate if *Serratia* spp. mastitis is suspected in a herd. Regardless of the teat antiseptic product used in a herd, thoroughly wash and air dry teat dip cups between milkings to reduce exposure to these pathogens.

Additional information

Serratia spp. often cause chronic infections lasting several lactations. Infection rates are higher during the dry period than lactation. *Serratia* spp. infections respond poorly to antibiotic therapy.

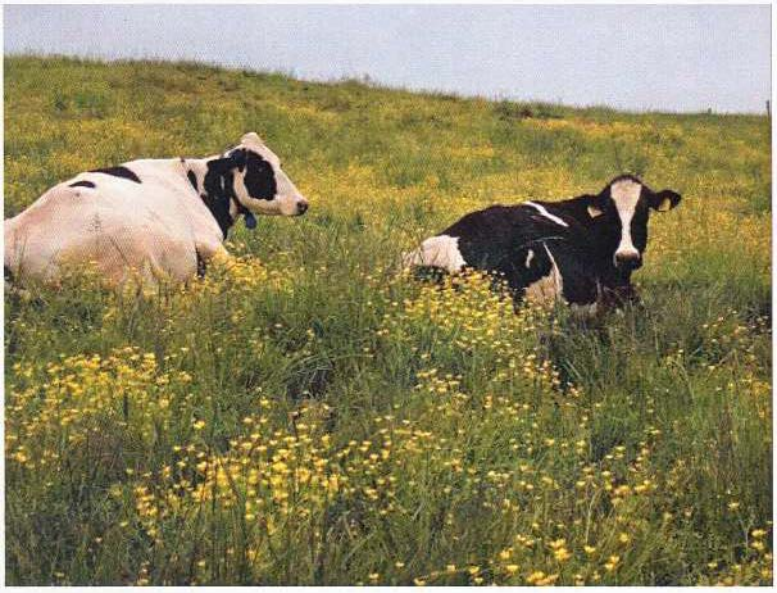


Figure 7.8
Serratia spp. are environmental pathogens that commonly inhabit soil and plants.

Laboratory identification procedures

Appearance on blood agar – Colonies are 2 to 3 mm in diameter, yellow-gray, and may resemble staphylococci, or may be red, depending on strain. Red pigment is more likely to develop at incubation temperatures <37°C. Approximately 20% of isolates from bovine intramammary infections are hemolytic.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Negative

Lactose fermentation – Negative

Appearance on MacConkey agar – Translucent colonies, often with red pigment.

Triple Sugar Iron reaction – Alkaline slant, acid butt, no gas

Growth on Simmons Citrate agar – Positive

Motility – Positive

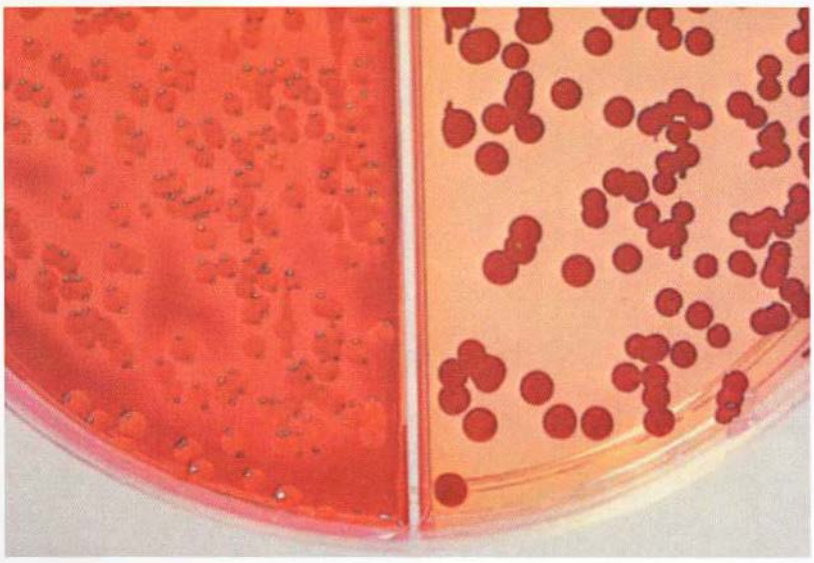


Figure 7.9
Serratia spp. on split plate with blood agar on left and MacConkey agar on right. The red color is due to colony pigment, not lactose fermentation.

Pseudomonas spp.

Source

Pseudomonas spp. are environmental pathogens commonly found in water and wet bedding.

Means of spread

Pseudomonas spp. gain access to teat ends following contact with environmental sources, such as wet bedding and contaminated water used to prepare teats for milking.

Basic prevention and control measures

Controlling *Pseudomonas* spp. mastitis is based on reducing exposure to contaminated water. Effective environmental sanitation practices include the use of inorganic bedding, avoiding overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicidal pre-dip and thoroughly drying teats prior to milking may reduce new infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce incidence and severity of clinical cases caused by many Gram-negative, mastitis-causing bacteria.

Miscellaneous information

Pseudomonas spp. typically cause chronic infections that respond poorly to antibiotic therapy. Infected cows are frequently culled. *Pseudomonas* spp. are resistant to certain sanitizers and may contaminate milking parlor drop hoses, even when disinfectants are added to wash water.

Figure 7.10
Pseudomonas spp. may contaminate milking parlor drop hoses even when disinfectants are added to wash water.

Laboratory identification procedures

Appearance on blood agar – *Pseudomonas* spp. produce colonies 2 to 4 mm in diameter. Colonies are white-gray with irregular edges. *Pseudomonas* spp. are usually hemolytic and produce a distinctive grape-like odor.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Positive

Lactose fermentation – Negative

Appearance on MacConkey agar – Translucent, shiny colonies

Triple Sugar Iron reaction – Alkaline slant, alkaline butt, no gas

Motility – Positive

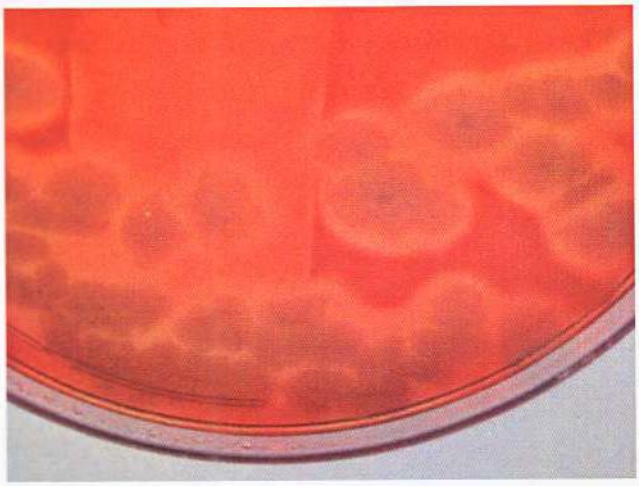


Figure 7.11a
Pseudomonas spp. on blood agar streaked with *S. aureus* beta-hemolysin.

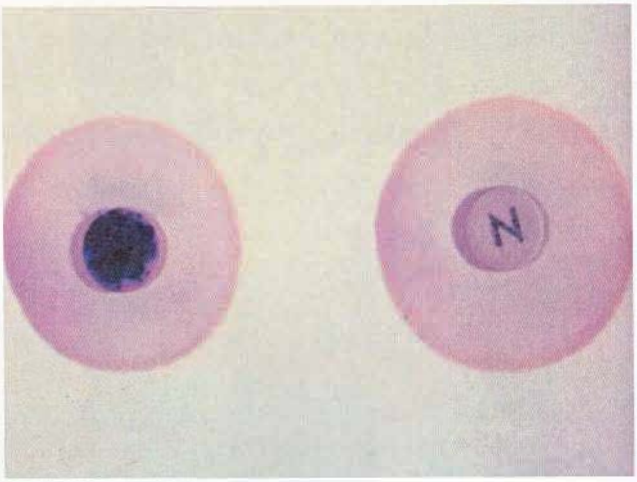


Figure 7.11b
Oxidase test - left - positive; right - negative.

Proteus spp.

Source

Proteus spp. may reside in the cow's environment in bedding, feed, and water.

Means of spread

Contact of *Proteus* spp. with teat ends occurs primarily between milkings.

Basic prevention and control measures

Little information is known regarding the pathogenesis of *Proteus* spp. Control procedures effective against other Gram-negative bacteria appear to be effective against *Proteus* spp. Effective environmental sanitation practices include the use of inorganic bedding, avoiding overcrowding of cows, frequent removal of manure and urine, and preventing access to muddy lots or corrals, wet areas under shade, marshy areas, and pools of standing water. The use of an effective germicidal pre-dip and thoroughly drying teats prior to milking may reduce infections during lactation. Immunization of cows with rough mutants, such as *E. coli* J5, can reduce incidence and severity of clinical cases caused by many Gram-negative mastitis causing bacteria.

Additional information

Proteus spp. are not common mastitis pathogens in most herds. Often, the presence of these bacteria on a standard milk culture is the result of contamination. Careful attention to the guidelines for determining a contaminated sample should be followed. *Proteus* spp., in some instances, can cause herd outbreaks. Infections tend to be chronic and clinical cases are often severe. These infections respond poorly to antibiotic therapy.

Figure 7.12
Proteus spp. may reside in the cow's environment on bedding and in feed and water.

Laboratory identification procedures

Appearance on blood agar – *Proteus* spp. produce gray swarming colonies that can cover the entire surface of media in a Petri plate. Isolates produce a putrid odor and colonies are not hemolytic.

Gram stain – Gram-negative rods

KOH reaction – Positive

Cytochrome oxidase – Negative

Lactose fermentation – Negative

Appearance on MacConkey agar – Translucent growth

Triple Sugar Iron reaction – Alkaline slant, acid butt, hydrogen sulfide positive

Motility – Positive

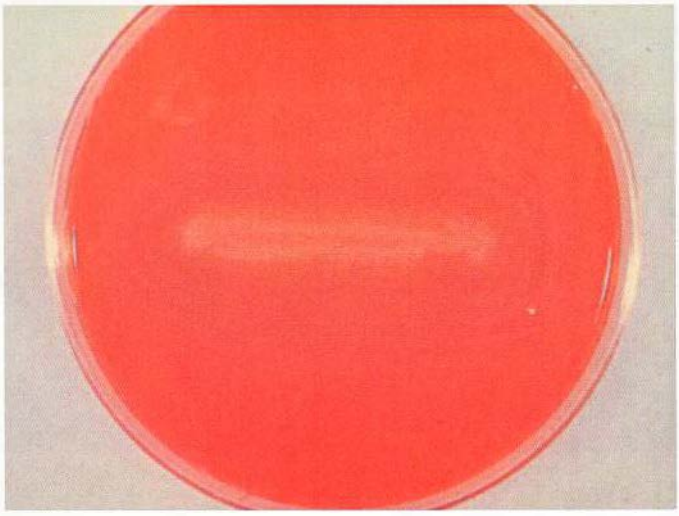


Figure 7.13
Proteus spp. on blood agar showing swarming or spreading over surface of agar.

Pasteurella spp.

Source

The source of *Pasteurella* spp. is the upper respiratory tract of mammals and birds.

Means of spread

The means of spread is unknown, probably from cow to cow. *Pasteurella* spp. from the respiratory tract of cows could be transported to the udder via bloodstream or lymphatic system, and cause intramammary infections under suitable conditions.

Basic prevention and control measures

Prevent teat injuries. If infection is confirmed, remove affected cows from the herd.

Additional information

Pasteurella spp. are seldom reported as a cause of bovine mastitis. However, outbreaks in individual herds may occur. *Pasteurella* spp. may cause acute, severe mastitis. Affected quarters may produce a creamy-yellow, thick, and viscous secretion, sometimes with a foul odor. Agalactia may develop.

Despite *Pasteurella* spp. sensitivity to several antibiotics in vitro, mastitic cows usually do not respond to intramammary or parenteral treatments. Death may result from endotoxemia.

Figure 7.14
Antibiotic therapy generally has no effect on *Pasteurella* spp. mastitis.

Laboratory identification procedures

Appearance on blood agar – *Pasteurella* spp. produce colonies 2 to 4 mm in diameter. Colonies are gray, very mucoid, and often confluent. Irregular, rough colonies may occur. *Pasteurella* spp. are usually hemolytic and produce a musty odor.

Oxidase – Positive (slow)

Catalase – Positive

Microscopic examination of culture growth – Short, ovoid, plump, Gram-negative rods. Bi-polar staining often seen. Capsules around the bacterial cell are frequently present.

Motility – Negative

Comments – With the exception of *P. haemolytica*, pasteurellas do not grow on MacConkey agar.

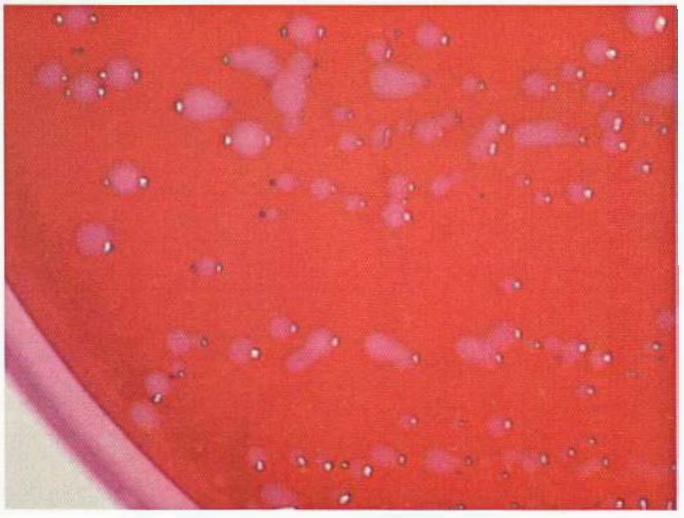


Figure 7.15
Pasteurella spp. on blood agar showing lobate edges on most colonies

Table 7.1

Notable characteristics of Gram-negative growth on blood-esculin and MacConkey agar

Organism	Blood-esculin Agar	MacConkey Agar
Escherichia coli	Fecal odor	Pink, bile precipitate
Klebsiella spp.	Mucoid	Pink-yellow mucoid
Enterobacter spp.	Fecal odor	Pink
Serratia spp.	Resemble staphylococci, ~20% pigmented	Translucent or red pigment
Pseudomonas spp.	Grape-like odor, often hemolytic	Shiny, translucent, metallic sheen
Proteus spp.	Swarming	Translucent
Pasteurella spp.	Confluent growth	No growth or translucent

Table 7.2

Differentiation of the most frequently isolated Gram-negative bacteria

Organism	Lactose	Citrate	Motility	Oxidase	Triple Sugar Iron (TSI)
Escherichia coli	+	-	+	-	A/A, G
Klebsiella spp.	+	+	-	-	A/A, G
Enterobacter spp.	+	+	+	-	A/A, G
Serratia spp.	-	+	+	-	K/A
Pseudomonas spp.	-	+/-	+	+	K/K
Proteus spp.	-	-/+	+	-	K/AS+
Pasteurella spp.	-	-	-	+	A/A

+ Means majority of strains are positive.

- Means majority of strains are negative.

+/- Means more strains are positive than negative.

-/+ Means more strains are negative than positive.

"A" Means acid.

"K" Means alkaline.

"G" Means gas production.

"S+" Means hydrogen sulfide production.

Chapter 8
Mycoplasmas

Mycoplasma spp. are bacteria that lack a cell wall and these types of bacteria are often termed: L-forms. *Mycoplasma* spp. are considered members of a class of bacteria referred to as mollicutes. They have fastidious growth requirements and are slower to replicate than most mastitis pathogens. Their lack of a cell wall and slow growth enable them to be refractory to many intramammary antibiotic therapy regimens.

There are more than 100 species of *Mycoplasma* identified and at least 11 have been identified as causing mastitis. The five most common species associated with dairy cattle mastitis are: *M. alkalescens*, *M. bovis*, *M. bovigenitalium*, *M. californicum*, and *M. canadense*. *Mycoplasma bovis* is the most prevalent and accounts for the majority of mycoplasma mastitis cases. Care must be taken to differentiate between *Mycoplasma* spp. and *Acholeplasma* spp. The *Acholeplasma* are a genus with very similar characteristics to *Mycoplasma* but are not normally considered mastitis pathogens. *Acholeplasma* spp. can be isolated from milk, on occasion, and are often considered as contaminants. Although rare, outbreaks of mollicute mastitis have been attributed to *Acholeplasma* spp.

Mycoplasma spp.

Sources

The primary sources of *Mycoplasma* spp. that cause mastitis are the mammary gland and respiratory and urogenital tracts. The presence of *Mycoplasma* spp. in the mammary gland is indicative of infection. However, animals may carry *Mycoplasma* spp. at other body sites without clinical signs of disease, i.e., asymptomatic carriage. Addition of animals raised outside the dairy premise can be the source of new strains of *Mycoplasma* spp. to the herd.

Means of spread in the herd

Mycoplasma spp. are contagious mastitis pathogens. It is generally believed that most transmission of mycoplasma mastitis occurs during milking time by contact with contaminated equipment. However, evidence suggests that mycoplasma mastitis agents are transmitted between animals outside the milking parlor. Transmission may occur via aerosols, nose-to-nose contact, and contaminated treatment devices and other fomites. In several recently reported outbreaks of mycoplasma mastitis, transmission of mycoplasma pathogens among animals likely occurred outside of the milking parlor; aerosol transmission was suspected.

Basic prevention and control measures

Biosecurity is an essential element of controlling mycoplasma mastitis. Comingling of youngstock with replacement cattle from other farms should be done in a manner that avoids overcrowding and "stress." Importation of replacement cattle from farms with current or recent history of mycoplasma diseases (mastitis, arthritis, pneumonia, or metritis/vaginitis) should also be avoided.

Strict milking time hygiene practices should be used, which include: use of disposable latex gloves by milkers, effective application of disinfectants (teat dips) before and after milking, use of single-service towels to clean and dry udders, and milking unit disinfection between milkings. Mycoplasma mastitis is generally refractory to intramammary therapy.

Identification of cows with mycoplasma mastitis and segregation and/or culling of those cows have been advocated. Several reports document the success of eliminating mycoplasma mastitis by using a strict test and cull protocol of confirmed mycoplasma mastitis cows. However, there are reports that suggest that segregation of mycoplasma mastitis cows from herdmates and milking those cows last can be used to control the spread of the disease. Either strategy necessitates careful and consistent monitoring of the herd-level mycoplasma mastitis status. Some herds have noted what appears to be spontaneous recovery from mycoplasma mastitis and thus control may be achieved without a test and slaughter control program.

Monitoring mycoplasma mastitis

Consistent culturing of bulk tank milk and examination of cultures for *Mycoplasma* spp. is commonly used to monitor the mycoplasma mastitis status of a herd. A positive culture of *Mycoplasma* spp. from bulk tank milk is generally assumed to be indicative that the herd has cow(s) with mycoplasma mastitis. In follow-up to a positive bulk tank milk culture, often collection and culture of milk samples from cows with clinical mastitis, cows with multiple mammary quarters with clinical mastitis, cows whose milk secretion has changed dramatically, or cows with elevated milk somatic cell counts can be used to identify the infected cows. When such targeted sampling does not lead to discovery of the infected cow(s), pooled sampling might be necessary where pooled pen or milking string samples are collected and cultured, like bulk tank samples. In larger herds, this pooled sampling strategy should lead to a smaller number of cows that need to be screened at follow-up samplings. It has been shown that once a group has been identified that includes the cow(s) with mycoplasma mastitis, then cows can be further divided into groups of five animals. Individual milk samples should be collected from all five cows but pooled together for culture. If milk from the pooled group of five cows has the mycoplasma pathogen, then individual cows from the group of five must be resampled to determine which cow(s) is infected. This approach of reducing the pool of cows to five has been shown to be an economically effective way to identify mycoplasma mastitis cows. It should be noted that some cows, at times, will shed low to undetectable numbers of mycoplasma organisms in milk from infected glands. Thus, culture of bulk tank and/or milking string samples must be consistent and frequent to determine the true mycoplasma mastitis status of the herd.

Laboratory identification procedures

Culture on growth media – *Mycoplasma* spp. will not reliably grow on blood agar incubated aerobically. The standard culture method for *Mycoplasma* spp. is a direct spread of milk on Modified Hayflick Agar (see Appendix 2). Plates should be incubated for 7 to 10 days in a moist chamber at 37°C, 5 to 10% CO_2. The experience of some mastitis laboratories is that colonies can be first seen at 3 days. Cultured colonies are rarely visible with the naked eye and should be examined with stereo-microscope at 15 to 25 X magnification. Colonies will have a characteristic "fried egg" appearance, Figure 8.1a. Milk samples with high concentrations of *Mycoplasma* spp. may show confluent growth, making it difficult to observe individual colonies. It may be necessary to punch out a section from the agar, pass it through mycoplasma broth media, and use it to establish a new culture on agar, using purity streak isolation, such that growth may be more visible.

Conversely, a milk sample might be thought to be positive for *Mycoplasma* spp., but culture results do not confirm that suspicion. In such cases, it is advisable to pass the milk through a *Mycoplasma* spp. broth media with incubation as described above for 4 days. A portion of the cultured broth can then be spread on Modified Hayflick Agar and incubated for 7 to 10 days. This enrichment procedure can be used before the primary culture on agar and can be used for isolation of *Mycoplasma* spp. from swabbing solutions as well as milk.

Several laboratories use a candle jar to create an enriched CO_2 atmosphere. It is difficult to obtain a 10% CO_2 atmosphere with a candle jar and thus this procedure may yield false-negative results.

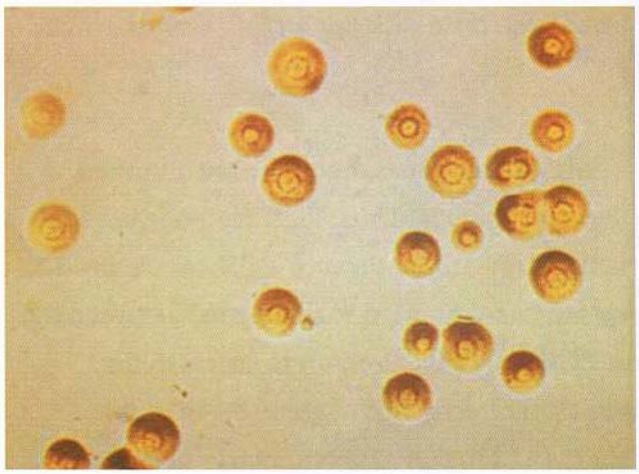


Figure 8.1a
Mycoplasma spp. colonies with typical fried egg appearance (50 X).

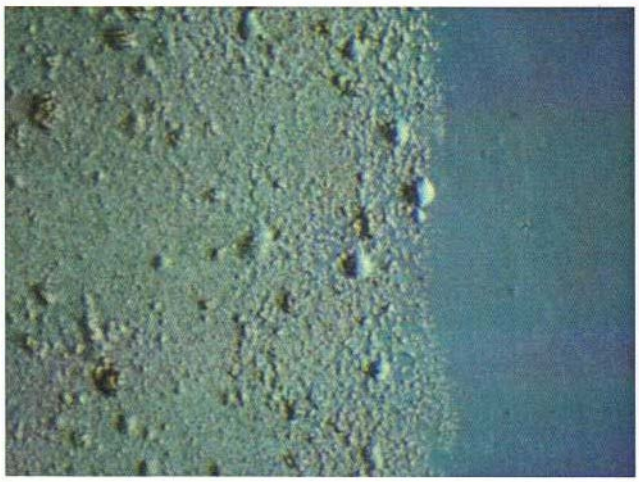


Figure 8.1b
Confluent growth of *Mycoplasma* spp. at edge of milk film.

Non-culture methods to detect *Mycoplasma* spp. from milk – Molecular techniques amplifying and then detecting specific nucleic acid segments of the *Mycoplasma* spp. genome have been developed. These systems rely on polymerase chain reactions, PCR. The limitation of PCR techniques used to determine the presence of *Mycoplasma* spp. is the lack of focus on a specific species. For bovine samples, the primary effort has been to detect *M. bovis*. If a PCR platform is chosen for diagnosis of mycoplasma mastitis, then it is advisable that the one chosen will identify *Mycoplasma* spp. in general, or better yet identifies *M. bovis* and other species. Indeed, *M. bovis* is the major mycoplasma mastitis pathogen. Multiplex PCR and other combined methods will improve the sensitivity of PCR used as a diagnostic for mycoplasma mastitis. An advantage of PCR is its ability to rapidly amplify nucleic acid, reducing the time to detection of mycoplasma mastitis to hours, rather than the several days, which is characteristic of standard mycoplasma culture.

Genus and species identification – Culture methods can be used to presumptively distinguish *Mycoplasma* spp. from *Acholeplasma* spp. Either the digitonin and/or the nisin disk assays can be used to distinguish between *Mycoplasma* spp. and *Acholeplasma* spp. *Mycoplasma* spp. are more sensitive to digitonin, a detergent, than *Acholeplasma* spp. Contrarily, nisin, an antimicrobial peptide, is more active against *Acholeplasma* spp. than *Mycoplasma* spp. When a mycoplasma-like organism is identified on primary culture on Modified Hayflick Agar, representative colonies can be transferred to broth and cultured until visible growth in broth is seen, usually at 3 to 5 days. A portion of that culture can be spread uniformly on a new agar plate to create a lawn of growth. Digitonin or nisin disks (Appendix 2) can be placed on the agar plate before incubation. *Mycoplasma* spp. will characteristically have a zone of no growth around the digitonin disk but will grow closely around the nisin disk. *Acholeplasma* spp. reacts in the opposite way, with a zone of no growth around the nisin disk but growth closely around the digitonin disk.

Antibody-based techniques have been developed to speciate mycoplasmas, but there are a limited number of laboratories that have the resources to use these techniques. Thus, PCR methods have the most promise to differentiate *Mycoplasma* spp. that cause mastitis. Limitations of PCR are the need to amplify and distinguish among several species of *Mycoplasma* and the need to distinguish between the genus *Mycoplasma* and *Acholeplasma*. Additionally, most PCR tests have been more successful in identifying and speciating *Mycoplasma* from agar cultures than directly from organisms in milk. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) spectrometry is being developed for speciation of mastitis cultures, including *Mycoplasma* spp.

Storage of milk samples – Recent research has demonstrated that freezing milk samples will result in reduced recovery of *Mycoplasma* spp. over time. In general, with each week of storage there is an approximate 10-fold loss in viable colony-forming units. Addition of 10 to 30% v/v glycerol to the milk prior to freezing will improve viability during frozen storage. Refrigerating milk up to 3 to 5 days does not seem to affect viability of *Mycoplasma* spp. Hence, culture of fresh milk is preferred for the best recovery and increased sensitivity of detection of *Mycoplasma* spp.

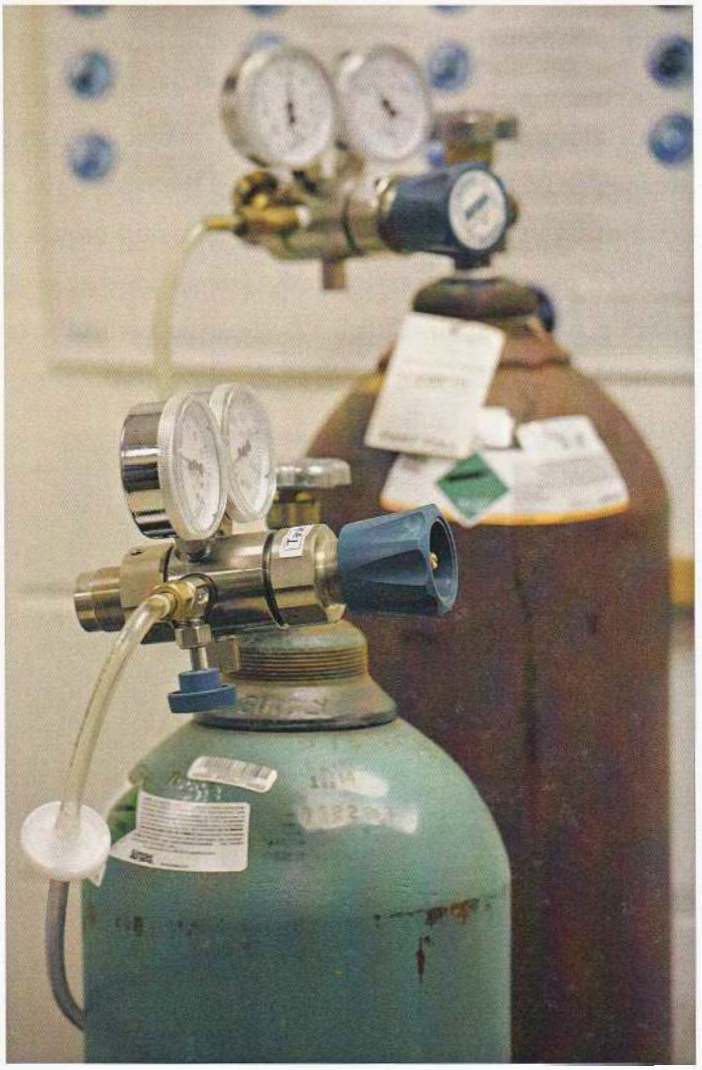


Figure 8.2
Mycoplasma spp. must be incubated in a 5 to 10% CO_2 atmosphere on mycoplasma-specific media.

Chapter 9
Miscellaneous Organisms

It has been reported that there are at least 137 microbial species, subspecies, and serovars that have been isolated from the milk of bovine mammary glands and are implicated in causing mastitis. The mastitis pathogens that are generally considered most prevalent and of significant importance have been considered and described in previous chapters. It is difficult, then, to choose which of the more than 100 remaining pathogens should be discussed in this chapter. Based on recent surveys of the prevalence of mastitis in herds around the world, the miscellaneous pathogens chosen for brief description in this chapter include: yeasts and molds, *Nocardia species*, *Prototheca species*, *Corynebacterium bovis*, *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*), *Mycobacterium* spp., *Bacillus* spp., and other Gram-positive bacilli. This list is consistent with the miscellaneous organisms described in Chapter 7 of the second edition of the Handbook, with the exception of *Mycoplasma* spp., which now have their own chapter (Chapter 8).

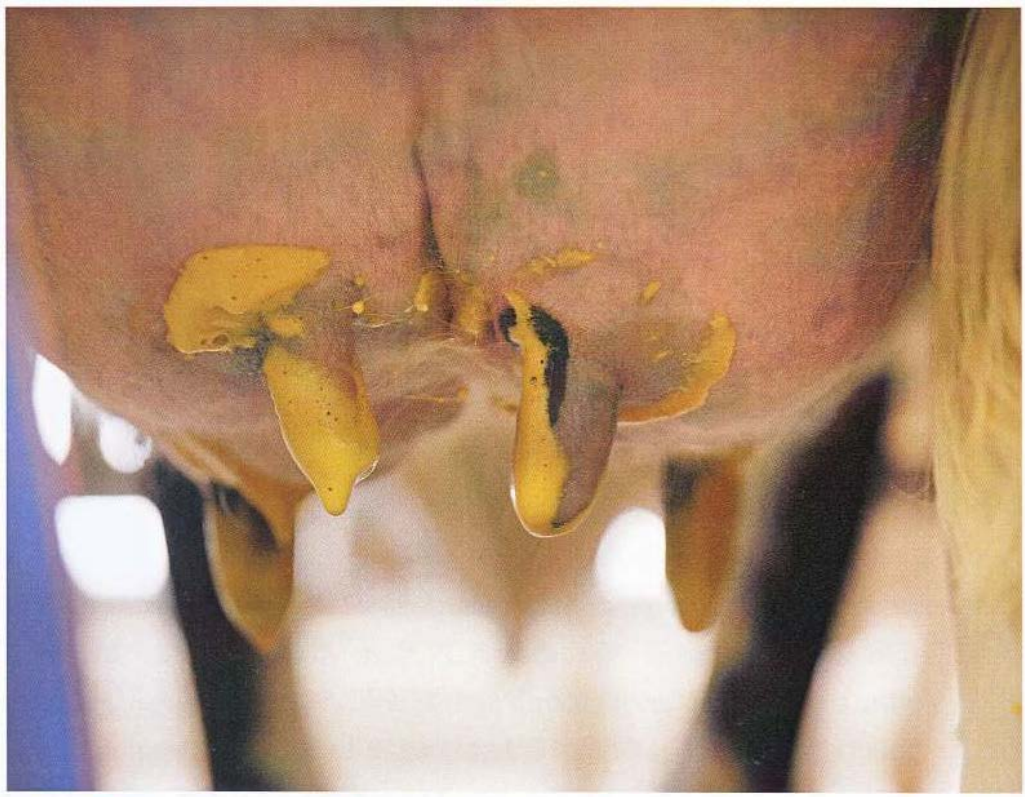


Figure 9.1
Improper teat disinfection prior to intramammary infusion of antibiotics can be the origin of intramammary infection due to *Nocardia* spp., *Prototheca* spp., and yeasts.

It is important in an investigation of a mastitis problem in a dairy herd to identify the causative agent properly and to determine the source of the pathogen and the risk factors associated with its transmission. Because many of these miscellaneous agents are present in the environment, they are often considered contaminants, rather than a true cause of an intramammary infection, especially in cases of subclinical mastitis. Yet, when clinical mastitis is a problem and/or several cows are involved in an outbreak, special care must be taken to ensure that the miscellaneous pathogens are not discounted as the inciting cause of mastitis.

The source and route of transmission of the miscellaneous pathogens must be determined when the causative agent is identified. Many miscellaneous organism outbreaks have been linked to contaminated treatment devices or treatment preparations, especially homemade preparations, and improper teat disinfection. Additionally, environmental sources have often been implicated in several outbreaks and isolation or removal of these sources has been associated with abatement of the outbreak. Exceptions include *C. bovis* and *T. pyogenes*, which may be contagious.

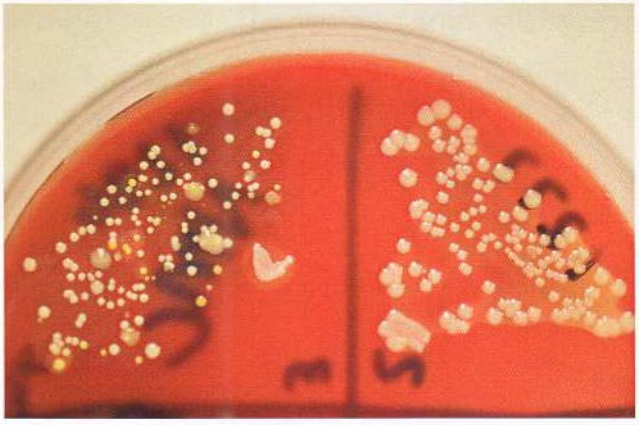


Figure 9.2
Laboratory personnel must be able to differentiate between contaminated cultures and cultures representing an intramammary infection.

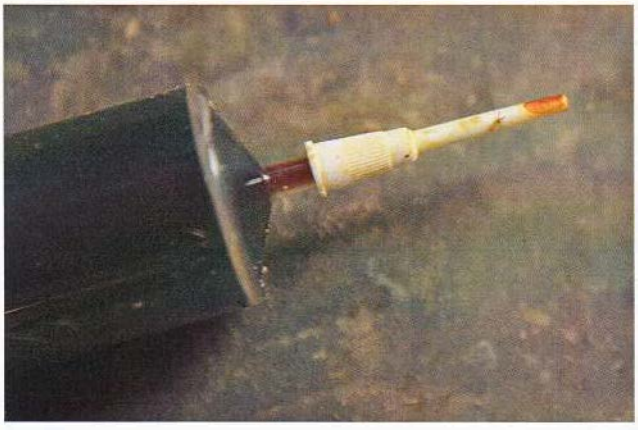


Figure 9.3
Contaminated treatment devices, such as syringes and cannulas, are common sources of yeasts and molds.

Yeasts and Molds

Yeasts and molds are mycotic agents that live freely in the environment. Although there are many different mycotic agents that can cause bovine mastitis, the most prevalent belong to the *Candida* genus and are usually considered to be opportunistic pathogens. Excessive contamination of the teats and contaminated mammary therapy procedures (Figure 9.3) are considered the major risk factors associated with mastitis caused by these agents. It has been suggested that poor teat hygiene, either during milking or prior to intramammary therapy, may increase the likelihood of mastitis. Thus, cleaning teats thoroughly followed by an alcohol scrub of the teat end before intramammary therapy administration will avoid many of these infections. Use of sterile product preparations and delivery devices (syringes, needles, etc.) is critical. Transmission of yeast and mold to cows leading to outbreaks of mastitis might occur during milking. Contagious spread is thought to occur when excessive pathogen loads from infected mammary quarters contaminate potential fomites (milking units, milkers' hands, teat dip containers, and implements used to wash and dry teats). Thus, strict milking time hygiene techniques cannot be overlooked when a herd experiences a mastitis outbreak involving yeasts and molds.

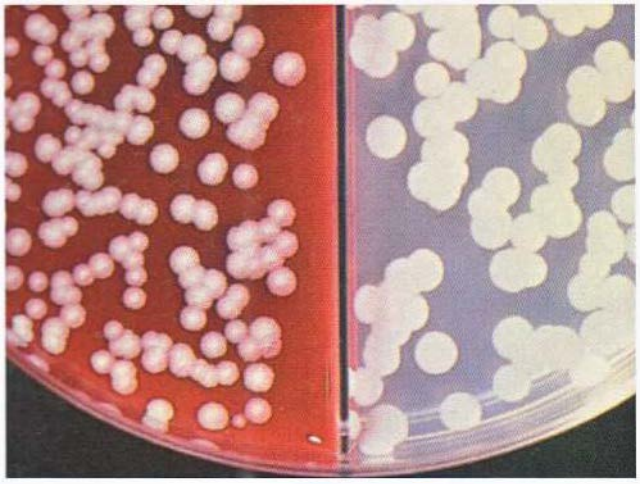


Figure 9.4a
Yeast culture on split plate with blood agar on left and Sabouraud Dextrose Agar on right (large, dry colony type).

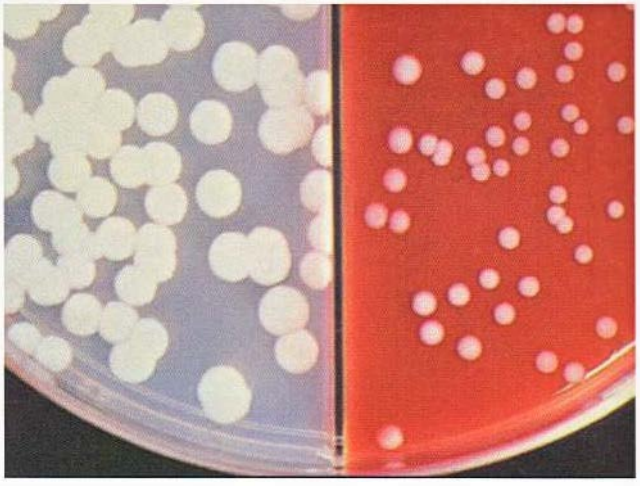


Figure 9.4b
Yeast culture on split plate with Sabouraud Dextrose Agar on left and blood agar on right (moist colony type).

Laboratory identification procedures

Culture on growth media – Colony characteristics of yeasts are highly variable, but are often waxy or mucoid, and either white or off white in color. Molds will often exhibit the characteristic visible hyphae, appear fluffy, and are gray on agar cultures. These mycotic agents will generally grow initially on blood agar at 37°C. Some yeast colony characteristics on blood agar may be similar to some coagulase-negative staphylococci. Subcultures and future cultures of the mycotic agents should be made on Sabouraud Dextrose Agar, a selective agar for fungi. This medium has an acid pH (5.6) and is considered nutritionally poor. Thus Sabouraud Dextrose Agar is often used as a selective agar that does not encourage the growth of non-mycotic mastitis pathogens. It should be noted that mycotic agents are potentially harmful to humans and therefore examination and tests of cultured organisms should be done under a biological safety hood.

Gram stain – A Gram stain of the yeast culture should be conducted, given the similar colony characteristics with other bacteria on blood agar. Yeasts will appear as Gram positive. When examined at 1,000 X magnification, they will be notably larger than bacteria and often appear as "American football" shaped. The mycotic agents will be characteristically visible under 400 X magnification of a saline wet mount. A second sample's culture from an apparent mastitic quarter should be taken to confirm the presence of the mycotic agent as the causative pathogen.

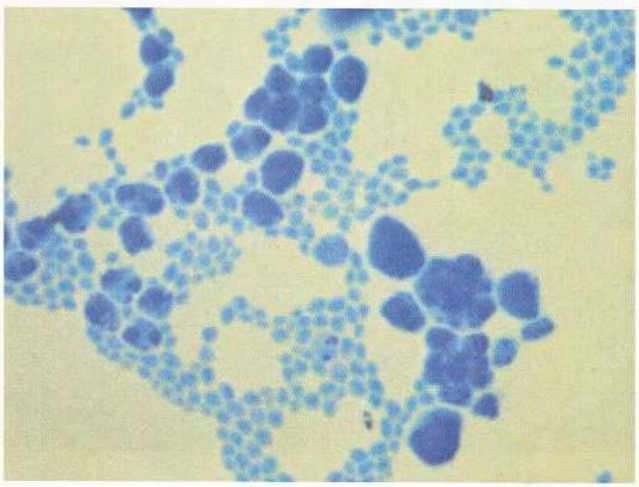


Figure 9.5
Methylene blue stained smear of yeast (small) and *Prototheca* spp. (large).

Nocardia spp.

Nocardia spp. are Gram-positive, weakly acid-fast, actinomycetes. A wet mount can be used to presumptively identify the pathogen group. Under microscopic examination, the pathogen will often appear filamentous. The primary sources of the agent are soil, water, air, grass, and skin. It is generally considered an environmental mastitis pathogen. Seven species have been associated with nocardia bovine mastitis. Yet, there are two prevalent species: *N. nova* and *N. farcinica*. *Nocardia nova* appears to be the most prevalent and may account for >80% of all *Nocardia* spp. mastitis cases. Mammary nocardiosis is marked by granulomas, where often treatment failure leads to culling of the infected cattle.

Laboratory identification procedures

Culture on growth media – *Nocardia* spp. will grow on blood agar and appear as dry, usually white or yellow or orange, rough powdery or smooth colonies. Visible colony growth may require 2 to 7 days incubation at 37°C. Colonies will burrow into the agar and thus isolation can require removal of the colony and the agar from the plate and re-culture of the colony in broth before re-plating. Samples should be cultured as soon as possible, as *Nocardia* sp. often do not survive storage.

Catalase – Positive

Caution! – *Nocardia* spp. have the ability to cause human disease. Extreme caution in culture, examination, and testing of the *Nocardia* spp. must be maintained, given that the organism forms airborne aerial hyphae that can be transferred to the skin or mucosal surfaces. Thus, strict care must be applied to all colonies that resemble *Nocardia* spp. Use of biological safety hood is highly recommended. Cultures should be taped shut to avoid accidental aeration. Colonies should be killed by formalin fumigation prior to opening for morphologic examination. It may be most advisable to allow experienced laboratories to work with samples and cultures suspected of containing *Nocardia* spp.

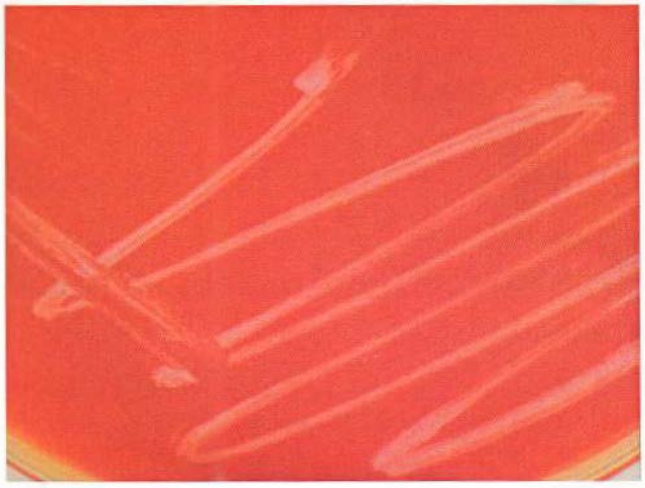


Figure 9.6a
Nocardia spp. culture at 72 hours on blood agar showing powdery appearance.

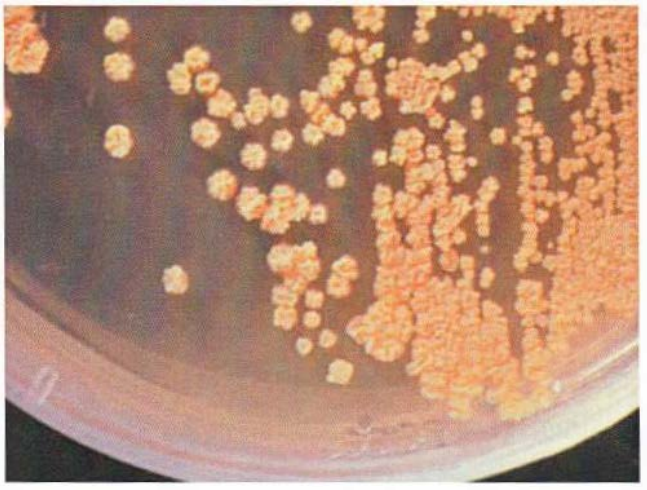


Figure 9.6b
Nocardia spp. culture on Brain Heart Infusion agar.

Prototheca spp.

Prototheca spp. are unicellular, achlorophyllous algae that can cause acute clinical as well as subclinical and chronic mastitis. As non-photosynthetic, they rely heavily on nutrients from the environment for their metabolic processes. Reproduction is asexual, involving the production of endospores within parent sporangia, which rupture and release the endospores into the environment.

The most common species associated with mastitis is *P. zopfii* and the most prevalent strain of *P. zopfii* is genotype II. *Prototheca blaschkeae* and *P. wickerhamii* have also been associated with cases of mastitis. *Prototheca* spp. are generally regarded as environmental mastitis pathogens, with several reports indicating they can be found on dairies in fecal matter, water sources, and bedding. Moreover, the occurrence of *Prototheca* spp. in the environment may not be more prevalent on dairy farms that have experienced an outbreak than those that have had no *Prototheca* spp. mastitis. Thus, it would seem logical that the recommendation for the control of prototheca mastitis would be keeping the cow's environment clean and dry. *Prototheca* spp. are not susceptible to common antibiotics, as they are not bacteria. It has been suggested that cows with intramammary infections of *Prototheca* spp. should be culled, as the infections cannot be successfully treated and teat cups may become contaminated with the pathogen. Additionally, *Prototheca* spp. have been found to be slightly resistant to disinfectants and data suggest that dilute solutions of disinfectants, such as those used in unit backflush systems, could fail to kill the agent. Reports that indicate the effectiveness of culling of cows with chronic prototheca mastitis to control the disease, and its potential transmission at milking time, in aggregate, could suggest that *Prototheca* spp. should also be classified as a contagious mastitis pathogen. Four critical points to control prototheca mastitis have been suggested: 1) maintain excellent hygiene in all housing areas, including the milking parlor; 2) pasteurize milk before feeding or discarding raw milk that is normally fed to calves; 3) separate infected animals and do not house with healthy ones, especially during the periparturient period; and 4) do not treat, as treatment is rarely effective; instead, cull infected animals as soon as possible.

Laboratory identification procedures

Culture on growth media – Cream-white or grayish-white, pasty colonies form after 24 to 36 hours incubation at 37°C. Colonies on blood agar can be confused with yeasts and coagulase-negative staphylococci. *Prototheca* spp. grow well on Potato Dextrose Agar and Sabouraud Dextrose Agar at 25 to 37°C. Selective enrichment medium (Prototheca Isolation Media), not commercially available, can be used to enhance isolation from environmental sources and from milk.

Microscopic examination of culture growth – *Prototheca* spp., as colorless algae, can be confused microscopically with other microorganisms. A fixed smear of *Prototheca* spp. colonies stained with Methylene Blue or Gram stain will show spherical-oval sporangia, with or without endospores. A matrix of ruptured cells and released smaller daughter cells are often observed. The quick saline wet mount described previously for yeasts, molds, and fungi may also be used to identify cellular morphology.

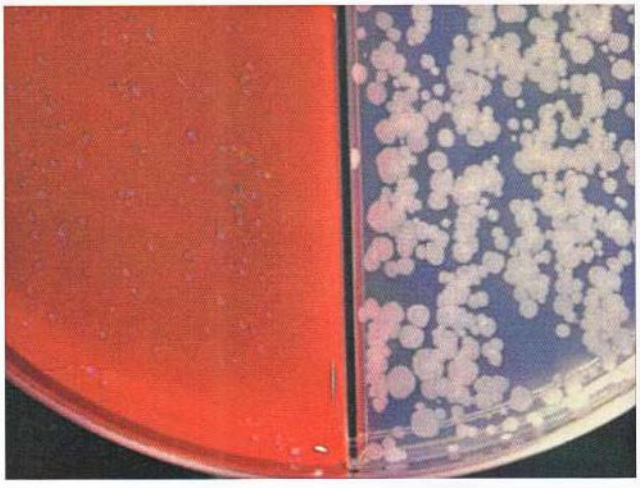


Figure 9.7a
Prototheca spp.culture at 48 hours on split plate; blood agar on left and Sabouraud Dextrose Agar on right, showing dry, granular surface of colony.

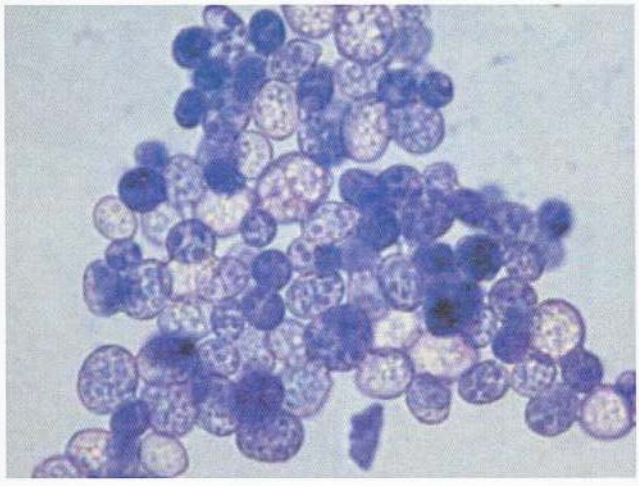


Figure 9.7b
Methylene blue stained smear of *Prototheca* spp. cells.

Corynebacterium bovis

Corynebacterium bovis primarily colonize on the teat canal and are generally thought to be mildly pathogenic. *Corynebacterium bovis* may cause intramammary infections, but they are usually only associated with a mild inflammatory response. Spread is from cow to cow at milking and this organism can be highly contagious. It is commonly believed that *C. bovis* may not truly be the cause of intramammary infections and mastitis, but rather their culture from milk is the result of streak canal colonizers being washed into the milk during sample collection. *Corynebacterium bovis* can be easily controlled by milking time hygiene techniques that include teat dipping. Dry cow therapy has been found to be effective in the elimination of this pathogen. Thus, herds with excellent control of contagious mastitis can effectively eliminate this disease agent and may report no or a very low incidence of *C. bovis* mastitis.

Figure 9.8
Proper post-milking teat disinfection will control the spread of *Corynebacterium bovis*

Laboratory identification procedures

Culture on growth media – Traditionally, *C. bovis* have been identified by presumptive identification from milk cultures on blood agar. They normally appear as white or off white, opaque, small, non-hemolytic, pinpoint colonies. Apparent growth generally requires 48 hours of incubation at 37°C. They have a high predilection for lipids and can be found associated with milk fat on culture. They can easily be differentiated from *Streptococcus* spp., as they are catalase positive.

Microscopic examination of culture growth – Short, pleomorphic, Gram positive, rod-shaped oval, or spherical.

Note: It has been suggested that presumptive identification is not very precise and that secondary phenotypic or genotypic tests should be used to ensure a greater likelihood of proper identification when precision is required. Other *Corynebacterium* spp. can be isolated from milk in some herds.

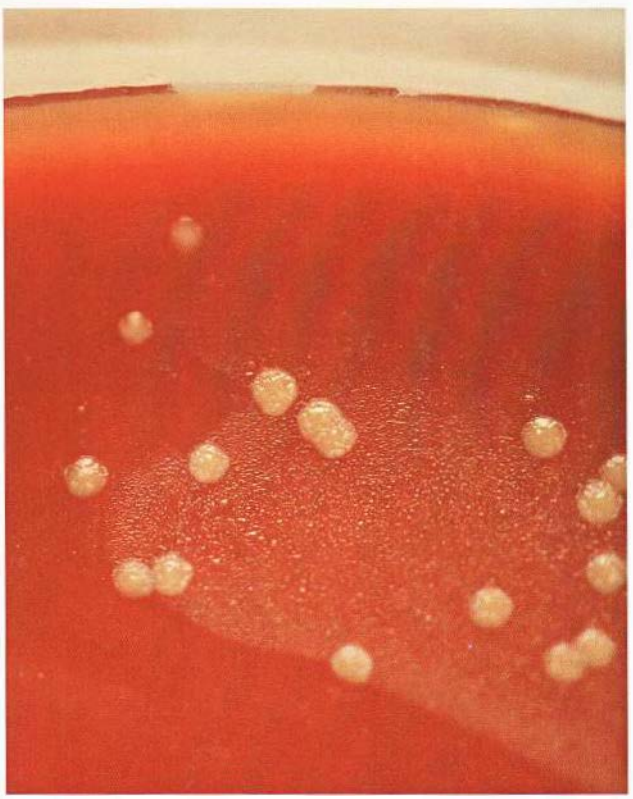


Figure 9.9
Corynebacterium bovis culture at 48 hours on blood agar.

Trueperella pyogenes

Trueperella pyogenes have been classified in several other bacterial genera over the years, including *Arcanobacterium pyogenes*, *Actinomyces pyogenes*, and *Corynebacterium pyogenes*. The reservoir for *T. pyogenes* can be the cow, as the organism is often found in wound infections and abscesses. It is not unusual for *T. pyogenes* intramammary infections to be simultaneously present with wounds and abscesses. Environmental sources have been suggested as reservoirs. Flies may be vectors for infection. *Trueperella pyogenes* contribute to "summer mastitis," a syndrome that occurs in northern Europe in pastured cattle. Several pathogens contribute to the etiology of summer mastitis and biting flies have been implicated in transmission of the disease. If detected early, antimicrobial therapy may be effective in eliminating intramammary infections caused by *T. pyogenes*; otherwise, the pathogen is very difficult to eliminate from the mammary gland.

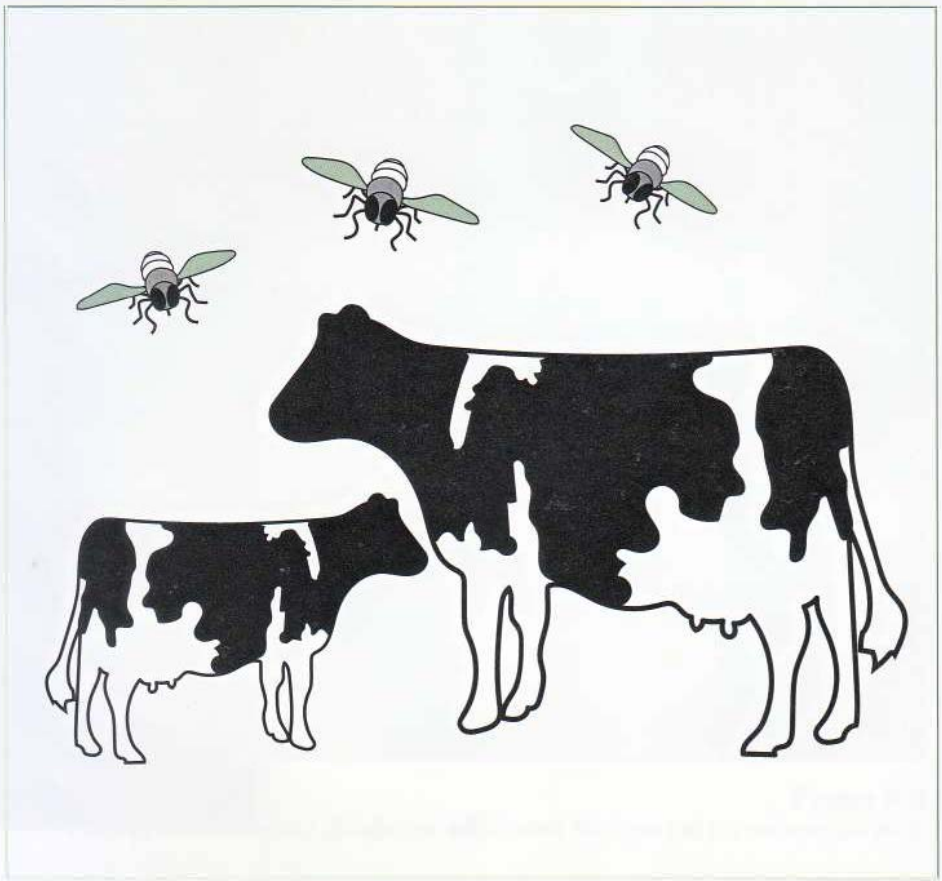


Figure 9.10
Control measures for *Trueperella pyogenes* include fly control and maintaining cows in a clean and dry calving area.

Laboratory identification procedures

Culture on growth media – *Trueperella pyogenes* grow well on blood agar at 37°C. Colonies resemble *C. bovis* on agar, but often are hemolytic, producing a translucent zone within 48 hours of incubation. They are catalase negative.

Microscopic examination of culture growth – *Trueperella pyogenes* appear as Gram positive, rod shaped, or coccobacilli.

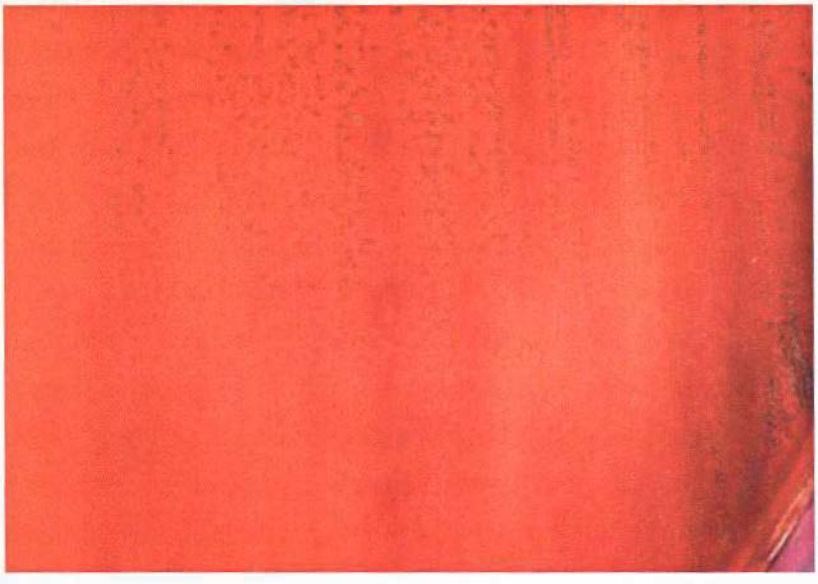


Figure 9.11
Trueperella pyogenes culture at 48 hours on blood agar, showing pinpoint colonies with typical hemolysis.

Mycobacterium spp.

Mycobacterium spp. have been associated with mastitis and may be a greater problem in developing, rather than developed, countries. *Mycobacterium tuberculosis* is generally a human pathogen and *M. bovis* is a pathogen of cattle. *Mycobacterium* spp. are zoonotic and thus extreme caution must be used during culture and identification. This is a reportable disease and hence suspected cases should be reported to the appropriate regulatory body. In the United States, this is usually the state veterinarian's office. The regulatory agency will then decide on the animal's and herd's disposition. The agent is resistant to many antibiotics. Airborne transmission among cattle, followed by internal organ transmission by lymph and blood fluids, is a likely course for establishing intramammary infections. Contaminated treatments and treatment devices have also been suggested as part of the transmission pathway for *M. bovis* mastitis.

Laboratory identification procedures

Culture on growth media – Mycobacteria isolated from intramammary infections are usually from the Runyon Group IV and grow in less than 1 week at ambient temperature (25°C) or at 37°C on blood agar. On blood agar, they appear as small off-white, raised, rough, or smooth colonies. Colonies can be confused with *Nocardia* spp. and coryneforms. However, the long incubation time can be used to differentiate between *Mycobacteria* spp. and other mastitis pathogens. Care must be taken to ensure sufficient culture incubation time has elapsed before ruling out mycobacterial mastitis.

Microscopic examination of culture growth – *Mycobacteria* spp. are acid-fast bacilli and can be fixed in a smear with Kinyoun acid-fast or the Ziehl-Neelsen stains. Gram staining of *Mycobacteria* spp. will often fail.

Caution! – Cultures are potentially very dangerous to humans and thus care in culturing and handling of isolates must be maintained. Cultures should be handled in a biological safety hood and plates and tubes containing cultures should be kept tightly and firmly closed.

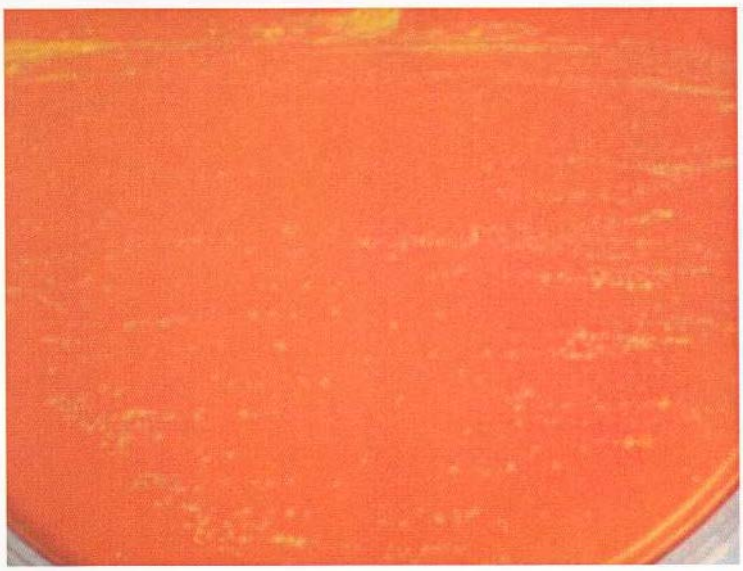


Figure 9.12
Mycobacterium spp. on blood agar after 5 days incubation at room temperature. Colony characteristics vary among species.

Bacillus spp. and Other Gram-positive Bacilli

Bacillus spp. are often found as contaminants on milk cultures and may indicate poor teat end preparation prior to sample collection. *Bacillus cereus* has been reported to cause mastitis. Its common occurrence in the soil, water, dust, air, feces, vegetation, feedstuffs, wounds, and abscesses suggest the reservoir for infection is the environment. It is likely to account for <1% of all mastitis cases. Thus, exposure of the cow to significant loads of this pathogen in the environment may be the greatest risk factor for its occurrence in mastitis. Contaminated treatments and treatment devices have also been suggested as part of the transmission pathway. *Bacillus cereus* has also been reported several times to cause acute and fatal gangrenous mastitis.

Other species of *Bacillus* as mastitis agents are rare, but *Bacillus anthracis* has been reported as being isolated from a cow with mastitis in a herd experiencing an outbreak of anthrax. And, *B. subtilis* has been reported to cause bovine mastitis.

Laboratory identification procedures

Culture on growth media – On blood agar, *Bacillus cereus* colonies are large, slightly gray with irregular edges, and often surrounded by a clear zone of hemolysis after 24 to 48 hours at 37°C. *Bacillus subtilis* colonies are greenish-gray with a ground glass appearance.

Catalase – Positive.

Microscopic examination of culture growth – *Bacillus* spp. are Gram positive, rod shaped, and tend to form chains.

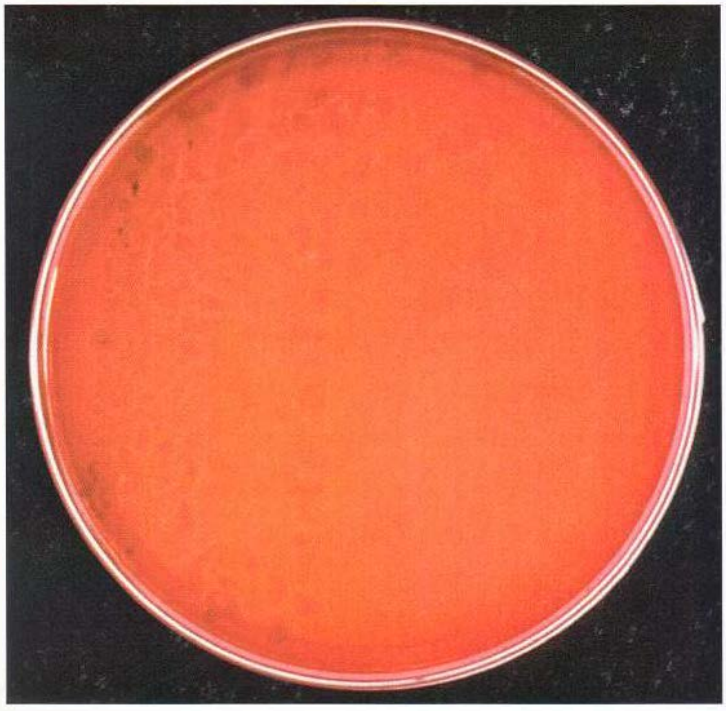


Figure 9.13
Bacillus spp. on blood agar.

Chapter 10
Somatic Cell Count

This section covers screening tests for detecting and enumerating somatic cells (SCC) in milk. Milk for SCC can be collected from individual quarters, a composite of all quarters (cow level), or the bulk tank. Interpretation of test results and the relationship of SCC to milk production and resultant financial loss are discussed.

Diagnostic thresholds

Milk SCC are convenient and useful indicators of udder health and milk quality. Somatic cells are predominantly white blood cells (leukocytes) in milk, but small percentages of epithelial cells are sometimes found. Somatic cell counts >200,000 cells/ml generally indicate infection by one or more microorganisms. The range of SCC in normal, uninfected udders represents a continuum that overlaps with that of infected udders. Thus, it is not possible to set a specific SCC that absolutely predicts the presence or absence of infection. However, approximately 50% of uninfected quarters have SCC <100,000 cells/ml. The probability that an infection is present also increases as SCC increases. Setting a threshold of 200,000 cells/ml has indicated the presence of an intramammary infection with a sensitivity and specificity of 73% and 86%, respectively, and is routinely used as a diagnostic threshold indicative of intramammary infection. Factors such as stage of lactation or parity generally do not result in significant increases in SCC >200,000 cells/ml when the gland is not infected. Of note, when collecting samples from individual mammary quarters, the first few streams or strippings of milk have higher cell concentrations and should be discarded.

Bulk tank SCC

Monitoring udder health by SCC in bulk tank milk is a useful tool for monitoring subclinical mastitis, with bulk tank SCC (BTSCC) being impacted by the number quarters/cows infected, the intensity of the inflammatory response, and the volume of milk contributed by each cow. As the bulk tank SCC increases, the likelihood of isolation of contagious pathogens, such as *S. aureus* and *S. agalactiae*, increases. Episodic increases in bulk tank SCC may be indicative of an environmental mastitis problem. Furthermore, as BTSCC increases and the prevalence of infection increases, losses in production also increase (Table 10.1). Categories of low (<200,000 cells/ml), medium (200,000 to 400,000 cell/ml), and high (>400,000 cells/ml) can be used to help categorize herds. Within herds, BTSCC that fall outside a 95% confidence interval surrounding the mean can indicate a problem within the herd. When interpreting BTSCC, be aware that extremely high SCC milk from only a small percentage of the total cows in the herd may dramatically elevate BTSCC. Such effects are more pronounced in smaller herds.

Herds that have controlled contagious pathogens and have bulk tank SCC <300,000 cells/ml may experience more frequent and severe clinical mastitis and losses due to environmental pathogens, such as *E. coli* and *Klebsiella* spp., without elevating the bulk tank SCC for extended periods of time. This lower BTSCC may occur because the overall prevalence of environmental pathogen infections are low in the herd, infections are short in duration, and/or infections are associated predominantly with the dry period and calving. Occasional clinical mastitis outbreaks in such herds may cause an elevation in BTSCC. However, milk from cows with clinical mastitis should not be entering the bulk tank.

Table 10.1

Estimated infection prevalence and losses in milk production associated with elevated BTSCC

BTSCC/ml	Infected Quarters in Herd (%)	Production Loss (%)*
200,000	6	0
500,000	16	6
1,000,000	32	18
1,500,000	48	29

*Production loss calculated as a percent of production expected at 200,000 cells/ml.

California Mastitis Test

Uses and limitations

The California Mastitis Test (CMT) is an inexpensive, easy-to-use, cowside screening test that estimates SCC of milk but does not provide an exact SCC (Table 10.2). The CMT reactions are related *broadly* to the number of somatic cells in milk and positive reactions indicate mastitis. Dairy producers can use this to monitor subclinical mastitis in their herd. The producer should keep a running record of the results and develop a history on each cow for maximum benefit. Although the procedure calls for visual determinations at five levels, scores of negative, suspicious, or positive may be adequate to assist the average dairy producer in monitoring udder health.

Sample storage

The CMT must always be performed on fresh milk (not frozen or preserved). The CMT should be performed before milking or from an inline sampler or weigh jar, as results can be less accurate on milk samples collected at the end of milking.

Procedures

Procedures can be obtained from package inserts supplied with the commercially available CMT reagent solution.

Table 10.2

California Mastitis Test scores and estimates of somatic cell levels that may be associated with each

CMT Score	Relative Range of Somatic Cell Level	Interpretation
Negative	<200,000 cells/ml	Healthy quarter
Trace	150,000 to 500,000 cells/ml	Suspicious
1	400,000 to 1,500,000 cells/ml	Mastitis positive
2	800,000 to 5,000,000 cells/ml	Mastitis positive
3	Generally, >5,000,000 cells/ml	Mastitis positive

Wisconsin Mastitis Test

Uses and limitations

The Wisconsin Mastitis Test (WMT) is a semi-quantitative laboratory adaptation of the CMT and is used to estimate SCC of fresh milk samples (Table 10.3). The WMT is also considered a screening test that results in an estimated SCC but will not give a precise count. The procedure is conducted using calibrated tubes and caps, accurate measurement of milk and reagent volumes, and specific timing of reaction. The reaction of the reagent and cells in the milk results in a viscous mixture; the viscosity is positively correlated to cell content. Tubes must be inverted for exactly 15 seconds; and the greater the cell content, the greater the volume of mixture left in the tube. The fluid column in the tube is measured in millimeters (mm) giving an objective, rather than subjective, test result. The reading is then compared with a chart that converts millimeters to approximate SCC. Numerous conversion tables have been published over the years. For greatest confidence, each laboratory running the WMT should develop its own conversion table by comparing WMT millimeter readings with the known SCC of the samples determined by electronic somatic cell counters or by direct microscopic SCC.

Sample storage

Fresh samples <36 hours old and stored at 0 to 4°C are required for the test. Frozen samples cannot be used.

Procedures

Procedures can be obtained from package inserts supplied with the commercially available reagent solution or found in the book "Standard Methods for the Examination of Dairy Products" (2004), published by the American Public Health Association, Washington, DC.

Table 10.3

Examples of the relationship between the WMT reading and SCC

WMT Score (mm)	Approximate Somatic Cell Counts (cells/ml)
0 to 5	<200,000
6 to 12	200,000 to 500,000
13 to 21	500,000 to 1,000,000
22 or greater	>1,000,000

Direct Microscopic Somatic Cell Count

Uses and limitations

The Direct Microscopic Somatic Cell Count (DMSCC) is a labor-intensive method of arriving at SCC by performing a microscopic count of stained cells on a slide. A precisely measured volume of milk is spread within a calibrated area of a microscope slide, the milk film is dried, stained, and the visible (stained) cells are counted within a defined area of the film. The resultant count is multiplied by a conversion factor, which is determined by the degree of magnification used and the area counted to arrive at a cell count per millimeter of milk. The procedure requires a high quality microscope and thorough training of personnel to gain proficiency.

Sample storage

Use fresh or refrigerated samples no more than 2 days old. Frozen samples cannot be used.

Procedures

Procedures may be found in the book "Standard Methods for the Examination of Dairy Products" (2004), published by the American Public Health Association, Washington, DC.

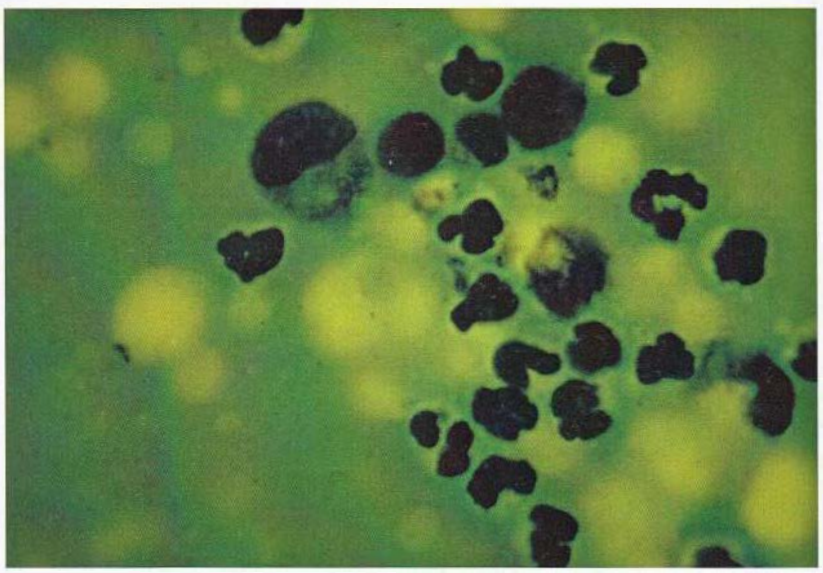


Figure 10.1
Leukocytes stained for DMSCC.

Electronic Somatic Cell Count

Uses

Automated electronic cell counters (Foss, North America, Inc., Eden Prairie, MN, or Somacount, Bentley Instruments, Inc., Chaska, MN) and computerized records make possible periodic, usually monthly, SCC reports on all milking cows in the herd by the Dairy Herd Information Association (DHIA) or similar organizations. Portable counters, such as the DeLaval Direct Cell Counter, also provide an accurate system that can be used to test SCC in a lab or on farm. In addition, differential cell counts can now be performed on the farm using a unit distributed by Advanced Animal Diagnostics (Raleigh, NC).

Bulk tank SCC are also provided on a regular basis by the organization purchasing milk from the dairy farmer. The performance of an electronic SCC has the distinct advantages of speed and repeatability, compared with DMSCC. The study of SCC records is helpful in monitoring progress and revealing deficiencies in mastitis control.

Somatic Cell Score

DHIA has adopted an SCC scoring system that divides the SCC of composite milk into 10 categories from zero to nine, known as somatic cell score (SCS). DHIA programs can determine the SCC of each lactating cow monthly and report the SCC and/or the SCS. The formula for calculating SCS = log2 (SCC/100) + 3 and is outlined in Table 10.4. The SCS provides a linear relationship between SCC and milk yield. The relationship between successive increases in SCS and milk yield are illustrated in Table 10.5.

Table 10.4

Calculation of milk somatic cell score using formula SCS = log2 (SCC/100) + 3

Somatic Cell Score (SCS)	Example: SCC = 200 (x 10^3)
1. Divide the reported SCC by 100*.	200/100 = 2
2. Take the log2 of the result.	log2 (2) = 1
3. Add "3" to the result.	1 + 3 = 4 SCS

*If the SCC is expressed as cells per milliliter with the thousands place not truncated (200,000 instead of 200 as an example), divide by 100,000 and not 100: (200,000/100,000) = 2.

Milk loss

The inflammation leading to the rise in SCC also lowers milk production. This response is evident with a lactation SCC average of 100,000 cells/ml and becomes more pronounced with each doubling of the SCC (Table 10.5). In the table, the average SCC for the lactation most accurately reflects reduced milk yields. Production losses in older cows are about double those of first lactation cows. Table 10.6 demonstrates the relationship among the different methods of measuring SCC and predicted milk loss.

An alternative method of calculating milk loss is to assign a 1.5 lb loss per day for every SCS unit a cow is above an SCS of 2. For example, if SCS increases from 2 to 5, multiply 3 units x 1.5 lb = 4.5 lb loss per day. This number times the price of milk gives an estimate of the dollar value of milk lost per day. Determining the exact amount of milk lost at a specific SCC or SCS is not possible, but the relationships are real and these estimates can be a valuable tool for encouraging producers to improve mastitis control and for evaluating the response to improvements made.

Table 10.5

Estimated differences in lactation milk yield associated with each increase in the SCS

Lactation Average SCC Score (SCS)	Lactation Average SCC (x 1,000/ml)	Difference in Milk Yield Lactation 1 (lb/305 days)	Difference in Milk Yield Lactation 2+ (lb/305 days)
0	12.5	0	0
1	25	0	0
2	50	0	0
3	100	-200	-400
4	200	-400	-800
5	400	-600	-1,200
6	800	-800	-1,600
7	1,600	-1,000	-2,000

Table 10.6

Somatic cell counts as they relate to screening tests and estimated milk losses

CMT Score	WMT (mm)	Somatic Cell Score (SCS)	Somatic Cell Count (cells/ml)	Milk Loss (%)	Estimated Milk Production Loss per Cow/Year (lb)
Negative	2	3.0	100,000	3	400
	5	4.0	200,000	6	800
Trace	8	4.6	300,000	7	1,000
	10	5.0	400,000	8	1,200
	12	5.3	500,000	9	1,300
1	14	5.6	600,000	10	1,400
	16	5.8	700,000		1,500
	18	6.0	800,000	11	1,600
	20	6.2	900,000		1,650
	22	6.3	1,000,000	12	1,700
2	25	6.6	1,200,000	>12	>1,700

Chapter 11
Bulk Tank Cultures

Procedures for bulk tank milk (BTM) culturing are based upon limited scientific data; however, BTM culturing can provide two important types of information: 1) presence or absence of mastitis pathogens, and 2) quantitation of predominant bacterial groups pertaining to raw milk quality, including psychrotrophic (bacteria able to grow at cold temperatures) and thermoduric (bacteria able to survive pasteurization) bacteria.

Sampling Interval

The more often BTM is sampled, the more useful the information. Samples taken over consecutive days or weeks are most useful. Extreme caution should be taken when interpreting results from a single BTM sample.

Interpreting Bulk Tank Milk Cultures

Mastitis pathogens

Bulk tank milk analysis can be useful in determining if mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, or *Mycoplasma* spp. are present in the herd. Presence of these pathogens in BTM almost always indicates the presence of infected quarters in the herd. However, negative culture results do not necessarily mean that the herd is negative for infections caused by these pathogens.

Raw milk quality

Bulk tank analysis may also be used to determine raw milk quality based on quantitation of certain bacterial groups. Enzymes, such as lipases and proteases, secreted by bacterial species in raw milk can break down milk fats and proteins, respectively, resulting in reduced milk quality and possibly reduced shelf life. Theoretically, any bacterial isolate from BTM could arise from an intramammary infection, but elevated counts of certain bacterial groups may also result from milking wet udders, organic soil in milk lines, cracked inflations, inadequately heated wash water, and inadequate cooling of milk. Standard plate count (SPC) provides semi-quantitative measure of the total bacterial load in BTM. Laboratory pasteurization count (LPC) measures the number of thermoduric bacteria, species able to survive pasteurization, in BTM. Preliminary incubation count (PIC) measures the number of psychrotrophic bacteria, bacteria able to grow at cold temperatures. When carefully interpreted, data on predominant

bacterial groups obtained from BTM culturing can be used to indicate components of the milking process that may need attention in order to improve raw milk quality.

Caution! – Bulk tank milk cultures are not useful as indicators of mastitis prevalence in a herd. Bulk tank milk cultures can be valuable supplements to quarter- or cow-level milk samples, but never a substitute for determining infection incidence and prevalence based on quarter- or cow-level milk samples. Interpretive criteria pertaining to raw milk quality suggested here are based on data collected throughout Pennsylvania only and are simply meant to provide helpful guidelines for bulk tank monitoring and troubleshooting. Ideal counts given in this publication are not meant to be substituted for government standards.

Bulk Tank Milk Culture Procedures

No industry standard exists for BTM culturing. The procedure outlined below results in a semi-quantitative measure of common mastitis pathogens and bacterial groups pertaining to raw milk quality found in BTM.

1) Materials
   - Latex gloves
   - Sterile dipper
   - Sterile 50-ml tube or whirl-pack bags
   - 2 x sterile 15-ml tubes
   - Sterile spreaders or loops
   - Pipettor with 50-µl capacity and compatible sterile tips
   - 68.2°C water bath, 12°C refrigerator, 32°C incubator
   - Sterile phosphate buffered saline (PBS)
   - Media:
     - Plate count agar (PCA)
     - MacConkey's agar (MAC)
     - Edwards Modified agar supplemented with colistin sulfate and oxolinic acid (EMCO)
     - Baird-Parker agar (BPA)
     - Mycoplasma agar (MA)

2) BTM sample collection (Figure 11.1)

- Agitate milk in the bulk tank for at least 10 minutes prior to collection.
- Wear gloves while collecting BTM to prevent contamination of the sample.
- Always collect the sample from the top of the bulk tank (never the release valve), using a clean, sanitized dipper.
- Approximately 25 to 50 ml should be collected into a sterile tube or whirl-pack.
- Place samples on ice immediately or refrigerate until they can be analyzed. Samples must be stored cold (<5°C) but not frozen.

3) BTM sample analysis (Figure 11.2)

- Each BTM sample is plated on PCA, MAC, EMCO, BPA, and MA, using the spread to plate method:
  - Dispense 50 µl of milk onto the center of an agar plate.
  - Use a sterile spreader or loop to evenly spread the milk over the surface of the plate, avoiding the outer 1/4 inch of the plate. Continue to spread until the milk has been completely absorbed into the agar, taking care not to gouge the agar.
- Transfer 5 ml of BTM to each of 2 tubes labeled LPC and PIC.
  - Incubate the LPC tube for 30 min in a 62.8°C water bath.
    - Inoculate a PCA plate with 50 µl of milk from the LPC tube, using the spread to plate technique.

Figure 11.1a
Proper collection of a bulk tank milk sample.

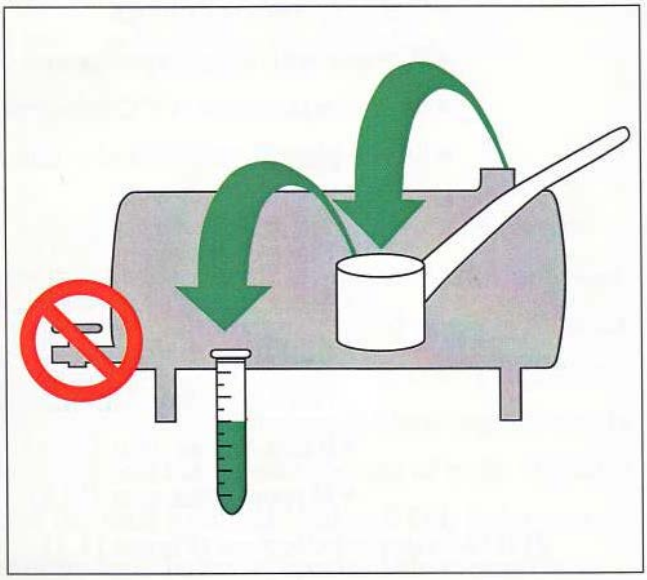


Figure 11.1b
Never obtain samples from a bulk tank's milk release valve.

- Incubate the PIC tube for 18 hours in a 12°C refrigerator.
    - Make two serial dilutions of the milk from the PIC tube.
        - 1:10 dilution: Transfer 1 ml milk from PIC tube to 9 ml sterile PBS, mix thoroughly.
        - 1:100 dilution: Transfer 1 ml of the 1:10 PIC dilution to 9 ml sterile PBS, mix thoroughly.
            - Inoculate three PCA plates using the spread to plate technique, inoculate one plate for each of the following:
                - 50 µl undiluted PIC milk
                - 50 µl 1:10 dilution
                - 50 µl 1:100 dilution
- Incubate all plates, except MA (see Chapter 8 for proper incubation conditions), at 32°C for 48 hours.

4) Recording culture results

- Count the colonies on respective agar plates as described below:
    - SPC: all colonies
    - PIC: all colonies
    - LPC: all colonies
    - MAC:
        - Non-coliforms (NC): clear/yellow colonies
        - Coliforms (CC): dark pink/purple colonies
    - BPA:
        - Coagulase-negative staphylococci (CNS): black colonies, no zone
        - *Staphylococcus aureus* (SA): black colonies with an opaque white zone around them
    - EMCO:
        - Streptococci (SS): all non-*Streptococcus agalactiae* colonies (see below)
        - *Streptococcus agalactiae* (SAG): small white colonies with a pearly blue hue and pinpoint beta-hemolysis
    - MA: count colonies, refer to Chapter 8 for identifying colonies
- Convert all colony counts to colony-forming units per ml (CFU/ml) and record in data sheet:

    Colony count x 20 = CFU/ml

    For PIC dilutions:

    Colony count x 20 x dilution factor = CFU/ml

Note: Not all PIC plates may be countable. If a plate has too many colonies to count, or no colonies, do not include data from these plates in the final results. Record the mean CFU/ml value of the countable plate(s) as the PIC in the data sheet.

If analyzing more than one sample from the same bulk tank, use the mean value for each bacterial count when interpreting results (Table 11.1).

Table 11.1

Suggested interpretive criteria for bulk tank milk culture results.

Bacterial Count	Ideal Counts (CFU/ml)	Interpretive Criteria
SPC	<5,000	IF >5,000 CFU/ml, milking system cleaning needs attention
PIC	<10,000	IF >10,000 CFU/ml, milking system cleaning and udder hygiene need attention
LPC	<100	IF >100 CFU/ml, milking system cleaning needs attention
CC	<50	IF >50 CFU/ml, check teat and teat end cleanliness, and check milking system cleaning
NC	<200	IF >200 CFU/ml, check teat and teat end cleanliness, and check milking system cleaning
SA	Not detected	IF detected in 2 out of 4 bulk tank samples, this suggests cows with *Staph. aureus* infection
SAG	Not detected	IF detected in 2 out of 4 bulk tank samples, suggests cows with *Strep. ag* infection
CNS	<500	IF >500 CFU/ml, check teat end cleanliness, teat dipping, bedding, and cows with mastitis
SS	<500	IF >500 CFU/ml, check teat end cleanliness, teat dipping, bedding, and cows with mastitis
MM	Not detected	IF detected in bulk tank sample, this suggests cows with mycoplasma infection

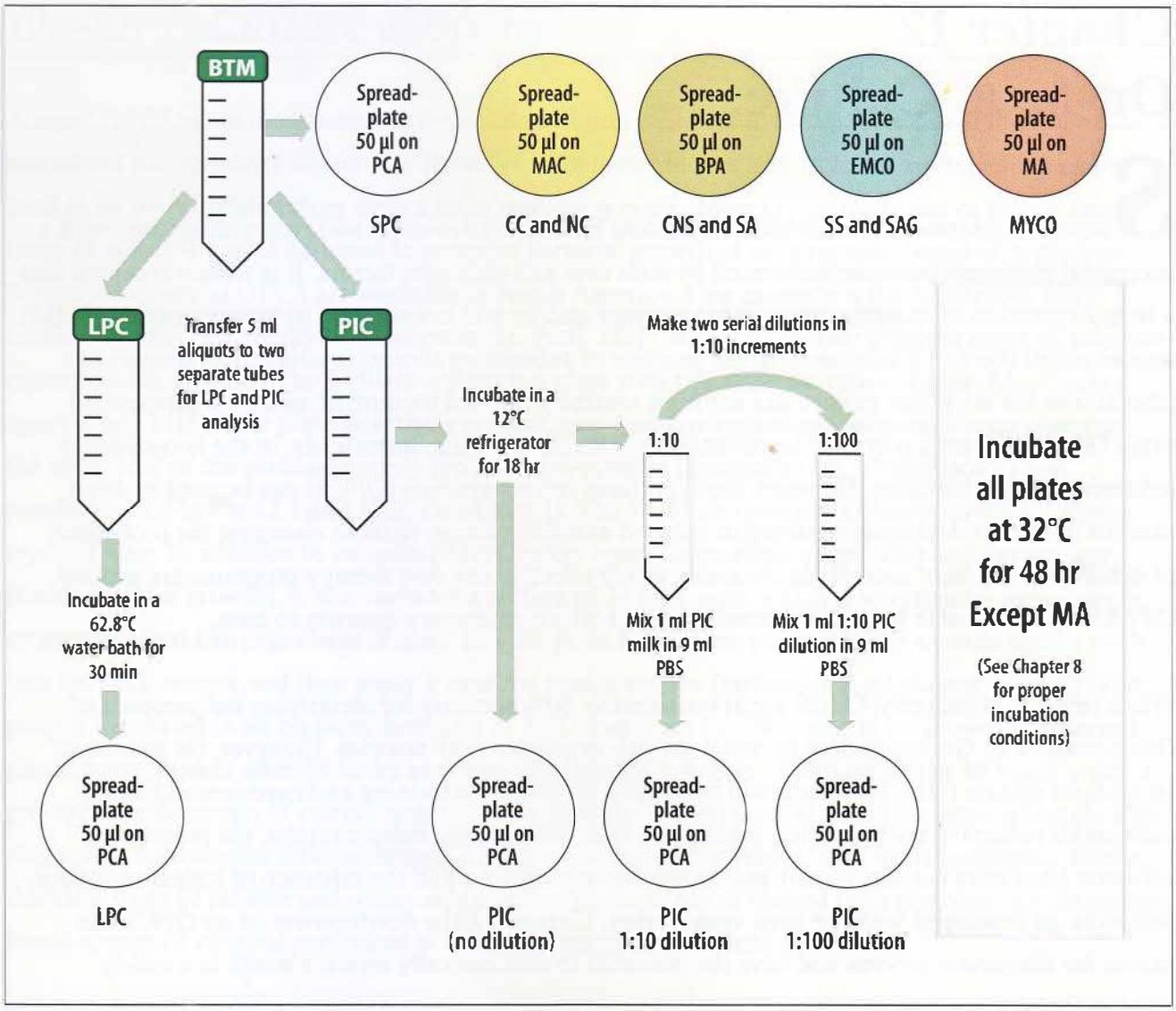


Figure 11.2
Schematic of bulk tank milk sample analysis protocol.

Chapter 12
On-farm Culture

Successful treatment of mastitis is dependent upon early detection and proper diagnosis, with a successful treatment outcome influenced by both cow and pathogen factors. It is widely accepted that a large proportion of mastitis cases do not warrant and/or will not respond to intramammary (IMM) antimicrobial therapy. Unfortunately, the practice of submitting clinical mastitis milk samples to laboratories for microbial culture has not been routinely utilized because of extended turnaround times (36 to 48 hours), cost, and inconvenience. This has resulted, historically, in the non-selective treatment of mastitis cases. However, rapid on-farm culture systems (OFCS) can be used to direct mastitis treatment decisions, resulting in reduced antibiotic usage, without changing the probability of achieving treatment success. In instances where selective dry cow therapy programs are utilized, OFCS may be valuable in making decisions about which mammary quarters to treat.

When performed properly, OFCS are approximately 80% accurate for identifying the presence of Gram-positive or Gram-negative bacterial growth in quarter milk samples. However, the success of an on-farm culture (OFC) program will be depend on adequate training and experience of those individuals collecting milk samples, plating the milk, interpreting culture results, the presence of adequate laboratory conditions and quality assurance programs, and the existence of logical treatment protocols, as developed with the herd veterinarian. Ultimately, the development of an OFCS can hasten the diagnostic process and have the potential to economically return a result in a timely manner to dairy managers.

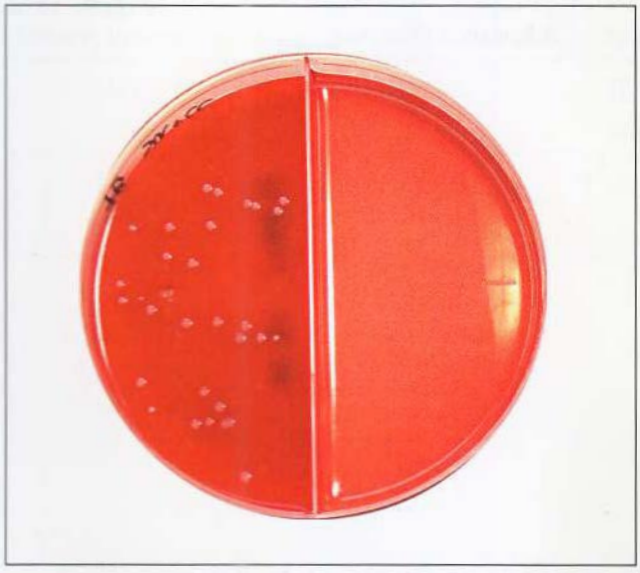


Figure 12.1
Growth on Factor media of Bi-plate indicates Grampositive bacteria (Minnesota Easy Culture System, 2013).

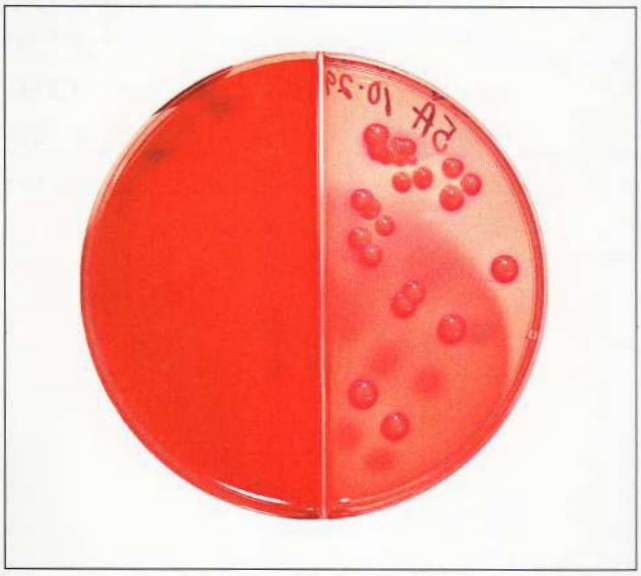


Figure 12.2
Growth on MacConkey media of Bi-plate indicates Gram-negative bacteria (Minnesota Easy Culture System, 2013).

On-farm Culture Systems

A rapid OFCS is not a replacement for nor does it provide all of the diagnostic capabilities of an accredited microbiology laboratory. However, simple techniques and tools are available that can be used in an on-farm laboratory or in a local veterinary clinic. Most OFCS make use of one or more types of selective media designed to promote bacterial growth of only certain classes of organisms. Several commercial OFCS are available in North America. One example is the Minnesota Easy Culture System (University of Minnesota. St. Paul, MN), which offers two different types of selective culture media systems. The Bi-Plate system is a plate with two different types of agar, MacConkey agar on one-half of the plate selectively grows Gram-negative organisms, whereas Factor agar on the other half of the plate selectively grows Gram-positive organisms (e.g., staphylococci and streptococci) (Figures 12.1 and 12.2; Flowchart 1). The Tri-Plate system is a plate with three different types of agar. In addition to including MacConkey agar (Gram-negative growth) and Factor agar (Gram-positive growth), it also includes a section of MTKT agar, which is selective for streptococci or streptococcal-like organisms (Figure 12.3-12.4). In both cases, producers dip a sterile cotton swab into the milk sample and then apply it over the media surface (estimate 0.1 ml plating volume). The plate is incubated in an on-farm incubator at 37°C and is read at 24 hours. If no growth is observed, plates are rechecked after 48 hours and then discarded (Flowchart 2). No growth at 48 hours yields a presumptive diagnosis of culture negative. The Bi-plate and Tri-plate system have approximately 80% diagnostic accuracy for differentiating Gram-positive and Gram-negative mastitis pathogens. Hence, this OFCS can be reliable and reasonably accurate in the hands of trained farm personnel for diagnosing broad groups of mastitis pathogens to facilitate treatment decisions.

Figure 12.3
Growth on only the Factor media of the Tri-plate indicates a Gram-positive organism and likely *Staphylococcus* spp. The zone of hemolysis likely indicates *S. aureus* (Minnesota Easy Culture System, 2013).

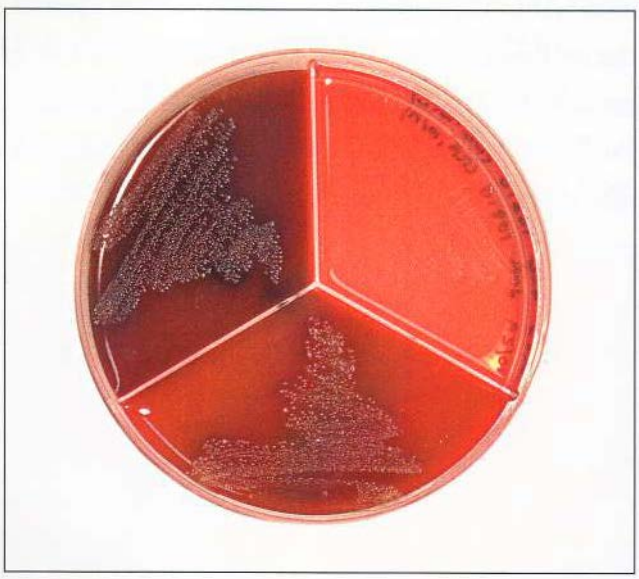


Figure 12.4
Growth on both the Factor media and MTKT media of the Tri-plate indicates Gram-positive organisms, likely *Streptococcus* spp. or streptococcal-like organisms (Minnesota Easy Culture System, 2013).

The Petrifilm™ system (3M Microbiology, St. Paul, MN) has also been used for on-farm diagnosis of mastitis pathogens. The Petrifilm™ system offers a variety of selective plates, including an Aerobic count plate, Coliform count plate, and the Staph Express count plate (Figures 12.5 and 12.7). This system is very similar to the University of Minnesota agar plate system in terms of using selective and differential media. The Petrifilm™ Staph Express count plate contains chromogenic, modified Baird-Parker medium, which is selective and differential for *Staphylococcus* spp. Milk is diluted 1:10 with sterile water, a 1-mL aliquot is plated onto the Petrifilm™ plate, and then plates are incubated at 35°C for 24 hours. If growth is detected on the Staph Express count plate, then confirmation of *S. aureus* can be performed by applying a disk (STX disk) and incubating an additional 3 hours. The STX disk contains deoxyribonuclease and a dye that reacts to produce a distinct pink zone around *S. aureus* colonies. Thus, the Petrifilm™ offers the ability to better distinguish *S. aureus* from other staphylococci, but at the cost of additional time, technique, and labor. The need to dilute the milk is an extra step that requires aseptic technique and could increase the error rate. Researchers at the University of Minnesota concluded that the Petrifilm™ System was not as accurate as the Bi- and Tri-plate agar system. Variability in results from farm to farm and often the fact that the milk had to be diluted 1:10 in sterile water before plating on the Petrifilm™ System added to the difficulties of implementing the Petrifilm™ system on farms with the same degree of success achieved with the Minnesota Easy Culture System.

Both systems (Petrifilm™ and Tri-plate) are reasonably effective in differentiating between Gram-positive and Gram-negative organisms, and in distinguishing streptococci from staphylococci. Both systems can be used to identify *S. aureus*, although the Petrifilm™ system with the addition of the STX disk may be more accurate. In general, neither system is designed to identify pathogens to the species level. Another shortcoming is that neither system can effectively recognize pathogens that are outside the common mastitis pathogens discussed. For example, a sample of milk from a quarter with an intramammary infection caused by *Mycoplasma* spp. would yield a false-negative result. Alternatively, *Prototheca* spp. would grow on all agars included in the Bi- or Tri-plates, and thus might easily be misclassified or considered a contaminant.

Figure 12.5
Bacterial growth on Aerobic Count Plate (Petrifilm System).

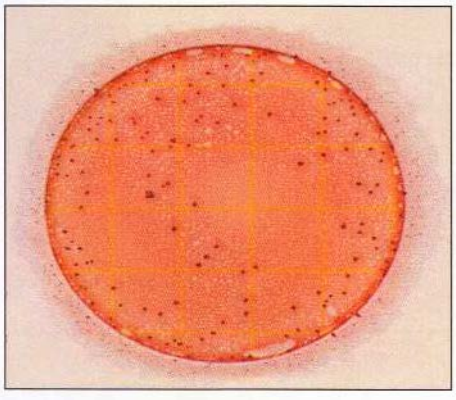


Figure 12.6
Growth of Coliform bacteria on Coliform Count Plate (Petrifilm System).

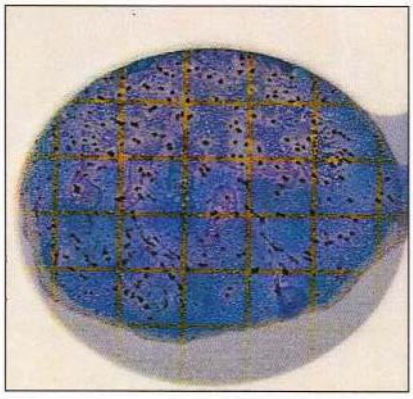


Figure 12.7
Bacterial growth on Rapid Staph aureus Count Plate (Petrifilm System).

Use of the On-farm Culture System

Selective lactating cow therapy

The major utility of OFCS, even as a primary level of diagnosis, is to target those cows/infections that would more likely respond to antibiotic treatment. Most commercial intramammary treatment products labeled for use in lactating cattle in the United States have little to no efficacy against the Gram-negative pathogens, with the possible exception of ceftiofur (Spectramast LC®, Zoetis). Hence, lactating cow therapy for clinical mastitis is generally aimed at and most effective against Gram-positive (*Staphylococcus* spp. and *Streptococcus* spp.) intramammary infections. Producers can use OFCS to decide which mammary quarters are likely to respond to antibiotic treatment. Selective lactating cow therapy has significant advantages. This program will save on the cost of antibiotic, cost of labor and management involved in treatment, decrease the amount of discarded milk, and potentially reduce the risk of an antibiotic residue violation. The major negative factor of OFCS may be the time needed to determine the outcome of the milk culture, which is generally 24 hours. Culture time can delay treatment, which may reduce the success of therapy of sensitive pathogens or may prolong the cow's duration of infection. However, the consensus is that OFCS program positives outweigh the negatives, with respect to targeted therapy. Also, recent data suggest that a 24-hour delay in treatment does not appreciably impact overall outcomes in udder health.

Selective dry cow therapy

Globally, there is interest in moving away from blanket dry cow therapy approaches, where all mammary quarters of all cows receive intramammary treatment just before dry-off, to selective programs that only treat subclinically infected mammary quarters. Diagnosis of an infection can be done indirectly by monitoring signs of inflammation (e.g., milk somatic cell count) or directly by culture. The advantage of using OFCS prior to dry-off would be to identify the specific pathogen groups (Gram-positive versus Gram-negative) or genera to make informed treatment decisions in consultation with the herd veterinarian. Mammary quarters yielding no growth may be protected from new intramammary infections during the dry period by use of an internal or external teat sealant.

On-farm Culture Laboratory

The quality of results, and therefore the success of OFCS, will rely on several factors, including the application of correct techniques for milk sample collection, handling and storage to maintain high quality samples for culture, proper plating methods, proper incubation conditions (i.e., temperature, humidity, and time), and experience of the reader interpreting the results. In a study of the accuracy of the Petrifilm™ Staph Express Count system, variation was noted in the accuracy of results among different readers and emphasized the importance of adequate training. A more detailed description on collecting high quality milk samples, setting up and operating an on-farm culture laboratory, and interpreting on-farm culture results are provided in the Minnesota Easy Culture System User's Guide (2013). However, a summary of these requirements is provided below.

Laboratory space

Producers will need a clean, heated, designated space, free of barn traffic (manure, dust, etc.) to set up an OFC laboratory. The lab area should be insulated against large fluctuations in room temperature and free of drafts. The workspace countertop must be clear, free of food or drink, and easily disinfected. The lab worker(s) should always wear clean disposable gloves when working with mastitic milk samples or culture plates.

Incubator setup and operation

Laboratory incubators are available from a variety of laboratory supply sources, with many options (size and price) to choose from. Even low-cost egg incubators can be made to work on smaller dairies to incubate a small number of plates. The on-farm incubator must maintain a temperature of 37°C (98.6°F). It should have a thermometer that is regularly checked, plus a dish of water inside to maintain constant humidity. Temperature and water level in the incubator should be checked daily, as variation can result in erroneous culture results, including "no bacterial growth." Because OFC media have a limited shelf life (e.g., approximately 1 month for the Minnesota Easy Culture System), it will be important that the lab manager ensures that fresh media is always available for use. Stored media should be refrigerated, not frozen.

Milk sample collection and handling

Supplies needed to collect milk samples or complete lab work include disposable gloves, single-use milk sample vials, cotton balls or gauze squares soaked in 70% alcohol, a cooler with ice or freezer packs, racks for holding sample vials, germicidal teat disinfectant for cleaning teats prior to sampling, and paper or cloth towels. Farm staff must be trained in aseptic milk sample collection techniques, as described elsewhere in this handbook.

Sample preparation and plating procedures

If the sample cannot be plated within 1 hour of collection, refrigerate or freeze it until later. If a sample is frozen, allow it to thaw completely in the refrigerator before plating. Mix the sample well by gently inverting the vial several times before plating. Plating procedures may differ among different OFCS. For example, the Petrifilm™ system may require dilution of milk prior to plating. As such, users must refer to manufacturer guidelines for the specific OFCS in place on the farm. The following is an example for the Minnesota Easy Culture system. After washing hands and putting on new disposable gloves, turn the culture plate upside down and label the bottom of the plate (cow ID, quarter, date). Use a new sterile disposable cotton swab, place the swab into the milk sample for approximately 10 seconds or until it is saturated with milk (Figure 12.8). Streak the milk over the entire surface of the culture media. Re-dip the swab in the milk sample between each different section of the culture plate. Once finished swabbing, immediately place the lid back onto the media plate and reseal the lid on the milk sample. Freeze the milk sample immediately in case it is needed for confirmatory testing at a later date. Place the plate in the incubator upside down (i.e., place the plate inverted lid-side down) so that any condensation on the lid will not drip onto the plated sample. Once plating is completed, dispose of any garbage (e.g., used swabs) and clean and disinfect the workspace.

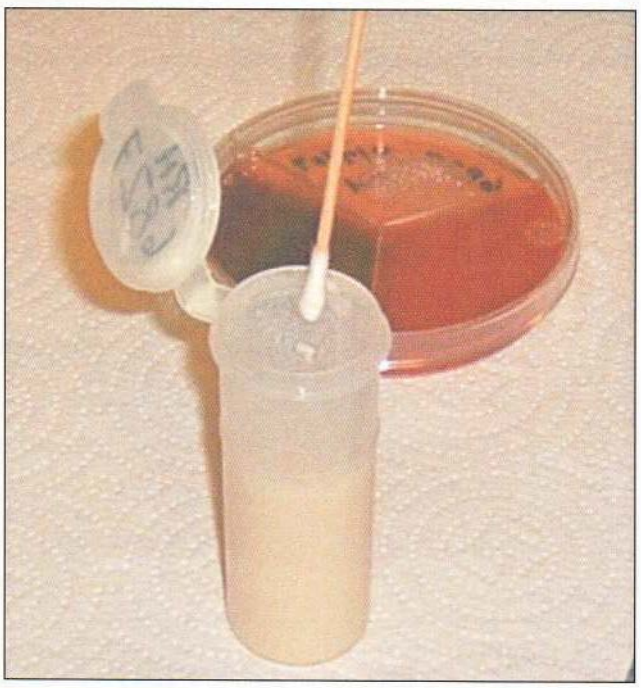


Figure 12.8
Using sterile disposable cotton swab to plate milk onto Tri-plate.

Plate Interpretation and Record Keeping

The plates should remain in a 37°C incubator for up to 48 hours (Figure 12.9). After 18 to 24 hours, the plate can be read for the first time. For interpretation, refer to the guidelines recommended by the manufacturer of the culture system being used. Examples of interpreting culture results from the Minnesota Easy Culture System and Petrifilm™ system are presented in Figures 12.1-12.7. A record keeping system should be in place to record, at a minimum, the sample date, cow ID, quarter affected, and final culture result. Culture results may later be transferred into an on-farm computerized record keeping system (e.g., Dairy Comp 305, Valley Agricultural Software). Such records are necessary to monitor the types of organisms causing mastitis on a given dairy and to evaluate treatment success. If OFCS results are suspicious, not easily interpreted, or there is routine failure of mastitis cases to respond to appropriate therapy, then frozen stored milk samples should be submitted to a commercial mastitis laboratory for routine culture and verification of OFCS results.

No growth results

If no bacterial growth is evident after 48 hours, then the sample is considered a "No Growth," as shown in Figure 12.10. Approximately 20 to 40% of samples from quarters with clinically abnormal milk will produce a "No Growth" result. Explanations for this could include:

1. The quarter sampled was not infected (true negative).
2. The quarter sampled was infected, but the cow's immune system had already eliminated the bacteria before the sample was collected (true negative). This is believed to be common in Gram-negative infections.
3. Equipment failure or errors in sample collection, storage, or culture technique resulted in a false-negative result.
4. The type of bacteria or other organism causing the mastitis does not grow under the conditions of the culture system (false negative; e.g., *Mycoplasma* spp.)

Figure 12.9
Example of an incubator for on-farm culture system.

If >40% of OFC results are "No Growths," or if a cow continues to show clinically abnormal milk (failure to cure), despite "No Growth" results, consult with your veterinarian or a reference laboratory to determine the cause.

Contaminated samples

A milk sample is considered "Contaminated" when three or more types of bacteria are identified on the Minnesota Easy Culture System media (Figure 12.11). Typically, when individual quarter milk samples are collected properly, only a single mastitis pathogen is identified. When a milk sample is "Contaminated," it is uncertain which, if any, of the bacteria found are causing disease and which are merely environmental bacteria that contaminated the milk sample during the collection process. Consideration must be given to the types of bacteria isolated, the number of colonies appearing on the plate, and the stage of infection. Dairy producers are encouraged to consult with their herd veterinarian and should consider resampling the affected quarters using very clean sampling, handling, and plating techniques. With proper techniques, <5% of individual quarter samples should be "Contaminated." If contamination is encountered more frequently (>10% of samples), review milk sampling technique, sample handling, and culturing procedures with farm staff involved. Frozen milk samples may also be submitted to a microbiology laboratory for confirmatory testing.

Figure 12.10
No bacterial growth on Bi-plate (Minnesota Easy Culture System, 2013).

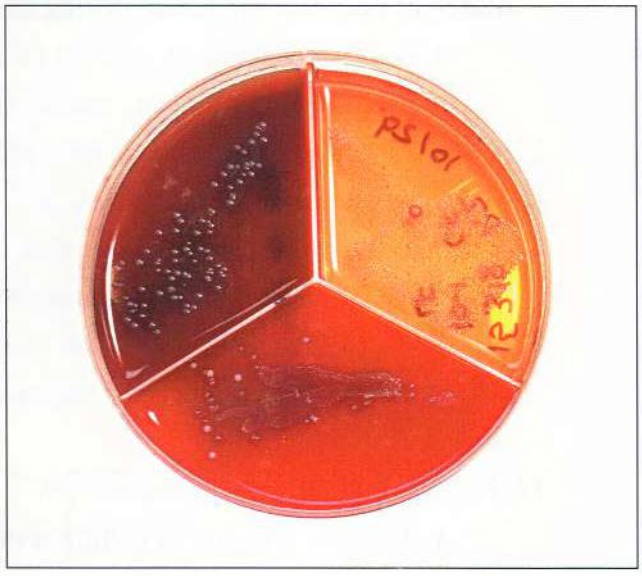


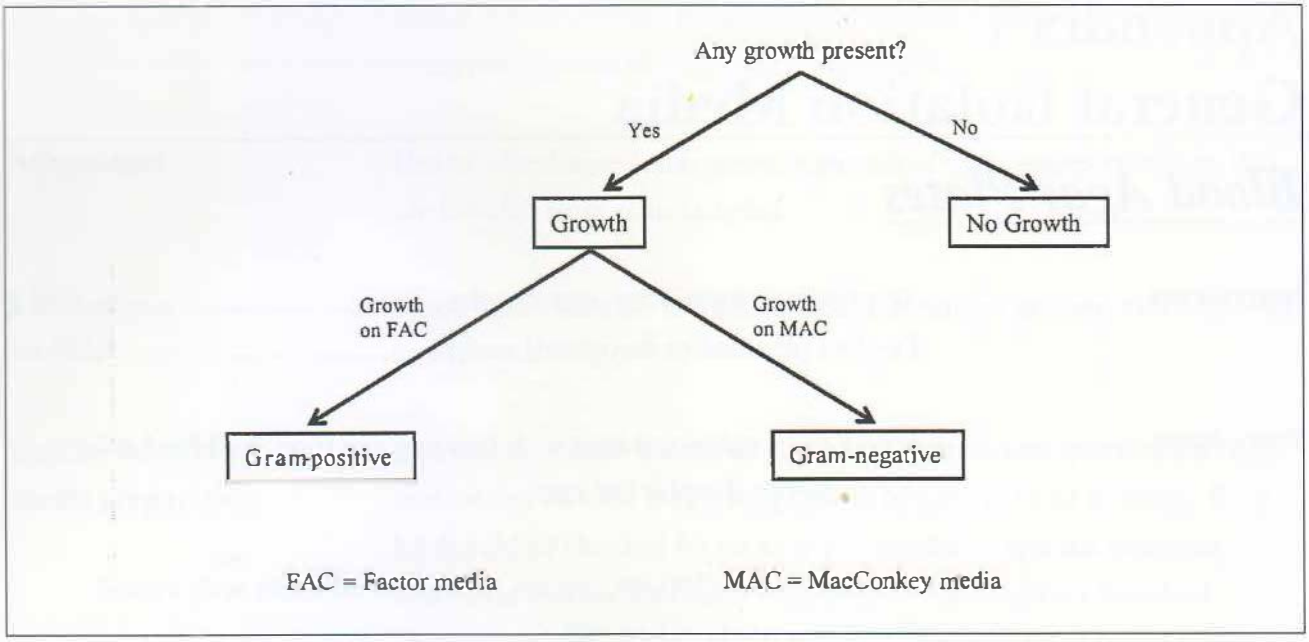
Figure 12.11
Growth of three or more different types of bacteria from a quarter milk sample indicate contamination (Minnesota Easy Culture System, 2013).

Quality Assurance

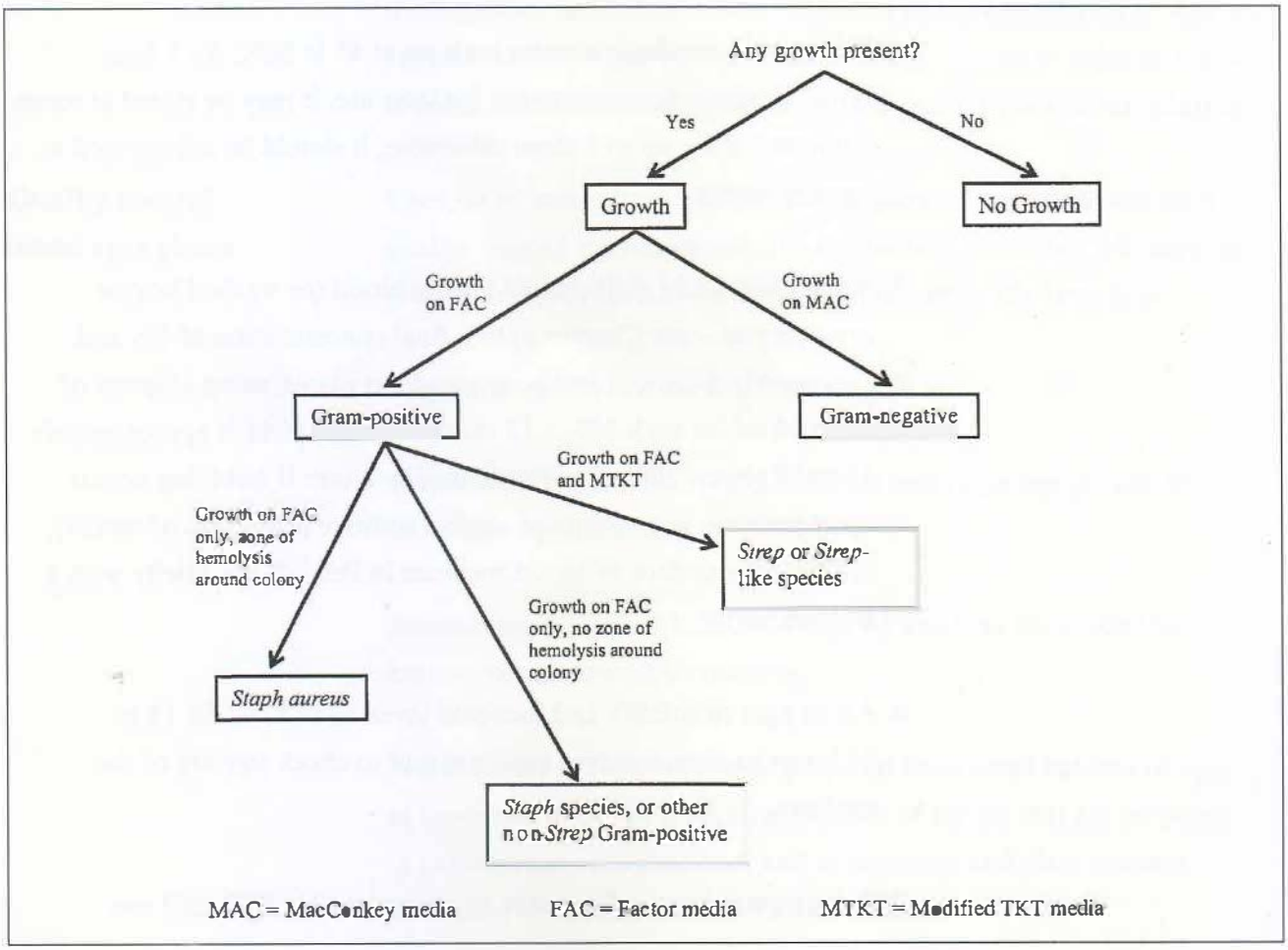
Ongoing quality assurance is a crucial part for the success of OFCS. Veterinarians or other qualified professionals should visit the dairy regularly and work with farm staff (i.e., weekly or biweekly basis) to review:

1. Milk sample collection and storage (i.e., aseptic technique, plating, or refrigeration).
2. Storage of new plates (i.e., refrigerator temperature, plate stacks placed upside down).
3. Labeling of culture plates (i.e., cow ID, quarters, and date).
4. Incubator functioning:
    a. Thermometer reading of 37°C (98.6°F).
    b. Presence of water source in incubator to maintain relative humidity at about 75%.
5. Plating technique (i.e., no milk chunks, well demarcated swabbing between samples).
6. Placing of plates in the incubator (i.e., upside down, not stacking more than two plates).
7. Time plates spend in the incubator.
8. Reading of plates from that day with on-farm culture technician.
9. Used plates should be stored in a refrigerator (different from the one where storing new plates) to:
    a. Evaluate agreement with the on-farm culture technician results.
    b. Additional testing (e.g., colonies suspicious of being *S. aureus*, *Streptococcus agalactiae*, *Klebsiella* spp., *Prototheca* spp., etc.).
    c. Evaluate plating technique.
    d. Identify contaminated samples.
10. Calculate the frequency of contaminated cultures (goal should be <10% of samples).
11. Observe hygiene practices (e.g., no food or drinks in the laboratory or refrigerator, workers wear clean clothes and disposable gloves, laboratory benches are cleaned and disinfected, etc.).
12. Records evaluation:
    a. Accurate entering of culture results.
    b. Matching between culture results and treatment decisions.
13. Discarding of plates (i.e., autoclaving or contracting disposal services).

It is recommended that farm staff working with OFCS periodically verify their culture results by regularly reviewing and interpreting plates with the regular herd veterinarian or by sending frozen milk samples to a local diagnostic laboratory for culture, and then compare culture results with those obtained on farm. Frequent verification should be used.



Flowchart 1
Identification of mastitis organisms using the Bi-Plate (Minnesota Easy Culture System, 2013).



Flowchart 2
Identification of mastitis organisms using the Tri-Plate (Minnesota Easy Culture System, 2013).

Appendix 1
General Isolation Media

Blood Agar Plates

Ingredients

Trypticase or tryptic soy agar powder ..... 40 g
Purified (distilled or deionized) water ..... 1,000 ml

Procedures

1. Mix thoroughly and heat with frequent agitation; boil for 1 minute to completely dissolve the agar.

2. Dispense into 200-ml aliquots, using 250-ml flasks with vented stoppers or aluminum foil.

3. Sterilize by autoclaving at 15 lb pressure (121°C) for 15 minutes.

4. Place sterile media in a water bath set at 45 to 50°C for 1 hour. Note: If agar is being prepared for later use, it may be stored at room temperature for up to 5 days; otherwise, it should be refrigerated at approximately 6°C.

5. After 1 hour, add defibrinated bovine blood (or washed bovine erythrocytes – see Chapter 2) to a final concentration of 5% and swirl gently. Mix well and pour into Petri plates, using aliquots of 12 to 14 ml for each 100- x 15-mm plate. The yield is approximately 15 to 18 plates/200 ml of sterile media. Note: If bubbling occurs upon pouring, use pourite or similar additive (1 drop/L of media), or flame the surface of liquid medium in Petri dishes briefly with a Bunsen burner.

6. Allow agar to solidify and incubate inverted at 37°C for 18 to 24 hours to reduce excess moisture and to check sterility of the medium.

7. Store inverted in a refrigerator at approximately 6°C until use.

8. Use within 2 weeks because plates become dehydrated and do not support growth of some microorganisms.

Advantages

Use of blood agar plates permits growth of most microorganisms that are isolated from milk samples.

Limitations

Blood agar plates do not detect esculin reactions or support the growth of mycoplasmas.

Quality control of media preparation

Dehydrated media should be purchased in sufficient quantities so that one lot can be used over a long period of time (6 to 12 months). Each lot should be checked for its ability to produce expected reactions, according to the American Public Health Association's Standard Methods for the Examination of Dairy Products. Each bottle of dehydrated media should be labeled, indicating the date received and opened, and stored in a cool, dry location protected from light, or in a refrigerator or freezer, if recommended by the manufacturer. Any expired media should be discarded or if any change in color or texture is noted. Complete mixing of media is necessary prior to autoclaving.

Quality control blood agar plates

Each lot of newly prepared blood agar plates should be tested with quality control microorganisms to ensure that the media will support growth and provide correct differential reactions for hemolysis.

Suggested quality control microorganisms:

Streptococcus dysgalactiae ATCC 27957 to ensure proper growth of fastidious streptococci.

Staphylococcus aureus ATCC 29749, alpha and beta toxin positive to ensure correct hemolytic patterns.

Note: Blood agar plates can be prepared from small batches of agar, as described above or from large batches of media that are prepared in commercial agar sterilizers and with automated plate pourers, according to the manufacturer's instructions.

Blood-esculin Agar Plates

Ingredients

Trypticase or tryptic soy agar powder ..... 40 g
Esculin ..... 1 g
Purified (distilled or deionized) water..... 1,000 ml

Procedures

1. Mix thoroughly and heat with frequent agitation; boil for 1 minute to completely dissolve the agar powder.

2. Dispense into 200-ml aliquots into 250-ml flasks with vented stoppers or aluminum foil.

3. Sterilize by autoclaving at 15 lb pressure (121°C) for 15 minutes.

4. Place sterile media in a water bath set at 45 to 50°C for 1 hour. Note: If agar is being prepared for later use, it may be stored at room temperature for up to 5 days; otherwise, it should be refrigerated at approximately 6°C.

5. After 1 hour, add defibrinated bovine blood (or washed bovine erythrocytes – see Chapter 2) to a final concentration of 5% and swirl gently. Mix well and pour into Petri plates using aliquots of 12 to 14 ml for each 100- x 15-mm plate. The yield is approximately 15 to 18 plates/200 ml of sterile media. Note: If bubbling occurs upon pouring, use pourite or similar additive (1 drop/L of media), or flame surface of liquid medium in Petri dishes briefly with a Bunsen burner.

6. Allow agar to solidify, and incubate inverted at 37°C for 18 to 24 hours to reduce excess moisture and to check sterility of the medium.

7. Store inverted in a refrigerator at approximately 6°C until use.

8. Use within 2 weeks because plates become dehydrated and do not support growth of some microorganisms.

Advantages

Use of blood-esculin agar plates permits esculin hydrolysis by streptococci on primary isolation.

Limitations

Alpha hemolysis of some *Streptococcus agalactiae* isolates may be confused with esculin splitting. Blood-esculin agar plates are available commercially from some vendors.

Quality control of media preparation

Dehydrated media should be purchased in sufficient quantities so that one lot can be used over a long period of time (6 to 12 months). Each lot should be checked for its ability to produce expected reactions according to the American Public Health Association's Standard Methods for the Examination of Dairy Products. Each bottle of dehydrated media should be labeled indicating the date received and opened, and stored in a cool, dry location protected from light, or in a refrigerator or freezer if recommended by the manufacturer. Expired media should be discarded or if any change in color or texture is noted. Complete mixing of media is necessary prior to autoclaving.

Quality control of blood-esculin agar plates

Each lot of newly prepared blood-esculin agar plates should be tested with quality control microorganisms to ensure that the media will support growth and provide correct differential reactions for hemolysis and utilization of esculin.

Suggested quality control microorganisms:

Streptococcus dysgalactiae ATCC 27957 to ensure proper growth of fastidious streptococci.

Streptococcus uberis ATCC 27958, esculin positive to ensure proper esculin reaction.

Staphylococcus aureus ATCC 29749, alpha and beta toxin positive to ensure correct hemolytic patterns.

Note: Blood-esculin agar plates can be prepared from small batches of agar as described above or from large batches of media that are prepared in commercial agar sterilizers and with automated plate pourers, according to the manufacturer's instructions.

Blood Agar Plates with Staphylococcus aureus Beta-Hemolysin

Procedure

Prepare blood agar plates and use one of the two methods listed below to apply beta-hemolysin. Allow the hemolysin to dry before streaking samples.

Swab method

Saturate a sterile swab with beta-hemolysin and streak a single line across the diameter of blood agar plate. If the plate is to contain four milk samples, streak a second line perpendicular to the first.

Drop method

With a sterile syringe and small (27-gauge) needle, place 6 drops of beta-hemolysin across the diameter of a blood agar plate. If four samples are to be streaked on the plate, add another 6 drops on the perpendicular axis.

Advantages

Development of CAMP reaction by streptococci on primary isolation.

Limitations

CAMP-positive *Streptococcus uberis* and *Streptococcus canis* may be confused with *Streptococcus agalactiae*.

Preparation of Beta-Hemolysin

I. Chloroform method

Two methods are available to prepare beta-hemolysin and are described below.

Ingredients and supplies

Staphylococcus aureus colonies showing beta-hemolysin production.
Blood agar plates.
Brain Heart Infusion or tryptose broth.
Chloroform.

Procedure

1. Transfer an isolated *S. aureus* colony onto blood agar several times to ensure that it consistently produces beta-hemolysin.

2. Transfer one typical colony to broth. The volume of broth depends on the quantity of beta-hemolysin desired.

3. Incubate at 37°C for 3 to 4 days.

4. Add 1 ml of chloroform per 10 ml broth culture (this will kill the *S. aureus* present but will not destroy the beta-hemolysin).

5. Incubate at room temperature for 18 to 24 hours.

6. Store at room temperature (or refrigerate) for up to 6 weeks.

7. Swab or drop onto blood agar surface, as described above or incorporate into medium at a 1% concentration. Care should be taken not to remove chloroform portion when removing beta-hemolysin. (Chloroform will be the bottom layer.)

8. After each use, swirl the chloroform throughout the hemolysin to remove contaminants, which may have entered the supply during use.

II. Filtration method

Reagents and equipment

Staphylococcus aureus colonies showing beta-hemolysin production.
Blood agar plates.
Brain Heart Infusion or tryptose broth.
Centrifuge.
Filters with 0.45- to 0.50-µm pore size.
Vacuum or air supply.

Procedures

1. Transfer an *S. aureus* isolate onto blood agar several times to ensure that the microorganisms consistently produce beta-hemolysin.

2. Transfer one colony to broth. The volume of broth depends on the quantity of beta-hemolysin desired.

3. Incubate at 37°C for 3 to 4 days.

4. Centrifuge at 1,000 x g for 30 minutes.

5. Filter supernatant through sterilizing filters with the aid of a vacuum or air supply.

6. Dispense in small quantities for use.

7. Store under refrigeration.

8. The preparation should be stable for several months.

Appendix 2
Mycoplasma Medium and Testing Procedures

Mycoplasma Medium

Stock agar ingredients

Mycoplasma agar base ..... 36 g
Distilled water ..... 1,000 ml

OR

PPLO Broth w/o crystal violet ..... 21 g
Agar No.1 (Oxoid) ..... 10 g
Distilled water ..... 1,000 ml

Procedures

1. Boil to dissolve the agar and distribute 250-ml volumes into flasks (500 ml) large enough to hold additives.

2. Sterilize by autoclaving at 15 lb pressure (121°C) for 20 minutes. Store at 4°C.

Complete working agar

1. 250 ml of stock agar melted and cooled to 50°C.

2. Add (warmed to 37°C):

Horse serum ..... 55 ml
Fresh yeast extract solution ..... 35 ml
Thallium acetate 1% (w/v) solution ..... 7 ml
DNA 0.2% (w/v) solution ..... 5 ml
Penicillin 200,000 IU/ml ..... 2 ml
Dextrose 50% (w/v) solution ..... 5 ml

3. Pour into 15- x 100-mm Petri plates, approximately 15 ml/plate.

4. Ready to use when agar surface is dry.

5. Store inverted at 4°C in plastic bags.

Ingredients

Thallium acetate

POISON – HANDLE WITH CARE!

Dissolve 1 g of thallium acetate in 100 ml distilled deionized water to make a 1% stock solution. Pass through a sterile, disposable, 0.22-µm pore size filter, dispense in 10-ml amounts, label, and store in a freezer (-30 to -70°C).

Fresh yeast extract

Purchase or prepare as follows: Heat 500 ml of distilled deionized water to 80°C on a hot plate and slowly add while stirring continuously 125 g of Fleischmann's dry yeast, type 2040. Keep the mixture at 80°C for 20 minutes. After cooling, centrifuge at 1,000 x g for 45 minutes. Remove the supernatant fluid and filter several times through decreasing pore size on filters (1.2, 0.8, 0.45 µm), dispense in convenient aliquots, and autoclave at 10 lb pressure (115°C) for 5 minutes or pass through a sterile, disposable, 0.22-µm pore size filter. Label and store in a freezer (-30 to -70°C).

Penicillin

Dissolve 5 million IU of potassium penicillin G in 25 ml of deionized distilled water. Pass through a sterile, disposable, 0.22-µm pore size filter, dispense in 2-ml amounts, label, and store in a freezer (-30 to -70°C).

DNA solution

Dissolve 0.2 g DNA (sodium salt from salmon testes) in 100 ml of deionized distilled water. Dispense in 5-ml aliquots and autoclave at 15 lb pressure (121°C) for 15 minutes. Label and store in a freezer (-30 to -70°C).

Dextrose solution

Dissolve 50 g of dextrose in 100 ml of deionized distilled water. Pass through a sterile, disposable, 0.22-µm pore size filter, dispense in 5-ml amounts, label, and store in a freezer (-30 to -70°C).

Horse serum

Purchase or prepare from whole blood as follows: Allow blood to clot at room temperature for several hours or overnight at 4°C. Break clots and separate serum by centrifugation at 2,000 x g. Pass through a sterile, disposable, 0.22-µm pore size filter, label, and store in sterile bottles in a freezer at -30°C.

Digitonin Disc Diffusion Assay

(adapted from Tully, 1983)

Procedures

1. Prepare digitonin stock solution by adding 75 mg of digitonin to 5 ml of 95% ethanol in a screw-capped tube.
2. Gently heat solution by immersing the tube in boiling water until digitonin powder is completely dissolved.
3. Store the stock solution at 4°C until use.
4. Make digitonin discs by adding 25 µl of the digitonin stock solution to each 6-mm paper disc.
5. Allow the digitonin discs to be dried overnight and store at 4°C until use.
6. Prepare a broth culture (4-day culture) of the isolate to test, or use a sample of fresh milk with the infectious mycoplasma isolate to test.
7. Pour and spread 200 µl of each liquid media (broth culture or test milk) on the surface of modified Hayflick agar plates. Allow the surface to dry.
8. Place a digitonin disc on the surface of agar.
9. Incubate the plate at 37°C with 10% CO_2 for 7 to 10 days.
10. Measure the zone of inhibition from the edge of the disc to the edge of the clear zone (mm) around the disc under the stereomicroscope.

Interpretation

- Inhibition zones of >5 mm = Positive (+) for *Mycoplasma* spp.
- Inhibition zones of 3-5 mm = Ambiguous result (+/-) for *Mycoplasma* spp.
- Inhibition zones of <3 mm = Negative (-) for *Mycoplasma* spp.

Nisin Disc Diffusion Assay

(adapted from Boonyayatra, 2010)

Procedures

1. Store nisin stock solution (5.16 mg/ml) at 5-7°C. This stock solution can be used for up to 24 months. Make a 10-fold dilution of nisin from stock solution with 10 mM citrate pH 3.5.

2. Nisin discs with two different concentrations of nisin are made by adding either 20 µl of the nisin stock solution (undiluted) or 20 µl of the 10-fold diluted solution to 6-mm paper discs. The two types of disc will contain 103.2 µg (undiluted) or 10.32 µg (diluted) of nisin, respectively.

3. Allow the nisin discs to be dried overnight and store at 4°C until use.

4. Prepare a broth culture (4-day culture) of the isolate to test, or use a sample of fresh milk with the infectious mycoplasma isolate to test.

5. Pour and spread 200 µl of each liquid media (broth culture or test milk) on the surface of modified Hayflick agar plates. Allow the surface to dry.

6. Place undiluted and diluted nisin discs on the surface of agar.

7. Incubate the plate at 37°C with 10 % CO_2 for 7 to 10 days.

8. Measure the zone of inhibition from the edge of the disc to the edge of the clear zone (mm) around the disc under the stereomicroscope.

Interpretation

- Presence of inhibition zones = Positive (+) for *Mycoplasma* spp.
- Absence of inhibition zones = Negative (-) for *Mycoplasma* spp.

Appendix 3
Other Media

MacConkey Agar

Ingredients

MacConkey agar powder	50 g
Distilled water	1,000 ml

Procedures

1. Mix solution and heat to dissolve.
2. Sterilize at 15 lb pressure (121°C) for 15 minutes.
3. Temper sterile media at 47 to 50°C for 1 hour.
4. Pour 13 to 15 ml per plate.
5. Store inverted in a refrigerator.

Triple Sugar Iron Slants

Ingredients

Triple sugar iron agar powder	59.4 g
Distilled water	1,000 ml

Procedures

1. Mix solution and heat to dissolve.
2. Dispense 5 ml per tube (tube size 16 mm ID).
3. Autoclave not over 118°C for 15 minutes.
4. Cool in a slanted position to obtain deep butt.
5. Store refrigerated.

Simmons Citrate Slants

Ingredients

Simmons citrate agar powder	24.2 g
Distilled water	1,000 ml

Procedures

1. Mix solution and heat to dissolve.
2. Dispense 5 ml per tube (tube size 16 mm ID).
3. Autoclave at 15 lb pressure (121°C) for 15 minutes.
4. Cool in a slanted position.
5. Store refrigerated.

Motility Test Medium

Ingredients

1% 2, 3, 5-triphenyltetrazolium chloride (TTC)	5 ml
Motility test agar (MTA) powder	20 g
Distilled water	1,000 ml

Procedures

1. Mix and dissolve MTA in distilled water.
2. Add TTC solution.
3. Dispense 5 ml into 13- x 100-mm tubes.
4. Autoclave at 15 lb pressure (121°C) for 15 minutes.
5. Store refrigerated.

CAMP-esculin Plates

Ingredients

Trypticase soy agar	40 g
Esculin	1 g
1% ferric citrate solution	10 ml
Sterile bovine or ovine blood*	50 ml
Distilled water	950 ml

Procedures

1. Mix and dissolve trypticase soy agar and esculin in distilled water.
2. Add 10 ml of 1% ferric citrate solution.
3. Autoclave at 15 lb pressure (121°C) for 15 minutes.
4. Temper media at 47 to 50°C for 1 hour.
5. Add blood* and gently swirl to mix well before pouring.
6. Pour 12 to 15 ml per plate.
7. Incubate plates at 37°C overnight to check for sterility.
8. Store inverted at 4 to 6°C.

*Washed bovine red blood cells or whole blood from selected animals may be used.

Carbohydrate Fermentation Media

Ingredients

Phenol red broth base ..... 16 g
Carbohydrate* ..... 10 g
*inulin, lactose, raffinose, mannitol, xylose, etc.
Distilled water ..... 1,000 ml

Procedures

1. Mix and dissolve ingredients.
2. Dispense 4 ml into 15- x 130-mm screw-top tubes.
3. Autoclave at 15 lb pressure (121°C) for 15 minutes.
4. Store refrigerated.

Modified Rambach Agar Medium

Ingredients

Propylene glycol	10 g
Peptone	5 g
Yeast extract	2 g
Neutral red	0.03 g
Beta D-gal*	0.1 g
*5-bromo-4-chloro-3-indolyl-beta, D-galactopyranoside	
Agar	15 g
Distilled water	1,000 ml

Procedures

1. Add peptone, yeast extract, neutral red, and agar to distilled water.
2. Sterilize by autoclaving at 15 lb pressure (121°C) for 15 minutes.
3. Temper sterile media at 45 to 50°C for 1 hour.
4. Add filter to sterilized propylene glycol and filter to sterilized galactopyranoside.
5. Pour 13 to 15 ml per plate.
6. Store inverted in refrigerator.
7. Media should be used within 5 days.

Sabouraud Dextrose Agar Plates

Ingredients

Sabouraud Dextrose Agar ..... 65 g
Distilled water ..... 1,000 ml

Procedures

1. Mix solution and heat to dissolve.
2. Sterilize at 15 lb pressure (121°C) for 15 minutes.
3. Temper sterile media at 47 to 50°C for 1 hour.
4. Pour 13 to 15 ml per plate.
5. Store inverted in refrigerator.

Trypticase Soy Broth

Ingredients

Trypticase soy broth powder ..... 30 g
Distilled water ..... 1,000 ml

Procedures

1. Mix and dissolve ingredients.
2. Dispense 4 ml into 15- x 130-mm screw-top tubes.
3. Autoclave at 15 lb pressure (121°C) for 15 minutes.
4. Store refrigerated.

6.5% NaCl Agar

Ingredients

Agar	15 g
Dextrose	10 g
NaCl	65 g
Bromocresol purple	0.02 g
Distilled water	1,000 ml

Procedures

1. Mix solution and heat to dissolve.
2. Dispense 5 ml per tube (tube size 16 mm ID).
3. Autoclave at 15 lb pressure (121°C) for 15 minutes.
4. Cool in a slanted position.
5. Store refrigerated.

Sodium Hippurate Medium

Ingredients

Infusion broth base	25 g
Sodium hippurate	10 g
Distilled water	1,000 g

Ferric chloride solution

Ferric chloride	12 g
Concentrated HCl	2.5 ml
Distilled water	97.5 ml

Procedures

1. Dissolve broth base and sodium hippurate in 1,000 ml distilled water.
2. Sterilize at 15 lb pressure (121°C) for 15 minutes.
3. Aseptically transfer 3 ml per sterile test tube and incubate 37°C overnight to test sterility. (Mark level of medium in a tube before incubating.)
4. Store broth solution refrigerated.
5. Store ferric chloride solution at room temperature.

Potato Dextrose Agar

Ingredients

Potato extract	4 g
Glucose	20 g
Agar	15 g
Distilled water	1,000 ml

Procedures

1. Mix solution, heat to dissolve, and adjust pH to 5.6 +/- 0.2.

2. Sterilize at 15 lb pressure (121°C) for 15 minutes.

3. Temper sterile media at 47 to 50°C for 1 hour.

4. Pour 13 to 15 ml per plate.

5. Store inverted in refrigerator.

Prototheca Isolation Medium

Ingredients

Potassium hydrogen phthalate	10 g
Sodium hydroxide	0.9 g
Magnesium sulfate	0.1 g
Potassium hydrogen phosphate	0.2 g
Ammonium chloride	0.3 g
Glucose	10 g
Thiamine hydrochloride	0.001 g
Agar	20 g
5-fluorocytosine	0.25 g
Distilled water	1,000 ml

Procedures

1. Suspend potassium hydrogen phthalate and sodium hydroxide in 1,000 ml distilled water.
2. Add remaining ingredients, except 5-fluorocytosine, heat to dissolve, and adjust pH to 5.0 to 5.2.
3. Sterilize at 15 lb pressure (121°C) for 15 minutes.
4. Temper sterile media at 47 to 50°C for 1 hour.
5. Add filter to sterilized 5-fluorocytosine.
6. Pour 13 to 15 ml per plate.
7. Store inverted in refrigerator.

Edwards Modified Medium

Ingredients

Lab-Lemco powder	10 g
Peptone	10 g
Esculin	1 g
Sodium chloride	5 g
Crystal violet	0.0013 g
Thallous sulfate	0.33 g
Agar	15 g
Sterile bovine or ovine blood	50 ml
Distilled water	950 ml

Procedures

1. Mix ingredients (except sterile blood) and heat to dissolve.

2. Sterilize at 15 lb pressure (121°C) for 20 minutes.

3. Temper at 50°C for 1 hour.

4. Add sterile blood and swirl gently.

5. Pour 13 to 15 ml per plate.

6. Store inverted in refrigerator.

Caution! – Thallous sulfate is poisonous.

Vogel-Johnson Agar

Ingredients

Tryptone	10 g
Yeast extract	5 g
Mannitol	10 g
Dipotassium phosphate	5 g
Lithium chloride	5 g
Glycine	10 g
Phenol red	0.025 g
Agar	16 g
Distilled water	1,000 ml
3.5% potassium tellurite	5.7 ml

Procedures

1. Mix solution, except for potassium tellurite, and heat to dissolve.

2. Sterilize at 15 lb pressure (121°C) for 15 minutes.

3. Temper sterile media at 47 to 50°C for 1 hour.

4. Add sterile potassium tellurite solution.

5. Pour 13 to 15 ml per plate.

6. Store inverted in refrigerator.

Appendix 4
Testing Procedures

CAMP-esculin Test

Inoculation

1. Inoculate a CAMP-esculin plate by streaking a culture of a beta-hemolysin producing *S. aureus* in a straight line across the center.
2. Streak streptococcal cultures perpendicular to and within 1 to 2 mm of the *S. aureus* streak.
3. Ten isolates may be tested per plate, with five on a side.
4. Incubate the plate inverted for 18 to 24 hours at 37°C.

Interpretation

Positive CAMP reaction

Complete clearing in a half-circle fashion (arrow-head) in the partial lysis zone of the *S. aureus* (e.g., *Streptococcus agalactiae*).

Positive esculin hydrolysis

Browning around organism growth (e.g., *Streptococcus uberis*).

Carbohydrate Fermentation

(for identifying streptococci and coliform bacteria)

Inoculation

1. With a wire loop, select isolated colonies from a blood agar plate.
2. Dip the wire loop into the broth.
3. Incubate the inoculated broth for 48 hours at 37°C.

Interpretation

Positive for fermentation

Acid production is indicated by yellow coloration.

Negative for fermentation

Broth remains red. Partial color change is interpreted as negative.

Esculin Hydrolysis

Inoculation

1. With a wire loop, select isolated colonies of Gram-positive, catalase-negative cocci from a blood agar plate.

2. Dip the wire loop into esculin broth.

3. Incubate 48 hours at 37°C.

Interpretation

Positive reaction

Esculin hydrolysis is indicated by a brown-black coloration of broth (e.g., *Streptococcus uberis*).

Negative reaction

No color change in broth is a negative reaction for esculin hydrolysis (e.g., *Streptococcus agalactiae*).

Sodium Hippurate Test

Inoculation

1. With a wire loop, select isolated colonies from a pure fresh culture of Gram-positive, catalasenegative cocci on a blood agar plate.

2. Dip the wire loop into sodium hippurate medium.

3. Incubate 48 hours at 37°C.

4. Centrifuge cultures for 15 minutes at 1,000 x g.

5. Pipette 0.8 ml of supernatant into clean test tube.

6. Add 0.2 ml of ferric chloride into supernatant and mix.

Interpretation

Positive reaction

A positive reaction is indicated by the persistence of a reddish-brown precipitate (e.g., *Streptococcus agalactiae*).

Negative reaction

Clearing of the initial precipitate after mixing indicates that the hippurate was not hydrolyzed and is considered a negative reaction (e.g., *Streptococcus uberis*).

Note – After 48 hours of incubation at 37°C, the concentration of the sodium hippurate must be exactly 1% or false positives may occur. Therefore, the level of broth in the tube should be marked with a wax pencil. If evaporation occurs, distilled water should be added after incubation and before the addition of the ferric chloride reagent.

Catalase Test

Procedures

1. Put a drop of 3% solution of hydrogen peroxide on a microscope slide.

2. Emulsify a colony in the peroxide.

Interpretation

Positive reaction

Bubbles are produced (e.g., staphylococci).

Negative reaction

No reaction (e.g., streptococci).

Caution! – Red blood cells produce catalase. Therefore, avoid picking blood agar with the colony as the red blood cells will give a false-positive reading. Never run tests directly on the blood agar plate.

Oxidase Test

Procedures

Using a sterile wooden applicator stick, transfer a colony from agar onto filter paper saturated with a drop of oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride).

Interpretation

Positive reaction

Oxidase-producing organisms will react within 30 seconds, turning the disc purple-black (e.g., pseudomonads).

Negative reaction

No color reaction (e.g., coliforms).

Note: Nichrome wire loops should NOT be used in performing the oxidase test, as they can cause a false-positive reaction. The oxidase test should NOT be performed using bacterial growth from selective media such as MacConkey.

Coagulase Tube Test

Inoculation

1. Inoculate 0.5 ml coagulase plasma with a heavy inoculum of staphylococci from a 24-hour plate culture. Use a wire loop or the end of a sterile applicator stick for inoculation.

2. Incubate tubes at 37°C in a water bath or an incubator for up to 24 hours.

Interpretation

Positive reaction

Semi-solid to solid gelation evident when tube is tipped (e.g., *Staphylococcus aureus*).

Negative reaction

Liquid state after 24 hours incubation (e.g., *Staphylococcus chromogenes*).

Note: The inoculum must be staphylococci of one type for uniform test results. If the culture is >24 hours old, a colony should be picked and placed in trypticase soy broth overnight. From the overnight culture, inoculate a coagulase tube with 0.05 ml of broth culture.

A positive test can appear within 2 to 4 hours or may take up to 24 hours. Positive results can be recorded when evident. Some *S. aureus* produce the enzyme staphylokinase that may lyse the clot if allowed to incubate too long. All negative tests must remain incubated for 24 hours. Coagulase-positive and coagulase-negative staphylococci controls should be run with each test to determine the stability of plasma.

KOH Test for Gram Staining Potential

A simple and effective method for determining the Gram staining reaction of bacteria is the KOH test. The only reagent required is a 3% aqueous solution of potassium hydroxide (KOH) and the results have been correlated closely with Gram staining results.

Procedure

1. Place a drop of 3% KOH onto a microscope slide.
2. Transfer one or more like colonies from the surface of solid medium into the KOH solution on the slide.
3. Mix and read within 60 seconds.

Interpretation

Positive reaction

Mixture becomes viscous or gels (e.g., Gram-negative bacteria).

Negative reaction

Mixture remains fluid (e.g., Gram-positive bacteria).

MacConkey Agar Reaction

Inoculation

Isolates are streaked zigzag over one-sixth to one-eighth of the plate divided in pie fashion.

Interpretation

MacConkey agar inhibits the growth of most Gram-positive organisms. Those colonies that ferment lactose turn pink and may be surrounded by a zone of precipitated bile salts (e.g., *Escherichia coli*).

Triple Sugar Iron Agar

Inoculation

1. Pick an isolated colony with a sterile wire.

2. Stab the butt to approximately 1/8 inch from the bottom of the tube.

3. Streak the slant.

4. Incubate inoculated tubes at 37°C for 18 to 24 hours.

Interpretation

Yellow = acid production (A)
Red = negative for acid production (K)
Black = positive for hydrogen sulfide production (+)

Reporting results

Acid slant/acid butt (A/A) due to lactose and/or sucrose fermentation (e.g., coliform bacteria).

Alkaline slant/acid butt (K/A) due to dextrose fermentation, not lactose fermentation (e.g., *Serratia* spp.).

Alkaline slant/alkaline butt (K/K) due to no lactose, sucrose, or dextrose fermentation (e.g., *Pseudomonas* spp.).

Black butt due to hydrogen sulfide production (e.g., *Proteus* spp. with K/K reaction).

Note: If tubes are incubated longer than 24 hours, false negatives may occur due to reversion of the medium to a neutral color.

Simmons Citrate Agar

Inoculation

1. Pick an isolated colony with a sterile wire.

2. Streak the surface of agar in slant or on plate.

3. Incubate at 37°C for 18 to 24 hours.

Interpretation

Positive reaction

Any blue color is an indication of citrate utilization (e.g., *Klebsiella* spp.).

Negative reaction

Agar remains green (e.g., *Escherichia coli*).

Motility

Inoculation

1. Using an inoculating wire, stab motility agar with an isolated colony.

2. Incubate tubes at 37°C for 18 to 24 hours.

Interpretation

Positive reaction

Motility is evidenced by turbidity throughout the medium or growth away from stab (e.g., *Escherichia coli*).

Negative reaction

Non-motile organisms grow only along stab (e.g., *Klebsiella* spp.).

Growth in 6.5% NaCl

Inoculation

1. Pick an isolated colony with a sterile wire.

2. Streak the surface of agar in slant or on plate.

3. Incubate at 37°C for 18 to 24 hours.

Interpretation

Positive reaction

Color change from blue-yellow is an indication of salt tolerance (e.g., enterococci).

Negative reaction

Agar remains blue (e.g., *Streptococcus uberis*).

Appendix 5
Stains

Methylene Blue Stain

Ingredients

Methylene blue.....0.3 g
Ethyl alcohol 95% .....30 ml
When dissolved, add distilled water.....100 ml

Store at room temperature in a dark dropper bottle.

Procedures

1. Flood heat-fixed smear of an organism with stain.

2. Allow stain to remain on slide for 10 seconds.

3. Rinse with tepid tap water.

4. Blot dry.

Interpretation

Nuclei will be stained blue, making them more observable under the microscope.

Dienes Stain

Ingredients

Methylene blue	2.4 g
Maltose	10 g
Azure II	1.25 g
Sodium chloride	0.25 g
Distilled water	100 ml

Store at room temperature in a dark dropper bottle.

Procedures

1. Apply a thin film (uniform and light) of stain to a clean coverslip with a cotton swab.

2. Treated coverslips are ready to use or may be stored indefinitely when dry.

3. A 1-cm square agar block containing suspected *Mycoplasma* spp. colonies is cut out and transferred to a microscope slide with colony side up.

4. Place a treated coverslip, stain side down, over the agar block.

5. Staining is complete within a few minutes.

Interpretation

Bacterial colonies, as well as *Mycoplasma* spp. colonies, will stain; however, the bacterial colonies (except L-forms, such as mycoplasmas) will reduce the methylene blue within 15 minutes. *Mycoplasma* spp. colonies retain the color. The preparation must be examined under 20 to 40 X magnification.

Gram Stain

Procedures

Make a slide from a PURE culture by mixing a small amount with a small drop of sterile distilled water or sterile broth. Mark slide with wax pencil to locate smear. Air dry and fix by lightly passing slide through flame, being careful not to burn. Flame-fixed slide should be able to be held on wrist without feeling too hot.

Staining

1. Apply crystal violet to smear by flooding slide for 30 to 60 seconds.
2. Wash off with tap water.
3. Apply Gram's iodine for 30 to 60 seconds. Drain it; do not wash.
4. Decolorize by continual gentle rinsing with 95% alcohol just until color is no longer present in runoff.
5. Rinse with tap water.
6. Apply safranin for approximately 1 minute.
7. Rinse away safranin stain with tap water.
8. Blot dry with bibulous paper and examine.

Caution! – ALWAYS RUN CONTROLS with known Gram-positive (e.g., *Staphylococcus* spp. - blue) and Gram-negative (e.g., coliform - red) organisms.

Stains should always be kept in brown bottles to prevent deterioration by light. Stains must be free of sediment and should be filtered every 2 to 3 weeks to avoid artifacts that confuse results. ALWAYS USE FRESH CULTURES, as old cultures may give erroneous results.

Note: Prepared solutions of stains are available commercially.

Acid-Fast Stain: Ziehl-Neelsen Method

Preparations

Carbol-fuchsin Stain

- Basic fuchsin ..... 0.3 g
- Ethanol 95% ..... 10 ml

This solution is mixed with:

- Phenol (melted crystals) ..... 5 ml
- Distilled water ..... 9.5 ml

Acid Alcohol

- Hydrochloric acid (concentrated) ..... 3 ml
- Ethanol 95% ..... 97 ml

Counterstain

- Methylene blue (certified) ..... 0.3 g
- Distilled water ..... 100 ml

Staining procedures

1. Prepare a think smear, dry, and fix by heat.
2. Place a strip of filter paper over the smear only.
3. Flood the slide with Carbol-fuchsin stain and heat until steam rises from stain. DO NOT BOIL.
4. Allow to stand for 5 minutes, remove the filter paper, and wash slide thoroughly under running tepid water.
5. Decolorize in acid alcohol until all traces of red have disappeared from the film. Decolorization should not be attempted in one stage; there should be intermittent washings in water and reapplication of acid alcohol.
6. Wash well in water when decolorization is complete.
7. Counterstain with methylene blue for approximately 30 seconds.
8. Wash and stand on end. DO NOT BLOT.

Interpretation

Positive – Acid-fast organisms stain red (e.g., *Mycobacterium* spp.).

Negative – Acid-fast negative bacteria stain blue (e.g., *Bacillus* spp.).

Note: Prepared solutions of stains are available commercially.

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