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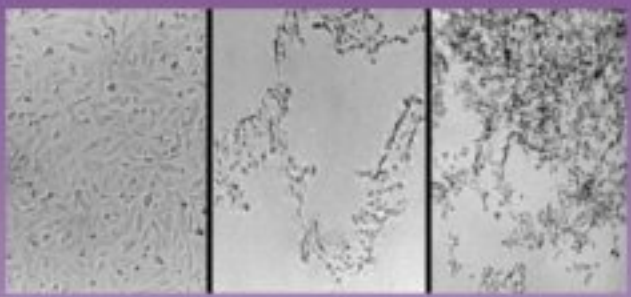
Food-Borne Pathogens

Methods and Protocols

Edited by

Catherine C. Adley



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Food-Borne Pathogens

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Preface

Advanced food manufacturing technologies have allowed food preparation to become a worldwide process rather than a local home industry. With this technological advancement, the control of food-borne pathogens, viruses, and parasites has become the responsibility of the manufacturers rather than the consumers. The worldwide distribution systems and storage of prepared food have also generated increased vigilance on the part of the manufacturers to control contamination by food-borne pathogens. Rapid, valid testing methods to detect and identify food-borne pathogens have therefore become a daily necessity for the food industry. Furthermore, surveillance and monitoring are a justifiable requirement, if confidence in the food we eat is to be maintained.

The contributions to *Food-Borne Pathogens: Methods and Protocols* present emerging molecular methods of analyzing food-borne pathogens. It contains methodologies for the laboratory isolation and identification of the three groups of organisms that cause food-borne disease: bacteria, viruses, and parasites. A review of toxin detection kits and the analysis by high performance liquid chromatography and bacterial storage conditions is also included. These methods demonstrate the direction in rapid identification systems presently being developed. The move from the use of biochemical tests and commercial miniaturized identification kits has been slow and will depend on the accuracy and validation of molecular methods. Cost will also be a factor in many instances.

This inclusion of *Food-Borne Pathogens: Methods and Protocols* in the food testing laboratory library will allow technologists access to both the methods currently being used and to new methodologies for testing organisms that might not have been attempted previously.

The importance of surveillance systems and risk assessment has also been highlighted and should not be underestimated by food testing personnel as an addition to their laboratory protocols.

It is envisioned that the methodologies presented in *Food-Borne Pathogens: Methods and Protocols* will be used on an ongoing basis by the food technologist and research scientist alike.

Catherine C. Adley

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I

The Bacteria

1

Detection of Hemolysins in *Aeromonas* spp. Isolates From Food Sources

PCR Analysis and Biological Activity

Rosabel Falcón, Tatiana d'Albuquerque e Castro, Maria das Graças de Luna, Angela Corrêa de Freitas-Almeida, and Tomomasa Yano

Summary

Aeromonas species are water-borne bacteria that are often found as environmental and food contaminants. They have been involved in human diarrhea disease and extraintestinal infections and are considered as emerging pathogens. These infections are probably acquired by food and water consumption, as there is a high prevalence of *Aeromonas* in the environment and food. From the species isolated, *A. hydrophila, A. veronii* biovar *sobria,* and *A. caviae* are the species most commonly implicated in human intestinal infections. The mechanism of pathogenesis is complex and not well understood. *Aeromonas* virulence is considered to be multifactorial. Toxins with hemolytic, cytotoxic, and enterotoxic activities have been described in many *Aeromonas* spp. The hemolytic activity of aeromonads is related to both hemolysin (*aer*A and *hly*A) and cytolytic enterotoxin *(aer)* genes. Several virulence factors have been identified in strains isolated from a number of sources. It is possible that more than one of the genes involved in hemolytic/enterotoxic activity occur in the same strain. One rational approach to determine whether *Aeromonas* strains have the potential to be virulent is to detect the presence of hemolysin and enterotoxin genes by polymerase chain reaction (PCR) assays. PCR results can be compared with biological assays to assess the expression of the hemolytic and cytotoxic effects.

Key Words: *Aeromonas; aer* gene; *aer*A gene; *hly*A gene; β-hemolysin; cytolytic enterotoxin; PCR; Vero cells; cytotoxicity assay; hemolytic activity.

1. Introduction

Because *Aeromonas* hemolysins have been correlated with food-borne gastrointestinal infections in immunocompetent humans, many attempts have been made to develop methods to detect the virulence factors involved in this process.

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The β-hemolytic activity in most *Aeromonas* isolates is often associated with the presence of *hly*A and/or *aer*A genes. The cytolytic enterotoxin gene *(aer)* has also been linked with β-hemolysis in *Aeromonas* ***(1)***. These genes can be easily identified by polymerase chain reaction (PCR), which provides a highly sensitive and specific tool for detecting hemolysins and cytotoxic enterotoxins in *Aeromonas.* In practice, however, biological methods, such as the production of cell-free hemolytic activity at 37°C and analysis of the cytotoxic effects in mammalian cells, are usually used to detect hemolytic activity ***(2–4)***. Together, these methods have contributed to the characterization of hemolysins, which are important virulence factors involved in the pathogenesis associated with *Aeromonas* spp.

This chapter will cover the most important aspects of bacterial DNA preparations, including the amplification of DNA sequences related to hemolysin expression and the subsequent analysis of PCR products. The biological characterization of *Aeromonas* hemolysin is presented at the end of the chapter.

2. Materials

1. *A. hydrophila* ATCC 7966 (hemolytic strain used as a positive control).
2. *A. caviae* ATCC 15468 (nonhemolytic strain used as a negative control).
3. Source of bacterial cells from which DNA will be extracted.
4. DNA/RNase-free distilled water (Invitrogen, cat. no. 10977-015).
5. 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl) (Invitrogen, cat. no. 18067-017).
6. 50 m*M* $MgCl_2$ (Invitrogen, cat. no. 18067-017).
7. Recombinant *Taq* DNA polymerase (100 units) (Invitrogen, cat. no. 10342-053).
8. Oligonucleotide primers (100 μ*M; see* **Table 1**).
9. dNTP set (dATP, dTTP, dGTP, and dCTP, each at a concentration of 100 m*M*) (Invitrogen, cat. no. 10297-018).
10. Nuclease-free light mineral oil (do not autoclave) (Sigma, cat. no. M3516).
11. 1X Electrophoresis buffer (TBE): 89 m*M* Tris-borate, 2 m*M* EDTA (for 1.0 L of 5X TBE: 54.0 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 *M* EDTA, pH 8.0).
12. Ultrapure agarose (Invitrogen, cat. no. 15510-019).
13. Ethidium bromide (10 mg/mL) **(highly toxic)** (Invitrogen, cat. no. 15585-011).
14. 10X Electrophoresis loading buffer (60% glycerol, 0.1% bromophenol blue, 1X TBE).
15. DNA molecular weight marker (100-bp DNA ladder, Invitrogen, cat. no. 15628-019).
16. Rat blood (use a fresh heparinized suspension) (CEMIB—UNICAMP).
17. 1X Phosphate-buffered saline (PBS): 137 m*M,* 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.4 m*M* KH_2PO_4, pH 7.3.
18. Vero cells (CCL81, American Type Culture Collection, Rockville, MD).

Table 1
Primer Sequences Used for PCR Amplification

Primer set	Target gene	Sequence (5′ to 3′)	Reference
Hem PF	*aer*	CCGGAAGATGAACCAGAATAAGAG	***(5)***
Hem PR		CTTGTCGCCACATACCTCCTGGCC	
Era-A1	*aer*A	GCCTGAGCGAGA AGGT	***(6)***
Era-A2		CAGTCCCACCCACTTC	
HlyA H1	*hly*A	GGCCGGTGGCCCGAAGATACGGG	***(6)***
HlyA H2		GGCGGCGCCGGACGAGACGGG	

19. Eagle's minimum essential medium (EMEM) (store at 4°C).
20. Fetal calf serum (store at –20°C).
21. 0.25% Trypsin/ 0.02% EDTA (store at –20°C).
22. 100,000 U of penicillin/L and 10 mg of streptomycin/L (store at –20°C).
23. 70% Ethanol in water **(prepare immediately before use).**
24. EMEM containing 10% fetal calf serum, 1000 U/mL of penicillin, and 250 µg/mL of streptomycin (Sigma).
25. Programmable thermal cycler.
26. Gel electrophoresis equipment.
27. Electrophoresis documentation and analysis system software.
28. Personal protective equipment (sterile gloves, laboratory coat, safety visor).
29. Water bath set to appropriate temperature.
30. Microbiological safety cabinet at appropriate containment level.
31. CO_2 incubator.
32. Pipets.

3. Methods

The methods described below outline (1) bacterial DNA preparation, (2) PCR, and (3) toxin characterization.

3.1. Bacterial DNA Preparation

Chromosomal DNA preparation from bacterial strains is done as described in **Subheadings 3.1.1.** and **3.1.2.**

3.1.1. Bacterial Strains and Growth Conditions

Bacterial strains are stored in 10% skimmed milk (Difco Laboratories, Detroit, MI) containing 10% glycerol at –70°C. An initial culture is grown aerobically in 3 mL of Standard II Nahr-Bouillon broth (Merck, Germany) by incubation at 28 to 30°C for 18 to 20 h. The bacterial growth obtained is trans-

ferred to agar plates containing 20 mL Standard II Nahr-Bouillon with the addition of 1% agar-agar in order to isolate the colonies. The plates are incubated aerobically for another 18 to 20 h at 28 to 30°C.

3.1.2. DNA Preparation

This procedure describes a rapid method for preparing bacterial DNA ***(3)***.

1. Collect three to five bacterial colonies from culture plates (*see* **Note 1**).
2. Suspend these colonies carefully in microcentrifuge tubes containing 900 µL of ultrapure, sterile distilled water.
3. Gently vortex the tubes for 10 s to ensure that the cell suspension is homogenous.
4. Place the tubes in a 100°C water bath for 10 min to lyse the cell membrane and release the DNA. At this temperature, the DNA filaments will also separate. After 10 min, immediately transfer the tubes to an ice-water bath in order to keep the filaments apart.
5. The PCR assays can be done immediately after this last step or the tubes can be stored at –20°C.

The DNA samples may be stored for up to 1 wk before the PCR.

3.2. PCR

This section outlines the methods used to amplify the hemolysin genes that may be present in bacterial DNA samples.

3.2.1. Primer Selection

Table 2 shows the target genes and the genome position of the primers analyzed by BLAST searches (http://www.ncbi.nlm.nih.gov/blast), and **Table 1** shows the oligonucleotide sequences of the primers that can be used to detect hemolysin/enterotoxin genes in *Aeromonas* spp. The lyophilized primers may be purchased from Invitrogen and are diluted in ultrapure, sterile distilled water to a final concentration of 100 µ*M*.

3.2.2. DNA Amplification

1. Thaw the bacterial DNA preparations at room temperature and centrifuge the tubes at 14,000*g* for 15 s to precipitate cellular debris. The DNA will be in the supernatant.
2. Prepare the PCR master mix (final volume, 45 µL) in a thin reaction tube:

Ultrapure, sterile distilled water	31 µL
10X PCR buffer	5 µL
50 m*M* $MgCl_2$	2 µL
100 m*M* solution of four dNTPs	4 µL
100 µ*M* of forward primer	0.5 µL

Table 2
Primers, Target Genes, and PCR Products

Primer	GeneBank accession number	Gene	Genome position	PCR product	Reference
Hem PF	M84709	*A. hydrophila* cytolytic	568–591	451 bp	***(1)***
Hem PR		enterotoxin *(Aer)*	1018–995		
AerA A1	M84709	*A. hydrophila* cytolytic	1653–1668	418 bp	***(1)***
AerA A2		enterotoxin *(Aer)*	2070–2056		
AerA A1	AF410466	*A. hydrophila* hemolysin	1165–1180	418 bp	***(7)***
AerA A2		(*Aer*A)	1582–1568		
HlyA H1	U81555	*A. hydrophila* hemolysin	2028–2006	595 bp	***(8)***
HlyA H2		(*hly*A)	1434–1454		

Table 3
PCR Cycles for Each Set of Primers

	Primer set		
Cycles	Hem PF–PR	*Aer*A A1–A2[a]	*Hly*A H1–H2[a]
Denaturing	95°C for 1 min	94°C for 30 s	94°C for 30 s
Annealing	55°C for 1 min	52°C for 30 s	62°C for 30 s
Extension	72°C for 1 min	72°C for 2 min	72°C for 2 min
	30 cycles	35 cycles	35 cycles

[a]After the last cycle, extend for an additional 1 min at 72°C (for the primer pairs *Aer*A A1–A2 and *Hly*A H1–H2).

100 µ*M* of reverse primer	0.5 µL
0.5 units of *Taq* polymerase/µL	2 µL

All reagents and the master mix should be kept in an ice bath to avoid degradation.

3. Add 5 µL of the DNA sample to each tube of the master mix (*see* **Notes 2** and **3**).
4. Add to the tubes a drop of nuclease-free light mineral oil. The addition of mineral oil is necessary only if the thermal cycler used does not have a heated lid to prevent the formation of condensation.
5. Place the tubes in the thermoblock and program the thermal cycler according to the primer used. The cycles for each set of primers are shown in **Table 3.**
6. Following the PCR, store the samples at 4°C until the electrophoretic analysis.

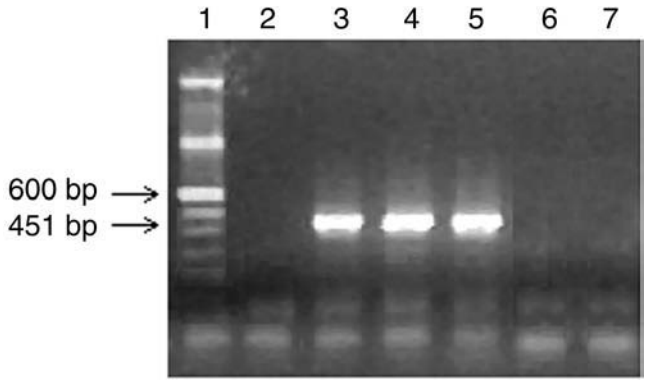

Fig. 1. Detection of the 451-bp amplicon for the primer pair Hem PF-PR after PCR. Lane 1, molecular weight markers (100 bp); lane 2, control reaction (all reagents except DNA); lane 3, *A. hydrophila* AH7; lane 4, *A. hydrophila* ATCC7966; lane 5, *A. caviae* AC28; lane 6, *A. caviae* AC41; lane 7, *A. caviae* ATCC15468.

3.2.3. Analysis of PCR Products

The DNA amplicons from the PCR assays are visualized after electrophoresis in agarose gels. The bands are visualized under ultraviolet (UV) light to detect ethidium bromide fluorescence.

1. Prepare a solution of ultrapure agarose (1%) in 1X TBE electrophoresis buffer. Heat the mixture in a microwave oven or boiling water bath until the agarose dissolves. Allow the mixture to cool to 55°C in a water bath. When the molten gel has cooled, ethidium bromide can be added to a final concentration of 0.5 µg/mL (*see* **Note 4**).
2. Pour the agarose solution onto the gel casting platform and immediately insert the gel comb. After polymerization of the gel, remove the comb and place the gel in the electrophoresis tank containing sufficient 1X TBE electrophoresis buffer to cover the gel.
3. Mix the samples of 45 µL DNA amplicon with 5 µL 10X electrophoresis loading buffer and load into the wells. Be sure to include DNA molecular weight markers (100-bp DNA ladder).
4. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode. The gel is run for 60 min at 10 V/cm. After this time, the bromophenol blue dye in the loading buffer should have migrated a sufficient distance to separate the DNA fragments.
5. Turn off the power supply and remove the gel from its platform. Wash the gel apparatus with water and rinse it with distilled water.

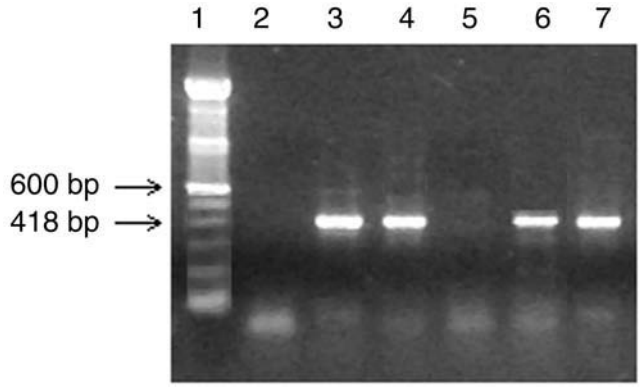

Fig. 2. Detection of the 418-bp amplicon for the primer pair AerA A1–A2 after PCR. Lane 1, molecular weight markers (100 bp); lane 2, control reaction (all reagents except DNA); lane 3, *A. hydrophila* AH7; lane 4, *A. caviae* AC36; lane 5, *A. caviae* ATCC15468; lane 6, *A. hydrophila* ATCC7966; lane 7, A. caviae AC37.

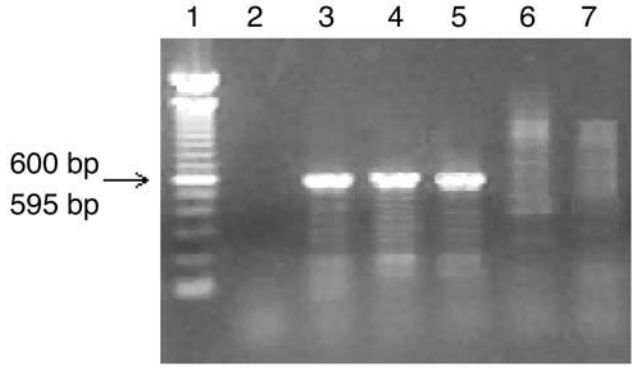

Fig. 3. Detection of the 595-bp amplicon for the primer pair HlyA H1–H2 after PCR. Lane 1, molecular weight markers (100 bp); lane 2, control reaction (all reagents except DNA); lane 3, *A. hydrophila* AH7; lane 4, *A. caviae* AC27; lane 5, *A. hydrophila* ATCC7966; lane 6, *A. caviae* ATCC15468; lane 7, *A. hydrophila* AH15.

6. After electrophoresis, photograph the gel under UV light.
   For this step, a Kodak Digital Science 1D image analysis system can be used.
7. Estimate the PCR product size by comparison with the DNA marker.

The PCR product should have the same size as the product obtained with a positive control (*A. hydrophila* ATCC 7966) (*see* **Figs. 1–3**).

3.3. Characterization of Toxin

The following sections **(Subheadings 3.3.1.–3.3.3.)** describe how to characterize the hemolysins present in *Aeromonas* isolates. The steps described include: (1) toxin preparation, (2) the assay for hemolytic activity, and (3) the test for cytotoxicity in cultured cells.

3.3.1. Toxin Preparation

Aeromonas spp. strains are cultured in 10 mL of trypticase soy broth (TSB) at 37°C for 18 h, with shaking at 110 rpm. The cultures are subsequently centrifuged (10,000*g,* 10 min, 4°C) and the supernatants are filtered through 0.2-μm filters ***(9)***.

The culture filtrates can stored at –20°C until the next step.

3.3.2. Hemolytic Activity

1. Wash rat erythrocytes with PBS, pH 7.4, and resuspend in this solution to a final concentration of 1% (v/v) (*see* **Notes 5** and **6**).
2. Prepare serial dilutions of supernatant cultures with PBS in a 96-well microtiter plate.
3. Add 50 μL of culture supernatant to an equal volume of rat erythrocyte suspension prepared in **step 1.**
4. Incubate the cells for 1 h at 37°C with gentle rocking.
5. Remove unlysed cells and membranes by centrifugation for 1 min.
6. Determine the amount of hemoglobin released based on the absorbance at 405 nm. The hemolytic activity is defined as [(A_{405} for the supernatant culture – A_{405} for the control without hemolysin) – 100/(A_{405} for total lysis caused by SDS – A_{405} for the control without hemolysin)] ***(2)***.

3.3.3. Cytotoxicity Assay in Cultured Cells

Aeromonas β-hemolysins cause damage to various types of mammalian cells ***(10)***. The cytotoxicity of hemolysins from culture filtrates of *Aeromonas* spp. provides an important means for assessing the potential toxicity of these virulence factors.

1. Place 5 mL of complete EMEM containing 10% fetal calf serum and 1% penicillin/streptomycin antibiotics into a new flask.
2. Count the Vero cells using a hemacytometer designed for tissue culture cells (*see* **Note 7**).
3. Each cell in a small square is equivalent to 10^4 cells/mL.
4. Seed 1–2 × 10^6 Vero cells into a new 25-cm^2 flask. Incubate at 37°C in a 5% CO_2 atmosphere. Feed the cells every 2 d with complete medium until they reach confluency.
5. Remove the medium and trypsinize the confluent monolayer of Vero cells (*see* **Note 8**).

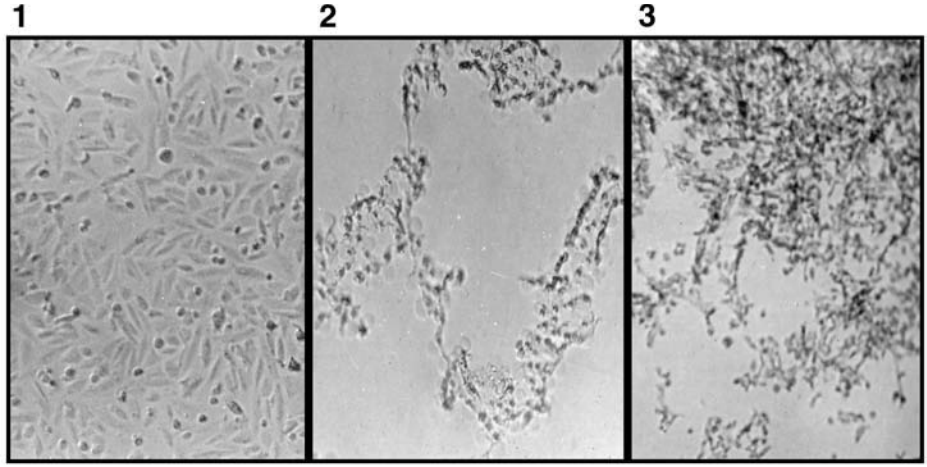

Fig. 4. Photographs showing the cytotoxicity of *A. hydrophila* culture filtrates on Vero cells. 1. Control culture showing Vero cells treated with a nonhemolytic *A. hydrophila* AH69 strain. 2. Vero cells treated with the culture filtrate of *A. hydrophila* ATCC7966 showing intracellular and morphological alterations. 3. Severe cell damage indicating cell death. Original magnification ×135.

6. Count the cells and seed 96-well tissue culture dishes with 1×10^5 cells/well in complete EMEM (*see* **step 1**).
7. Incubate the plate overnight in a CO_2 incubator at 37°C until confluency is reached.
8. Prepare nine twofold serial dilutions of the culture supernatant in fresh EMEM.
9. Remove the medium from the Vero cells and pipet the cells into duplicate wells using 100 µL of supernatant culture dilutions (*see* **step 6**).
10. Place the cells in a CO_2 incubator at 37°C for 48 h.
11. Inspect the plates daily and determine the cytoxicity titer. View the cultures using an inverted microscope to assess the degree of damage to the cells induced by the *Aeromonas* hemolysin (*see* **Fig. 4**).

4. Notes

1. Be sure that you have a pure *Aeromonas* spp. culture in order to avoid cross-contamination that could interfere with your results.
2. Use separate pipet tips for all additions and be careful not to cross-contaminate the samples. To avoid contaminating your reagents with DNA, prepare the PCR master mix in a special DNA-free chamber.
3. To facilitate the optimization and validation of the PCR, each reaction set must include the positive control *A. hydrophila* ATCC7966 and the negative control *A. caviae* ATCC15468. A control reaction in which the template DNA is omitted should always be done to confirm the absence of contamination.

4. **Caution:** Ethidium bromide is a potential carcinogen. Wear gloves when handling.
5. Although sheep, bovine, chicken, guinea pig, and horse erythrocytes also have been used in this assay, rat and rabbit erythrocytes are the most sensitive to *Aeromonas* species.
6. When doing the hemolytic assay, be sure to wear plastic gloves and a lab coat. Blood products are screened for a number of disease agents, but they should be handled as if they contained pathogenic agents. Do not forget to place all used materials in decontamination pans.
7. Personal protective equipment should be worn to prevent the contamination of cell cultures. The safety cabinet should be cleaned with 70% ethanol before and after use.
8. Although most cells will detach in the presence of trypsin alone, EDTA is added to enhance the activity of the enzyme. Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the cell monolayers with PBS.

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2

Detection and Purification of *Bacillus cereus* Enterotoxins

Toril Lindbäck and Per Einar Granum

Summary

Bacillus cereus causes two types of food poisoning, emetic and diarrheal. The emetic disease is caused by a small cyclic polypeptide (cereulide), and the diarrheal disease is caused by three different enterotoxins. Commercially available kits are used for detection of two of the enterotoxins. The enterotoxins are secreted by *B. cereus* in the early stationary phase and can be purified from the growth medium by chromatographic methods. The enterotoxins are membrane-active and the toxicity is tested on Vero cells, while the presence of the emetic toxin is detected using boar spermatozoa. Methods for detection and purification of enterotoxins are described, in addition to detection of the emetic toxin.

Key Words: *Bacillus cereus;* enterotoxins; emetic toxins; food poisoning.

1. Introduction

Bacillus cereus belongs to the taxonomically complex genus *Bacillus.* The bacteria belonging to this genus are aerobic, endospore-forming, Gram-positive rods commonly found in soil and water. The *Bacillus cereus* group comprises six separate species: *Bacillus cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis,* and *Bacillus anthracis* ***(1–3)***. The group is genetically similar but phenotypically very diverse. *B. cereus* was first recognized as a food-borne pathogen in 1949, after an outbreak of diarrheal food poisoning at a hospital in Oslo, Norway ***(4)***, and it has been isolated from a variety of foods, including rice, spices, meat, eggs, milk, and milk products ***(5,6)***.

1.1. Identification

The six species are all lecithinase-positive mannitol-negative, and V-P-positive, and are facultative anaerobes. Aerotolerance tests should be performed to rule

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Table 1
Criteria to Differentiate Between Members of *Bacillus cereus* Group

Species	Colony morphology	Hemolysis	Mobility	Susceptible to penicillin	Parasporal crystal inclusion
B. cereus	White	+	+	–	–
B. anthracis	White	–	–	+	–
B. thuringiensis	White/grey	+	+	–	+
B. mycoides	Rhizoid	(+)	–	–	–
B. weihenstephanensis	Separated from *B. cereus* by growth at <7°C and not at 43°C and can be identified rapidly using rDNA or cspA (cold shock protein A) targeted PCR ***(2).***				
B. pseudomycoides	Not distinguishable from *B. mycoides* by physiological and morphological characteristics. Clearly separable based on fatty acid composition, and 16S RNA sequences ***(3)***.				

From ref. *6.*

out anaerobic Gram-positive bacilli. BioMérieux recommends API CH50 used in conjunction with API 20E for identification of *B. cereus* ***(7)***. Criteria to differentiate among the members of the *B. cereus* group are listed in **Table 1.**

B. cereus is usually strongly β-hemolytic. *B. mycoides* are sometimes weakly β-hemolytic, with production of complete hemolysis only underneath the colonies. *B. anthracis* is usually nonhemolytic, but aging cultures may demonstrate weak γ-hemolysis. Proper precautions should be taken if a nonhemolytic colony is isolated. *B. cereus* can be differentiated from *B. anthracis* by penicillin resistance, distinct hemolysis on sheep blood agar, motility (at 35°C), rapid growth at 42°C, gelatine hydrolysis, and acid production from glucose, maltose, and salicin. Detection of the *B. anthracis* virulence genes by polymerase chain reaction (PCR) is recommended, although some strains may be negative (avirulent).

The genetic diversity of the *B. cereus* group has been studied using various methods, including multilocus enzyme electrophoresis (MEE), pulsed-field gel electrophoresis, and amplified fragment length polymorphism ***(8–10)***.

1.2. Isolation

B. cereus can be isolated from food by plating on blood agar and selective agar (*see* **Subheading 2.1.**). The selective agar contains mannitol and egg yolk medium in addition to a dye that changes color because of the lack of acid production from mannitol. Typical colonies of *B. cereus* will have a specific color

(blue or red, depending on the type of medium used), surrounded by an egg yolk reaction (lecithinase).

1.3. Sporulation

A number of Gram-positive genera—*Bacillus, Clostridium,* and *Sporosarcina*—are capable of developing dormant structures called endospores ***(11)***. These structures develop within vegetative cells and are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectants, and desiccation. With unfavorable environmental conditions, endospores can remain dormant for many years. Spores of *B. cereus* are ellipsoidal, centrally located, and do not disseminate the cells ***(12)***. Generally, *B. cereus* strains will sporulate on most agar plates after 1 to 3 d incubation at 20 to 37°C. The percentage of sporulated cells is estimated using phase-contrast microscopy (×1000).

1.4. Bacillus cereus *Toxins*

B. cereus produces toxins causing two different types of food poisoning: emetic and diarrheal syndromes ***(6)***. The diarrheal syndrome is caused by enterotoxins produced by the bacteria in the small intestine, which act on the epithelial cells, causing massive secretion of fluid into the intestinal lumen leading to diarrhea ***(13)***. *B. cereus* produces three different enterotoxins that are believed to be involved in food poisoning: hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) ***(6)***. Hbl and Nhe are both three-component enterotoxins, while CytK is composed of one single component. Hbl, originally believed to consist of one binding component, HblB, and two lytic components, $HblL_1$ and $HblL_2$, was the first *B. cereus* enterotoxin to be characterized ***(14,15)***. However, more recently, another model for the action of Hbl has been proposed, suggesting that the components of Hbl bind to target cells independently and then constitute a membrane attacking complex resulting in a colloid osmotic lysis mechanism ***(16)***. A 1:1:1 ratio of the three components seems to give the highest biological activity ***(17)***. Substantial heterogeneity has been observed in the components of Hbl, and individual strains produce various combinations of single or multiple variations of each component ***(18)***. This is probably due to multiple genes of *hbl* with sequence variation, but this must be established genetically. Hbl possesses a variety of biological effects such as dermonecrotic and vascular permeability activities, causes fluid accumulation in ligated rabbit ileal loops, and is a major contributor to *B. cereus* ocular virulence ***(18)***.

Nhe was characterized after an outbreak of food poisoning involving 152 people in Norway, caused by an *hbl*-negative strain ***(19)***. The three Nhe components, A, B, and C, differ from those of Hbl, although there are sequence sim-

ilarities. Nearly all tested *B. cereus* strains produce Nhe, while about 50% produce Hbl ***(20,21)***.

The newly discovered enterotoxin, cytotoxin K (CytK), is similar to the α-toxin of *Staphylococcus aureus* and the β-toxin of *Clostridium perfringens,* and was the cause of a severe outbreak of *B. cereus* food poisoning in France in 1998 resulting in three deaths ***(22)***. Two other enterotoxins have been proposed: enterotoxin T and enterotoxin FM ***(23,24)***. However, it was recently suggested that the *bce*T gene product does not possess biological activity and cannot contribute to food-borne diseases ***(25)***, and seems to be a cloning artifact ***(26)***. Nothing is known about the role of enterotoxin FM, but it has sequence homology to a cell wall hydrolase from *B. subtilis* ***(27)***, and is probably not an enterotoxin.

The emetic syndrome is caused by a cyclic dodecadepsipeptide, cereulide ***(28)***, which is heat-stable and resistant to proteolysis and extreme pH ***(29)***. The toxin is produced in food during vegetative growth, and after the toxin has been produced, no treatment can destroy this stable molecule, including stomach acid and the proteolytic enzymes of the intestinal tract ***(6,29)***. After release from the stomach into the duodenum, cereulide is bound to a 5-HT_3 receptor ***(30)***, and stimulation of the vagus afferent causes emesis (vomiting).

In addition to the enterotoxins and the emetic toxin, *B. cereus* produces a number of other membrane-damaging virulence factors. *B. cereus* produces at least three different phospholipase C proteins ***(31,32)***. Two of these, a sphingomyelinase and a phosphatidylcholin hydrolase, comprise the hemolysin cereolysin AB ***(31)***. Due to the presence of Ca^{2+} in the intestinal tract, phospholipase C is regarded as harmless to epithelial cells. In addition, three more hemolysins have been described ***(33–35)***.

2. Materials

2.1. Identification and Growth

1. *B. cereus* selective agar: *Bacillus cereus* selective agar base with *B. cereus* Selective Supplement from Oxoid, UK (blue colonies) or Bacto MYP Agar with Bacto Antimicrobic Vial P from Difco Laboratories, USA (pink colonies).
2. Blood agar plates: 7% bovine citrate blood in agar.
3. Brain heart infusion broth (BHI) (Oxoid, UK).
4. API CH50 and API 20E (BioMérieux, France).

2.2. DNA Isolation

1. SET buffer: 75 m*M* NaCl, 25 m*M* EDTA, 20 m*M* Tris-HCl, pH 7.5.
2. Lysozyme (lyophilized powder from chicken egg white, Sigma) in SET buffer.
3. RNase (Sigma).
4. Proteinase K (Sigma).

5. Chloroform, isoamylalcohol, NaCl, isopropanol (Merck).
6. Lauryl sulfate (SDS)(Sigma).

2.3. Detection of Enterotoxins

1. Bacillus Diarrhoeal Enterotoxin Visual Immunoassay (TECRA International Pty Ltd, Australia).
2. BCET-RPLA Toxin Detection Kit (Oxoid).
3. V-well microtiter plates (Greiner).

2.4. Purification of Enterotoxins

1. CGY: 2% casein hydrolysate (Merck), 0.4% glucose, 0.6% yeast extract, 0.2% $(NH_4)_2SO_4$, 1.8% K_2HPO_4, 0.2% KH_2PO_4, 0.1% sodium citrate, and 0.2% $MgSO_4$.
2. DEAE Sephacel (Amersham Biosciences, UK).
3. XK 16/20 columns (Amersham Biosciences).
4. Bio-Gel HT Hydoxyapatite, hydrated (Bio-Rad).
5. Resource Q column (1 mL) (Amersham Biosciences).
6. Gradient mixer GM-1 (Amersham Biosciences).
7. Fraction collector FRAC-100 (Amersham Biosciences).
8. Peristaltic pump P1 (Amersham Biosciences).
9. Bis-Tris/HCl (Sigma).
10. Triethanolaminhydrochloride (Merck).

2.5. Cell Culture and Toxicity Test

1. Vero cells: Vero C 1008 (vero 76 cloneE6) ATCC number: CRL-1586.
2. Minimum essential medium (MEM), with Earle's salts, with L-glutamine (Gibco, UK). The medium is supplemented with 5% fetal calf serum and 1X penicillin/streptomycin (cat. no. P11-010, PAA Laboratories Ltd., UK).
3. Low-leucine medium: MEM Powder cat. no. 074-90494 (made on specification by Gibco). The box (91.6 g) is dissolved in 4 L H_2O. Add 100 mL 200 m*M* L-glutamine (cat. no. M11-004, PAA Laboratories) and 400 mL 0.5 *M* HEPES buffer (pH 7.7), and adjust to 10 L with H_2O. Sterilize by filtration in 500-mL bottles (pH 7.4–7.5).
4. Trichloroacetic acid (Merck)
5. Tissue Culture Plate, 24 well (Falcon, France).
6. L-[U-^{14}C]Leucine, >300 mCi/mmol (Amersham Biosciences).
7. Scintillation cocktail (Ultima Gold, Packard BioScience, US).
8. Scintillation counter.

2.6. Testing for Emetic Activity

1. Boar spermatozoa: Porcine AI company in each country can supply boar spermatozoa.
2. Microscope (×1000) with heating block to keep the temperature at 37°C.

3. Methods

3.1. Isolation of DNA From B. cereus

This is a quick method for isolation of genomic DNA from Gram-positive bacteria *(36)*. The DNA is suitable for cloning and PCR.

1. Grow bacteria at 37°C overnight in BHI.
2. Centrifuge 3.0 mL culture to pellet the cells.
3. Resuspend the cell pellet in 495 µL SET-buffer.
4. Add 50 µL freshly made lysozyme (10 mg/mL) and 10 µL RNase (10 µg/mL), and incubate with occasional inversion for 1 h at 37°C.
5. Add 50 µL 10% SDS and 5 µL proteinase K (25 mg/mL), and incubate for 2 h at 55°C.
6. Add 200 µL 5 *M* NaCl and 700 µL chloroform:isoamylalcohol (24:1) and incubate at room temperature with frequent inversions for 30 min.
7. Centrifuge for 30 min at 4500*g* and transfer the aqueous phase to a fresh tube.
8. Precipitate the DNA with an equal volume of isopropanol by centrifugation for 10 min at maximum speed in a tabletop centrifuge. Wash the precipitate with 70% ethanol. Let the pellet air-dry.
9. Resuspend the DNA in 100 µL H_2O.

3.2. Detection of Genes Encoding Enterotoxins

PCR is used for detection of genes encoding the *B. cereus* enterotoxins. At time of writing, the nucleotide sequence of three *B. cereus* strains and five *B. anthracis* strain genomes are available in public genomic databases (Genbank, EMBL DDBJ). The available nucleotide sequences are used to produce specific primers to identify the genes encoding the enterotoxins in other strains. Standard PCR programs—e.g., 95°C for 1 min, 30 cycles of 95°C for 1 min, 48–52°C for 1 min (annealing temperature according to the specific primers) and 72°C for 1 min, followed by a final extension step of 72°C for 7 min—are used to amplify the toxin genes. For both Nhe and Hbl, three different PCR reactions are necessary to ensure the presence of all three genes **(Note 1)**.

3.3. Detection of Enterotoxins

Two different immunological tests, from Oxoid (UK) and TECRA (Australia), are commercially available for detection of the enterotoxins Hbl and Nhe of *B. cereus* (*see* **Note 2**). The kit from Oxoid uses antibodies reacting with the L_2 component of Hbl, while the kit produced by TECRA, *Bacillus* diarrheal enterotoxin (BDE) visual immunoassay (VIA), detects NheA *(37,38)*.

For detection of enterotoxins using the TECRA kit, the bacteria should be cultured in BHI broth with 1% glucose for 6 to 8 h at 32°C with shaking. Cells are removed by centrifugation and the culture supernatants are added to wells coated with high-affinity antibodies against NheA. Captured enterotoxins are

detected with conjugate (enzyme-labeled antibodies) converting a colorless substrate into green.

The BCET-RPLA detection kit from Oxoid uses polystyrene latex particles sensitized with purified antiserum taken from rabbits immunized with purified *B. cereus* diarrheal enterotoxin. The test is performed in V-well microtiter plates. Dilutions of food extract or culture supernatants are made in wells and the latex particle suspension is added to each well. If *B. cereus* Nhe enterotoxin is present, agglutination occurs due to the formation of a lattice structure. After settling, this forms a diffuse layer on the base of the well. If *B. cereus* enterotoxin is absent or is at a concentration below the assay detection level, no such lattice structure can be formed, and a tight button will be observed. There is, at the time of this writing, no kit available for the detection of CytK.

3.4. Purification of Enterotoxins

The culture medium used for purification of enterotoxins is a modification of CGY medium ***(15,39)*** (*see* **Notes 3** and **4**).

1. 100 mL *B. cereus* overnight culture is used to inoculate 2 L CGY. The culture is grown with shaking at 32°C (*see* **Note 5**) for 6 to 7 h.
2. Extracellular proteins are separated from cells by centrifugation (10,000*g* at 4°C for 20 min).
3. The supernatant is concentrated by precipitation with 70% saturated $(NH_4)_2SO_4$ overnight at 4°C with mixing. The precipitated proteins are pelleted by centrifugation at 10,000*g* for 20 min at 4°C. The pellet is then resuspended in 25 mL H_2O, and dialyzed at 4°C against 25 m*M* Bis-Tris-HCl (pH 5.9)/1 m*M* EDTA.
4. The concentrated protein solution is applied to DEAE-Sephacel packed in a 1.6-cm diameter column (10 cm high) with peristaltic pump.
5. Proteins are eluted with a linear gradient of 0–0.5*M* NaCl in 25 m*M* Bis-Tris-HCl (pH 5.9) in 20 fractions over 200 mL.

When purifying Hbl and CytK, the fractions can be tested for hemolytic activity, while in purifying Nhe, the fractions must be tested for cytotoxic activity in a Vero cell assay. In addition, fractions can be visualized on silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Different Nhe and Hbl components will appear in different fractions, so combinations of fractions must be tested to obtain hemolytic or cytotoxic activity. When using a DEAE column to purify Nhe, NheB will elute at 25–75 m*M* NaCl, while NheA will elute at 200–300 m*M* NaCl. NheC has never been purified directly from *B. cereus* culture supernatant, probably because it interacts with either NheA or NheB (*see* **Note 6**). NheC is also produced in small amounts (1/10) compared to the production of NheB.

6. Following DEAE, selected fractions are pooled and applied directly to a column (1.6 cm diameter, 6 cm height) of Bio-Gel HT hydroxyapatite, equilibrated with 10 m*M* NaCl.
7. Proteins are eluted with a linear gradient of sodium phosphate buffer (pH 6.8) from 0 to 0.24 *M* in 20 fractions over 100 mL in 10 m*M* NaCl.
8. Selected fractions are dialyzed overnight at 4°C against 20 m*M* triethanolamine, containing 1 m*M* EDTA. The pH of the buffer is 8.1 for fractions containing NheB and CytK, and 7.8 for fractions containing the other proteins.
9. The dialyzed fraction is applied to a Resource Q column.
10. Proteins are eluted with a linear gradient of NaCl from 0 to 0.5 *M* in 20 fractions over 40 mL in 20 m*M* triethanolamine.

3.5. Test for Hemolytic Activity

Add 10 µL of the sample and 100 µl 2% bovine citrate blood (in 0.9% NaCl) to each well of a microtiter plate, and observe. No hemolytic activity will result in a tight button of blood cells at the bottom of the well.

3.6. Toxicity Test Using Vero Cells

Vero cells are grown in MEM medium supplemented with 5% fetal calf serum. Cells are seeded into 24-well plates 2 to 3 d before testing. Before use, check that the growth of the Vero cells is confluent. If so, remove the medium and wash the cells once with 1 mL preheated (37°C) MEM medium.

1. Add 1 mL preheated (37°C) low-leucine medium to each well and then add the toxin to be tested (max 100 µL).
2. Incubate the cells for 2 h at 37°C.
3. Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37°C) low-leucine medium. Mix 8 mL (enough for 24 wells) preheated low-leucine with 16 µL ^{14}C-leucine and add 300 µL of this mixture to each well.
4. Incubate the cells for 1 h at 37°C.
5. Remove the radioactive medium and add 1 mL 5% trichloroacetic acid (TCA) to each well, and incubate at room temperature for 10 min.
6. Remove the TCA, and wash the wells twice with 1 mL 5% TCA.
7. After removing the TCA, add 300 µL 0.1 *M* KOH and incubate at room temperature for 10 min. Transfer the content of each well to liquid scintillation tubes with 2 mL liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 min.
8. Percentage inhibition of protein synthesis is calculated using the following formula (*see* **Note 7**):

$$[(\text{Neg. ctrl} - \text{sample}) / \text{Neg. ctrl}] \times 100$$

The negative control is Vero cells from wells without addition of sample.

3.7. Testing for Emetic Activity

Boar sperm motility is inhibited by exposure to cereulide (emetic toxin), and boar sperm is useful for detecting cereulide concentrations toxic to humans. The threshold concentration of cereluide provoking visible damage in boar sperm in vitro is 2 ng cereulide/mL boar sperm ***(40)***. Exact concentration of the emetic toxin can be measured by LC-MS ***(41)***.

1. Spread the bacteria on an agar plate and incubate 1 to 3 d at 22°C.
2. Pick three colonies and dissolve in 200 µL methanol (use glass equipment with tight capsule).
3. Boil for 10 min in a water bath, and cool to room temperature.
4. Preheat boar sperm, pipet tips, and microscope slides to 37°C.
5. Add 5 to 10 µL cooled extract to 200 µL boar sperm.
6. Incubate for 10 min at 37°C.
7. The motility of the exposed sperm cells is estimated using phase-contrast microscopy at 37°C.

4. Notes

1. All three genes encoding the three components of Nhe or Hbl must be present for production of active enterotoxins. Even so, with positive PCR results there might be strains that are enterotoxic-negative resulting from lack of or mutation in the PlcR regulator, or mutation in the toxin genes.
2. The two commercially available immunological kits for enterotoxin detection test for only one out of three components in the enterotoxin complex, while all three components must be present for biological activity. A positive TECRA or Oxoid test does not necessarily mean that active enterotoxin is produced.
3. When purifying proteins from culture supernatants, the use of a strain producing only one of the enterotoxins is highly preferable. The properties of the toxin components in the two different three-component enterotoxin complexes are similar so they will copurify in most cases. Positive strain, NVH 0075-95, produces exclusively Nhe and NVH 0391-98 produces exclusively CytK and may be requested from the authors.
4. For purification of entertoxins from culture supernatant, CGY is chosen, as CGY contains fewer large proteins than BHI.
5. Expression profiles of the enterotoxins will vary for each strain at different growth temperatures. The growth temperature for optimal enterotoxin expression has to be established for each strain.
6. Small amounts of NheC will often be purified together with NheA and NheB, as NheC seems to be associated with NheA and NheB in the culture supernatant. To obtain NheA and NheB absolutely pure of NheC, they should be expressed recombinantly.
7. The correlation between percentage inhibition of protein synthesis in Vero cells and concentration of toxin are linear in the range from about 30 to 75%, so minimum or maximum toxicity measurements should be kept within this range.

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3

Campylobacter

Isolation, Identification, and Preservation

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Summary

Globally *Campylobacter* has been recognized as a leading cause of human gastroenteritis, generating considerable interest in the development of special selective techniques for optimal growth, isolation, and preservation of *Campylobacter* from clinical and environmental sources. *Campylobacter* is a microaerophilic micro-organism sensitive to natural levels of oxygen found in the environment, thus requiring specific conditions for growth. The methods described herein apply a microaerophilic environment complemented with supplements such as blood, charcoal, and ferrous sulfate, sodium metabisulfate, and sodium pyruvate (FBP), which are thought to act by quenching toxic oxygen derivatives that develop over time in the media. Biotyping is the establishment of a characteristic biochemical pattern and is a simple but comprehensive method for identification of *Campylobacter.* The BioMérieux API Campy system is employed as the biotyping method of choice in the procedures described in this chapter. Three methods for long-term preservation of *Campylobacter* are described herein: (1) FBP medium, (2) 15% glycerol, and (3) Cryobank Microbial Preservation System using defibrinated lysed horse blood and glass beads.

Key Words: *Campylobacter;* isolation; identification; biotyping; long-term preservation; cryopreservation; FBP.

1. Introduction

Campylobacter can cause a wide spectrum of infections including diarrheal disease, reproductive disorders in domestic animals, and opportunistic infections in humans ***(1,2)***; it has also been suggested to play a role in the initiation of Guillain-Barré syndrome ***(2,3)***. Globally *Campylobacter* has been recognized as a leading cause of human gastroenteritis ***(4–7)***. This worldwide recognition has generated considerable interest in the development of special selective techniques for optimal growth and isolation of *Campylobacter* from clinical and environmental sources ***(1,8–10)***.

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A major drawback with isolation and long-term storage of *Campylobacter* is that some 72 h after primary isolation, this micro-organism can become viable but nonculturable (VBNC) ***(11)***. When it enters into this dormant phase, the organism degenerates into a nonmotile coccoid form ***(12)***; this degenerating property of *Campylobacter* makes its isolation and preservation difficult in the laboratory ***(13)***. Sensitivity to oxygen is another problem during isolation and storage of *Campylobacter.* Toxic oxygen derivatives such as superoxide anions, hydroxyl radicals, singlet oxygen, and hydrogen peroxide are detrimental to *Campylobacter* ***(14–16)***, and are formed by the reduction of oxygen during cellular metabolism, auto-oxidation, or photochemical oxidation in the environment ***(15)***. Supplements such as blood, charcoal, and ferrous sulfate, sodium metabisulfate, and sodium pyruvate (FBP) are thought to act by quenching these toxic oxygen derivatives that develop over time in the media ***(14,15)***.

The following procedures summarize selective techniques for the isolation of *Campylobacter* using selective and differential media, identification by biotyping, and successful long-term storage techniques for *Campylobacter* spp., based on a comprehensive study of long-term preservation of *Campylobacter jejuni* ***(17)***.

2. Materials

1. Columbia agar base (Oxoid, Basingstokes, UK).
2. Defibrinated lysed horse blood (Unitech, Dublin, Ireland).
3. Campy*Gen*™ Microaerophilic sachets (Oxoid).
4. Anaerobic jar (Oxoid).
5. Nutrient Broth no. 2 (Oxoid).
6. Modified Preston *Campylobacter* selective supplement (Oxoid).
7. FBP *Campylobacter* growth supplement: 0.025% ferrous sulfate (w/v), 0.025% sodium metabisulfite (w/v) 0.025% sodium pyruvate (w/v) (Oxoid).
8. Stomacher Seward 400 (Seward, London, UK).
9. Stomacher Lab system bags (Seward).
10. Cefoperzone charcoal desoxycholate agar (CCDA) (Oxoid).
11. CCDA selective supplement (Oxoid).
12. Hydrogen peroxide (H_2O_2) (BDH, Poole, England).
13. API CAMPY (BioMérieux, Marcy l'Etoile, France).
14. API CAMPY reagents: NIT 1; NIT 2; Fast Blue (FB); Ninhydrin (NIN) (BioMérieux).
15. Bacteriological agar (Oxoid).
16. Yeast extract (Oxoid).
17. Glycerol (R.B. Chemicals, Tallaght, Dublin, Ireland).
18. Cryobank Microbial Preservation System (MAST Diagnostics, Merseyside, UK).

2.1. Preparation of Columbia Blood Agar

1. Columbia blood agar (CBA) is freshly prepared with deionized water in accordance with manufacturer's instructions.

2. Following autoclaving the medium is allowed to cool to approx 65°C and supplemented with 5% (v/v) defibrinated lysed horse blood.
3. All plates must be allowed to solidify before use.
4. Store plates in a light-proof container and use either on the day of plating or the next day (*see* **Note 1**).

2.2. Preparation of Preston Enrichment Broth

1. Nutrient Broth no. 2 is freshly prepared with deionized water in accordance with manufacturer's instructions.
2. Following autoclaving the broth is allowed to cool to approx 65°C and supplemented with modified Preston *Campylobacter* selective supplement, FBP *Campylobacter* growth supplement, and 5% (v/v) defibrinated horse blood.
3. Use freshly prepared broth either on the day of plating or the next day (*see* **Note 1**).

3. Methods

3.1. Determination of Campylobacter *spp.*

As noted previously, *Campylobacter* is a fastidious micro-organism; thus, a few techniques must first be highlighted prior to isolation of this bacterium.

Columbia blood agar (CBA) is used frequently for culturing *Campylobacter,* as blood is an undefined medium containing iron and detoxifying enzymes such as catalase, peroxidase, and superoxide dismutase, which have been shown to reduce toxicity of media ***(15)***. Blood has been recognized as an excellent supplement in the growth and recovery of *Campylobacter* ***(14,18)***. *Campylobacter* is a microaerophilic micro-organism requiring 5% O_2, 10% CO_2, and 85% N_2 ***(19)*** and is therefore sensitive to high levels of oxygen ***(8,13)***, thus requiring appropriate conditions for incubation.

3.2. Appropriate Conditions for Incubation

1. Incubation is carried out at either 37°C or 42°C (*see* individual methods) for 48 h under microaerophilic conditions.
2. Microaerophilic conditions are achieved by placing samples in an anaerobic jar and adding a Campy*Gen* microaerophilic sachet before placing on the lid.

3.3. Sample Collection and Preparation

Two types of sample collection are explained for maximum recovery of *Campylobacter* using selective and differential media. Samples can be collected either by using a swab, e.g., when collecting a sample of a surface area, or by collecting a whole sample, e.g., food or stool samples.

3.3.1. Swab Sample Collection

1. Using a sterile swab moistened with Preston Enrichment Broth (PEB), swab an area of approx 50 cm^2.

2. Replace the swab into a sterile universal tube containing 10 mL of PEB.
3. Analyze for the presence of *Campylobacter* as described in **Subheading 3.4.**

3.3.2. Whole-Sample Collection

1. Aseptically transfer 25 g of sample into 225 mL of PEB in a stomacher bag and stomach (Seward Stomacher 400) for 2 min on full power.
2. Samples are then analyzed for the presence of *Campylobacter* as described in **Subheading. 3.4.**

3.4. *Isolation of* Campylobacter

1. PEB, containing the swab/whole sample, is incubated at 42°C ± 1°C under microaerophilic conditions for 18 to 24 h.
2. Following incubation the PEB culture is subcultured onto modified Cefoperazone charcoal deoxycholate agar (CCDA) supplemented with CCDA selective supplement and incubated at 42°C ± 1°C in a microaerophilic atmosphere for 48 h ± 3 h.
3. Characteristic colonies (*see* **Note 2**) are subcultured onto CBA plates **(Subheading 2.1.)** that are incubated at 42°C ± 1°C under microaerophilic conditions for 48 h ± 3 h.
4. Presumptive *Campylobacter* species are tested for catalase **(Subheading 3.5.1.)** and confirmed by biochemical identification using an API CAMPY biotyping identification system.

3.5. Campylobacter *spp. Identification*

3.5.1. Catalase Test

Some bacteria can reduce diatomic oxygen to hydrogen peroxide or superoxide, both of which are toxic to bacteria. *Campylobacter,* however, possess a defense mechanism in which the enzyme catalase catalyzes the conversion of hydrogen peroxide and superoxide into diatomic oxygen and water. The following is a quick, simple test to determine the presence of catalase ***(20)***.

1. Place three to four colonies from a fresh presumptive *Campylobacter* culture onto a clean glass slide.
2. Add two to three drops of H_2O_2 onto the culture.
3. The production of O_2 bubbles represents a positive catalase test; the absence of O_2 bubble formation is indicative of a negative result ***(20)***.

3.5.2. Biotyping

Biotyping is the establishment of a characteristic biochemical pattern ***(21)***. A strain exhibiting a particular biochemical pattern is termed a *biovar* or a *biotype.* The best known biotyping schemes for *Campylobacter* spp. include those of Skirrow ***(22)***, Lior ***(23)***, and Preston ***(24)*** and have been applied in various studies ***(25,26)***. Skirrow and Benjamin ***(22)*** were the first to present a *Campylobacter* biotyping scheme that includes three biochemical tests: hippurate hydrolysis

(HH), rapid hydrogen sulfide (H_2S) test, and resistance to nalidixic acid (Na), resulting in the differentiation of *Campylobacter* into three groups: *C. jejuni* (HH+; H_2S–; Na–), *C. coli* (HH–; H_2S–; Na–), and *C. lari* [HH–; H_2S–; Na+].

In 1982, based on hippurate hydrolysis, rapid H_2S test, and DNA hydrolysis, Lior further discriminated *C. jejuni* into four biotypes and *C. coli* and *C. lari* into two biotypes each ***(23)***; however, this scheme was not applicable to other clinically important strains of *Campylobacter,* e.g., *C. fetus, C. hyointestinalis,* and *C. upsaliensis* ***(27)***. A more comprehensive speciation and biotyping scheme was provided by the Preston scheme, which utilized 11 resistotyping and 4 basic biochemical tests to provide a numerical code ***(24)***. Today, commercially available kits for *Campylobacter* identification are based on this type of scheme, resulting in a numerical code, which can be entered into a database e.g., BioMérieux API CAMPY (http://biomerieux-usa.com) and the MAST ID Camp Biotyping Scheme http://www.mastgrp.com.

3.5.2.1. Biochemical Identification of *Campylobacter* using BioMérieux API CAMPY

Biochemical identification of *Campylobacter* using BioMérieux API CAMPY must be performed according to manufacturer's instructions. The BioMérieux API CAMPY strip consists of 20 microtubes containing dehydrated substances, with each microtubule corresponding to an individual test. The 20 tests are divided into two parts.

The first part is composed of enzymatic and conventional tests. The dehydrated media are reconstituted with the addition of a bacterial suspension. During incubation aerobically at 37°C for 24 h, metabolism results in color changes that are either spontaneous or revealed by the addition of reagents. Spontaneous reactions include the tests for urease, esterase, and the reduction of chloride to triphenyl tetrazolium. The reduction of nitrates requires the addition of the reagents NIT 1 & NIT 2; hippurate hydrolysis reaction required the addition of NIN reagent and γ-glutamyl transferase, pyrrolidonyl arylamidase, L-aspartate arylamidase, and alkaline phosphatase; and the production of H_2S requires the addition of the FB reagent.

The second part of the API CAMPY strip involves assimilation or inhibition tests. The bacteria grow if they are capable of utilizing the corresponding substrate that includes glucose, succinate, acetate, proprionate, malate, and citrate or if they are resistant to the antibiotics tested, which include nalidixic acid, cefazoline, and erythromycin.

On the results sheet provided the tests were separated into groups of three and a number, 1, 2, or 4, was indicated for each. The numbers corresponding to a positive reaction are added and a seven-digit numerical profile is obtained. Using the BioMérieux Analytical Profile Index software, version 3.3.3 ***(28)***, this seven-digit number corresponds to a bacterial species.

3.6. Preservation Methods

Long-term preservation of *Campylobacter* at –20°C is most successful using the FBP medium method; similarly, long-term storage in 15% glycerol is quite sucessful. Where freezing temperatures of –85°C are available, these two techniques, as well as the Cryobank Microbial Preservation System using defibrinated lysed horse blood and glass beads, are very successful long-term preservation techniques for *Campylobacter* ***(17)*** (*see* **Note 3**).

3.6.1. Preparation of FBP Medium: Nutrient Broth No. 2/Glycerol/FBP Medium

1. This medium is prepared by autoclaving Nutrient Broth No. 2, 0.12% (w/v) bacteriological agar, 15% (v/v) glycerol, and 0.1% (w/v) yeast extract.
2. The medium is cooled to approx 50°C and FBP enrichment supplement aseptically added.
3. The medium is gently mixed and 4-mL amounts dispensed aseptically into sterile 15-mL universal tubes.
4. Viable cultures of organisms are inoculated into 5 mL Nutrient Broth no. 2 and incubated at 37°C under appropriate conditions **(Subheading 3.2.)**.
5. Following incubation, 500 µL of culture broth is inoculated into each of two vials of FBP medium.
6. One vial is stored at –20°C and the other at –85°C.
7. Recovery of the organism requires complete thawing of the medium. A sterile 10-µL loop of culture is streaked onto a CBA plate, which is then incubated at 37°C under appropriate conditions **(Subheading 3.2.)**.

3.6.2. Preparation of 15% Glycerol

1. Viable cultures of organisms are inoculated into 5 mL Nutrient Broth no. 2 and incubated at 37°C under appropriate conditions **(Subheading 3.2.)**.
2. Following incubation, 850 µL of culture broth is aseptically transferred into two vials of 150 µL sterile glycerol.
3. The mixtures are emulsified by vortexing and one stored at –20°C and the other at –85°C.
4. Organisms are recovered by streaking a 10-µL loop of culture onto CBA plates and incubating at 37°C under appropriate conditions **(Subheading 3.2.)**.

3.6.3. Preparation of Cryobank Microbial Preservation System Using Defibrinated Lysed Horse Blood and Glass Beads (see ***Note 4***)

1. Remove all hypertonic cryopreservative solution from the MAST vials and replace with 750 µL lysed horse blood.
2. Viable cultures of organisms are plated on 2X CBA plates and incubated at 37°C under appropriate conditions **(Subheading 3.2.)**.
3. Following incubation the surface culture of each plate is aseptically harvested into two sterile cryogenic preservation vials.

4. The caps are replaced and the culture mixed carefully by inverting the tube to completely distribute the organism.
5. With a sterile pipet as much of the defibrinated horse blood as possible is removed from each vial and one stored at –20°C and the other at –85°C.
6. Recovery of the organism is performed by removing a single bead from the vial and streaking immediately over the surface of a CBA plate and incubating under appropriate conditions (*see* **Note 5**).

4. Notes

1. *Campylobacter* is a microaerophilic micro-organism and therefore sensitive to high levels of oxygen ***(8,19)***. Toxic oxygen derivatives can develop in the media and previous studies have found that storage conditions and the age of the media are very important for successful recovery of *Campylobacter* ***(14–16)***. Basal medium without supplements stored in light and air for just 48 h developed toxicity, and colony counts were much reduced when compared with media stored in a dark reduced atmosphere for the same period of time ***(14)***. Similar results were obtained when Brucella agar without supplements was illuminated ***(15)***. However, when FBP was added, *Campylobacter* counts were much higher, and growth at higher oxygen levels was also observed. The effects of aging on dehydrated and hydrated Brucella media found that aging greatly affected the aerotolerance and viable counts of *Campylobacter* ***(16)***. However, when 0.01% sodium bisulfite was added to the aged media, these inhibitory effects were diminished. These results indicated a need for addition of supplements to the media for successful recovery of *Campylobacter.*
2. *Campylobacter* form characteristic gray moist colonies on CCDA, *C. jejuni* are generally flat-spreading, whereas *C. coli* are slightly raised ***(29)***.
3. Long-term preservation of micro-organisms is required for storage of quality control strains, teaching, research, epidemiological purposes, and quantitative and qualitative analyses ***(7)***. Common preservation techniques, e.g., 50:50 glycerol:culture stock, are not suitable for such fastidious micro-organisms as *Campylobacter* ***(30)***. A number of published reports have described simple short-term preservation techniques for *Campylobacter* ***(18,31,32)***. However, long-term preservation of *Campylobacter* described in the literature involves liquid drying ***(30)***, liquid nitrogen, and freeze-drying ***(10,23)***. These methods require equipment not available to all scientific laboratories. The methods described here are simple, inexpensive techniques for long-term preservation of *Campylobacter* ***(17)***.
4. The MAST Cryobank Microbial Preservation System contains hypertonic cryopreservative solution and glass beads, however, for this method we have replaced the cryopreservative solution with 750 µL lysed horse blood. Due to the nature of the blood it was not possible to determine a density equivalent to McFarland 3 or 4 standard as suggested in the manufacturer's instructions; therefore, a fresh culture plate was surface-scraped from CBA plates and added to each vial.
5. The repetitive action of freeze–thawing can cause stress on the stored bacterial samples resulting in detrimental effects over time. The use of cryogenic glass beads

eliminates the effects of freeze–thawing, as an individual bead can be removed from the vial, with the remaining beads in the vial being immediately replaced under freezing conditions.

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4

Detection of *Clostridium botulinum* by Multiplex PCR in Foods and Feces

Miia Lindström, Mari Nevas, and Hannu Korkeala

Summary

Clostridium botulinum is a diverse group of anaerobic spore-forming organisms that produce lethal botulinum neurotoxins (BoNT) during their growth. BoNTs cause a paralytic condition, botulism, to man and animals. The most common forms of human botulism include the classical foodborne botulism due to ingestion of BoNT preformed in food, and infant botulism due to spore germination, growth, and toxin production in the infant's intestine. Botulism is diagnosed by detecting BoNT and/or *C. botulinum* in the patient and in suspected food samples. There are several drawbacks related to the diagnostics of botulism; the standard bioassay for toxin detection employs the use of laboratory animals, making it laborious and expensive and possessing ethical concern. Selective media for culturing the organism are not available. Neurotoxin gene-specific PCR has facilitated the detection of *C. botulinum.* In this chapter a multiplex PCR for simultaneous detection of *C. botulinum* types A, B, E, and F in foods and feces is described. The method involves sample dilution and homogenization, and two-step enrichment followed by cell wash, cell lysis, and multiplex PCR. Quantification is obtained by the most-probable-number technique. Depending on the type of sample material, the detection limit of the assay varies from 10^{-2} to 10^{3} spores per gram of sample material.

Key Words: *Clostridium botulinum;* botulism; botulinum neurotoxin; BoNT; *bot;* multiplex PCR.

1. Introduction

Clostridium botulinum is a diverse group of Gram-positive spore-forming organisms that produce botulinum neurotoxins (BoNT) during their growth. BoNTs are the most potent toxins known, and when entering human or animal tissues and subsequently blood circulation, they block neurotransmitter release from nerve endings, causing a neuroparalytic condition known as botulism. Based on their serological properties, BoNTs are classified as types A–G, with types A, B, E, and F causing disease to humans. The human pathogenic

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C. botulinum strains are divided into groups I (proteolytic and mesophilic) and II (nonproteolytic and psychrotrophic) based on their phenotype

The most common forms of human botulism include classical food-borne botulism ***(1)*** and infant botulism ***(2)***, the former being an intoxication following ingestion of BoNT preformed in food and the latter being an infection resulting from *C. botulinum* spores germinating, growing, and producing toxin in an infant's intestine. Other types of human botulism include wound botulism ***(3)*** owing to spore germination and subsequent growth and toxin formation in deep wounds; adult infectious botulism ***(4)***, a condition equal to infant botulism that may follow heavy antibiotic treatments or abdominal surgery; inhalation botulism ***(5)***; and iatrogenic botulism as a consequence of the use of botulinum toxin as a therapeutic agent ***(6)***.

The diagnosis of botulism is based on the detection of BoNT in the patient's feces or serum and in suspected food items ***(7)***. The presence of *C. botulinum* in these samples supports the diagnosis. The most reliable—and currently the only standard—method for toxin detection is the mouse bioassay, where sample extractions are injected intraperitoneally into mice ***(7)***. In the event of a positive result the mice develop typical symptoms of botulism. The toxin type is determined by seroneutralization with specific antibodies. In spite of being sensitive and specific, the method is expensive and time-consuming, and above all possesses ethical concern due to the use of laboratory animals. *C. botulinum* has traditionally been detected by demonstrating toxigenesis in a growing culture by the mouse test. The species includes a variety of physiologically diverse organisms, a common denominator of which being merely the BoNT production. Therefore there are no selective media available that would support the growth of all *C. botulinum* strains.

Neurotoxin gene *(bot)*-specific polymerase chain reaction (PCR) has provided a valuable tool in the diagnostics of botulism, and a number of reports on PCR detection of *C. botulinum* in various sample materials have been published during the last decade ***(8–11)***. However, the disadvantage of these methods is that only one of the seven toxin genes may be detected at a time, and more than one separate reaction is required to investigate a sample for the presence of all the *bot* genes. A multiplex PCR method enables the simultaneous detection of more than one *bot* gene ***(12)***. A protocol for the simultaneous detection of the human pathogenic strains of *C. botulinum* types A, B, E, and F in food and feces is described.

2. Materials

2.1. Sample Preparation and Culture

1. NaCl-peptone (0.9% NaCl, 1.0% peptone) or peptone (1.0%) water.
2. Tryptose-peptone-glucose-yeast extract (TPGY) medium ***(7)***. Before sample inoculation, the tubes containing TPGY medium are steamed in a boiling water bath for

Table 1
Oligonucleotide Primers Used in Multiplex PCR

Target gene	Primer direction	Primer sequence (5′–3′)	Expected PCR fragment size (bp)
*bot*A	Forward	AGC TAC GGA GGC AGC TAT GTT	
	Reverse	CGT ATT TGG AAA GCT GAA AAG A	782
*bot*B	Forward	CAG GAG AAG TGG AGC GAA AA	
	Reverse	CTT GCG CCT TTG TTT TCT TG	205
*bot*E	Forward	CCA AGA TTT TCA TCC GCC TA	
	Reverse	GCT ATT GAT CCA AAA CGG TGA	389
*bot*F	Forward	CGG CTT CAT TAG AGA ACG GA	
	Reverse	TAA CTC CCC TAG CCC CGT AT	543

15 min in order to remove the oxygen from the medium (*see* **Note 1**). Thereafter the tubes should be stored under anaerobic conditions (*see* **Note 2**).

3. For investigation of liquid samples: Filter membranes with pore size 0.45 µm and diameter of 47 mm (Millipore, Bedford, MA). Vacuum pump (Vacuum/Pressure Pump, Millipore) and filter holder (Analytical Stainless Steel Filter Holder, Millipore).
4. Anaerobic jars with gas generation kits (Anaerogen, Oxoid, Basingstoke, UK)/gas change facility (Anoxomat, Mart Microbiology, Lichtenvoorde, The Netherlands), or anaerobic work station.

2.2. Cell Lysis and Multiplex PCR

1. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
2. PCR-grade water (Sigma Aldrich, Poole, UK).
3. DNA polymerase (DynaZyme, Finnzymes, Espoo, Finland) with appropriate buffer (Finnzymes) (*see* **Note 3**).
4. 25 m*M* $MgCl_2$ stock solution (Roche Diagnostics Ltd., Lewes, UK).
5. Oligonucleotide primers specific for *bot*A, *bot*B, *bot*E, and *bot*F **(Table 1)**.
6. Deoxynucleoside triphosphates (dNTP) dATP, dCTP, dGTP, and dTTP (dNTP Mix, Finnzymes). Prepare small aliquots of diluted stocks for a suitable number of samples. The dNTPs should not undergo more than five freeze–thaw cycles. Always store frozen and when used, keep on ice.
7. Agarose (e.g., I.D.NA agarose, Bio Whittaker Molecular Applications, Rockland, ME).
8. Molecular weight marker (100-bp DNA Ladder, Promega, Southampton, UK) (*see* **Note 4**).
9. Ethidium bromide (EtBr) (10 mg/mL ethidium bromide solution, molecular grade, Promega). Protective gloves must be worn every time when handling this mutagenic agent (*see* **Note 5**).

10. Loading buffer: 6X Blue-Orange Loading Dye, Promega.
11. Gel electrophoresis buffer (TAE) buffer: 0.04 *M* Tris-acetate, 1.0 m*M* EDTA, pH 8.0.

3. Methods

3.1. Sample Preparation and Culture

1. *Sample collection:* Obtaining sufficient and representative samples when suspecting human botulism may prove difficult. In order to obtain a quantitative estimate of *C. botulinum* count in food and fecal samples, a minimum of 10 g of solid sample material and 10 mL of liquid material are desirable. With other types of material, such as environmental samples, a much larger sample size (100–1000 g) may be required, as *C. botulinum* is present in the environment in low numbers (*see* **Note 6**). Samples to be investigated immediately after collection should be kept at 0 to 3°C; otherwise they should be frozen until analyzed.
2. *Sample preparation:* Solid food samples may be either directly cultured into TPGY medium (see the next step), or, preferably, diluted (1:9) and homogenized in NaCl-peptone water or peptone water before inoculation into TPGY. The sample:TPGY ratio should be 1:100–1:10. Depending on the sample type and the expected number of *C. botulinum* spores or cells present, 0.1 to 10-g aliquots of sample material are inoculated into 10 to 100 mL of TPGY, respectively. Particularly fatty and protein-rich foods may interfere with PCR, and may require the greater dilution rate of 1:100. The number of subsamples investigated and the size (grams) of each subsample contribute to the cell count estimate obtained by most probable number (MPN) technique as described below (*see* **Subheading 3.3**). Competitive bacteria present in fecal samples, for example, may inhibit or retard the growth of *C. botulinum.* Heating the sample at 65 to 70°C for 15 min eliminates most vegetative bacteria. These include also vegetative *C. botulinum* cells, so heating the sample is only appropriate when *C. botulinum* spores are expected to be present.

If liquid samples are investigated, they may be inoculated in broth medium as such, or, if low *C. botulinum* count is expected, concentrated by filtering through membranes with 0.45-µm pores. These membranes may then be inoculated into 10 mL of TPGY broth ***(13)***. Viscous samples such as honey are first diluted in 1% Tween-80 (1:9) and heated at 65°C for 30 min, after which the samples are centrifuged for 30 min at 9000*g* ***(13)***. The supernatant is then filtered through the membranes and membranes are inoculated into TPGY. If the filter membrane gets clogged, several filters may be used and inoculated into the same TPGY tube ***(13)***.

3. *Culture technique* (*see* **Note 7**). Only anaerobic medium should be used when culturing *C. botulinum.* Pipet and pipet tips should be made anaerobic before being brought into contact with the culture. All incubations are made anaerobically. Ideally, all sample material to be cultured for *C. botulinum* should be stored overnight under anaerobic conditions in order to remove excess oxygen. However,

particularly in suspected cases of human botulism, rapid analysis is essential and the samples should be processed and cultured as soon as possible. *C. botulinum* has been reported to grow at a positive Eh of approx 150 mV ***(14)***, and logarithmic cultures possess a good reducing capability, so anaerobic overnight storage of samples may be omitted if necessary.

The samples are optimally inoculated in an anaerobic work station by introducing the sample to the bottom of the medium. When expecting group I *C. botulinum,* the tubes are incubated at 37°C, while the group II strains require a milder temperature of 26 to 30°C. If enough sample material is available, replicate cultures incubated at both temperatures give the most reliable result, since incubation at a nonoptimal growth temperature may inhibit or retard the growth of some *C. botulinum* strains. The tubes are incubated for 1 to 5 d (*see* **Note 8**), followed by transfer of 1 mL of each culture to 10 mL of fresh TPGY, preferably prewarmed to the appropriate incubation temperature, and overnight incubation (14–16 h) at the same temperature (*see* **Note 8**). The two-step enrichment is employed to ensure the optimal growth of *C. botulinum* that grows poorly in the presence of competitive bacteria, and to confirm that DNA released from lysed cells does not interfere with PCR.

3.2. Cell Lysis and Multiplex PCR

1. *Cell harvest:* Cells from 1 mL of overnight culture are spun down in Eppendorf tubes at a maximum speed for 3 min, and the cell pellet is resuspended in 1 mL of TE (10:1) buffer. The tubes are incubated at 37°C for 1 h followed by centrifugation as described above. The cell pellet is resuspended in 1 mL of sterile distilled H_2O, heated at 95°C for 5 min to release the DNA, and spun down to concentrate the cell debris that might interfere with PCR (*see* **Note 9**). The supernatant is used as a template in PCR.
2. *PCR reaction mixture:* Prepare one large batch of reaction mixture for all samples (*see* **Note 10**). Add water first, then 10X PCR buffer. Adjust the final $MgCl_2$ concentration to 4.8 m*M*. Add 0.25 µ*M* of each primer and 220 µ*M* of each dNTP. Add 1 µL of template per 50-µL reaction (*see* **Note 11**). To avoid any nonspecific activity, add DNA polymerase last. Keep tubes on ice until loaded in the thermocycler with a heated lid.
3. *PCR conditions:* A total of 28 cycles of denaturation, 30 s at 95°C, primer annealing 25 s at 60°C, and extension 85 s at 72°C are followed by final extension of 3 min at 72°C.
4. *Gel electrophoresis:* Prepare a 2% agarose gel in TAE buffer. Load samples, molecular weight marker, and control samples (*see* **step 5**) into the gel wells using 6X loading buffer (1:6), and depending on the gel size, run at 80 to 120 V for 40 to 120 min.
5. *Control samples:* Negative and positive control reactions are essential when evaluating the final multiplex PCR results. A negative control contains all the other reaction components except for the template (an equal amount of PCR-grade water may

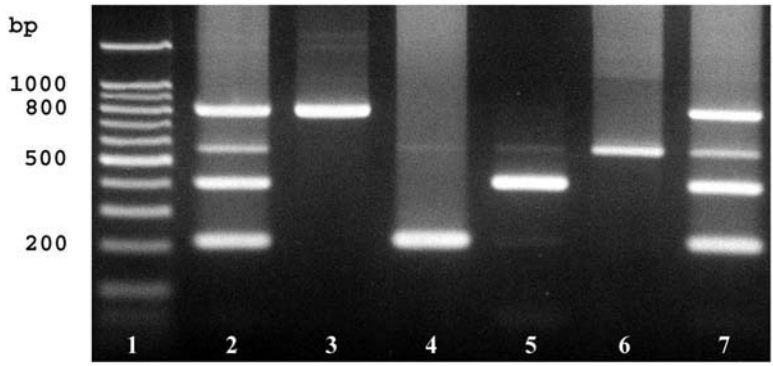

Fig. 1. Multiplex PCR detection of *Clostridium botulinum* types A, B, E, and F. Lane 1, molecular weight marker (100-bp DNA Ladder); lanes 2 and 7, *C. botulinum* types A, B, E, and F; lane 3, *C. botulinum* type A; lane 4, *C. botulinum* type B; lane 5, *C. botulinum* type E; lane 6, *C. botulinum* type F.

be added in the reaction mixture); this tests for the possibility of reaction contamination by the template sequence. The positive control contains all the four PCR products (*bot*A-, *bot*B-, *bot*E-, and *bot*F-specific fragments), and it tests that the PCR conditions are optimal for the reaction (*see* **Notes 4** and **12**).

6. *Data analysis:* The sizes of the expected fragments in a positive sample are presented in **Table 1** and in **Fig. 1** (*see* **Notes 13** and **14**).

3.3. Estimation of Clostridium botulinum *Count by Most Probable-Number Technique*

The number of *C. botulinum* present in the sample material can be established using the most-probable-number (MPN) technique. In suspect cases of human botulism, when only a limited amount of sample material is often available, an MPN technique for an unusual series of dilutions ***(7)*** described by Thomas ***(15)*** is appropriate. This can be applied in combination with PCR detection ***(16)***:

$$MPN/g = P/\sqrt{(TN)}, \text{ where}$$

P = the number of PCR-positive sample tubes
N = the amount (g) of sample material in all PCR-negative tubes
T = the total amount (g) of sample material tested by PCR.

4. Notes

1. Alternatively, sealed tubes containing anaerobic medium may be used.
2. Care should be taken when handling sodium thioglycolate, which is used as a reducing agent in the TPGY medium, as it may cause irritation to skin. Autoclaved

material containing sodium thioglycolate should be handled under a fume hood, as exposure may cause respiratory stress.

3. As with any PCR-based protocol, the reaction conditions need to be reoptimzed if other types of DNA polymerases are to be used.
4. The positive control reaction containing all the four *bot*-specific PCR fragments is a useful additional marker to be employed at the extreme right and left hand lanes of a gel **(Fig. 1)**.
5. For safety, EtBr may be replaced by less toxic and/or less mutagenic compounds such as DAPI (4′,6-diamidino-2-phenylindole dihydrochloride:hydrate) and SYBR Green I (Molecular Probes Eugene, OR) ***(17)***, but these solutions are more expensive than EtBr.
6. The detection limit of the method varies depending on the sample material ***(12)***, which should be taken into account when estimating the sample size. For example, the detection limit for *C. botulinum* types A, B, E, and F in meat and fish was shown to be 10^{-2} to 10^{-1} spores/g, while that in feces was as high as 10^3 spores/g ***(12)***.
7. BoNTs are highly potent neurotoxins. Liquid cultures containing *C. botulinum* may contain high concentration of BoNTs, and thus handling of the cultures requires restricted-containment laboratory facilities and well-trained, immunized personnel. Only tubes, bottles, and other equipment made of unbreakable materials should be used in a laboratory handling *C. botulinum.*
8. In most cases a 3-d incubation is appropriate, but particularly with fecal samples containing a high number of competitive microflora, a 5-d incubation may be required ***(12)***. In some cases it might be beneficial to subculture the sample and perform PCR analysis on several subsequent days (e.g., after 1, 3, and 5 d of incubation) in order to avoid a false-negative result due to slow growth, or to ensure the early detection of fast-growing bacteria that lyse soon after they have reached stationary phase.
9. As an alternative for the heating procedure, if a large number of samples are to be investigated, 1 µL of unheated cell suspension may be added directly to the reaction mixture. The PCR tubes are then heated in the thermocycler during an additional heating step (95–98°C, 10 min) prior to adding the DNA polymerase and starting the first PCR cycle.
10. To avoid contamination, always prepare the PCR reaction mixture in a place separate from sample preparation and gel electrophoresis facilities.
11. The template concentration can be varied depending on the sample, but usually the two-step enrichment ensures that there is a sufficient number of *C. botulinum* cells present in the template. Too-high DNA concentration may alter the activity of DNA polymerase, and thus cause false-negative results.
12. In addition to negative and positive controls, the use of internal controls in all detection PCR tests has been recently proposed to control the stability of reaction conditions that may be altered by trace amounts of sample material ***(18)***. This employs the use of an additional primer pair with similar annealing properties as the actual primers, and a template DNA matching with the control primers but not with the actual test primers. In a multiplex reaction, however, any additional primer

set and template DNA increase the risk of primer dimer formation as well as non-specific annealing and amplification. This should be taken into account when designing internal controls.

13. Care should be taken in estimating the fragment size. As the *bot* gene sequences are conservative, PCR fragments with only a slightly different size from the expected size should be regarded as non-specific. This has been concluded from the analysis of such samples in parallel with another set of *bot*-specific primers ***(8)***. If in doubt of fragment size, the electrophoresis can be repeated in a 3% agarose gel for an extended time (120–150 min) for more efficient resolution of fragments with similar sizes. Alternatively, sequencing of the PCR fragment or Southern blot analysis with a specific probe will naturally provide a reliable result.
14. The method has been shown to be 10 times more sensitive for type B strains than for types A, E, and F strains ***(12)***. This is probably due to the small PCR product size, making product formation more probable for type B than for the other types.

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5

Multiplex PCR for Specific Identification of Enterohemorrhagic *Escherichia coli* Strains in the O157:H7 Complex

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Summary

The "O157:H7 complex" is comprised mostly of enterohemorrhagic *Escherichia coli* (EHEC) strains, with serotype O157:H7 being the prototypic and predominate pathogenic strain in the complex. However, several phenotypic O157:H7 variants and genetically closely related O55:H7 serotype enteropathogenic *E. coli* (EPEC) are also included in the complex. The EHEC strains in the complex share many of the same virulence factors, but can exhibit diverse phenotypic profiles. As a result, identification of the various EHEC strains in the "complex" often requires multiple assays. Using PCR primers that are specific for four characteristic EHEC virulence genes (stx_1, stx_2, γ-*eae,* and *ehx*A) and to a single nucleotide polymorphism (+92 *uid*A) gene marker that is highly conserved among strains in the complex, a multiplex PCR assay was developed that simultaneously detects these five markers and allows the identification of the pathogenic EHEC strains in the O157:H7 complex.

Key Words: Enterohemorrhagic *E. coli;* O157:H7 complex; multiplex PCR.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as an important pathogen that causes hemorrhagic colitis (HC), which may progress into the more severe hemolytic uremic syndrome (HUS) ***(1)***. EHEC are distinguished from other pathogenic *E. coli* by their trait virulence factors, most notable of which is the production of Shiga toxins (Stx) ***(2,3)***. There are more than 200 serotypes of Shiga toxin-producing *E. coli* (STEC), but not all have been implicated in human illness; therefore, EHEC are a small subset of STEC that is comprised of strains that have the same clinical, epidemiological and pathogenic features ***(3)***. Other trait EHEC virulence factors include the chromosomal *eae* gene that encodes for intimin, a protein essential for cellular attachment,

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and the presence of a 90-kb plasmid that carries several putative virulence factors, including the *ehx*A gene that encodes the EHEC hemolysin or enterohemolysin ***(4)***.

Although several serotypes, including O111:H8, O26:H11, O103:H2, O113:H2, O104:H21, have caused human illness and are recognized as EHEC ***(5)***, serotype O157:H7 remains the most important strain and is most often implicated in human EHEC infections worldwide. Cluster analysis has identified an "O157:H7 complex" that is composed of several genetically related strains ***(6)***. EHEC O157:H7 is the prototypic and dominant strain in the complex, but the complex also includes several Stx-producing variants, such as nonmotile O157:H7 strains ***(7)***, β-glucuronidase-positive O157:H7 strains ***(8)***, the sorbitol-fermenting (SF) O157:H^- variants that have emerged as an important pathogen in Europe ***(9)***, and the non-Stx-producing serotype O55:H7 EPEC strains ***(10)***. Because the EHEC strains in the complex exhibit phenotypic, serologic, and genetic diversity, multiple assays are often required to identify the various EHEC strains in the O157:H7 complex. We describe a multiplex PCR assay that simultaneously detects five virulence and trait genetic markers that enable identification of EHEC strains in the O157:H7 complex and differentiates these from the other STEC and EHEC strains.

2. Materials

1. Thermocycler.
2. Oligonucleotide primers.
3. HotStarTaq™ DNA Polymerase and reaction buffer (Qiagen, Valencia, CA).
4. Agarose gel electrophoresis equipment.
5. Transilluminator and gel documentation equipment.
6. Micropipets.

3. Methods

The following sections describe the specific genetic targets and primers used in developing the multiplex PCR. Also provided are detailed procedures on template preparation, PCR assay setup, and the specific amplification parameters, as well as analysis and interpretation of results.

3.1. Primer Design and Sequences

The primer sequences, genetic targets, and the expected sizes of the amplification products from the multiplex PCR assay are shown in **Table 1**. The *stx*$_1$ and *stx*$_2$ are phage-encoded genes that encode for Shiga toxin 1 (Stx$_1$) and Stx$_2$, respectively. Also known as verotoxins (VT), these toxins inhibit cellular protein synthesis by interfering with the functions of the 23S rRNA ***(3)***. Stx$_1$ is virtually identical to the Shiga toxin produced by *Shigella dysenteriae* type I;

Table 1
Primers Used in Multiplex PCR

Gene	Primer	Sequence	Amplicon
stx$_1$	LP30	5′-CAGTTAATGTGGTGGCGAAGG-3′	348 bp
	LP31	5′-CACCAGACAATGTAACCGCTG-3′	
stx$_2$	LP43	5′-ATCCTATTCCCGGGAGTTTACG-3′	584 bp
	LP44	5′-GCGTCATCGTATACACAGGAGC-3′	
+92 *uid*A	PT-2	5′-GCGAAAACTGTGGAATTGGG-3′	252 bp
	PT-3	5′-TGATGCTCCATCACTTCCTG-3′	
γ-*eae*A	AE22	5′-ATTACCATCCACACAGACGGT-3′	397 bp
	AE20-2	5′-ACAGCGTGGTTGGATCAACCT-3′	
*ehx*A	MFS1Fb	5′-GTTTATTCTGGGGCAGGCTC-3′	166 bp
	MFS1R	5′-CTTCACGTCACCATACATAT-3′	

hence, the LP30/LP31 primers will detect both EHEC *stx*$_1$ and the *S. dysenteriae* toxin gene. Both Stx_1 and Stx_2 toxins, individually or in combination with each other, are most often produced by EHEC strains causing illness. However, Stx_2 seems to be implicated more often in cases of HUS and, therefore, may be more important in human infections. The LP43/LP44 primers will detect Stx_2 and several Stx_2 variants (*stx*$_{2c}$, *stx*$_{2d}$, and *stx*$_{2e}$) ***(11)*** (*see* **Note 1**).

The *uid*A (*gus*A) gene encodes for β-glucuronidase (GUD) and is expressed by most *E. coli,* except for O157:H7. The O157:H7 *uid*A gene carries a T to G transversion mutation at +92 that is highly conserved ***(12)*** and an unique marker that, so far, has been found only in the EHEC strains of the O157:H7 complex ***(6)*** (*see* **Note 2**). The PT2/PT3 primer pair is highly specific for the +92 *uid*A base mutation and is a critical component of this multiplex PCR assay for identifying EHEC strains in the complex.

The *eae* gene, which resides on the locus for enterocyte effacement (LEE) pathogenicity island, encodes for the intimin protein that is involved in causing the attachment/effacing lesions characteristic of both EHEC and EPEC ***(2)***. There are several intimin alleles, which may be carried by various EHEC strains. Primers AE22/AE20-2 ***(13)*** are specific for γ-intimin (γ-*eae*), which is found in the O157:H7 serotype and its phenotypic variants, EPEC O55:H7 and a few other rare serotypes, and therefore, will detect all these strains (*see* **Note 3**).

The *ehx*A gene that encodes for enterohemolysin resides on the 90-kb EHEC plasmid, referred to as pO157, carried by strains of the O157:H7 serotype. This putative virulence factor, which is found in most O157:H7 strains, is not necessarily carried by EHEC strains of other serotypes ***(14)***. Furthermore, although the *ehx*A gene is highly conserved, there are 2 distinct *ehx*A genetic alleles that separate the various hemolysin-positive EHEC strains into two clusters. The

MFS-1R ***(13)*** and MFS-1Fb ***(15)*** primer pair used in the multiplex PCR assay detects the *ehx*A gene of both clusters (*see* **Note 4**).

3.2. Sample Preparation

Clinical and environmental isolates of *E. coli,* including EHEC, STEC, and EPEC strains, were obtained from various sources worldwide and are part of the in-house collection at the Division of Microbiological Studies of the Food and Drug Administration (FDA). The template DNA used in the PCR was prepared by suspending a single colony in 100 µL of water that was heated for 5 min in a boiling water bath, centrifuged to remove debris, and kept frozen at –20°C until used. For each amplification reaction, 2 µL of this preparation was used.

3.3. Multiplex PCR—Reaction Setup

The 10 primers shown in **Table 1** were pooled and premixed as a stock solution and added to each reaction to attain a final concentration of 300 n*M* for each primer. Each 50-µL reaction mix also contained 200 µ*M* of each deoxynucleotide triphosphate, 1X PCR buffer, 3 m*M* $MgCl_2$, template DNA, and 0.5 µL (2.5 U) of HotStarTaq™ DNA polymerase.

3.4. Multiplex PCR—Amplification Parameters

All reagents sufficient for the number of samples to be tested were batch mixed and aliquoted to appropriately identified reaction tubes. The template DNA was added and the reaction was initiated with a single incubation at 95°C for 15 min to activate the HotStarTaq DNA polymerase. Subsequently, target amplification was achieved with 25 successive cycles in a Perkin-Elmer GeneAmp 2400 thermocycler. Each cycle consisted of a denaturation step of 1 min at 94°C, an annealing period of 1 min at 56°C, and a extension period of 1 min at 72°C. Amplification was terminated with a single incubation of 7 min at 72°C.

3.5. Agarose Gel Electrophoresis of Multiplex PCR Amplicons

Following amplification, 8 µL of each reaction was examined by agarose gel (1%) electrophoresis in Tris-borate EDTA buffer, pH 8.2, at 100 V for 1.5 h. A 123-bp ladder was used as a molecular size ladder. Results for selected strains are shown in **Fig. 1**. The products amplified from virulence and trait genetic markers and the expected sizes (bp) of these products are shown with arrows at left of **Fig. 1**. The EHEC strains from the O157:H7 complex are shown in Fig.1, lanes 1, 4, and 8. Lane 7 shows a Stx_2-producing serotype O55:H7 strain, which is also in the O157:H7 complex, but is not an EHEC. The production of some of these virulence factors were verified using phenotypic assays (*see* **Note 5**).

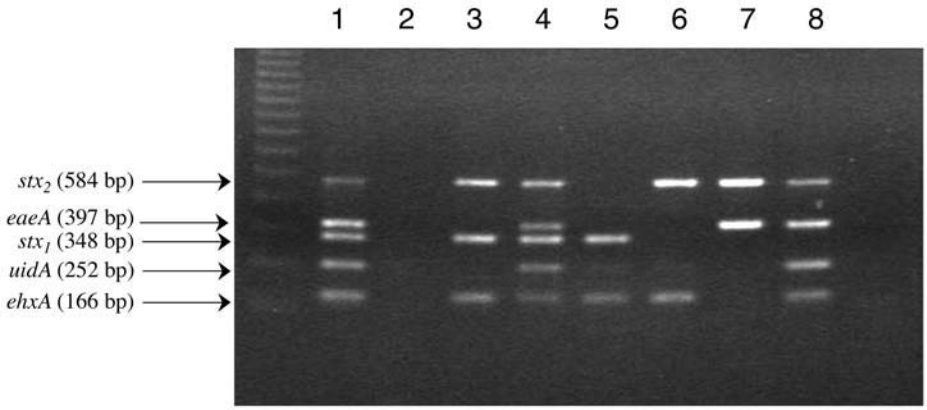

Fig. 1. Agarose gel electrophoresis of DNA fragments amplified from various EHEC strains by multiplex PCR. The products, with the expected sizes (bp) are indicated by arrows at left. The strains, with the expected markers in parentheses, are: lane 1, O157:H7 (stx_2, γ-*eae,* stx_1, +92 *uid*A, *ehx*A); lane 2, *E. coli* (none); lane 3, O111:H8 (stx_2, stx_1, *ehx*A); lane 4, O157:H7 (stx_2, γ-*eae,* stx_1, +92 *uid*A, *ehx*A); lane 5, O45:H2 (stx_1, *ehx*A); lane 6, O104:H21 (stx_2, *ehx*A); lane 7, O55:H7 (stx_2, γ-*eae*); and lane 8, O157:H$^-$ (stx_2, γ-*eae,* +92 *uid*A, *ehx*A).

3.6. Interpretation of Multiplex PCR Results

The results from the analysis of various STEC, EHEC, and EPEC strains are summarized in **Table 2**. With the exception of the O55:H7 strains that do not produce Stx (*see* **Note 6**), almost all the strains from the O157:H7 complex as well as the other EHEC and STEC strains examined carried stx_1, stx_2, or both toxin genes. Many of the other STEC and EHEC serotypes also carried the *ehx*A gene for enterohemolysin, with almost all the EHEC strains in the "O157:H7 complex" carrying the gene. There were, however, ten O157 strains in the "complex" that did not carry *stx* and one that did not have *ehx*A, suggesting that the strains have lost these virulence factors that are carried on mobile genetic elements (*see* **Note 6**).

Our studies have shown that the γ-*eae* allele is found in all the strains from the O157:H7 complex, including the O55:H7 strains (**Table 2**). Genetic analysis showed that O157:H7 is closely related to and postulated to have evolved from the O55:H7 strains ***(6,16,17)***. Although the other EHEC and STEC strains examined did not have γ-*eae,* these strains may be carrying other types of *eae* alleles.

The presence of the +92 *uid*A mutation, as evidenced by the 252-bp amplicon, is found only in the O157:H7 and its Stx-producing phenotypic variants

Table 2
Summary of Multiplex PCR Analysis of Various *E. coli* Strains Showing Numbers of Strains That Exhibited Identical Genotypic Patterns

			Patterns Observed				
Cluster	Serotype	Number	*stx*$_1$	*stx*$_2$	*uid*A	*eae*A	*ehx*A
O157:H7	O157:H7	14	+	+	+	+	+
complex		10	–	+	+	+	+
		1	+	–	+	+	+
		6	–	–	+	+	+
	O157:H$^-$/NM	7	+	+	+	+	+
		25	–	+	+	+	+
		1	+	–	+	+	+
		1	–	+	+	+	–
		4	–	–	+	+	+
	O55:H7[a]	9	–	–	–	+	–
		1	–	+	–	+	–
Other	various	4	+	+	–	–	+
EHEC[b]		11	+	–	–	–	+
		7	–	+	–	–	+
		2	+	+	–	–	–
STEC[b]	various	3	+	+	–	–	+
		8	+	–	–	–	+
		3	–	+	–	–	+
		3	+	+	–	–	–
		3	+	–	–	–	–
		1	–	+	–	–	–
		3	+	+	–	–	–

[a]Enteropathogenic *E. coli* strains
[b]EHEC, enterohemorrhagic *E. coli;* STEC, Shiga toxin-producing *E. coli.*

within the O157:H7 complex **(Table 2)**. Although O157:H7 and O55:H7 are closely related, this mutation is postulated to have occurred during the evolutionary emergence of O157:H7 from O55:H7 ***(6)***; hence, it is absent in the latter serotype **(Table 2)**. Extensive analysis of STEC, EHEC, and other enteric bacteria showed that the +92 *uid*A mutation is unique to O157:H7 and its variant strains; therefore, it is a reliable marker for its identification ***(12)***.

With the exception of the +92 *uid*A marker, which is found only in O157:H7, the other gene markers used in the multiplex PCR assay are present in various combinations in other EHEC and STEC serotypes. In the interpretation of multiplex PCR results, therefore, EHEC strains from the O157:H7 complex are dis-

tinguished from the other EHEC and STEC by the presence of the γ-*eae* allele and the +92 *uid*A marker. Within the complex, the γ-*eae* allele is carried by all strains, but the EHEC strains can be differentiated from O55:H7 by the production of Stx (*see* **Note 6**), enterohemolysin, and the presence of the +92 *uid*A mutation.

4. Notes

1. There are several variants of Stx_2 (Stx_{2c}, Stx_{2d}, Stx_{2e}, Stx_{2f}, etc.) ***(18)*** and although many of these are produced by animal or environmental STEC isolates, recent evidence suggests that, in addition to Stx_2, some Stx_2 variants may also play a role in causing human illness.
2. Serotype O157:H7 strains carry the *uid*A gene, but produce a nonfunctional GUD enzyme due to a double G insertion at +686 that caused a frame-shift mutation in the *uid*A structural gene ***(19)***. Within the O157:H7 complex, the O157:H7-type strain and its nonmotile variants do not exhibit GUD activity. However, the complex also contains other variants of O157:H7, as well as SF O157:H^- strains, both of which do not carry the double G insertion in *uid*A and, therefore, express functional GUD enzymes. Most importantly, the +92 T to G transversion mutation in the *uid*A gene, which is incidental to GUD expression ***(19)***, is found in both the GUD-negative and GUD-positive EHEC strains in the O157:H7 complex and, so far, seems to be exclusive to this group ***(6)***.
3. The LEE pathogenicity island is a virulence factor of both EHEC and EPEC. There are several *eae* alleles (α, β, γ, δ, etc.) that may be carried by various EPEC and EHEC strains ***(20)***. Although the alleles exhibit significant homogeneity, there are genetic differences that can be used to design allele-specific PCR primers. Since the same *eae* allele may be found in both EHEC and EPEC strains, allele-specific primers will detect both pathogenic groups. EPEC strains, however, may be differentiated from EHEC by the lack of *stx* genes ***(3)***.
4. The two distinct *ehx*A genetic alleles share 98% nucleic acid homology ***(4)***. EHEC serotypes O157:H7, O26:H11, O111:H8, and O103:H2 maintain the cluster I allele, while cluster II is found in the EHEC serotypes O113:H21 and O104:H21 ***(4)***. Although the two clusters share a great deal of homology, cluster-specific PCR primers can be designed to distinguish the two clusters. The primer pair used in the multiplex assay detects both clusters.
5. Some of the genotypic results of multiplex PCR were verified by serological and phenotypic assays. The production of Stx_1 and Stx_2 were tested using the Verotox-F test (Denka Seiken, Tokyo, Japan), a reverse passive latex agglutination test that distinguishes these two toxins. The enterohemolytic activity was tested for on tryptic soy agar plates containing 5% sheep blood (washed three times in phosphate-buffered saline) supplemented with 10 m*M* $CaCl_2$ (pH 7.3).
6. Because the stx_1 and stx_2 genes are phage-encoded in *E. coli,* these may be lost during cultivation. As a result, strains of STEC and O157:H7 that have lost *stx* genes are known to exist ***(21,22)***. Conversely, *stx* genes can also be transmitted to other enteric bacteria. For instance, EPEC strains are typically distinguished from EHEC

by the absence of Stx;however, rare strains of EPEC O55:H7 that produce Stx have been isolated (**Fig. 1,** lane 7). Similarly, plasmids are mobile genetic elements that may be lost during routine subculture, and plasmid loss has been reported to occur in *E. coli **(23)***.

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6

PulseNet's Step-by-Step Laboratory Protocol for Molecular Subtyping of *Listeria monocytogenes* by Macrorestriction and Pulsed-Field Gel Electrophoresis

Lewis M. Graves and Balasubramanian Swaminathan

Summary

Subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis (PFGE) provides sensitive and epidemiologically relevant discrimination between strains and allows public health officials to detect potential common source outbreaks of listeriosis. Fundamental to the method is the delicate process of isolating intact genomic DNA from bacterial cells embedded in a gel matrix within a reasonable time period (3–4 h) and results are available within 24 to 48 h. The intact DNA is digested with an infrequently cutting restriction endonuclease (*Asc*I and *Apa*I). PFGE technology is based on separation of large fragments (20–1000 kb) of microbial chromosomal DNA. The digested DNA is incorporated into a gel matrix and allowed to migrate by alternating the electric field between spatially distinct pairs of electrodes. This causes the DNA fragments to reorient and migrate through the pores in the agarose gel at rates proportional to their size.

Key Words: *Listeria; Listeria monocytogenes;* subtyping; pulsed-field gel electrophoresis; macrorestriction; PulseNet.

1. Introduction

Bacteria of the species *Listeria monocytogenes* are Gram-positive, rod-shaped, and non-spore-forming. They are found in a variety of environments, including soils, water, silage, sewage, and plant and animal food products. The genus *Listeria* is composed of six species, with *L. monocytogenes* as the primary cause of human infections ***(1)***. In human food-borne listeriosis cases, the incidence of serious illness and death in affected individuals is high. Groups at highest risk of acquiring infection are pregnant women, neonates, immunocompromised patients, and the elderly. However, listeriosis may occasionally occur in persons who have no predisposing underlying condition; up to 30% of

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adults with listeriosis may be immunocompetent ***(2)***. Listeriosis occurs as sporadic disease as well as epidemic outbreaks ***(3–8)***. The infectious dose for listeriosis has not been determined and it may depend, in part, on the susceptibility of the host. An estimated 2500 *L. monocytogenes* infections occur in the United States each year ***(9)***.

In 1996, PulseNet was established in the US PulseNet is a national network of public health and food regulatory agencies in the United States that perform standardized PFGE subtyping of bacteria that are causative agents of foodborne disease. PulseNet laboratories are able to rapidly compare PFGE patterns with a national electronic database of PFGE patterns maintained at the Centers for Disease Control and Prevention ***(10)***. Current PulseNet protocols for PFGE subtyping include *Escherichia coli, Salmonella* spp., *Shigella* spp., *Listeria monocytogenes,* and *Campylobacter* spp. ***(11,12)***. PulseNet has evolved into an international network with participants from Europe, the Asia-Pacific region, and Latin America. Participating countries have adapted the PulseNet standardized protocols. The method presented here is a step-by-step *Listeria monocytogenes* PFGE protocol that includes the use of a specific strain of *Salmonella* ser. Braenderup as a universal molecular size standard ***(13)***. The following is a complete self-contained description of the protocol.

2. Materials

2.1. Preparation of the Salmonella *ser. Braenderup and* L. monocytogenes *Bacterial Cell Suspensions*

1. Bacteria strains: *Salmonella enterica* serotype Braenderup (CDC no. H9812, standard reference strain) (American Type Culture Collection [ATCC] no. BAA-664 *Salmonella choleraesuis* subsp. *choleraesuis* [Smith] Weldin serotype Braenderup); *L. monocytogenes* (CDC no. H2446, control strain), and three *L. monocytogenes* test strains (T1, T2, and T3).
2. Water: All water used in this protocol is reagent-grade or equivalent, except where indicated. In our laboratory, we use distilled, deionized WFI quality, 0.2-µm sterile-filtered water (Mediatech, Herndon, VA, cat. no. 25-055-CM).
3. 1 *M* Tris-HCl, pH 8.0 (Gibco/BRL, Bethesda, MD, cat. no. 15568-025).
4. 0.5 *M* EDTA, pH 8.0 (Amresco, Solon, OH, cat. no. E177-500ML).
5. Cell suspension buffer (CSB): 100 m*M* Tris-HCl, 100 m*M* EDTA, pH 8.0.
6. TE buffer (0.01 *M* Tris/EDTA Buffer), (Mediatech, cat. no. 99-937-CM).
7. 1.2% SeaKem Gold agarose (Cambrex Bio Sciences, Rockland, ME, cat. no. 50150) in sterile reagent-grade water. Prepared by dissolving 0.12 g of SeaKem Gold agarose in 10 mL of water in a 125-mL screw-cap Wheaton bottle or flask. Microwave the agarose until it melts completely; keep in a 54.5°C (±1°C) water bath (*see* **Note 1**).
8. SSP solution: 1.2% SeaKem Gold:1% sodium dodecyl sulfate: 0.2 mg/mL proteinase K.

9. *N*-Lauroyl-sarcosine, sodium salt (SDS) (Sigma, cat. no. L-9150); 10% (w/v) in sterile water.
10. Lysozyme (Sigma, cat. no. L-6876); 10 mg/mL dissolved in sterile water. Prepare 1-mL aliquots and store at –20°C. Thaw and keep on ice until ready to use.
11. Proteinase K (Roche Diagnostics, Indianapolis, IN, cat. no. 745 723); 20 mg/mL dissolved in sterile water. Prepare 0.5-mL aliquots and store at –20°C. Thaw and keep on ice until ready to use.
12. Cell lysis solution: 50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, pH 8.0, 1% *N*-lauroyl-sarcosine.

2.2. Lysis of Cells in Agarose Plugs and Washing of Agarose Plugs After Cell Lysis

1. Cell lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, pH 8.0, 1% *N*-lauroyl-sarcosine, 0.15 mg/mL proteinase K; proteinase K is added just before use.
2. Reagent-grade water for washing agarose plugs.
3. TE buffer.
4. Two reusable 10-well PFGE plug molds, 2 cm × 1 cm × 1.5 mm (Bio-Rad, cat. no. 170-3622).
5. Five green screened caps (Bio-Rad, cat. no. 170-3711).

2.3. Preparation of Restriction Enzyme Buffers and Restriction Enzyme Mix for Digestion of DNA in Agarose Plugs and Cutting of Plug Slices

1. *Asc*I Restriction Endonuclease, 2500 units with Buffer 4 (New England Biolabs, Beverly, MA, cat. no. R0558L).
2. *Apa*I Restriction Endonuclease, 20,000 units with Buffer A (Roche Diagnostics, cat. no. 703 753).
3. *Xba*I Restriction Endonuclease, 20,000 units with Buffer H (Roche Diagnostics, cat. no. 1 047 663).
4. Microcentrifuge tube rack, 80-well (Daigger, Vernon Hills, IL, cat. no. EF29025A).

2.4. Casting Agarose Gel and Loading Restricted Plug Slices on Comb

1. Tris-Borate EDTA (TBE) buffer 0.5X: dilute 25 mL of 10X Tris-borate EDTA (Sigma, cat. no. 4415) with 500 mL of deionized water.
2. SeaKem Gold agarose (1%) in 0.5X TBE: dissolve 1.0 g of SeaKem Gold agarose in 100 mL of 0.5X TBE in a 500-mL screw-cap flask. Microwave the agarose until it completely melts; swivel gently to mix and place in a 54.5°C (±1°C) water bath (*see* **Note 1**).
3. 10-Well comb, 14 cm wide, 1.5 mm thick (Bio-Rad, cat. no. 170-4326).
4. Standard casting stand, with 14 × 13 cm frame and platform (Bio-Rad, cat. no. 170-3689).

2.5. Preparation of Pulsed-Field Electrophoresis Chamber

1. Running buffer 0.5X TBE: dilute 110 mL of the 10X TBE with 2.2 L of deionized water in a measuring cylinder.

2. Use frame that came with standard casting stand.

2.6. Staining and Documentation of PFGE Agarose Gel

1. Prepare ethidium bromide (Sigma, cat. no. E-1510): dilute the 10 mg/mL stock solution 1:10,000 in deionized water (*see* **Note 2**).
2. Bio-Rad Gel Doc 2000 or equivalent documentation system that is equipped with a charge-coupled device (CCD) camera that can provide IBM-compatible uncompressed TIFF images with resolution of ≥768 × 640 pixels, and that will allow comparison of images with BioNumerics software (Applied Maths, Inc. Sint-Martens-Latem, Belgium).

3. Methods

The method is composed of eight major steps/modules for *L. monocytogenes* and *Salmonella* ser. Braenderup: (1) Preparation of the bacterial cell/lysozyme or cell suspension; (2) lysis of the bacterial cells embedded in an agarose gel matrix; (3) removal of cellular debris and interfering substances from the intact genomic DNA in the agarose gel matrix; (4) digestion of the genomic DNA with appropriate rare cutting restriction endonucleases that produce simple profiles (10–20 bands); (5) electrophoresis of the digested DNA using optimized parameters and running conditions; (6) ethidium bromide staining of DNA fragment separated by PFGE; (7) capturing the DNA fingerprint using imaging equipment; and (8) computer analysis of PFGE fingerprints. A critical step in the protocol is lysozyme treatment. Treatment of the bacterial cells with lysozyme for 10 min at 37°C sufficiently weakens the cell wall of *L. monocytogenes* cells without lysing them. Following lysozyme exposure, treatment with the lysis solution leads to complete lysis of the bacterial cells suspended in the agarose matrix.

3.1. Preparation of Salmonella ser. Braenderup and Listeria monocytogenes Bacterial Cell Suspensions

Label five brain heart infusion agar plates as follows: T1, T2, T3, H2446, and H9812. Inoculate each plate with the appropriate bacteria strain and incubate in a 37°C incubator for 16 to 18 h.

3.1.1. Salmonella ser. Braenderup (H9812)

1. Label one tube (Falcon 2057, 14 mL—17 × 100 mm) H9812. Add 3 mL of cell suspension buffer to the tube. Use a sterile polyester-fiber or cotton swab that has been moistened with cell suspension buffer to remove bacteria from the plate. Suspend the cells in the cell suspension buffer by gently spinning the swab so that cells will be evenly dispersed.
2. Use a MicroScan Turbidity Meter (Dade Behring, Inc., Deerfield, IL, cat. no. B1018-66) to adjust the cell suspensions to 0.70 (±0.02) for H9812 (for cell sus-

Table 1
SSP Preparation

Number of strains	SDS (10%)	SeaKem Gold Agarose (1.2%)	Proteinase K (20 mg/mL)
1	30 µL	267 µL	3 µL
10	300 µL	2.67 mL	30 µL

pensions in Falcon 2057 tubes). The graduated marking on the Falcon 2057 tube should face the front of the tube to avoid deflecting the light path (*see* **Note 3**).
3. Transfer 300 µL of the adjusted H9812 bacterial suspension to appropriately labeled 1.5-mL microcentrifuge tube (*see* **Note 4**).
4. Hold the bacterial cell suspension at room temperature or on ice until *L. monocytogenes* cell/lysozyme suspensions are prepared or continue with the steps in **Subheading 3.2.** if only preparing H9812 bacterial cell suspensions.

3.1.2. Listeria monocytogenes

1. Label four tubes (Falcon 2057, 14 mL—17 × 100 mm) as follows: T1, T2, T3, and H2446. Add 3 mL of TE to each tube. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile TE to remove bacteria from the plate. Suspend the cells in TE by gently spinning the swab so that cells will be evenly dispersed.
2. Use a MicroScan Turbidity Meter to adjust the cell suspensions to 0.80 (±0.02) for *L. monocytogenes* cells (for cell suspensions in Falcon 2057 tubes). The graduated marking on the Falcon 2057 tube should face the front of the tube to avoid deflecting the light path (*see* **Note 3**).
3. Transfer 240 µL of each *L. monocytogenes* bacterial suspension to appropriately labeled 1.5-mL microcentrifuge tubes (1 = T1, 2 = T2, 3 = T3, and 4 = H2446).
4. Add 60 µL of lysozyme solution (10 mg/mL) to each bacterial suspension and mix by pipetting up and down two to three times, then gently swirling the microcentrifuge tube briefly. **Do not vortex**.
5. Incubate in a water bath at 37°C for 10 min. The *L. monocytogenes* cell/lysozyme suspension should be processed immediately, without delay.
6. Continue with **Subheading 3.2**.

3.2. Preparation of SSP Solution

1. Prepare 10 mL of 1.2% SeaKem Gold agarose in sterile reagent-grade water and incubate in a 54.5°C ±1°C water bath.
2. Prepare enough SSP solution (1.2% SeaKem Gold:1% sodium dodecyl sulfate: 0.2 mg/mL proteinase K) for 10 cell suspensions (*see* **Table 1**).
3. Add 300 µL of 10% SDS to a 50-mL polypropylene screw-cap tube; place the tube inside a beaker containing water in a 54.5°C water bath. The water in the beaker should be at 54.5°C before placing the tube containing the 10% SDS in the beaker.

Table 2
Cell Lysis Buffer

Number of samples	50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, pH 8.0, 1% *N*-lauroyl-sarcosine	Proteinase K (20 mg/mL)
1	4 mL	30 µL
5	20 mL	150 µL

4. Add 2.7 mL of agarose to the tube containing 10% SDS; mix by swirling.
5. Add 30 µL of proteinase K just before the SSP solution is ready for use; mix by swirling and keep the tube in the water inside the beaker at 54.5°C.
6. Plug preparation: Remove *L. monocytogenes* cell/lysozyme suspensions in 1.5-mL microcentrifuge tubes from water bath (37°C) and place them in the rack with the *Salmonella* ser. Braenderup bacterial cell suspension at room temperature.
7. Remove SSP agarose from 54.5°C ±1°C water bath; keep in a beaker of warm water so that the agarose will stay warm while making plugs. Work quickly so that the agarose will not begin to solidify before the plugs are made (*see* **Note 5**).
8. Add 300 µL of SSP agarose solution to the first 300 µL cell suspension; mix by **gently** pipetting mixture up and down a few (2–3) times.
9. Immediately, dispense part of mixture into the appropriate well of a plug mold; repeat the procedure to prepare a second plug of the same mixture. Do not allow bubbles to form. Repeat for remaining samples. Allow plugs to solidify for 5 to 10 min at room temperature.

3.3. Lysis of Cells in Agarose Plugs and Washing of Agarose Plugs After Cell Lysis

1. Label five 50-mL polypropylene screw-cap tubes with cell suspension numbers (T1, T2, T3, H2446, and H9812) and add 20 mL of cell lysis solution to the first 50-mL tube.
2. Add 150 µL of proteinase K stock solution to the tube containing 20 mL of cell lysis solution; the final concentration of proteinase K in the cell lysis buffer is 0.15 mg/mL. Mix the tube well. **Table 2** shows the calculations for making the cell lysis buffer.
3. Add 4 mL of cell lysis buffer to each of the other labeled 50-mL polypropylene screw-cap tubes (this will leave 4 mL in the original tube).
4. Add plug(s) to appropriately labeled tubes containing the cell lysis buffer. Two plugs of the same strain can be lysed in the same 50-mL tube.
5. Trim excess agarose from top of plug with a scalpel. Dispose of the scalpel in an appropriate biohazard container.
6. Reusable Plug Molds: Open mold and transfer plugs from mold with a 5–6-mm-wide spatula to appropriately labeled tube. If tape is used to label the reusable mold, remove the tape from the reusable mold and immerse both sections of plug mold and spatulas in a container with 10% bleach solution (*see* **Note 6**).

7. Recap all tubes; confirm that plugs are submerged in the buffer and not on the side of the tube. **All steps up to this point in the protocol should be done without delay in sequence as outlined.**
8. Place tubes in the rack in 54°C (±1°C) shaking air incubator or water bath; incubate for 2 h with constant agitation.
9. Place the flask (bottle) of reagent-grade water (200 mL) and the flask (bottle) of TE (350 mL) in a 50°C (±1°C) water bath.
10. After 2 h, remove tubes containing plugs from the incubator or water bath and lower the temperature to 50°C (±1°C). Remove caps and replace with green screened caps. Carefully pour off cell lysis buffer into a discard container. Touch the top of the cap onto an absorbent paper towel so that most of the liquid during this and subsequent wash steps is removed.
11. Add 15 mL of reagent-grade water that has been preheated to 50°C to each tube and screw original cap on top of green screened cap.
12. Confirm that plugs are under water and not on the side of the tube or in the green cap; return to shaking incubator (50°C). Shake tubes for 10 min.
13. Pour off water and repeat wash step with preheated water **(step 2)** one more time.
14. Pour off water; add 15 mL prewarmed (50°C) TE, mix, and shake in 50°C incubator for 15 min.
15. Pour off TE and repeat TE wash step three more times.
16. Pour off TE, add 15 mL TE; use immediately or leave overnight at room temperature (*see* **Note 7**).
17. On the following morning, pour off TE; add 10 mL of room-temperature TE.
18. Use plugs immediately or store them at 4 to 6°C.

3.4. Buffer Preparation for Restriction Digestion of DNA in Agarose Plugs

Read the instructions in this section very carefully before continuing. Three different restriction enzymes and their respective buffers will be used. Plugs from the four *Listeria* strains will be restricted with New England Biolabs (NEB) *Asc*I (Buffer 4), three of the four *Listeria* strains with Roche *Apa*I (Buffer A), and the PulseNet Standard/reference strain, *Salmonella* ser. Braenderup H9812 will be restricted with Roche *Xba*I (Buffer H). Label all tubes carefully. *See* the Microcentrifuge Tube Rack Template that shows how the tubes should be labeled and arranged in the rack **(Fig. 1)**.

1. Label the 1.5-mL microcentrifuge tubes according to the rack template and place them in the rack.
   Microcentrifuge tubes labeled A, B, C, and D will be used for *Asc*I.
   Microcentrifuge tubes labeled E, F, and G will be used for *Apa*I.
   Microcentrifuge tubes labeled S1, S2, and S3 will be used for *Xba*I.
   Microcentrifuge tubes labeled Buffer 4, Buffer A, and Buffer H are for 1X buffer mix.

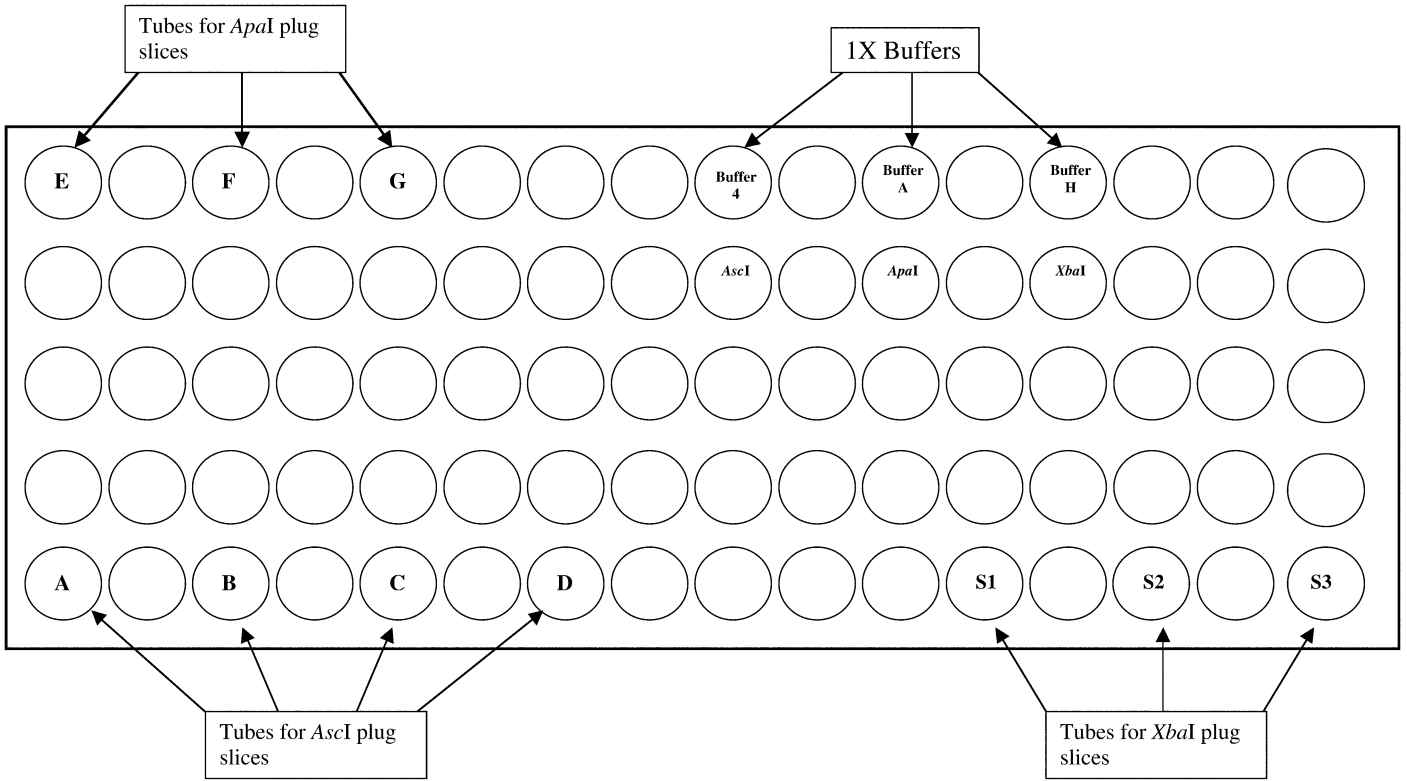

Fig. 1. Microcentrifuge tube rack template schematic diagram showing the arrangement of microcentrifuge tubes.
Tubes S1–S3 = *S.* Braenderup (H9812) plug slice
Tubes A, B, C, and E, F, G = *L. monocytogenes* test strains plug slice; T1–T3
Tube D = *L. monocytogenes* reference strain (H2446) plug slice

Microcentrifuge tubes labeled *Asc*I, *Apa*I, and *Xba*I are for 1X buffers for restriction enzyme mix.

2. Prepare 1X buffer for plug slice preincubation and enzyme mix from the stock 10X buffers according to the tables below and mix the tube well by inverting. Twice the amount of 1X buffer will be prepared for each plug to be tested. Part of the 1X buffer solution will be used for the plug slice preincubation and part will be used for the enzyme mix. Wear gloves when handling buffers and enzymes.
3. NEB 1X Buffer 4: Measure sterile reagent-grade water and 10X Buffer 4 (New England Biolabs) into labeled Buffer 4 microcentrifuge tube according to the following table to make a 1:10 dilution of the buffer. Measure reagents carefully; prepare enough for 9 plug slices. Mix well.

Reagent	µL/Plug slice	µL/9 Plug slices
Sterile reagent-grade-water	135 µL	1215 µL
NEB Buffer 4	15 µL	135 µL
Total volume	150 µL	1350 µL

4. Using a 1000-µL pipet and tip, add 737 µL of 1X NEB Buffer 4 to the 1.5-mL microcentrifuge tube labeled *Asc*I and store it on ice. This 1X NEB Buffer 4 will be used to prepare the *Asc*I enzyme mix.
5. Add 150 µL of 1X NEB Buffer 4 to microcentrifuge tubes A, B, C, and D.
6. Roche 1X Buffer A: Measure sterile reagent-grade water and 10X Buffer A into the labeled Buffer A microcentrifuge tube according to the following table to make a 1:10 dilution of the buffer. Measure reagents carefully; prepare enough for 8 plug slices. Mix well.

Reagent	µL/Plug slice	µL/8 Plug slices
Sterile reagent-grade water	135 µL	1080 µL
Roche Buffer A	15 µL	120 µL
Total volume	150 µL	1200 µL

7. Using the 1000-µL pipet and tip add 580 µL of Roche 1X Buffer A to the 1.5-mL microcentrifuge tube labeled *Apa*I and store it on ice. This 1X Buffer A will be used to prepare the *Apa*I enzyme mix.
8. Add 150 µL of 1X Buffer A to microcentrifuge tubes E, F, and G.
9. Roche 1X Buffer H: Measure sterile reagent-grade water and 10X Buffer H into the labeled Buffer H microcentrifuge tube according to the following table to make a 1:10 dilution of the buffer. Measure reagents carefully; prepare enough for 6 plug slices. Mix well.

Reagent	µL/Plug slice	µL/6 Plug slices
Sterile reagent-grade water	180 µL	1080 µL
Roche Buffer H	20 µL	120 µL
Total volume	200 µL	1200 µL

10. Using the 1000-µL pipet and tip, add 585 µL of Roche 1X Buffer H to the 1.5-mL microcentrifuge tube labeled *Xba*I and store it on ice or at refrigerator temperature. This Roche 1X Buffer H will be used to prepare the *Xba*I enzyme mix.
11. Add 200 µL of 1X Buffer H to microcentrifuge tubes S1, S2, and S3.

3.5. Cutting of Plug Slices

1. Carefully remove *Salmonella* ser. Braenderup (H9812) plug from tube containing TE with wide end of spatula and place in a sterile disposable petri dish (*see* **Note 8**).
2. Cut three 2- to 2.5-mm-wide slices from H9812 plug with a razor blade and transfer one slice to each of the three tubes that contain 1X Buffer H (tubes S1, S2, and S3). Be sure plug slices are under buffer. Replace the remaining piece of plug in the original tube (*see* **Note 9**).
3. Use the same procedure to cut slices from plugs in tubes labeled T1, T2, T3, and H2446. Cut two slices each from plug T1, T2, and T3. One plug slice will be added to the microcentrifuge tubes containing 1X Buffer 4 (A = T1, B = T2, and C = T3) and 1X buffer A (E = T1, F = T2, and G = T3). Cut one plug slice from H2446 and place it in the microcentrifuge labeled D (D = H2446). Also, *see* **Fig. 1** for letter designation for corresponding plugs.
4. Incubate all microcentrifuge tubes at 37°C for 5 to 10 min.
5. After incubation time is up, carefully remove the 1X buffer from each tube by turning the tube on its side and inserting a pipet fitted with a 200–250-µL tip all the way to bottom of the microcentrifuge tube to aspirate the buffer. Be careful not to cut the plug slice with the pipet tip, and that the plug slice is not discarded with the tip.

3.6. Preparation of AscI, ApaI, and XbaI Restriction Enzymes

1. Prepare NEB *Asc*I enzyme mix according to the following table, using the previously prepared *Asc*I microcentrifuge tube containing 737 µL of 1X Buffer 4.
2. Add 13 µL (10 units/µL) *Asc*I enzyme.
3. Mix well and keep on ice.
4. Add 150 µL of the *Asc*I enzyme mix to the microcentrifuge tubes labeled A, B, C, and D.

Reagent	µL/Plug slice	µL/5 Plug slices
1X NEB Buffer 4	147.5 µL	737 µL
NEB *Asc*I Enzyme (10 U/µL)	2.5 µL	13 µL
Total volume	150 µL	750 µL

5. Prepare Roche *Apa*I enzyme mix according to the following table using the previously prepared *Apa*I microcentrifuge tube containing 580 µL of 1X Roche Buffer A.
6. Add 20 µL of 40 units per µL *Apa*I enzyme.
7. Mix well and keep on ice.
8. Add 150 µL of the *Apa*I enzyme mix to the microcentrifuge tubes labeled E, F, and G.

Reagent	µL/Plug slice	µL/4 Plug slices
1X Roche Buffer A	145 µL	580 µL
Roche *Apa*I Enzyme (40 U/µL)	5 µL	20 µL
Total volume	150 µL	600 µL

9. Prepare Roche *Xba*I enzyme mix according to the following table using the previously prepared *Xba*I microcentrifuge tube containing 585 µL of 1X Roche buffer H.
10. Add 15 µL (40 U/mL) of *Xba*I enzyme.
11. Mix well and keep on ice.
12. Add 200 µL of the *Xba*I enzyme mix to the microcentrifuge tubes labeled S1, S2, and S3.

Reagent	µL/Plug slice	µL/3 Plug slices
1X Roche Buffer H	195 µL	585 µL
Roche *Xba*I Enzyme (10 U/µL)	5 µL	15 µL
Total volume	200 µL	600 µL

13. Incubate microcentrifuge tubes A, B, C, D, S1, S2, and S3 in a 37°C water bath for at least 3 h or overnight.
14. Incubate microcentrifuge tubes E, F, and G in a 30°C water bath for at least 5 h or overnight.

3.7. Casting Agarose Gel and Loading Restricted Plug Slices on Comb

1. Confirm that the gel casting stand (standard casting stand, with 14 × 13 cm frame and platform, Bio-Rad, cat. no. 170–3689) is level on the leveling table 20 × 30 cm (Bio-Rad, cat. no. 170-4046). Use the leveling bubble if necessary to level the leveling table. Place a 10-well comb in 14-cm-wide gel form. Confirm that the front of the 10-well comb, 14 cm wide, 1.5 mm thick (Bio-Rad, cat. no. 170-4326) holder and teeth face the top of the casting stand and that the teeth of the comb touch the bed of the casting platform.
2. Remove the restricted plug slices from the 30°C and 37°C water baths.
3. Remove enzyme/buffer mixture from each plug slice with pipet and tip. Insert pipet fitted with a 200–250-µL tip all the way to the bottom of the tube and aspirate the buffer. Be careful not to cut the plug slice with the pipet tip and that the plug slice is not discarded with the tip.
4. Add 200 µL of 0.5X TBE to each plug slice.
5. Place the comb on the bench top or on the casting mold with the comb facing down and load the plug slices on the bottom edge of the comb teeth. Starting from the left side of the comb, place the *Salmonella* ser. Braenderup (H9812) standard plug slices in microcentrifuge tubes S1, S2, and S3 on teeth 1, 6, and 10, respectively.
6. Load *Asc*I restricted plug slices in microcentrifuge tubes A, B, C, and D on teeth 2, 3, 4, and 5, respectively.
7. Load *Apa*I restricted plug slices in microcentrifuge tubes E, F, and G on teeth 7, 8 and 9, respectively.

8. Remove excess buffer with edge of lint-free tissue; allow the plug slices to air-dry for approx 2 to 3 min.
9. Position the comb in the gel casting platform, confirm that plug slices are correctly aligned, and carefully pour 100 mL of molten 1% SKG agarose (54.5°C) into the gel form. Remove any bubbles that form with a clean pipet tip. Allow the gel to solidify for 10 to 20 min before removing the comb (*see* **Note 10**).

3.8. Preparation of Pulsed-Field Electrophoresis System

1. These instructions assume the use of a Bio-Rad CHEF Mapper XA System or CHEF-DR III Variable Angle System fitted with a cooling module and variable speed pump (Bio-Rad, Hercules, CA). Confirm that the electrophoresis chamber is level using the leveling bubble supplied with the unit; adjust the leveling screws on the bottom of the unit, if necessary. Seat the 14 × 13 cm black gel frame (supplied with the standard casting stand) in the electrophoresis chamber; avoid touching the electrodes.
2. Add 2.2 L of running buffer (0.5X TBE); close the cover of electrophoresis chamber (*see* **Note 11**).
3. Turn on the power supply and pump; confirm that the pump is at the appropriate setting between 70 and 90 (calibrate the buffer flow to approx 1 L/min) and that buffer is circulating through the tubing.
4. Turn on the cooling module and confirm that temperature setting is 14°C. Check the buffer temperature in the electrophoresis chamber by pressing ACTUAL TEMP on the cooling module panel. It takes approx 20 min for the buffer to cool to 14°C.

3.9. Electrophoresis of Restriction Digests in PFGE Gel

1. Unscrew and remove end gates from the gel form; remove excess agarose from sides and bottom of the casting platform with a tissue. Keep the gel on the black casting platform and carefully place gel inside the casting frame in the electrophoresis chamber. The running buffer should cover the gel. Close the cover on the chamber.
2. Use the following electrophoresis conditions for digested *L. monocytogenes* DNA plugs slices when using the Chef Mapper electrophoresis unit:
   Select Auto Algorithm on the Chef Mapper key pad.
   Enter 30 kb for the Low MW; enter 700 kb for the High MW.
   Select default values by pressing "Enter."
   Change run time to 19 h; press Enter.
   Change initial switch time to 4.0 s.
   Change final switch time to 40.0 s.
3. Press "Start Run"; gas bubbles should begin to form at the electrodes.

3.10. Staining and Documentation of PFGE Agarose Gel

1. When the run is over, turn off chiller, pump, and Chef Mapper (3 power switches), open the lid, and remove the gel.
2. Place the gel in a covered plastic container that contains 40 µL ethidium bromide/400 mL deionized water. (Stock solution is 10 mg/mL; it is diluted 1:10,000 for staining) (*see* **Note 2**).

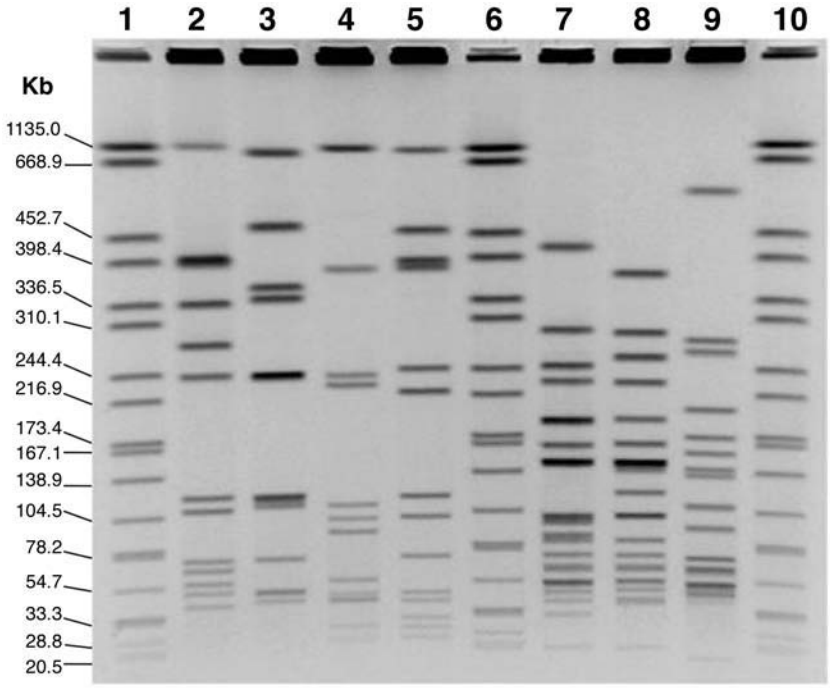

Fig. 2. Pulsed-field gel electrophoresis separation of *Asc*I (T1, T2, T3, and H2446; lanes 2–5) and *Apa*I (T1, T2, and T3; lanes 7–9) macrorestriction fragments of *L. monocytogenes* genomic DNA. Lanes 1, 6, and 10 *Xba*I digest of *Salmonella* ser. Braenderup standard/reference strain (S1, S2, and S3).

3. Place the container with gel on a rocker for 20 to 30 min.
4. Drain buffer from the electrophoresis chamber into large discard flask.
5. Rinse the electrophoresis chamber with 1 L deionized water and drain into large discard flask.
6. After gel has stained for 30 min, carefully pour out ethidium bromide solution into labeled bottle. **Wear gloves.**
7. Rinse gel with deionized water; discard wash. Add 500 mL water and place on the rocker to destain for 60 to 90 min; change water every 20 to 30 min, if possible.
8. Capture the image on Bio-Rad Gel Doc 2000 or ChemiDoc Documentation System or an equivalent documentation system that is equipped with a CCD camera that can provide IBM-compatible uncompressed TIFF images with resolution of ≥768 × 640 pixels **(Fig. 2)**.
9. If background interferes with resolution, destain the gel for an additional 30 to 60 min.
10. Analyze .tif image (file) using BioNumerics software **(Fig. 3)**.

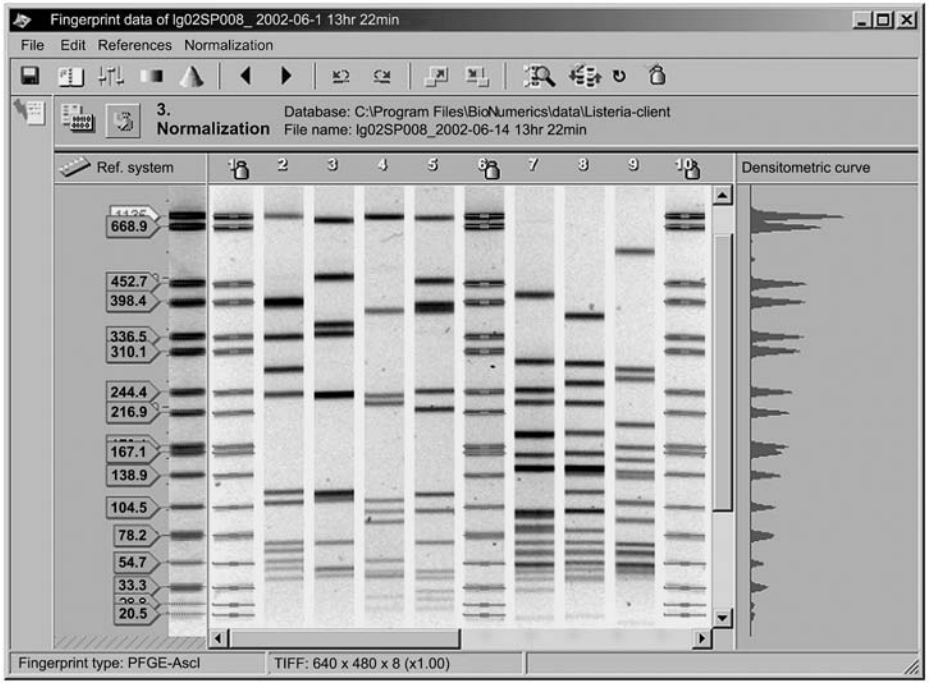

Fig. 3. TIFF image of PFGE patterns from **Fig. 2** after normalization against the PulseNet global reference standard using BioNumerics software version 3.5 (PulseNet customized version).

4. Notes

1. The agarose should be completely dissolved. Particles of agarose that are not dissolved will cause undesirable results, e.g., specks will be seen when the gel is stained with ethidium bromide.
2. Ethidium bromide is toxic and a mutagen. Store the stain according to the directions of the manufacturer and the concentrated solution should be stable for several years. If the dilute solution (1:10,000) is protected from light during storage, it can be reused six to eight times before discarding, according to your institution's guidelines for hazardous waste. Destaining bags (Amresco, cat. no. E732) are available to effectively and safely remove ethidium bromide from solutions and gels.
3. The MicroScan Turbidity Meter requires a blank tube. The blank tube should contain the solution (TE or CSB) in which the bacterial cells are suspended. Falcon 2054 tubes (Becton Dickinson) may be used; the MicroScan Turbidity Meter measurements for *Salmonella* ser. Braenderup and *L. monocytogenes* are 0.50 (±0.02) and 0.60 (±0.02), respectively. Alternately, a spectrophotometer (610 nm wavelength) may be used to adjust bacterial cell suspensions: *Salmonella* ser. Braenderup

and *L. monocytogenes* absorbance (optical density) are 1.35 (range of 1.3–1.4) in CSB and 1.3 (range of 1.25–1.35) in TE, respectively.

4. The *Salmonella* ser. Braenderup bacterial cell suspension may be held at room temperature for a short period of time (10–15 min). If the bacterial cell suspension is not used within a short time, it should be placed on ice until ready to use and then allowed to warm to room temperature.
5. If a shallow 54.5°C water bath is used, it may be possible to leave the beaker and tube containing the SSP solution in the water bath and work from the water bath. This will prevent the SSP solution from solidifying prematurely.
6. When reusable plug molds (2 cm × 1 cm × 1.5 mm) are used, up to two plugs can be made from these amounts of cell suspension and agarose: When disposable plug molds, 1.5 mm × 10 mm × 5 mm, (Bio-Rad, cat. no.170–3713) are used, four to six plugs can be made.
7. An automatic plug washing apparatus (includes pump, water bath, 30 screen caps, and connectors) is available from Lead Biotech (Taiwan). Information on the apparatus may be obtained via email, leadbiot@ms62.hinet.net.
8. A fine-point permanent mark can be used to mark two parallel lines 2.5 mm apart on the outside bottom of a Petri dish. Use these two parallel lines as a guide for cutting 2- to 2.5-mm plug slices inside the Petri dish.
9. The shape and size of the plug slice to be cut will depend on the size of the teeth on the comb used for casting the gel. Gel wells that are cast using combs with 10-mm-wide teeth will require a different size plug slice than those cast with combs with smaller teeth (5.5 mm). The number of slices that can be cut from the plugs will also depend on the skill and experience of the operator, integrity of the plug (e.g., whether it tore while doing the lysis and washing steps), and whether the slices are cut vertically or horizontally (5 mm × 10 mm plug).
10. Restricted plug slices may be loaded into the wells in a 1% SeaKem Gold agarose gel that has been poured in the gel casting platform with the comb holder positioned so that the teeth face the top of the gel casting platform and that the height of the comb's teeth is 2 mm above the floor of the gel platform. Positioning the comb in this orientation allows maximum distance for DNA fragments to migrate. The comb should be carefully removed after the gel has solidified for at least 20 to 30 min.
11. The brand (Sigma, Gibco BRL, or homemade) of stock 10X TBE used to prepare 0.5X TBE will affect the electrophoresis running time. The run time (19 h) used in this protocol is based on the equipment and reagents used at the CDC. Running times in your laboratory may vary (faster or slower) and will have to be determined empirically. A general rule is that the lowest band in the standard should migrate within 1 to 1.5 cm from the bottom of the gel.

References

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7

Plesiomonas shigelloides

Detection by PCR

Ivan Ciznar, Carlos González-Rey, Karel Krovacek, and Anna Hostacka

Summary

Plesiomonas shigelloides is a micro-organism involved in gastroenteritis infections and food poisoning. Because there is a lack of specific and sensitive methods of detection and identification of this bacterium in clinical diagnostic laboratories, the pathogen has usually been overlooked. This chapter describes a polymerase chain reaction (PCR) protocol for identification of this potential food-borne pathogen. For the diagnostic purposes, two primers were designed targeting part of the *23S rRNA* gene. The method is robust and easily performed in a standard laboratory equipped with a thermocycler, microcentrifuge, and agarose gel electrophoresis equipment. Applying this protocol, we could prove that PCR method is a suitable tool for a rapid and sensitive identification of *P. shigelloides* from different environmental and clinical samples.

Key Words: *Plesiomonas shigelloides;* identification; food-borne; detection; PCR; *23S rRNA* gene.

1. Introduction

Plesiomonas shigelloides is a Gram-negative, motile capsulated, flagellated, and non-spore-forming bacillus. Originally placed in the family *Vibrionaceae,* the genus *Plesiomonas* was recently transferred to the family *Enterobacteriaceae* due to molecular studies indicating the phylogenetic similarity of this species with *Proteus* ***(1–3)***. The primary reservoir for this bacterium is aquatic environment. *P. shigelloides* has been isolated from both freshwater (rivers, creeks, lakes, etc.) and estuarine (brackish) water, as well as from seawater ***(4–7)***.

Most of the reports on isolation of *P. shigelloides* are from countries in tropical or subtropical areas ***(8)***. The high incidence of this bacterium in Japan, Thailand, and, more recently, China has given the acronym "Asian" to this micro-organism. However, studies in Africa ***(9–19)***, among others, show that

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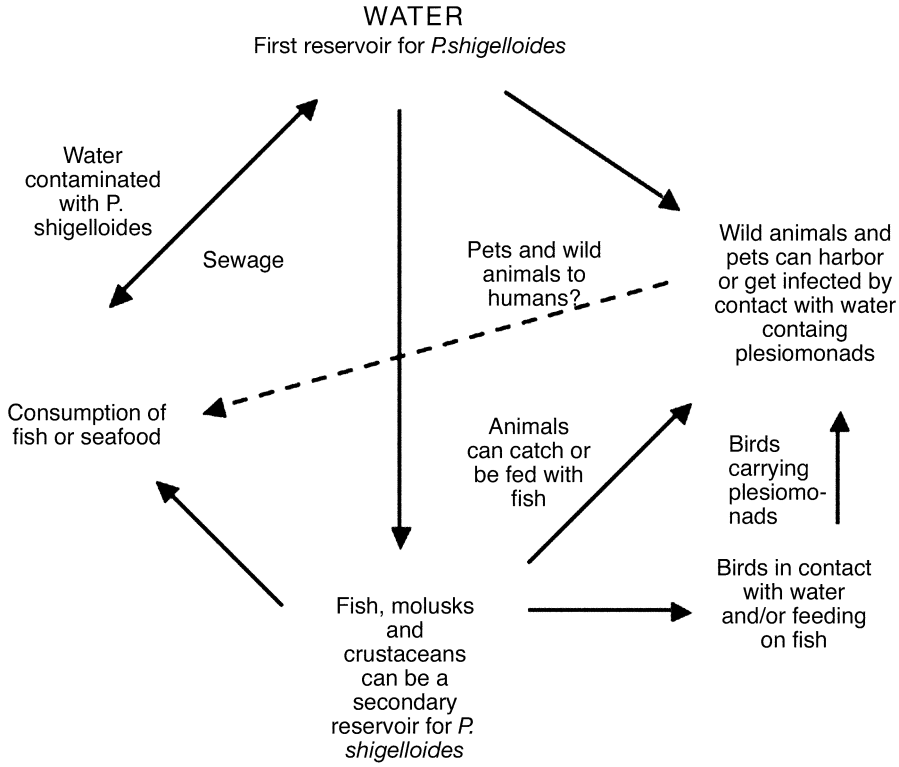

Fig. 1. *Plesiomonas shigelloides* routes of infectious pathways.

P. shigelloides is globally distributed. Surprisingly, we were able to isolate plesiomonads from a lake situated north of the Polar Circle ***(20)***.

P. shigelloides has been implicated as an agent of human gastroenteritis for more than a half century, and there are increasing numbers of reports describing infections caused by this microrganism ***(8)***. The most important vehicle for transmission of *P. shigelloides* to humans appears to be seafood ***(21,22)***, though recently transmission through contaminated vegetables was described in the literature ***(11)***. The route of entry into the human gastrointestinal tract is through the ingestion of contaminated food or water **(Fig. 1)**. Symptoms associated with gastroenteritis caused by *P. shigelloides* include diarrhea, vomiting, abdominal pain, and nausea, although chills, headache, and fever may also occur. Several virulence factors have been studied and described in the literature. They include enterotoxins, adhesins, invasines, enzymes, and other products such as tetrodotoxin and histamine that may be implicated in seafood poisoning

(23–25). The role and contribution of these factors to the overall pathogenic potential of this micro-organism are not fully elucidated yet.

Definitive diagnosis of bacterial infections requires the identification of the causative agent. Thus, an adequate bacterial identification of gastrointestinal infections is of great value to determine the correct therapy and management of the clinical cases and outbreaks. It is well known that classical bacteriological methods for isolation and identification of *P. shigelloides* are tedious and lengthy. Most clinical diagnostic laboratories concentrate on recovery of classical etiological agents of gastroenteritis, such as *Salmonella, Shigella, and Escherichia coli,* and *P. shigelloides* may thus be overlooked in a routine examination of stool samples. In latest years, polymerase chain reaction (PCR) has become a powerful tool in bacteriological research laboratories. However, unlike the latter these novel techniques have not fully reached diagnostic laboratories. The lack of standard PCR-based methods and the variety of equipment and reagents have strongly influenced its delay. Nevertheless, initiatives such as FOOD-PCR (http:/www.pcr.dk) ***(26)***, funded by the European Union, aim to establish standardized PCR-based detection methods for five major food-borne pathogens (*Salmonella* spp., *Campylobacter* spp., enterohemorrhagic *E. coli* (EHEC), *Listeria monocytogenes,* and *Yersinia enterocolitica*) can encourage diagnostic laboratories to adopt these techniques in their diagnostic routines.

Sequences originated from *23S* or *16S rDNA* are frequently used in the identification protocols for bacterial pathogens. The chances of identifying the etiological agents using species-specific sequences are high due to their highly conservative character.

We have applied a PCR protocol based on the 23S rDNA sequences for identification of *P. shigelloides* from environmental and clinical material. The PCR technique is a simple, rapid, and highly sensitive procedure for identification of the *Plesiomonas shigelloides.* It is recommended that the sample first be cultivated on a blood agar plate overnight and oxidase-positive colonies picked up and further processed according to the PCR protocol.

2. Materials

2.1. Specimen Preparation

1. Micropipets and sterile tips.
2. Sterile inoculation loops.
3. Reference strain of *Plesiomonas shigelloides* 29480 (or any other *P. shigelloides* reference strain; *see* **Note 1**).
4. Peptone water: 10 g peptone, 10 g NaCl, 1000 mL distilled water, pH 8.6 (sterilized by autoclaving; *see* **Note 2**).
5. Luria-Bertani broth: 1% Bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, pH 7.5 (sterilized by autoclaving; *see* **Note 2**).

6. Plastic plates.
7. Blood agar: 40 g blood agar base, 1000 mL distilled water. Autoclave. Let temperature go down to 45 to 50°C and add 5% sterile defibrinated bovine blood (*see* **Note 2**).
8. 1.5-mL microcentrifuge tubes.
9. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 (sterilized by autoclaving).
10. Oxidase test.
11. Heating block.
12. Microcentrifuge.

2.2. Polymerase Chain Reaction

1. 0.2-mL PCR tubes.
2. 10X PCR buffer (GeneAmp 10X PCR Buffer II, Perkin-Elmer Corp., Boston, MA, USA); store at –20°C.
3. 25 m*M* dNTPs mixed solution (Ultrapure dNTP Set, 100 m*M* each, Pharmacia Biotech, Piscataway, NJ); store in aliquots at –20°C (*see* **Note 3**).
4. Oligonucleotide primers: Prepare 5 µ*M* work solutions, store at –20°C. Sequence:
   PS23FW3: CTC CGA ATA CCG TAG AGT GCT ATC C
   PS23RV3: CTC CCC TAG CCC AAT AAC ACC TAA A
5. AmpliTaq DNA polymerase (Perkin-Elmer AmpliTaq DNA Polymerase, Perkin-Elmer Corp.), store at –20°C.
6. 25 µ*M* $MgCl_2$ (Perkin-Elmer Corp.), store at –20°C.
7. Double-distilled sterile water.
8. Thermal cycler.

2.3. Detection of PCR Product

1. Agarose.
2. 10X TBE buffer: 890 m*M* Tris-borate, 20 m*M* EDTA, pH 8.3.
3. Ethidium bromide, 0.5 µg/mL.
4. Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in TE.
5. Electrophoresis unit and power supply.
6. Molecular weight marker such as "Mixed Ladder" (Invitrogen, Groningen, The Netherlands) (*see* **Note 3**).
7. Ultraviolet light such as "Ultraviolet Transilluminator" (Ultraviolet Products, Ltd., Cambridge, UK) (*see* **Note 3**).
8. Polaroid camera or any other documentation system, such as ImageStore 5000 Annotator System (Ultra Violet Products Ltd., Cambridge, UK).

3. Methods

3.1. Specimen Preparation

1. Sample material is streaked with an inoculating sterile loop onto the surface of blood agar containing 5% of bovine erythrocytes. Inoculate simultaneously 200 mL of enrichment media with the same sample (usually a cotton swab inserted in

a transport medium is sufficient). The agar plates and the enrichment broth are then incubated overnight at 37°C. Reference strain should also be cultivated in order to have a visual example of the morphology and appearance of a single true *P. shigelloides* colony (*see* **Note 4**).
2. Colonies are tested for oxidase production by means of the oxidase test reagent. Each individual colony that might be suspected of being *P. shigelloides* because of the color and morphological similarity to the reference strain should be examined.
3. Oxidase-positive colonies are marked for further studies and for the PCR assay.
4. Add 200 µL TE to a microcentrifuge tube.
5. Resuspend each single oxidase-positive colony in the tube containing 200 µL TE (one colony per tube). Incubate at 95°C for 20 min.
6. Centrifuge in a microcentrifuge for 5 min at 13,600*g*.
7. The supernatant is transferred to a new sterile tube and can be used further or stored for 1 or 2 d at –20°C in a freezer.
8. If there are no isolated oxidase-positive colonies on the blood agar plate, take a loop of enrichment medium, strike a new blood agar plate, and follow the cultivation at 37°C again. Follow the procedure as in step 1.

3.2. PCR Assay and Visualisation of Products

1. For each colony to be tested, mix 5 µL of 10X PCR buffer, 0.8 µL of a 25 m*M* dNTPs mixed solution, 5 µL of each primer from a 5 µ*M* work solution, 5 µL of $MgCl_2$, 22.2 µL of double-distilled sterile water, 5 µL of the supernatant, and 2 U of AmpliTaq DNA polymerase. Total volume of reaction will be 50 µL.
2. Place the PCR tubes into microcentrifuge and spin briefly to be sure that the reaction mix is at the bottom of the tube. Each run should contain a reference strain DNA sample and a negative control (replace 5 µL of the supernatant with 5 µL of double-distilled sterile water).
3. Place tubes in a thermal cycler and program the profile as follows: 1 cycle at 95°C for 5 min followed by 35 cycles with a denaturation step at 94°C for 1 min, an annealing temperature of 68°C for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C is done at the end of 35 cycles for 10 min.
4. Prepare a 2% agarose gel with TBE buffer. Warm the mix in the microwave oven until no agarose particles are visible (*see* **Note 5**). Let the agarose cool down to 65°C. At this point EtBr can be added (*see* **Note 6**). Pour the agarose in the sealed tray, place the comb on the top, and let the gel solidify.
5. Pour the TBE buffer into the electrophoresis chamber; place the gel in the chamber and make sure the buffer covers the upper surface of the gel. Remove the comb.
6. Mix 5 to 8 µL of the PCR reaction with 2 to 3 µL of gel loading buffer and load into the wells of the gel. At this point the DNA molecular weight marker should be loaded in at least one well.
7. Run electrophoresis at 5 V/cm until the dye approaches the end of the gel.
8. Place the gel under the UV light and record the result by photography or gel documentation system. In case of positive result a 284-bp band should be visible (*see* **Fig. 2**).

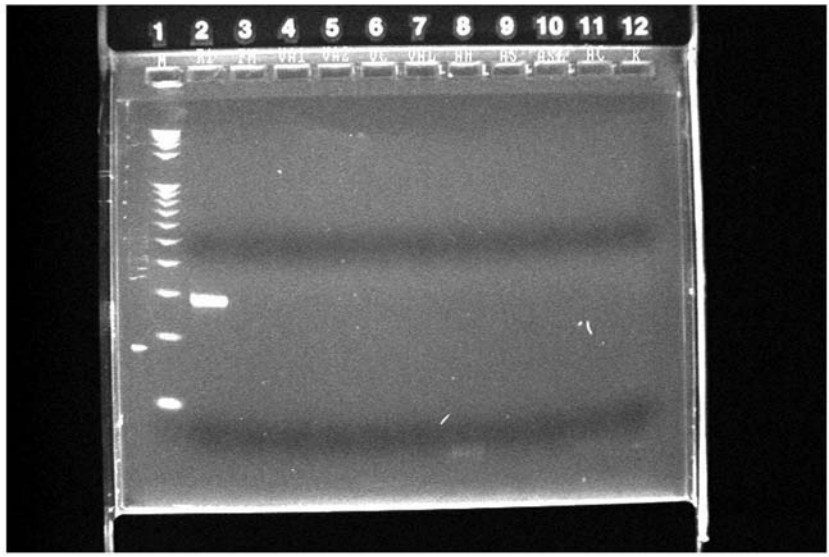

Fig. 2. Picture with the band on lane 2 (positive result) and the lack of bands from other closely related bacteria (negative result) lanes 3–12, *P. mirabilis, V. anguillarum, V. cholerae, V. alginolyticus, A. hydrophila, V. vulnificus, A. sobria, A. salmonicida, A. caviae.*

3.3. Concluding Remark

The method described in this protocol has already been checked for sensitivity and specificity confirming the reliability at the level as little as 100 fg of sample DNA detected. On the other hand, closely related bacterial species have also been tested for a false-positive signal and no amplification was observed (*see* **Fig. 2**). The method is robust and easily performed in a standard laboratory equipped with a thermocycler, microcentrifuge, and agarose gel electrophoresis equipment. Any technically skilled personnel can easily perform this laboratory procedure as described in this protocol. The key to the whole procedure is to avoid contamination at all steps. For that purpose, the pre-PCR procedure should be physically separated from the area where post-PCR is performed.

4. Notes

1. *P. shigelloides* reference strain should be identified and characterized by a certified reference laboratory.
2. Any commercial media (broth and agar) are suitable for this purpose.
3. Products of other companies can be utilized for the reaction.

4. A typical colony of *P. shigelloides* on a blood agar plate has a flat, round appearance with a smooth edge. Color of the colony may vary from white to gray.
5. Control the heating of the agarose in microwave oven. After turning off the oven let it stand inside for a few minutes in order to avoid an explosive boiling.
6. Handle EtBr carefully because it is a carcinogenic substance. EtBr can be added to agarose when its temperature is 65°C or it can be added to the running buffer. Another possibility is to prepare a bath and wash the agarose gel for 20 min when electrophoresis is finished.

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8

Pulsed-Field Gel Electrophoresis As a Molecular Technique in *Salmonella* Epidemiological Studies

Rachel Gorman and Catherine C. Adley

Summary

Salmonella are one of the most widespread micro-organisms found in the global food chain; they are frequently isolated from raw meats, poultry, and milk. They are responsible for a number of clinical syndromes, including gastroenteritis. Pulsed-field gel electrophoresis (PFGE) has been recognized as a powerful tool for molecular typing, and has been the method of choice applied in numerous epidemiological studies of *Salmonella.* The methods described herein outline (1) *Salmonella* culture preparation, (2) preparation of agarose-embedded bacterial DNA, (3) restriction endonuclease digestion of DNA-embedded agarose plugs, (4) gel electrophoresis of PFGE plugs, (5) determination of the size of restriction fragments in the PFGE pattern, and (6) chromosomal DNA restriction pattern analysis.

Key Words: *Salmonella;* pulsed-field gel electrophoresis; epidemiology; restriction endonuclease; DNA.

1. Introduction

Salmonella species are members of the Enterobacteriaceae. They are responsible for a number of clinical syndromes, including the most common type of salmonellosis, gastrointestinal infections, and septicemia and typhoid fever. Additional sequelae, which are less common, include arthritis, appendicitis, meningitis, and urinary tract infections ***(1,2)***. *Salmonella* are common in the global food chain, as they are frequently isolated from raw meats ***(3)***, poultry ***(4)***, poultry products ***(5)***, raw milk ***(6)***, pasteurized milk ***(7)***, and ready-to-eat vegetables ***(8)***. In an outbreak situation, epidemiological analysis is important to identify the spread of a clone of *Salmonella;* pulsed-field gel electrophoresis (PFGE) has been the molecular typing method of choice applied in numerous epidemiological studies of *Salmonella* from local, national, and international outbreaks ***(9,10)***. In 1998 the use of PFGE to confirm the chain of transmission

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of a genetically indistinguishable strain of *S. javiana* from restaurant food handlers to leftover food and customers, who were epidemiologically linked to the *Salmonella* outbreak, was reported ***(11)***.

PFGE has been recognized as a powerful tool and the "gold standard" of molecular typing methods ***(12–14)***, and has been very successfully applied in epidemiological studies of *Salmonella,* offering the advantages of interpretation of the entire bacterial genome in a single gel ***(15)***, high discrimination, reproducibility, and typability ***(9,10,16–19)***, as well as consensus guidelines for interpretation of PFGE results prepared by Tenover et al. ***(20)***, offering a distinct advantage over other genotyping methods.

The idea of PFGE originated from the fact that in conventional gel electrophoresis, DNA molecules pass through an agarose gel matrix in an electric field where its migration is inversely proportional to the log of its size ***(21)***. Smaller fragments move through the gel matrix faster than larger ones, and very large molecules express the same mobility, resulting in poor resolution of bands. To overcome this and to allow separation of large DNA molecules, David Schwartz applied the idea that once the electric field has been removed the DNA returns to its relaxed state, thus changing the orientation of the electric field at regular intervals. This would force the DNA molecules in the gel to relax on removal of the first field and elongate to align with a new field, a process that is size-dependent ***(22)***. This technique of PFGE involves embedding the organism in an agarose plug, thus reducing shearing of the DNA, lysing the organism *in situ,* and digesting the chromosomal DNA with an appropriate restriction enzyme ***(20,23)***. PFGE was first applied to the separation of the yeast chromosome, which is several hundred kilobases in length ***(24)***.

There are a number of electrophoresis systems commercially available, which are a variation of the original pulsed-field electrophoresis system designed by Schwartz and Cantor ***(24)***, some of which include the contour-clamped homogenous electric field (CHEF), transverse alternating field electrophoresis (TAFE), field-inversion gel electrophoresis (FIGE), and orthogonal-field alternation gel electrophoresis (OFAGE) ***(22,23,25,26)***.

2. Materials

1. Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK).
2. Chloramphenicol (Sigma, St. Louis, MO).
3. 100% molecular-grade ethanol (BDH, Poole, UK).
4. Contour-clamped Homogenous Electrophoresis Field (CHEF)™Bacterial Genomic DNA Plug Kit (Bio-Rad Laboratories, Hercules, CA), which contains the following ***(21)***:
   a. Cell suspension buffer: 10 m*M* Tris-HCl (pH 7.2), 20 m*M* NaCl, 50 m*M* EDTA (di-sodium-ethylenediaminetetraacetic acid•$2H_2O$).
   b. Plug molds.

Table 1
Restriction Endonucleases

Restriction enzyme	Sequence	Appropriate temperature (°C)	SuRE/Cut restriction endonuclease buffer (REB)	Vol. activity (units/µL)
*Xba*I	T↓CTAGA	37	H	10
*Bln*I	C↓CTAGG	37	H	10
*Spe*I	A↓CTAGT	37	H	10

   c. Proteinase K (25 mg/mL) (*see* **number 5** below).
   d. Proteinase K reaction buffer: 100 m*M* EDTA (pH 8.0), 2.0% sodium deoxycholate, 1% *N*-lauroyl-sarcosine sodium salt.
   e. Lysozyme.
   f. Lysozyme buffer: 10 m*M* Tris-HCl (pH 7.2), 50 m*M* NaCl, 0.2% sodium deoxycholate, 0.5% *N*-lauroyl-sarcosine sodium salt.
   g. 1X Wash buffer: 20 m*M* Tris-HCl (pH 8.0), 50 m*M* EDTA.
5. Proteinase K (Roche, Dublin, Ireland): Reconstitute 1 mg of proteinase K with 1 mL of 50 m*M* Tris-HCl (pH 8.0), 1 m*M* $CaCl_2$ buffer.
6. Phenylmethanesulfonyl fluoride (PMSF) (Sigma).
7. Restriction endonucleases *Xba*I, *Bln*I, and *Spe*I (Roche) (*see* **Table 1**).
8. 0.5X Tris-borate EDTA (TBE) buffer: 45 m*M* Tris-borate, 1 m*M* EDTA.
9. Ultrapure electrophoresis-grade agarose (Gibco BRL, Life Technologies, Paisley, Scotland, UK).
10. Pulsed-field gel electrophoresis marker D2416, fragment size: 48.5, 97, 145.5, 194, 242.5, 291, 339.5, 388, 436.5, 485, 533.5, 582, 630.5, 679, 727.5, 776, 824.5, 1018.5 kb (Sigma).
11. PFGE CHEF system (Pharmacia, Piscataway, NJ).
12. Ethidium bromide (Sigma).
13. Shortwave UV light source (Ultraviolet Products Inc.).
14. Scientific imaging system (Eastman Kodak).

3. Methods

The methods described below outline (1) *Salmonella* culture preparation, (2) preparation of agarose-embedded bacterial DNA, (3) restriction enzyme digestion of plugs, (4) gel electrophoresis of PFGE plugs, (5) determination of the size of bands in the PFGE pattern, and (6) chromosomal DNA restriction pattern analysis.

3.1. Salmonella *Culture Preparation*

1. Inoculate 5 mL of Luria-Bertani (LB) broth with four to five fresh colonies of *Salmonella* and incubate overnight (16 h) at 37°C, 150 rpm.

2. Adjust overnight broth cultures to $O.D._{600}$ of 0.8–1.0 (~5×10^8 cells/mL). This may require either additional incubation or dilution with sterile LB broth until the desired O.D. is reached.
3. When the desired O.D. has been reached, chloramphenicol is added to a final concentration of 180 µg/mL (*see* **Note 1**) and incubation continued for 1 h. Chloramphenicol is used to synchronize ongoing rounds of chromosomal replication and inhibit further rounds of replication.

3.2. Preparation of Agarose-Embedded Bacterial DNA

1. Centrifuge 1.25 mL of adjusted inoculum (this is equivalent to 6.25×10^8 cells/mL) at 14,000*g* for 3 min in a microcentrifuge (*see* **Note 2**).
2. Discarding the supernatant, resuspend the pellet in 50 µL of ice-cold cell suspension buffer (*see* **Note 3**).
3. Equilibrate the cell suspension at 50°C in a water bath for 5 to 10 min.
4. Prepare a 2% agarose and allow equilibration at 50°C in a water bath.
5. Using a sterile pipet tip pre-equilibrated at 50°C, combine 50 µL of 2% agarose and 50 µL of cell suspension into a sterile Eppendorf tube, which has also been pre-equilibrated at 50°C (*see* **Note 4**).
6. Mix the cell/agarose mixture gently but thoroughly using the pipet tip.
7. Immediately transfer the cell/agarose mixture to plug molds using a sterile transfer pipet.
8. Allow the mixture to solidify at 20°C for 2 to 3 min and then transfer to 4°C for a further 10 to 15 min.
9. Once the plugs are solid, push them into a sterile 15-mL universal tube containing 250 µL of lysozyme buffer and 10 µL of stock lysozyme per plug, incubate at 37°C, 50 rpm for approx 4 h.
10. Following incubation remove the lysozyme solution and wash the plug twice in 1 mL of sterile purified water per plug at 37°C, 5 rpm for 5 min.
11. Following the washing step transfer the plugs to another sterile 15-mL universal tube containing 250 µL of proteinase K buffer and 10 µL of proteinase K stock per plug and incubate at 50°C, stationary, for 24 h.
12. Wash the plugs in 1 mL of 1X wash buffer per plug at room temperature (27°C), 50 rpm for 1 h.
13. For the second wash replace the 1X wash buffer with 1 m*M* phenylmethanesulfonyl fluoride (PMSF) (*see* **Note 5**) to inactivate residual proteinase K.
14. The plugs can then be stored at 4°C in a minimal volume of 1X wash buffer (~350 µL per plug) where they are stable for up to 3 mo.

3.3. Restriction Endonuclease Digestion of Plugs

The choice of restriction endonuclease is influenced by (1) the G + C content of the bacterial species; DNA of low G + C content will cut infrequently when treated with restriction endonucleases with a G + C-rich sequence and vice versa, and (2) whether a frequent or infrequent cutter is required. The frequen-

cy of cutting depends on both the length of the recognition sequence and the base composition; the same restriction endonuclease will cut with different frequencies in the genomes of different bacterial species ***(22,23,25,26)***. For example, some common restriction endonucleases used for PFGE digestion of *S. typhimurium,* which has a G + C content of approx 53%, include *Xba*I, *Bln*I, and *Spe*I.

The following procedure is based on the restriction endonuclease digestion of a whole plug; however, one may require the digestion of just a portion of the plug. In this case place the *Salmonella* plug on a piece of parafilm and, using a sterile scalpel, slice approximately one-fourth of the plug per restriction endonuclease digestion and place in a 1.5-mL sterile Eppendorf tube. Perform the following procedure using proportionate quantities of buffer and restriction endonuclease. In this way up to four different single-restriction endonuclease digestions can be performed using just a single plug.

1. Wash the plug twice in 1 mL of 0.1X wash buffer per plug at 27°C, 50 rpm for 1 h.
2. A final wash in 0.5 mL of 0.1X wash buffer per plug is performed to reduce EDTA concentration, thus allowing for faster equilibration with the restriction enzyme buffer (REB).
3. Remove the wash buffer and replace with 1 mL per plug of appropriate 1X REB **(Table 1)** and incubate at 27°C, 50 rpm for 1 h.
4. Replace the 1X REB with 0.3 mL of fresh 1X REB.
5. Add 20 U of restriction endonuclease per plug and incubate overnight in a water bath at 37°C overnight (16–20 h).

3.4. Gel Electrophoresis of Salmonella *PFGE Plugs*

When performing gel electrophoresis, a portion of the DNA embedded plug (approximately one-fourth) will be sufficient for DNA analysis. If a whole plug has been digested, then a portion of the plug is cut as described previously, and the remainder can be stored in minimal 1X wash buffer at 4°C for up to 3 mo. The PFGE size marker D2416 is applied to both ends of the gel and, if a large number of samples are being applied, then to the center as well. This allows for normalization of the gel for computer documentation and analysis.

1. PFGE is carried out using a contour-clamped homogenous electrophoresis field (CHEF) system. The running buffer, 0.5X TBE (*see* **Note 6**), is recirculated in the system for approx 2 h prior to running the gel in order to ensure a uniform temperature of approx 14°C during the gel run (*see* **Note 6**).
2. Following digestion wash the plug twice with 0.1X wash buffer at 27°C, 50 rpm for 1 h.
3. Replace the 0.1X wash buffer with 0.5X Tris-borate EDTA (TBE) buffer to equilibrate the plug prior to running in the PFGE system.

4. Prepare a 1.2% agarose gel (*see* **Note 6**) and allow to solidify completely.
5. Load a portion of the DNA-embedded agarose plug in each well.
6. Also load a segment of the PFGE marker to the extremes of the gel. Depending on the number of plugs being analyzed, the marker should be run every five lanes (*see* **Note 7**). Gel electrophoresis is performed using a 180 V, 125 mA pulsed ramp 1 to 50 s for 42 h for *Xba*I restriction endonuclease digestion and 1 to 50 s for 25 h for *Bln*I and *Spe*I (*see* **Note 6**).
7. Carefully remove the gel from the PFGE system and stain in 250 mL of 0.5X TBE containing 25 µL of 10 mg/mL ethidium bromide for approx 4 h.
8. Destain in 250 mL of 0.5X TBE for a further 4 h (this may be left overnight) before positioning over a UV light source.

Depending on the scientific imaging system available in the laboratory it may be possible to determine the unknown size of the restriction fragments in the PFGE profile; however, this may also be determined manually and requires the ability to take a photograph of the gel.

*3.4.1. Determination of Size of Restriction Fragments in PFGE Profile **(27)***

From the gel photograph, the distance that the molecular weight marker of known band size has moved in the gel can be determined.

1. Using a ruler the distance is measured from the bottom of the gel to the leading edge of the marker band and is expressed in millimeters.
2. Using the Microsoft Excel computer program, a calibration curve can then be plotted of the $\log_{10}$ kb vs the distance migrated in millimeters.
3. Apply the best-fit line to the graph and determine the unknown from the equation of the line, e.g., generally the best-fit line is a power line where the equation of the line is $y = mx^c$, where y is the unknown, m is a constant, x is the distance migrated by the unknown, and c is a constant.

3.5. Interpretation of PFGE Gel

Chromosomal DNA restriction patterns produced by PFGE are interpreted based on the Tenover et al. ***(20)*** criteria for bacterial strain typing. This method is based on genetic events that affect the banding pattern. Based on the banding pattern, each isolate is assigned to one of four categories:

Indistinguishable: An isolate is designated genetically indistinguishable if the restriction patterns had the same number of bands and the corresponding bands were the same apparent size.

Closely related: An isolate is considered closely related to the outbreak strain if its PFGE pattern differed from the outbreak strain by a single genetic event, i.e., a point mutation or an insertion or deletion of DNA.

Possibly related: An isolate is considered to be possibly related to the outbreak strain if its PFGE pattern differed from the outbreak strain by two inde-

pendent genetic events, i.e., 4 to 6 band differences that could be explained by a point mutation or simple insertions or deletions of DNA.

Unrelated: An isolate is considered unrelated to the outbreak strain if its PFGE pattern differed from that of the outbreak strain by three or more independent genetic events giving rise to seven or more band differences.

3.6. Statistical Analysis of PFGE Gel Interpretation

The Dice coefficient quantifies the similarity between two items ***(28)***. Initial interpretation of the PFGE gel by the Tenover et al. criteria ***(20)*** uses visual interpretation of the banding pattern; the Dice coefficient quantifies this interpretation. In this case the Dice coefficient quantifies the similarity between two isolates of *Salmonella,* one being the outbreak strain. The Dice coefficient is expressed algebraically as:

$$2*n/(a + b)$$

where n is the number of restriction fragments that both isolates have in common;

a is the number of restriction fragments observed for the outbreak strain;

b is the number of restriction fragments observed for the isolates being compared to the outbreak strain.

The closer the Dice coefficient is to 1, the greater the similarity between the *Salmonella* strains.

4. Notes

1. Chloramphenicol must be dissolved in 100% molecular-grade ethanol.
2. Bio-Rad manufacturer's instructions suggests using 5×10^8 cells for each mL of agarose plug to be made, i.e., 1 mL of adjusted inoculum. However, for *Salmonella* PFGE we found stronger band visualization when this was increased to 6.25×10^8 cells/mL, i.e., 1.25 mL of adjusted inoculum.
3. A critical factor in obtaining high yields of intact chromosomal DNA that will efficiently undergo restriction enzyme digestion is to prevent spontaneous autolysis and DNA degradation prior to incubation in the lysis buffer. This is accomplished by processing the cultures rapidly into cold resuspension buffer and holding the cells on ice prior to agarose plug preparation ***(25)***.
4. Owing to the viscous consistency of agarose, it is necessary to remove the narrows-bore tip from the pipet tips prior to autoclaving. Also, the pipet tips must be equilibrated at 50°C prior to use. These two techniques will allow easy transfer and mixing of the agarose with the culture preparation.
5. Prepare a stock solution of 100 m*M* PMSF dissolved in 100% isopropanol. A working solution of 1 m*M* PMSF is prepared with sterile distilled water.
6. When performing a PFGE separation, the ultimate aim is for best resolution in the quickest time possible. There are a number of parameters that affect both: (a) the

voltage gradient: different voltage gradients are used for different size ranges of DNA, large molecules prefering lower voltages, which require a longer time period, and vice versa; (b) agarose: a durable, low electroendosmosis (EEO), high-purity agarose should be used for PFGE, many of which are commercially available; agarose concentration will affect the speed of separation and the size range of fragments resolved; (c) running temperature: cooled systems at about 14°C achieve best resolution; (d) running buffer: high resolution is obtained with buffers of good buffering capacity and low ionic strength, e.g., TBE used in this procedure; (e) an orientation angle of 120° will provide good resolution and separation of digested chromosomal DNA ***(22,23,25,26)***.

7. It is essential that at least the first and the last lane on the gel be reference lanes. The bands on these lanes are used as external reference positions to normalize the gel. No normalization algorithm, if using a computer documentation and analysis system, can work with only one reference lane per gel.

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9

Kits for Detection of Food Poisoning Toxins Produced by *Bacillus cereus* and *Staphylococcus aureus*

Moira M. Brett

Summary

Some strains of *Bacillus cereus* and *Staphylococcus aureus* produce toxins that cause food poisoning. Bacterial toxins can be detected using tissue culture assays or biochemical techniques; however, these methods are expensive and may be slow to give a result. Commercial immunoassay kits that detect bacterial toxins are easy to use and quick to produce results. Kits that detect these toxins are used in manufacturing to monitor food quality, and are also utilized in public health investigations. These uses may have different priorities for sensitivity and specificity of an assay. This chapter discusses factors to be considered when using immunoassay-based kits, some of the limitations and problems that may be encountered, and quality control procedures.

Key Words: EIA; RPLA; kits; bacterial toxins detection; quality control.

1. Introduction

Bacterial toxins are important causes of a variety of human and animal diseases. Bioassays (using either animals or tissue culture) that detect biological activity have been used to identify toxins, together with classical biochemical techniques such as mass spectrometry and high-performance liquid chromatography, which characterize such toxins. All these methods are expensive, are labor-intensive, require experience to perform and interpret, and need expensive equipment or facilities. Commercial kits for the detection of toxins offer ease of use and a short time to result, and are simple to perform. These kits will be used in different situations, with different requirements. Kits may be used to screen large numbers of foods in manufacturing, with follow-up testing to confirm presumptive positives, or they may be used to test small numbers of foods or clinical specimens in cases of illness. These two uses may have different priorities regarding the occurrence of false-positive and false-negative results.

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2. Immunoassays and Detection of Biological Activity

The commercial kits that are available in the United Kingdom for the detection of *Bacillus cereus* and *Staphylococcus aureus* enterotoxins are immunoassays, which use antibodies to detect the presence of antigen(s) of the toxin. These immunoassays have the limitation that the immunological activity may not parallel the biological activity. There are several possible reasons for this.

1. A general limitation of all immunoassays is the possibility of cross-reaction, which can occur when other compounds are present that are of sufficiently similar antigenic structure to the toxin of interest. These other compounds will react in the assay and give a false-positive result even if toxin is absent, or give a falsely high result if toxin is present. This cross-reaction is more likely to occur in complex matrices, such as food or feces, than in less complex analytes such as culture supernatants.
2. The stability of immunological activity may be different from the stability of biological activity. An example of this is the finding that the serological activity of staphylococcal enterotoxin is more heat-labile than the biological activity. This can lead to false-negative results in food that has been heated and contains toxin that is still biologically active but has lost immunological activity due to heating ***(1)***. Conversely, it has been reported that active and heat-inactivated *B. cereus* enterotoxins were not differentiated by the BCET-RPLA (*see* **Subheading 5.**), so that a heated food could give a false positive result for the presence of biologically active toxin ***(2,3)***.
3. The antigenic epitope used in generating the antibodies may not be the active site of the toxin, or there may several different functional domains in the molecule, all of which are needed for biological activity. Changes in the toxin molecule (for example, partial proteolytic degradation) can occur so that the toxin is no longer active, but these changes may not be detected by an immunoassay.
4. Immunoassays are designed to detect known antigens or toxins and will not detect unknown toxins. Thus kits for the detection of staphylococcal enterotoxins (SEs) detect SEA, SEB, SEC, SED, and SEE. There is molecular evidence for further SEs; however, these will not be detected by immunoassays.

3. Format of Immunoassays

3.1. Reversed Passive Agglutination Assay

1. In this assay, latex beads coated with specific antibody ("sensitized" beads) or with normal serum ("control" beads) are added to doubling dilutions of the test sample in a microtiter plate.
2. If antigen is present, the sensitized latex beads (coated with antibody) form a diffuse layer due to antigen–antibody reactions and so produce a lattice.
3. If the antigen is absent, the sensitized latex beads do not form a lattice and so produce a tight button.
4. The control beads (coated with normal serum) do not form a lattice, because there is no antigen–antibody reaction and so either form a tight button, or possibly a smaller diffuse layer because of nonspecific interference.

5. The difference between sensitized and control latex reactions is read by eye.
6. Because of the errors inherent in a series of doubling dilutions, it is usual to require that the sensitized latex gives a positive reaction for at least two dilutions (wells) greater than the control latex before the result is considered positive.

3.2. Sandwich Enzyme-Linked Immunosorbent Assay

1. This comprises a microtiter plate (or tube) coated with antibodies that react with the toxin antigen.
2. When toxin is added, it is bound by the antibodies on the plate.
3. Unbound material is thoroughly washed off.
4. A second antibody, also specific for the antigen, is added. This second antibody has an enzyme attached.
5. Unbound second antibody is thoroughly washed off.
6. If the second antibody has bound to the antigen on the plate, enzyme will be present. Addition of substrate for the enzyme produces a color that gives a measure of the amount of antigen present.
7. The result may be read by eye (a simple presence or absence), or the optical density may be read in a plate reader.
8. A standard curve of toxin will make the assay more quantitative. This standard curve can be produced in the laboratory from a series of dilutions of a culture supernatant of a known toxigenic strain.

4. Quality Control

4.1. Quality Control of Kit Before Use

Quality control (QC) of kits will have been performed by the manufacturer; however, batch-to-batch variation does occur. The manufacturer's controls for immunoassays may be an antigenic mimic and not the toxin of interest, and these controls may not pick up suboptimal performance with "real" toxin.

4.1.1. Additional Controls

1. Each kit should be tested with a known positive sample by the laboratory before the kit is used and must give satisfactory results. A suitable positive sample would be the cell-free culture supernatant of a known toxin-producing strain.
2. Each kit should be tested with a known negative sample by the laboratory before the kit is used and must give satisfactory results. A suitable negative sample would be the most frequently tested type of sample (e.g., feces, cheese).

4.2. Recovery of Toxin and Testing Spiked Samples

The efficiency of the extraction method will affect the sensitivity of any assay. The toxin may be extracted with lower efficiency from some food matrices than from others. The efficiency of recovery can be anything from 30 to apparently more than 100%. In general, recovery will be less effective at low concentrations of toxins than at high toxin concentrations.

4.2.1. Determination of Recovery of Toxin

1. Add a known amount of the toxin or of a cell-free culture supernatant of a toxigenic strain(s) to a food sample similar to that being tested.
2. The concentration of toxin added should be similar to the expected level in test samples or, if that is not known, chosen to be approximately five times the lower level of detection.
3. This spiked sample is then taken through the extraction process and assay in parallel with the test sample.
4. Comparison of the actual amount of toxin detected with the amount of toxin added will give information on the recovery of the entire process (the extraction and the assay).

4.3. Nonspecific Interference and Quality Control of Assay

Nonspecific interferences by the extract on the antibody–antigen reaction will affect the overall sensitivity and specificity of reversed passive agglutination assay (RPLA) and enzyme-linked immunosorbent assay (EIA) formats. Interference may arise because high levels of some constituents (e.g., lipids or proteins) in foods reduce the efficiency of the interaction of antibodies with the toxin antigen.

Interference can also occur in EIAs if the extract contains an enzyme—for example, peroxidase—that is the enzyme label used in the kit. In this situation the peroxidase naturally present in the extract increases the cleavage of the substrate. This increases the amount of color developed and the apparent concentration of toxin present. This has been shown to occur with peroxidase that is present naturally in some food (e.g., pickles) and interferes with an EIA produced by TECRA for the detection of staphylococcal enterotoxins in which the label used is peroxidase ***(4)***.

4.3.1. Internal Quality Control

1. **Negative control:** Use with each batch of tests. Use a food of the same type as that being tested (e.g., soft cheese, salami) and that is expected to be negative for the toxin(s) being tested for. This control should give a negative result in the assay and will control for any nonspecific interference of the food in the assay, and for any laboratory contamination.
2. **Positive control:** Use with each batch of tests. This will detect slow changes in assay results that may arise from aging reagents, incorrect storage temperature, or contamination. This QC of the kit is distinct from spiking and estimating recovery of the toxin in the extraction as discussed above.

5. Kits for Detection of *Bacillus cereus* Enterotoxin

B. cereus diarrheal syndrome is caused by enterotoxin that is released in the intestine following ingestion of large numbers (thought to be greater than 10^5

CFU/g food) of organisms. Laboratory confirmation of *B. cereus* diarrheal food poisoning requires detection of more than or equal to 10^5 CFU/g food or feces or the detection of enterotoxin in feces ***(5)***. *B. cereus* is widespread in a wide range of foods; some strains are psychrotrophic and able to produce enterotoxin at low temperatures ***(3)***. Enterotoxigenic activity is thought to be due to a tripartite protein of hemolytic, cytotoxic dermonecrotic, and vascular permeability factors ***(6)***. The enterotoxin has been identified provisionally, and the relative molecular weights of the three proteins are thought to be hemolysin > enterotoxin > lecithinase ***(3,7)***.

Two commercial kits are available in the United Kingdom. The TECRA sandwich EIA is manufactured by Bioenterprises Pty Ltd. (Roseville, Australia) and marketed in the United Kingdom by TECRA Diagnostics (Batley, UK). It is a sandwich EIA and can be read either visually or by a plate reader. Oxoid (Basingstoke, UK) markets the BCET-RPLA, which is manufactured by Denka, Japan.

5.1. Comparison of Kits for Detection of B. cereus *Enterotoxin:* Specificity

The results of the TECRA and RPLA kits were compared with those of Chinese hamster ovary tissue culture, which detects biological activity. There were discrepancies between the results of the different methods. Enterotoxin was detected in the culture supernatants of 13 strains of *B. cereus* using the TECRA kit, but in only 6 with the BCET-RPLA. One of the seven strains that was negative in the BCET-RPLA had been shown to produce diarrheal toxin in a monkey feeding test ***(8)***. Other studies have found no correlation between the results of the two kits, but the TECRA EIA was more sensitive than the BCET-RPLA ***(9)*** and more closely correlated with tissue culture cytotoxicity ***(10)***.

In cell-free culture superantants that had been heated at 100°C for 5 min, a treatment that will destroy the biological activity of enterotoxin, the TECRA kit and tissue culture assay did not detect enterotoxin, but toxin was detected by the BCET-RPLA ***(2,3)***. Thus the immunological activity detected by the BCET-RPLA may not give a direct measure of biological activity.

The differences in results between the two kits and tissue culture were shown to be due to the antigen detected by the two kits. Granum and coworkers ***(6)*** reported that the antiserum from the BCET-RPLA reacted against a 58-kDa component of the enterotoxin component that was cosecreted with the putative enterotoxin (the cytolytic activity) in 69 of 71 food poisoning strains and in all 87 isolates from dairy products. In contrast, the TECRA kit antiserum did not react with the enterotoxin but did react with two proteins (of 40 and 41 kDa) that were nontoxic in a vascular permeability assay ***(7)***. From these results it appears that the BCET-RPLA detects one component of the enterotoxin complex and the TECRA EIA detects two apparently nontoxic components. It is possible that the

components detected by the TECRA do participate in causing human diarrhea, but it is also possible that there may be false-negative or false-positive results.

6. Kits for Detection of *Staphylococcus aureus* Enterotoxin

Staphylococcal food poisoning is caused by the ingestion of enterotoxins (SEs) that are produced in foods by some strains of *S. aureus*. Any method for the analysis of food for SEs should be able to detect at least 0.4 ng/g food, as this concentration has caused human illness ***(11)***.

There are several kits for the detection of five SEs (SEA, SEB, SEC, SED, SEE). The SET-RPLA is supplied by Oxoid in the United Kingdom and EIAs are produced by TECRA (Batley, UK), RIDASCREEN (supplied by Quadratech, Epsom, UK) and VIDASCREEN (supplied by BioMériux, Basingstoke, UK).

6.1. Comparison of Kits for Detection of Staphylococcal Enterotoxins

6.1.1. Specificity

False-positive results have been caused in the TECRA EIA by endogenous peroxidase present in foods such as pickles and some brines ***(4)***. Pretreatment of the food with sodium azide inactivates endogenous peroxidase but also reduces the amount of SE detected by approx 20 to 30% ***(12)***.

Salami, mussels, and some other seafoods gave false-positive results in the TECRA EIA that were not due to endogenous peroxidase but were removed by treatment with normal serum or by heating at 70°C for 10 min. These false-positive results were not detected by the RIDASCREEN EIA or the SET-RPLA ***(12)***.

Nonspecific reactions have been reported with the SET-RPLA in cheese and onion pie, lasagne, bread roll, and raw frozen ravioli out of more than 300 foods tested ***(13–15)***.

6.1.2. Sensitivity

The number of SEs known to be produced by *S. aureus* is now thought to be greater than five (SEA–SEE), as gene fragments of further SEs have been detected using PCR. These SEs will not be detected by currently available kits.

The TECRA and VIDAS EIAs have the disadvantage that they use mixed reagents and do not identify the individual SEs. If either of these assays is used as a preliminary screen, a presumptive positive result should be confirmed by using another method, such as the RIDASCREEN EIA. The RIDASCREEN EIA will identify SEA to SEE; a collaborative trial of the RIDASCREEN with 12 laboratories gave essentially satisfactory results for both detection and identification of individual SEs ***(16)***. The sensitivity of the SET-RPLA is adequate for pure cultures but is too low for use in investigating suspect outbreaks of food poisoning ***(17)***.

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10

Microbiological and Molecular Methods to Identify and Characterize Toxigenic *Vibrio cholerae* From Food Samples

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Summary

Vibrios are Gram-negative γ-proteobacteria that are ubiquitous in marine, estuarine, and freshwater environments and encompass a diverse group of bacteria, including many facultative symbiotic and pathogenic strains. Toxigenic *Vibrio cholerae* strains belonging to the serogroups O1 and O139 are the etiologic agents of cholera. Apart from water-borne transmission, food plays an important role in the transmission of cholera. In the chapter, we present the basic methods used for isolation, identification, and PCR-based biotype differentiation, serotype confirmation, and detection of molecular markers of virulence. We also describe standardized methods to fingerprint the strains of *V. cholerae* by ribotyping and pulsed-field gel electrophoresis. These molecular typing techniques are now acknowledged as excellent tools in tracing the source of infection and tracking the spread of the disease.

Key Words: *Vibrio cholerae;* food analysis; cholera toxin; virulence genes; biotyping; ribotyping; pulsed-field gel electrophoresis.

1. Introduction

The second edition of *Bergey's Manual of Systematic Bacteriology* (2004) lists eight genera *(Allomonas, Catenococcus, Enterovibrio, Grimontia, Listonella, Photobacterium, Salinivibrio, Vibrio)* within the family Vibrionaceae, of which *Vibrio* has the largest number of species (*see* http://dx.doi.org/10.1007/bergeysoutline200310). Vibrios are Gram-negative γ-proteobacteria that are ubiquitous in marine, estuarine, and freshwater environments and encompass a diverse group of bacteria, including many facultative symbiotic and pathogenic strains. Of the 51 currently recognized species in the genus *Vibrio,* 10 are recognized as human pathogens. Among the 206 currently recognized O serogroups of *V. cholerae* ***(1)***, only the O1 and O139 serogroups are responsible for sporadic,

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epidemic, and pandemic cholera. Koch and coworkers discovered what is now known as *V. cholerae* O1 in Egypt in 1883, whereas O139 emerged in the Indian subcontinent in 1992. This is related to the observation that more than 95% of the strains belonging to the O1 and O139 serogroups produce cholera toxin (CT). In contrast, more than 95% of the strains belonging to non-O1 non-O139 serogroups do not produce CT. The explosive onset of symptoms in cholera is because of the effect of CT produced by multiplying vibrios within the gut ***(2)***. The potent enterotoxin binds monosialogangliosides (GM_1) present on the epithelial surface to cause loss of voluminous water and salt from the crypt cells in the form of life-threatening diarrhea. An enzyme-linked immunosorbent assay (ELISA)-based method for detection of in vitro production of CT by *V. cholerae* strains has been developed by exploiting its specific binding affinity to GM_1 ***(3)***.

Cholera is principally a water-borne disease; however, food has also been recognized as an important vehicle of transmission of cholera. The fecal-oral transmission of cholera usually occurs by the ingestion of fecally contaminated water by susceptible individuals. The disease is endemic in southern Asia and parts of Africa and Latin America, where outbreaks occur regularly, and it is particularly associated with poverty and poor sanitation.

The past few years have witnessed rapid advances in our understanding of the ecology, epidemiology, pathogenesis, and genetics of *V. cholerae.* An area that has witnessed particularly rapid strides is that on the molecular front. We now know that *V. cholerae* and probably all other vibrios have two circular chromosomes; we have the whole genome sequence of a strain of *V. cholerae* O1 of the El Tor biotype, and the genome of other strains of *V. cholerae* are being investigated. The stage is set for a new era of comparative genomics. The wealth of information that such studies will yield is enormous and the new information could bring about dramatic changes in how we identify strains and how we determine which strains have the potential to cause disease, epidemics, and pandemics.

2. Materials

2.1. Culture Media

Commercially available media; Thiosulfate citrate bile salts sucrose (TCBS) agar (Eiken, Japan), Luria-Bertani broth (LB, Difco), and Luria agar (LA, Difco).

Media that need to be prepared:

1. Alkaline peptone water broth (APW): 1% Bacto peptone (Difco) containing 1% sodium chloride (NaCl), pH 8.5. The medium is dispensed in 2.5-mL aliquots into 12×100 mm tubes and sterilized by autoclaving for 15 min.
2. AKI broth: 1.5% Bacto peptone, 0.4% yeast extract, and 0.5% NaCl containing 0.3% sodium bicarbonate ($NaHCO_3$). The medium is prepared by dissolving 1.5 g

Table 1
Ingredients of Multitest Medium Devised by Kaper *(5)*

Ingredients	Required amount
Bacto peptone	0.5 g
Yeast extract	0.3 g
Tryptone	1.0 g
Arginine hydrochloride	0.5 g
Dextrose	0.1 g
Inositol	1.0 g
Arabinose	1.0 g
Sodium thiosulfate	0.04 g
Ferric ammonium citrate	0.05 g
Sodium chloride	0.5 g
Bromocresol purple	0.004 g
Adjust the pH of the medium to 6.7 using 0.1 *N* NaOH	
Agar	2 g

All ingredients were dissolved in 100 mL of triple-distilled water, then the required amount of agar was added. The solution was mixed by vigorous shaking and agar was melted by placing the media in a boiling water bath. Four mL of molten media was dispensed into 12 × 100 mm glass tubes and sterilized at 121°C for 12 min. Following autoclaving, the tubes were placed on an inclined surface to develop a slant/butt and 30 min time was allowed for solidification of agar medium.

of Bacto peptone, 0.4 g of yeast extract, and 0.5 g of NaCl in 90 mL of water and autoclaving for 15 min. Sterilized medium is allowed to cool at room temperature and 10 mL of filter-sterilized 3% $NaHCO_3$ is added aseptically to prepare 100 mL of AKI broth ***(4)***.

3. Multitest medium: This medium was devised by Kaper ***(5)***. All ingredients **(Table 1)** were added to 100 mL of triple-distilled water, mixed by vigorous shaking, and the required amount of agar was added. The agar was melted by placing the medium in a boiling water bath or a microwave oven. The molten medium was dispensed (4 mL) into 12 × 100 mm glass tubes and sterilized by autoclaving for 12 min. Following autoclaving, tubes containing molten medium were placed on an inclined surface for 30 min for solidification of agar. Enough care was taken to generate at least 1 cm butt and an adequate slanted surface (*see* **Note 1**).

2.2. Additional Requirements

1. Oxidase reagent: 0.1 g of *N,N,N′,N′,*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) is dissolved into 10 mL of triple-distilled water and the solution is dispensed into small aliquots. All aliquots are stored at –20°C in the dark to avoid light exposure.
2. UV spectrophotometer (Cecil 3000, Cecil International, UK).

3. Stomacher 400 circulator (Seward Laboratory Systems, UK).
4. Microcentrifuges (Biofuge fresco, Heraeus, Germany).
5. Shaking, nonshaking incubators.

2.3. Serotyping Scheme for V. cholerae and CT Assay by GM_1-ELISA

1. Diagnostic antisera: *V. cholerae* O1 specific polyvalent antisera; monovalent Ogawa and Inaba antisera (Difco) and O139 antiserum. All these antisera are available from NICED, Kolkata, India.
2. Reference strains: Reference strains of *V. cholerae* O1 Ogawa classical strain O395 ***(6)*** and El Tor VC20 ***(7)***; *V. cholerae* O139 strain SG24 ***(8)***. Strains of the classical and El Tor biotypes and of O139 serogroup are available with all type culture collections and can substitute for the strains listed above.
3. Carbonate-bicarbonate buffer, pH 9.6: Dissolve 0.159 g of sodium carbonate and 0.293 g of $NaHCO_3$ in a volume of 100 mL triple–distilled water; pH of the buffer is autoadjusted to 9.6.
4. Phosphate-buffered saline (PBS), pH 7.2: The buffer is prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.425 g disodium hydrogen phosphate ($Na_2HPO_42H_2O$), and 0.2 g potassium dihydrogen phosphate (KH_2PO_4) in 1000 mL of triple-distilled water; pH is autoadjusted to 7.2.
5. PBS-T buffer: Phosphate-buffered saline, pH 7.2, containing 0.05% Tween-20 (Sigma). The buffer is prepared by dissolving 500 µL of Tween-20 in 1000 mL of PBS, pH 7.2.
6. Monosialoganglioside (GM_1): Working solution of GM_1 (2 µg/mL, Sigma) is prepared by diluting stock GM_1 (5 µg/mL) into 0.5 *M* carbonate-bicarbonate buffer, pH 9.6.
7. Cholera toxin: Purified cholera toxin was purchased from Sigma.
8. Bovine serum albumin (BSA): Fat-free BSA, Fraction V (Sigma). The BSA solution (3%, used in blocking the ELISA plate) was prepared by dissolving 0.6 g of BSA into 20 mL of PBS, pH 7.2, and 0.5% BSA solution (used for diluting antibody as well as conjugate) was prepared by dissolving 0.1 g BSA into 20 mL of PBS, pH 7.2.
9. 96-well polystyrene plates (Nunc, Denmark).
10. Rabbit anti-CT-antibody: The stock anti-CT antibody (Sigma) was diluted 1:20,000 in PBS, pH 7.2, containing 0.5% BSA.
11. Anti-rabbit IgG peroxidase conjugate: The stock conjugate (Jackson ImmunoResearch) was diluted 1:5000 in PBS, pH 7.2, containing 0.5% BSA. The specificity of the conjugate is specific to heavy and light chains of rabbit IgG.
12. 0.1 *M* Citrate buffer, pH 4.5: Dissolve 0.198 g of citric acid 0.047 m*M* and 0.312 g of trisodium citrate, 2 H_2O (0.053 m*M*) in 20 mL of triple-distilled water. The pH is autoadjusted to 4.5.
13. OPD: *O*-phenylene diaminedihydrochloride (Sigma). Dissolve 2 OPD tablets (1 mg/mL, Sigma) in 20 mL of citrate buffer, pH 4.5, containing 0.002% H_2O_2.
14. 6 *N* Sulfuric acid.
15. ELISA plate washer: ImmunoWash-1575 (Bio-Rad).
16. ELISA plate reader: Microplate reader-680 (Bio-Rad).

Table 2
Sequences of Primers Used to Detect Various Genes of *Vibrio cholerae* O1 and O139 of Diagnostic Importance

Target gene or encoding region	Primer type	Primer sequences (5′-3′)	Amplicon size (bp)
ompW	Forward	CACCAAGAAGGTGACTTTATTGTG	588
	Reverse	GAACTTATAACCACCCGCG	
ctxA	Forward	CTCAGACGGGATTTGTTAGGCACG	301
	Reverse	TCTATCTCTGTAGCCCCTATTACG	
O1 *wbe*	Forward	GTTTCACTGAACAGATGGG	192
	Reverse	GGTCATCTGTAAGTACAAC	
O139 *wbf*	Forward	AGCCTCTTTATTACGGGTGG	449
	Reverse	GTCAAACCCGATCGTAAAGG	
tcpA (cl)	Forward	CACGATAAGAAAACCGGTCAAGAG	618
	Reverse	ACCAAATGCAACGCCGAATGGAGC	
tcpA (El)	Forward	GAAGAAGTTTGTAAAAGAAGAACAC	472
	Reverse	GAAAGGACCTTCTTTCACGTTG	

cl, classical allele of *tcpA;* El, El Tor allele of *tcpA*.

2.4. PCR

1. Oligonucleotides: Oligonucleotides used for priming the PCR should be at least 16 nucleotides, and preferably 20 to 24 nucleotides in length. Primer pairs can be commercially purchased as desalted or high performance liquid chromatography (HPLC)-purified. All the primers are reconstituted in sterile triple-distilled water to a concentration of 10 μ*M*.
2. Primers: The primers used are listed in **Table 2**.
3. Standard PCR buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, and 1.5 m*M* $MgCl_2$ (*see* **Note 2**).
4. *Taq* DNA polymerase: The enzyme is commercially available from many sources. The supplier used in the methods below was from Takara, Japan. Approximately 1 unit of the enzyme is required to catalyze a typical reaction (*see* **Note 3**).
5. Deoxyribonucleoside triphosphates (dNTPs): This is a mixture of dGTP, dATP, dTTP, and dCTP at a saturating concentration of 200 μ*M* for each. This mixture is commercially available from many sources. The supplier used in the methods below was from Takara, Japan; pH of the dNTP solution should be 7.0.
6. Target sequences. Either the single- or double-stranded DNA containing the target sequences can be added to the PCR mixture (*see* **Note 4**).
7. Molecular-weight markers: DNA molecular-weight markers used in the methods below were λ *Hin*dIII, 100-bp DNA ladder and λ ladder for PFGE (New England BioLabs, USA).

8. *Escherichia coli* strain: Standard *E. coli* strain DH5α (Invitrogen) used as a negative control in PCR assays.

2.5. Ribotyping, Genomic and Plasmid DNA Isolation and Southern Hybridization

1. 1 *M* Tris-HCl buffer (pH 8.0): 121.1 g Tris base (Sigma) dissolved in 800 mL of triple-distilled water; pH is adjusted with concentrated hydrochloric acid (HCl) and volume is adjusted to 1000 mL with triple-distilled water and autoclaved.
2. 1 *M* Tris-HCl buffer (pH 7.4): 121.1 g Tris base dissolved in 800 mL of triple-distilled water; pH adjusted with hydrochloric acid (HCl) and volume adjusted to 1000 mL with triple-distilled water and autoclaved.
3. 0.5 *M* EDTA (pH 8.0): 18.61 g EDTA disodium salt dissolved with 2 g of NaOH pellet by gentle warming with triple-distilled water, keeping the final volume at 100 mL and pH adjusted to 8.0 with alkali. The solution is autoclaved for 15 min and stored at room temperature.
4. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, pH 8.0, solution containing 1 m*M* EDTA, pH 8.0.
5. SDS solution (10%): 10 g of sodium dodecyl sulfate (Sigma) dissolved in 100 mL of sterile, triple-distilled water and kept at room temperature.
6. Proteinase K solution: 20 mg of proteinase K (Sigma) dissolved in 1 mL of sterile triple-distilled water and stored at –20°C.
7. Cetyltrimethyl ammonium bromide (CTAB) solution (10% CTAB in 0.7 *M* NaCl): 4.1 g sodium chloride dissolved in 80 mL triple-distilled water; 10 g CTAB (Sigma) was added gradually with continuous stirring and heating at 65°C until it was completely dissolved. The final volume is adjusted to 100 mL with triple-distilled water.
8. RNase A solution: Pancraetic RNase (RNase A, Sigma) dissolved at a concentration of 20 mg/mL in 10 m*M* Tris-HCl (pH 7.5) and 15 m*M* NaCl, heated to 100°C for 15 min and allowed to cool slowly at room temperature, dispensed in aliquots of 0.5 mL and stored at –20°C.
9. Solution I: 50 m*M* glucose, 10 m*M* EDTA, and 25 m*M* Tris-HCl, pH 8.0. The reagent is autoclaved at 10 lbs pressure for 10 min and stored at 4°C.
10. Solution II: 0.2 *N* sodium hydroxide (NaOH), 1% sodium dodecyl sulfate (SDS). It is freshly prepared from the stock solutions of 10 *N* NaOH and 10% SDS.
11. Solution III: 3 *M* sodium acetate, CH_3COONa, pH adjusted to 4.8 with glacial acetic acid. Prepared solution is autoclaved and stored at 4°C.
12. 20X saline sodium citrate (SSC): 3 *M* NaCl, 0.3 *M* trisodium citrate, pH adjusted to 7.5. The reagent is autoclaved and stored at room temperature.
13. Depurination solution: 0.25 *N* HCl.
14. Denaturation solution: 1.5 *M* NaCl and 0.5 *N* NaOH.
15. Neutralization solution: 0.5 *M* Tris-HCl buffer, pH 7.4, containing 1.5 *M* NaCl.
16. Prehybridization buffer: The buffer is prepared with 100 mL of gold hybridization buffer (Amersham Pharmacia Biotech, USA) containing 2.92 g of NaCl and 5 g of blocking regent (Amersham Pharmacia Biotech). Briefly, the gold hybridization

buffer is warmed to 65°C and NaCl gradually added with constant stirring. Blocking reagent is gradually added with constant stirring and occasional heating until blocking agent is completely dissolved. It is stored at –20°C in 50-mL aliquots.
17. Primary wash buffer: The buffer is prepared by adding 7.5 mL of 20X SSC, 4 g SDS, and 360 g of urea with final volume of 1000 mL and stored at 4°C.
18. Secondary wash buffer: The buffer is prepared by diluting 100 mL of 20X SSC to 1000 mL in triple-distilled water and stored at 4°C.
19. Tris-acetate-EDTA (TAE, 50X): 242 g of Tris base is dissolved in a minimal volume of water. To this, 57.1 mL glacial acetic acid and 100 mL 0.5 *M* EDTA (pH 8.0) are mixed and the final volume adjusted to 1000 mL by adding triple-distilled water. The stock solution is autoclaved and stored at room temperature and electrophoresis performed with 1:50 dilution of the concentrated stock.
20. Tris-borate-EDTA (TBE, 10X): 108 g of Tris base and 55 g of boric acid is dissolved in a minimal volume of water. To this, 40 mL 0.5 *M* EDTA, pH 8.0, is added and volume was adjusted to 1000 mL with triple-distilled water. The stock solution is autoclaved and stored at room temperature and electrophoresis is performed with 0.5X TBE.
21. Restriction enzymes: *Bam*HI, *Bgl*I can be obtained from multiple suppliers. The source used in this chapter is Takara, Japan.
22. QIAquick gel extraction kit (Qiagen, Germany).
23. ECL™direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech).
24. UV crosslinker: GS Genelinker UV Chamber (BioRad).
25. UV transilluminator (Fisher Biotech).
26. X-ray film cassette: The cassette (Sigma) is suitable to accommodate X-ray film of 20.3 × 25.4 cm (Fuji Medical Film RX-U).
27. Hybron N^+ membranes (Amersham Pharmacia Biotech).
28. Phenol/chloroform/isoamyl alcohol (25:24:1).
29. Chloroform/isoamyl alcohol (24:1).
30. Ethanol: 95% stock ethanol (Wako, Japan) is used in the methods below. Another stock of 70% ethanol is prepared by diluting it accordingly in sterile triple-distilled water.
31. Vacuum pump: VacuGene pump (Amersham Pharmacia Biotech).

2.6. Pulsed-Field Gel Electrophoresis

1. Cell suspension buffer (CSB): 100 m*M* Tris-HCl, 100 m*M* EDTA, pH 8.0. The reagent is prepared by adding 10 mL of 1 *M* Tris-HCl, pH 8.0, and 20 mL of 0.5 *M* EDTA, pH 8.0, and volume made to 100 mL with triple-distilled water and sterilized by autoclaving for 15 min.
2. Cell lysis buffer (CLB): 50 m*M* Tris-HCl, 50 m*M* EDTA, pH 8.0, containing 1% sarcosine. The buffer is prepared by adding 5 mL of 1 *M* Tris-HCl, pH 8.0, 10 mL of 0.5 *M* EDTA, pH 8.0, while volume is made up to 90 mL with triple-distilled water. The reagent is autoclaved for 15 min, allowed to cool to room temperature, and 10 mL of 10% sarcosine (*N*-lauryl-sarcosine sodium salt) is added to prepare CLB.

3. Restriction enzymes: *Not*I, and accompanying buffer H (Takara, Japan).
4. Plug preparation agarose: 2% Sea-Kem HGT (FMC Bioproducts) agarose-containing 1% SDS; electrophoresis agarose 1% PFC grade (Bio-Rad).
5. Ethidium bromide: Working solution of ethidium bromide (Sigma) was prepared by diluting stock aqueous solution of ethidium bromide (10 mg/mL) to 0.5 µg/mL in triple-distilled water.
6. CHEF pulsed-field apparatus (Bio-Rad).
7. Gel documentation system: Gel Doc 2000 (Bio-Rad).
8. Hot plate: Dry bath incubator (Fisher Scientific).

3. Methods

The methods described below outline (1) the procedure for selective isolation of *V. cholerae,* (2), method for detection of cholera toxin, (3) detection of virulence genes by polymerase chain reaction (PCR), and (4) characterization of the strains by PCR-based assay, as well as DNA profiling by ribotyping and PFGE.

3.1. Culture Methods

3.1.1. Processing of Food Samples

Solid food samples were homogenized using a laboratory blender (Stomacher 400, Circulator) to form a homogenate. For this, 25 g of food specimen in 225 mL of normal saline (0.85% saline) is homogenized for about 1 to 2 min, then 0.1 mL of the homogenate is spread on TCBS directly and portions of the homogenate are enriched as described below, followed by plating of the enriched sample on TCBS.

3.1.2. Enrichment, Isolation, and Presumptive Identification of V. cholerae

1. A loopful of the homogenate is used to inoculate 3 mL of APW broth in a 12 × 100 mm test tube for selective enrichment of *V. cholerae.* All the tubes are placed in an incubator set to a temperature of 37°C for 6 h.
2. Using a sterile loop, a small portion from the surface of the enriched culture is streaked onto TCBS agar plate. TCBS plate is selective for *V. cholerae* and *V. cholerae* form typical yellow-colored colonies on TCBS due to sucrose fermentation ***(9)***.
3. After overnight incubation at 37°C, plates are examined for well-separated yellow colored colonies that are subsequently inoculated into the multitest agar medium ***(5,10)***. *V. cholerae* strains belonging to epidemic serogroups (O1 or O139) produce typical yellow-colored colonies with elevated centers.

3.1.3. Biochemical Characterization of Presumptively Identified V. cholerae

1. The multitest medium is inoculated by picking an isolated single colony from the TCBS plate. For each sample, at least five well-separated colonies are used to inoc-

ulate individual tubes containing the multitest medium, stabbing to the base of the tube with a straight needle and streaking the slant portion of the medium.

2. All the tubes are placed in an incubator set to a temperature of 37°C for 18 to 20 h, after which any change of coloration and gas production is recorded. The interpretation is based on the assumption that all *V. cholerae* strains (a) ferment glucose but not inositol and arabinose; (b) do not produce H_2S from thiosulfate and are negative for gas production; and (c) are negative for arginine dihydrolase activity.
3. Gas production will lift the medium from the bottom of the tube. Formation of H_2S can be interpreted by observing the blackening of the butt portion due to reaction of H_2S with the indicator ferric ammonium citrate. The typical reaction for *V. cholerae* is an acidic reaction at the butt portion and an alkaline reaction at the slant portion (K/A), which is observed as yellow and purple coloration at the butt and slant portion of the medium, respectively, with no gas production. The small amount of acid produced by fermentation of glucose by *V. cholerae* permits the top of the medium to revert to a purple color, while the butt portion remains acidic due to its inability to ferment arginine present in the medium.
4. A portion of the culture from the slant is used directly for the oxidase test; a positive reaction is recorded as immediate formation of deep-blue coloration upon addition of viable cultures to oxidase reagent-soaked wet filter paper.
5. Presumptive identification of *V. cholerae* is made based on the observed K/A reaction in this multitest medium, a positive oxidase test, and detecting the presence of indole in the multitest medium by the addition of Kovac's reagent to the culture present in the slant portion. Immediate formation of pink to red coloration indicates the presence of indole, and yellow coloration signifies the absence of indole.

3.1.4. Serotyping Scheme for V. cholerae

1. The antigenic specificity of the repetitive units of the polysaccharides (O antigen) of the lipopolysaccharide (LPS) of the smooth variants forms the basis of *V. cholerae* serotyping scheme.
2. Serogrouping is performed with commercially available polyvalent O1 and O139 antisera. A small portion of the culture from the slant portion of the multitest medium is taken and used for a slide agglutination test with different antisera. The agglutination is performed with *V. cholerae*-specific O1 polyvalent antiserum followed by O139 antiserum.
3. A positive agglutination within 30 s with either O1 or O139 antisera is recorded. If positive for agglutination with polyvalent O1 antiserum, the strain is further examined for agglutination with monovalent Ogawa or Inaba antisera.
4. *V. cholerae* strains that do not agglutinate with either O1 or O139 antisera are inferred to belong to the non-O1, non-O139 serogroup. These strains, if needed, can be serotyped by the somatic O antigen serogrouping scheme of *V. cholerae* developed at the National Institute of Infectious Diseases, Tokyo, Japan ***(11)***.
5. These smooth variants of *V. cholerae* are characterized by the presence of both smooth (S) O antigens and rough (R) antigens. In addition, there are *V. cholerae* strains that express only the R antigen and show strong agglutination with rough

specific antiserum ***(12,13)***. Rough strains can also be determined by agglutination of the strain nonspecifically with saline.

3.2. Cholera Toxin Expression by V. cholerae

3.2.1. Collection of Cell-Free Culture for CT Assay

Cholera toxin production by *V. cholerae* strains is determined in vitro by the GM_1-ELISA method.

1. A single colony from the nonselective LA plate is used to inoculate 10 mL of either AKI broth or LB medium, pH 6.5, taken in an Erlenmeyer flask. Organisms were allowed to grow for 16 h at 30°C under mild shaking conditions. Reference *V. cholerae* O1 classical strain O395 and El Tor VC20 were used.
2. The culture density is normalized to unit opacity at 540 nm by adding an appropriate amount of corresponding uninoculated medium.
3. Next, cell-free culture supernatant is collected by the removal of cells by centrifugation at 12,000*g* for 15 min at 4°C and subsequently used for CT assay.

3.2.2. CT Assay by GM_1-ELISA

1. Each well of a 96-well polystyrene plate is treated with 0.1 mL of 2 µg/ mL GM_1 solution prepared in 0.05 *M* carbonate-bicarbonate buffer, pH 9.6.
2. Following overnight incubation at 4°C, unbound materials are removed by washing five times with PBS-T.
3. Unbound sites were blocked by the addition of 0.1 mL of 3% fat-free BSA dissolved in PBS, pH 7.2, and plates are incubated at 37°C for 2 h.
4. Following removal of unbound BSA by five successive washes with PBS-T solution, 0.1 mL of cell-free culture supernatant is added to each well either neat or diluted 1:10 with uninoculated medium.
5. Plates are allowed to stand at 37°C for 2 h and washed five times with PBS-T solution.
6. Next, 0.1 mL of diluted (1:20,000) rabbit anti-CT antibody is added to each well; 2 h at 37°C are allowed for binding to GM_1-bound CT, if present, in the well.
7. After removal of unbound anti-CT antibody by washing five times with PBS-T solution, 0.1 mL of diluted (1:5000) anti-rabbit IgG peroxidase conjugate is applied to each well, and incubated at 37°C for 2 h.
8. Finally, the plate is washed with PBS-T solution five times and color developed by the addition of 0.1 mL of substrate solution containing 1 mg/mL of O-phenylene diaminedihydrochloride (OPD, Sigma) and 0.002% H_2O_2
9. Color reaction is stopped by the addition of 0.1 mL of 6 *N* sulfuric acid and absorbance measured in a micro-ELISA reader at 492 nm.

3.2.3. Estimation of CT Present in Culture Supernatant

1. For each set of determinations, varying amounts (0.001 µg to 1.0 mg) of purified CT in 0.1 mL of 0.5% BSA solution in PBS (pH 7.2) is used in GM_1-coated wells (which do not receive any culture supernatant).

Table 3
Detection of CT by *V. cholerae* O1 Classical and El Tor Strains Grown at Incubation Temperature of 30°C Under Varying Media and Different pH Conditions

	Culture conditions[a]		
Strains	Medium	pH	Amount[b] of CT (μg/mL/opacity unit) produced
O395[c]	LB	6.5	2.2
VC20[d]	LB	6.5	0.02
O395	AKI	7.4	0.13
VC20	AKI	7.4	0.6
E. coli (DH5α)	LB	6.5	<0.0001
E. coli (DH5α)	AKI	7.4	<0.0001

[a]Organisms were grown for 16 h under shaking conditions.
[b]Assayed by GM_1 ELISA method.
[c]Serogroup O1, biotype classical.
[d]Serogroup O1, biotype El Tor.

2. A standard curve is generated by plotting the logarithmic amount of CT in nanogram units to that of OD_{492} value. Estimation of the CT present in the test sample is computed by comparing the OD_{492} values obtained with the test samples to those of the control wells (containing purified CT of known amount). Amount of CT production is expressed as micrograms of CT produced per opacity unit (OD_{540}) of *V. cholerae* cells per milliliter.
3. Results are presented in **Table 3**, which shows that classical strain O395 produced more CT than the El Tor strain VC20 when grown in LB, pH 6.5. However, El Tor strain VC20 produced more CT in AKI medium. Results also showed that CT production by the classical strain is favored in LB, pH 6.5, while AKI conditions favored CT production by El Tor strain.

3.3. Molecular Methods for Detection of V. cholerae *Strains and Toxigenic Traits*

With the advent of molecular biology in the late twentieth century, several methods for detection of human pathogens have been developed and have improved the rapid detection of pathogens as compared with conventional methods. In the following section, PCR-based detection methods for *V. cholerae* O1 and O139 are described.

3.3.1. PCR-Based Detection

PCR-based identification or characterization relies on the amplification of a specific-sized amplicon of the target gene, which in turn is controlled by the use of a pair of single-stranded oligonucleotides specific to the target gene. This

single-stranded oligonucleotide is known as primer. The presence of a pair of primers specific to the target gene in the presence of DNA polymerase and the other reactants allows the amplification of the portion of the target gene bound by the relative positions of the primers within the same gene. In the multiplex PCR format, more than one pair of primers are used to detect the presence of more than one gene simultaneously in the test sample.

3.3.1.1. Preparation of Template for PCR

1. A portion of the culture from the multitest medium is inoculated into 3 mL of LB in a 12 × 100 mm tube and incubated for 3 to 4 h at 37°C under agitation.
2. Cells are harvested from 100 µL of culture by centrifugation at 8000*g* for 5 min at 4°C in a microfuge. The cell pellet thus obtained is resuspended directly into 200 µL of sterile triple-distilled water and treated in a boiling water bath for 5 min for the lysis of cells.
3. PCR assays are performed using 5 µL of these lysates as a source of template DNA.

3.3.1.2. Multiplex PCR Assay Targeting Genes *ompW* and *ctxA*

In the multiplex PCR format, simultaneous detection of both *ompW* and *ctxA* has been devised ***(14)***. The primer pair specific to *ompW* of *V. cholerae* origin has been shown to be species-specific. Amplification of a 588-bp band in the PCR assay with *ompW*-specific primers **(Table 2)** indicates the presence of *V. cholerae* in the test sample.

1. PCR assay is carried out with 5 µL of template DNA prepared as described above from limited dilution of the test culture.
2. Apart from 5 µL of template DNA, the PCR reaction consists of 2.5 µL of 10X reaction buffer provided by the manufacturer of *Taq* DNA polymerase, 2.5 µL of 0.25 m*M* dNTPs, and 1 U of *Taq* DNA polymerase in a reaction volume of 25 µL.
3. The concentration of primers specific to *ompW* is adjusted to 1.2 pmol/µL while primers specific to *ctxA* are adjusted to 0.25 pmol/µL.
4. The multiplex PCR assay to detect the presence of *ompW* and *ctxA* simultaneously was carried out with Mg^{2+} concentration of 20 m*M* in 10X reaction buffer.
5. PCR assay is carried out with an initial denaturation step of 5 min at 94°C followed by 30 complete PCR cycles consisting of denaturation at 94°C, annealing at 64°C, and polymerization at 72°C, allowing 30 s for each step.
6. After the completion of PCR cycles, 8 µL of the 25-µL PCR samples are separated on 1.5% agarose gels for analysis.
7. Results showed the presence of both 588-bp and 301-bp amplicons specific to *ompW* and *ctxA*, respectively, for reference O1 strains of classical biotype O395, El Tor biotype VC20 and O139 strain SG24 **(Fig. 1)**. *V. cholerae* strains of non-O1, non-O139 serogroups (lanes 4–8) also showed the presence of PCR amplicon specific to *ompW.* This result reconfirmed the identity of these strains as *V. cholerae.* Interestingly, two non-O1, non-O139 strains (lanes 6–7) also showed the presence of *ctxA* amplicon, thereby confirming their identity as toxigenic *V. cholerae* but

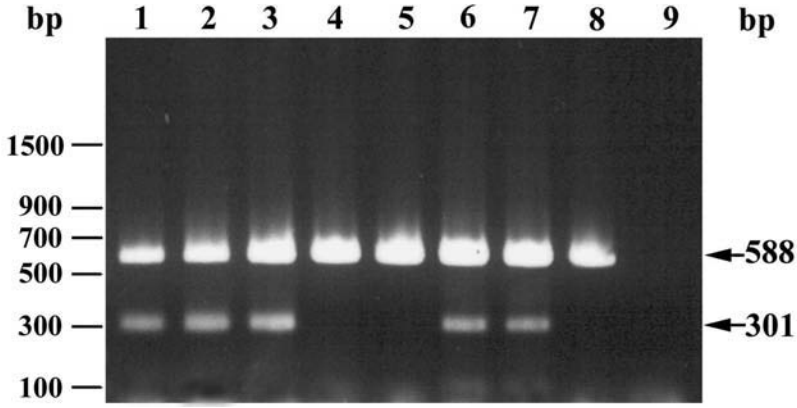

Fig. 1. Agarose gel electrophoresis patterns of PCR amplicons obtained with specific primers for *V. cholerae ompW* (for species-specific identification) and for *ctxA*. *V. cholerae* strains used were: lanes 1, O395 (O1); 2, VC20 (O1); 3, SG24 (O139); 4, SCE4 (O8); 5, SCE5 (O11); 6, SCE188 (O44); 7, SCE200 (O44); 8, 10325 (O34). *E. coli* strain DH5α was used as the negative control (lane 9). Amplicon sizes of *ctxA* (301 bp) and *ompW* (588 bp) are indicated by arrows. Positions of DNA fragments with known molecular mass are indicated in alternative from a 100-bp DNA ladder.

belonging to a serogroup other than O1 and O139. Such strains are rarely found in the environment and sometimes associated with human diarrhea.

3.3.1.3. Multiplex PCR Assay Targeting Gene Responsible for O1 and O139 Serotypes along with *ctxA*

The assay has shown to be specific and sensitive to *V. cholerae* strains belonging to either the O1 or the O139 serogroup ***(15)***.

1. The multiplex PCR assay is essentially carried out following the procedure described in **Subheading 3.3.1.2.**
2. PCR assay is performed with initial denaturation for 5 min at 94°C followed by 35 complete cycles consisting of denaturation at 94°C, annealing at 55°C, and polymerization at 72°C, allowing 1.5 min in each of these steps.
3. Primer pair specific to O1 *wbe* is used at a concentration of 1.5 pmol/μL in a reaction volume of 25 μL, while primer pairs for O139 *wbf* and *ctxA* **(Table 2)** are adjusted to 0.8 pmol/μL and 0.25 pmol/μL, respectively.
4. Results obtained with reference O1 strains of classical biotype O395, El Tor biotype VC20 and O139 strain SG24 are presented in **Fig. 2**. It is evident from the figure that both the O1 strains produced a 301-bp amplicon specific to *ctxA* as well as 192-bp amplicon specific to O1 *wbe,* while O139 strain SG24 showed the presence of 301-bp and 449-bp amplicons specific for *ctxA* and O139 *wbf,* respectively.

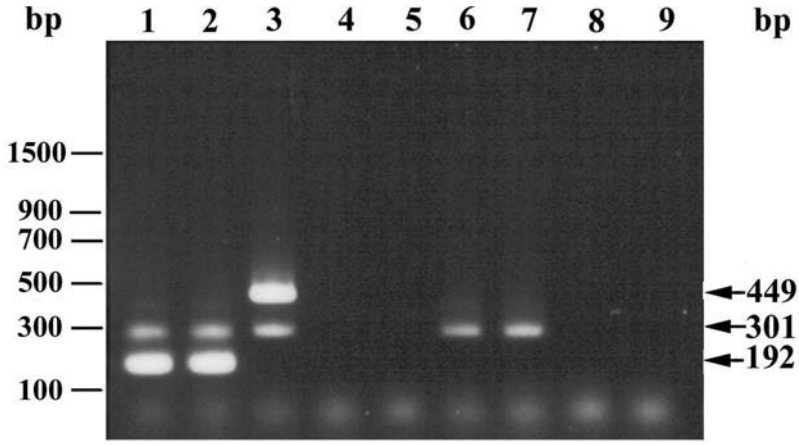

Fig. 2. Agarose gel electrophoresis patterns of PCR amplicons obtained with specific primers for *V. cholerae ctxA,* O1 *wbe* and O139 *wbf* in *V. cholerae* strains (lanes 1–8). *V. cholerae* strains used were: lanes 1, O395 (O1); 2, VC20 (O1); 3, SG24 (O139); 4, SCE4 (O8); 5, SCE5 (O11); 6, SCE188 (O44); 7, SCE200 (O44); 8, 10325 (O34). *E. coli* strain DH5α was used as the negative control (lane 9). Amplicon sizes of *ctxA* (301 bp), O1 *wbe* (192 bp), and O139 *wbf* (449 bp) are indicated by arrows. Positions of DNA fragments with known molecular mass are indicated in alternative from a 100-bp DNA ladder.

5. *V. cholerae* strains belonging to non-O1, non-O139 serogroups did not produce any amplicon specific to both O1 and O139 *wbe* or *wbf* (lanes 4–8), thereby confirming its identity as belonging to non-O1, non-O139 serogroup. Interestingly, two among these four *V. cholerae* non-O1, non-O139 strains (lanes 6–7) showed the presence of a PCR amplicon specific to *ctxA,* thereby confirming the presence of toxin genes in these strains.

3.4. Molecular Typing

3.4.1. PCR-Based Biotyping of V. cholerae *O1*

Biotyping of *V. cholerae* O1 strains using *ctxA-tcpA* multiplex PCR exploits the allele-specific nucleotide sequence differences within *tcpA* ***(16,17)***.

1. The assay is performed in 25 µL reaction volume, keeping each of the primers **(Table 2)** at a concentration of 1 pmol/µL.
2. PCR cycles consist of an initial denaturation step at 94°C for 5 min followed by 30 complete cycles of 1.5 min in each step, namely denaturation at 94°C, annealing at 60°C, and polymerization at 72°C, followed by an additional extension of 7 min at 72°C.
3. PCR amplicons (8 µL of 25 µL reaction volume) are separated on 1.5% agarose gel, stained with 0.5 µg/mL of ethidium bromide solution for 10 min, and viewed using a UV transilluminator **(Fig. 3)**.

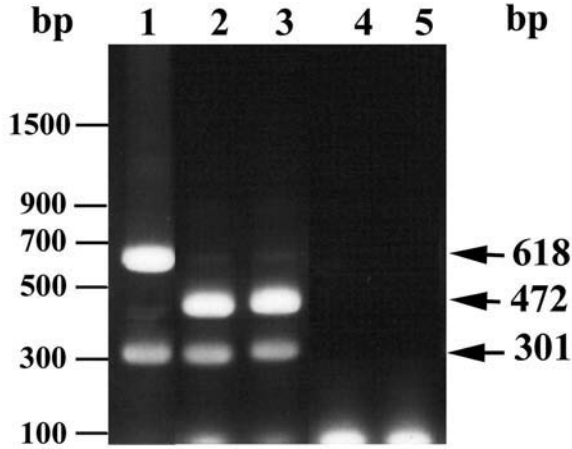

Fig. 3. Agarose gel electrophoresis patterns of PCR amplicons obtained with specific primers for *ctxA, tcpA* (classical), and *tcpA* (El Tor). *V. cholerae* strains used were: lanes 1, O395 (O1); 2, VC20 (O1); 3, SG24 (O139); 4, 10325 (O34). *E. coli* strain DH5α was used as the negative control (lane 5). Amplicon sizes of *ctxA* (301 bp), *tcpA* (classical, 618 bp), and *tcpA* (El Tor, 472 bp) are indicated by arrows. Positions of DNA fragments with known molecular mass are indicated in alternative from a 100-bp DNA ladder.

4. Results showed that *V. cholerae* O1 classical strain O395 (lane 1) and El Tor strain VC20 (lane 2) produced amplicons of 618 bp and 472 bp, respectively, specific for classical and El Tor *tcpA*. The results confirmed their identity as to classical and El Tor biotypes, respectively. Additionally, both strains produced the 301-bp amplicon specific to *ctxA* confirming their toxigenic trait. *V. cholerae* O139 strain SG24 (lane 3) produced an amplicon specific to El Tor *tcpA* (472 bp) as well as a 301-bp amplicon specific to *ctxA*. Therefore, *tcpA* polymorphism-based biotyping is applicable only to *V. cholerae* strains belonging to the O1 serogroup.

3.4.2. Ribotyping of V. cholerae

Ribotyping is a method that uses conserved sequences in the 16S and 23S ribosomal RNA genes to differentiate strains of bacteria on the basis of length of polymorphism in the 16S and 23S spacer region of the ribosomal RNA operon. This approach requires isolation of genomic DNA, enzymatic digestion, electrophoresis, Southern blotting, radioactive or fluorescent labeling of probe, hybridization, and detection of hybridized fragments by autoradiogram. The methods described below outline the different steps of standardized ribotyping of *V. cholerae* strains.

3.4.2.1. Isolation of Plasmid pKK3535

Plasmid DNA is purified following essentially the alkaline lysis method ***(18)***.

1. In brief, a single colony of *E. coli* strain DH5α containing the construct pKK3535 ***(19)*** is inoculated into 100 mL of LB broth containing 100 µg/mL of ampicillin and incubated at 37°C on a rotary shaker (200 rpm).
2. Bacterial cells are harvested by centrifugation at 8000*g* for 10 min at 4°C, suspended in 2 mL of ice-cold Solution I, and incubated on ice for 10 min.
3. Subsequently, 4 mL of Solution II is added in the same tube and gently mixed by inverting the tubes two or three times. The tube is kept on ice for 5 min.
4. 3 mL of Solution III is added. The reagents are mixed gently by inverting the tube 2 to 3 times and the tube is placed on ice for 10 min.
5. The lysate is clarified by centrifugation at 12,000*g* for 15 min at 4°C.
6. Supernatant is collected carefully from the tube without disturbing insoluble debris formed by the precipitation of denatured proteins and intact cells.
7. Two volumes of absolute ethanol are added to the collected supernatant, mixed well, and kept at –20°C for 2 h.
8. The plasmid DNA is recovered as an insoluble pellet after centrifugation at 12,000*g* for 15 min at 4°C.
9. The pellet is washed with 70% ethanol, vacuum-dried, and dissolved in 500 µL of TE containing DNase-free pancreatic RNase A (20 µg/mL) and kept at 37°C for 30 min.
10. The plasmid DNA is extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1).
11. The aqueous phase was collected in a fresh microfuge, mixed with 1/10 volume of 3 *M* sodium acetate (pH 4.8), and two volumes of absolute ethanol are added.
12. Plasmid DNA is finally recovered as insoluble pellet by centrifugation at 12,000*g* for 10 min at 4°C, washed with 70% ethanol, vacuum-dried, and dissolved in 100 µL of TE and stored at 4°C.
13. About 10 µL portion of the DNA solution is analyzed by spectroscopic measurement. DNA content was estimated from the OD_{260} value considering 50 µg equivalence to unit OD measured at 260 nm. Purity of the preparation is also estimated by calculating the ratio of OD_{260} to OD_{280}, which was 1.8, indicating that the sample was suitable for digestion with restriction enzymes.
14. The quality of the plasmid preparation was assured by agarose gel electrophoresis of 100 ng of the sample, which showed absence of any contaminating RNA and sheared genomic DNA in the plasmid preparation.

3.4.2.2. Restriction Digestion of pKK3535 With *Bam*HI and Elution of 7.5-kb Fragment as DNA Probe for Ribotyping

1. Plasmid DNA (5 µg) is digested in a reaction volume of 50 µL using restriction enzyme *Bam*HI for 2 h at 37°C.
2. Restricted fragments are separated by electrophoresis through 1% agarose gel using 1X TAE as electrophoresis buffer. A λ-*Hin*dIII molecular size marker is also electrophoresed alongside.
3. Following electrophoresis, gels are stained in 0.5 µg/mL of ethidium bromide solution for 20 min and viewed on a UV transilluminator.

4. The DNA fragment of interest is quickly cut with a sterile razor and transferred into a preweighed 1.5-mL microfuge tube.
5. Elution of the 7.5-kb DNA fragment from the gel slice is made by following the procedure of the QIAquick gel extraction kit. Briefly, three volumes of Buffer QG to 1 volume of gel slice (100 mg of gel, approx 100 µL) are added and the tubes were kept at 50°C for 10 min with occasional shaking for complete solubilization of the gel slice. Yellow coloration (similar to Buffer QG without dissolved agarose) indicates pH of the solution is 7.5. If the color of the mixture is orange or violet, add 10 µL of 3 *M* sodium acetate, pH 5.0, and mix well. The color of the mixture will turn to yellow.
6. Soluble material is applied to the QIAquick spin column set on a 2-mL collection vial and 30 s allowed for binding of the DNA material to the column.
7. DNA is recovered in the bound form to the column by a brief spin (1 min) at 12,000*g*. Flow-through is discarded and QIAquick columns are kept back on the same collection tube.
8. The columns are treated further with an additional 0.5 mL of Buffer QG and spun for 1 min to remove all traces of agarose.
9. Columns are taken back on the same collection tube and 0.75 mL of Buffer PE is applied to wash the resin bed, 1 min for equilibration allowed, and the columns are centrifuged for 1 min at 12,000*g* for the removal of unbound materials.
10. Flow-through is discarded and the columns are again centrifuged for an additional 1 min at 12,000*g* to remove residual Buffer PE.
11. The QIAquick columns with bound DNA are placed onto a fresh, sterile 1.5-mL microfuge.
12. To elute DNA, 50 µL of Buffer EB (10 m*M* Tris-HCl, pH 8.5) or sterile water is applied to the center of the QIAquick column and centrifuged for 1 min at 12,000*g*. Flow-through containing eluted DNA is collected in the fresh microfuge and used for subsequent application (*see* **Note 5**).

3.4.2.3. Nonradioactive Labeling of DNA Probe

Labeling of the DNA probes is carried out using the ECL™ Direct nucleic acid labeling and detecting system according to the manufacturer's instructions; all ingredients are supplied in the kit.

1. The probe DNA (100 ng) is diluted to a concentration of 10 ng/µL using sterile triple-distilled water and denatured by heating for 5 min in a boiling water bath followed by immediate cooling in ice for 5 min.
2. An equal volume of DNA labeling reagent (10 µL) is added to the chilled DNA solution and thoroughly mixed.
3. To this mixture, an equal volume (10 µL) of the glutaraldehyde solution is added, mixed thoroughly, and incubated for 20 min at 37°C for completion of the labeling reaction.
4. The labeled probe is stored at –20°C before being used for the assay.

3.4.2.4. Isolation of Genomic DNA

A modification of the method of Murray and Thompson ***(20)*** is used for DNA extraction.

1. Cells are harvested from 1.5 mL overnight culture by centrifugation at 12,000*g* for 5 min in a microfuge.
2. The cell pellet is resuspended in 567 µL of TE followed by the addition of 30 µL 10% SDS solution and 3 µL freshly prepared proteinase K solution (20 mg/mL).
3. The cell suspension is kept incubated for 1 h at 37°C.
4. Following incubation, 100 µL of 5 *M* NaCl, followed by 80 µL CTAB/ NaCl solution, are successively added.
5. The mixture is allowed further incubation at 65°C for 10 min and finally extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1).
6. The aqueous phase is transferred to a fresh microfuge tube and the DNA was precipitated with an equal volume of isopropanol.
7. The DNA pellet is washed with 70% ethanol, dried, and then dissolved in 500 µL of TE and treated with RNase A, keeping the concentration of RNase A at 20 µg/mL at 37°C for 30 min.
8. The treated DNA is extracted sequentially with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol following the procedures as described above.
9. The DNA is precipitated from the aqueous phase by adding two volumes of absolute ethanol, washed with 70% ethanol, vacuum-dried, and the DNA pellet is dissolved in 50 µL of TE. The DNA is stored at 4°C.

3.4.2.5. Gel Electrophoresis and Southern Transfer

1. For Southern blot, approx 5 µg of genomic DNA is digested with 10 U of *Bgl*I in a 25-µL reaction volume.
2. Digested material is electrophoresed on 0.8% agarose gel (1X TAE electrophoresis buffer) along with λ-*Hin*dIII molecular weight marker.
3. After completion of the run, the gel is soaked in depurination solution for 15 min. Then the gel is soaked in denaturation solution for 30 min, followed by neutralization for 30 min in neutralization solution.
4. DNA fragments are transferred from the gel to a Hybond N^+ membrane under vacuum. For this, vacuum is applied through the membrane and transfer is aided by slow addition of 20X SSC for 90 min.
5. After the transfer, the membrane is rinsed with 6X SSC and the transferred DNA is immobilized on the membrane using an UV crosslinker.

3.4.2.6. Hybridization and Stringency Washes

The ECL™ nucleic acid labeling kit is used according to the manufacturer's instructions. A specially optimized hybridization buffer, "gold hybridization buffer," is supplied with the kit.

1. The membrane is soaked in the hybridization buffer (preheated to 42°C) for 1 h.
2. Following prehybridization, the labeled probe is added to the prehybridization buffer and the blot is incubated overnight at 42°C with gentle agitation.
3. The prehybridization buffer contains 6 *M* urea, having equivalence to 50% formamide in reducing the T_m of the probe-target hybrid, thereby ensuring specific hybridization even at 42°C and incubation for 14 to 16 h.

3.4.2.7. Signal Generation and Detection

1. Following an incubation period of 14 to 16 h at 42°C, the membrane is washed twice for 20 min each at 42°C with primary wash buffer (preheated to 42°C) followed by two washes with secondary wash buffer at room temperature for 5 min each.
2. The DNA-carrying side of the membrane is then overlaid with a solution containing equal volumes of detection reagent 1 and detection reagent 2 supplied with the kit. Detection reagent 1 decays to generate H_2O_2, the substrate for peroxidase. Reduction of H_2O_2 by the enzyme is coupled to the light-producing reaction by detection reagent 2, which contains luminol. Excess detection reagent is drained off, and the membrane was wrapped in cling wrap (*see* **Note 6**).
3. The membrane, with the DNA side up, is then placed in the film cassette and exposed to X-ray film for 15 min, after which it is developed according to the manufacturer's instructions.

3.4.2.8. Interpreting Results

Ribotype patterns of *V. cholerae* strains of O1 and O139 serogroups are presented in **Fig. 4**. For this assay, *V. cholerae* O1 El Tor strains VC20, CO840, and BD213 were included along with O139 strain VO1. It is evident from **Fig. 4** that strain CO840 (lane 2) and BD213 (lane 3) generated identical profiles to each other; this profile is identical to the referred standardized *Bgl*I ribotype profile RIII of *V. cholerae* O1 El Tor strains that were isolated after the emergence of *V. cholerae* O139 in 1992 ***(7)***. Although the strains CO840 and BD213 were isolated in 1996 and 2003 respectively, they appeared to be clonal in nature. On the other hand, O1 El Tor strain VC20 generated a profile (lane 1) identical to the standardized *Bgl*I ribotype profile RI of *V. cholerae* O1 El Tor strains that were isolated before the emergence of *V. cholerae* O139 in 1992 ***(7)***. Interestingly, the ribotype profile of O139 strain VO1 (lane 4) did not show any match to either the RI or RIII profile of O1 El Tor strains. Although it was proposed that O139 strains evolved from O1 El Tor strains, VO1 belongs to a different clone as compared with RI and RIII clones. The ribotype profile of O139 strain VO1 is identical to that of the BII ribotype proposed for *V. cholerae* O139 ***(21)***. In fact, based on the standardized *Bgl*I ribotype profiles, *V. cholerae* O1 El Tor strains can be classified into three types, namely RI, RII, and RIII, while six ribotype profiles, BI to BVI, are known for O139 strains.

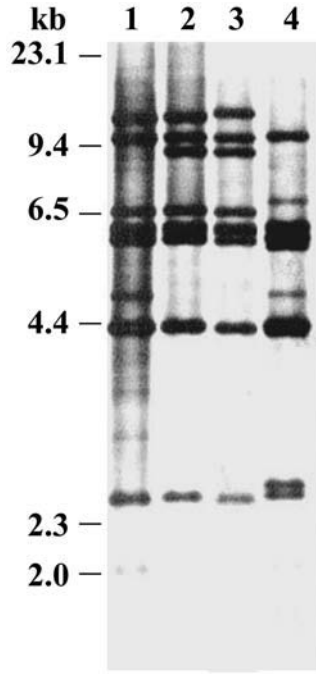

Fig. 4. Southern hybridization analysis of *Bgl*I-digested genomic DNA from *V. cholerae* strains (lanes 1–4) with a 7.5-kb *Bam*HI fragment obtained from pKK3535. *V. cholerae* strains used were: lanes 1, VC20 (O1, RI); 2, CO840 (O1, RIII); 3, BD213 (O1, RIII); 4, VO1 (O139, BII). Positions of DNA fragments obtained with λ-*Hin*dIII molecular weight marker are indicated.

3.4.3. Pulsed-Field Gel Electrophoresis

Development of pulsed-field gel electrophoresis (PFGE) techniques revolutionized the concept of separating DNA fragments on agarose gels. The PFGE-based assay relies on the comparative profiles of well-separated DNA fragments, up to 2 megabase pairs in length, generated with appropriate restriction enzyme. Similarity or closeness among different strains can be interpreted by comparing restriction patterns or similarity profiles of the DNA fragments when generated with restriction enzyme(s), which is known to be suitable for particular bacterial species. In this method, pulsed, alternating, orthogonal electric fields are applied to the gel preloaded with agarose-embedded, restriction-digested genomic DNA. Large DNA molecules become trapped in gels every time the direction of the electric field is altered and can make no further progress through the gel until they have reoriented themselves along the new axis of the electric field. Molecules of DNA whose reorientation times are less

than the period of the electric pulse will therefore be fractionated according to size. The limit of resolution of PFGE depends on (a) the degree of uniformity of the two electric fields,(b) the absolute lengths of the electric pulses,(c) the ratio of the length of the electric pulses used to generate the two electric fields,(d) the angles of the two electric fields to the gel, and (e) the relative strengths of the two electric fields.

3.4.3.1. Preparation of PFGE Plugs

As a consequence of improvements in the PFGE technique, resolution of DNA molecules larger than 5000 kb can now be achieved (*see* **Note 7**). Very long DNA molecules are extremely fragile and cannot withstand the mechanical shearing forces in the process of manipulation using standard molecular biological techniques. To prevent mechanical shearing during extraction of large DNA molecules, bacterial cells are lysed *in situ* in an agarose plug. Intact bacterial cells are resuspended in molten, low-melting-point agarose and solidified in blocks whose size matches the thickness of the loading slot of the gel. Depending on the organism, any of a variety of substances are infused into the plug to cause lysis of the cells and removal of proteins from the DNA. These procedures yield DNA that is both intact and susceptible to cleavage by restriction enzymes.

1. Test organisms are streaked on Luria agar (LA) plates to prepare a lawn of bacterial culture after overnight incubation at 37°C.
2. Cells are scraped from the plate with the aid of a bent clean sterile glass rod to prepare a cell suspension using 10 mL of cell suspension buffer (CSB).
3. The cell suspension is washed with an equal volume of CSB by centrifugation at 8000*g* for 10 min at 4°C.
4. Washed cells recovered as bacterial pellet are resuspended to an opacity of 1.3 to 1.4 at 600 nm.
5. Molten agarose for plug preparation in a volume of 200 µL is kept ready in separate 1.5-mL microfuges on a hot plate set at 55°C for equilibration to this temperature.
6. To each of the microfuges containing molten agarose, an equal volume (200 µL) of prewarmed (55°C), opacity-adjusted bacterial suspension is added and mixed gently after the addition of 10 µL of proteinase K (20 µg/µL).
7. The mixture is subsequently poured into blocks and allowed to solidify at room temperature for 20 min.
8. Upon solidification, blocks are taken into appropriate buffer (CLB) for subsequent lysis and isolation of intact genomic DNA in agarose-embedded form.

3.4.3.2. Isolation of Agarose-Embedded Intact Genomic DNA

1. Preformed agarose blocks containing embedded bacterial suspension are taken into 2.0-mL microfuges filled with 1.5 mL of CLB containing 40 µL of proteinase K (20 µg/µL).

2. Agarose plugs are incubated at 54°C in a water bath for 1.5 to 2 h under very mild shaking conditions.
3. Following incubation, all the buffers are removed carefully without disrupting the plugs and washed three times with 1 mL sterile water with a washing period of 15 min at 54°C under very mild shaking conditions.
4. Subsequently, plugs are also washed with 1 mL prewarmed (54°C) TE, pH 8.0, three times in the same way as described above. After washing the plugs with TE for the fourth me, they either can be stored at 4°C for later use or can directly be stabilized in appropriate restriction enzyme buffer for digestion with *Not*I restriction enzyme.

3.4.3.3. Restriction Digestion of Agarose-Embedded DNA

1. The plugs are stored at 4°C immersed in TE, pH 8.0. Before being used, stored plugs are kept at room temperature for 30 min.
2. TE is removed carefully and plugs are immersed in 1 mL of restriction enzyme reaction buffer (Buffer H for *Not*I, Takara, Japan) for 1 h under mild shaking at room temperature.
3. In the subsequent steps the buffer is replaced with 600 µL of fresh reaction buffer and 50 U of *Not*I restriction enzyme (Takara, Japan) is added, mixed gently, and incubated overnight at 37°C in a water bath.

3.4.3.4. Casting of Gel and Electrophoresis

1. The gel casting platform is leveled and the casting plate is placed with the comb. To cast 21-cm-wide gel, 180 mL of 1% agarose taken in 0.5X TBE is melted. Molten agarose is kept in a 65°C water bath for 15 min before being poured on the casting plate-comb assembly that is firmly placed on the gel casting platform. Enough care is taken to avoid formation of air bubbles within the gel and it is allowed to stand at room temperature for 1 h to solidify. Next, the gel comb is removed and the formed wells are washed with 0.5X TBE buffer.
2. In the subsequent steps, plugs are taken out from the incubator and the enzyme/buffer mix is replaced with equal volume of 0.5X TBE buffer. The plugs are kept immersed with 0.5X TBE buffer for 30 min under mild shaking conditions. Restricted DNA-embedded plugs are removed from the tube with the tapered end of a spatula and gently pushed into the appropriate wells, avoiding trapping of any air bubbles.
3. Wells are sealed with molten 1% low-melting agarose and allowed to solidify for at least 5 min.
4. Next, the gel is placed carefully inside the corner posts in an electrophoresis chamber that is filled with 3000 mL of 0.5X TBE prechilled to 14°C.
5. The pump dial is set to 60 to 70 while the cooling module temperature is set to 14°C. Electrophoresis is performed at the following fixed settings; these parameters are maintained for subsequent runs. It was possible to compare profiles generated in one run to another run as we kept the run parameters fixed.
6. **Run parameters:** Auto Algorithm is pressed and lower and higher molecular weight inputs are set to 20 and 300 kb, respectively. The calibration factor is kept fixed at 1.5 and all other parameters, including initial and final switch time, are

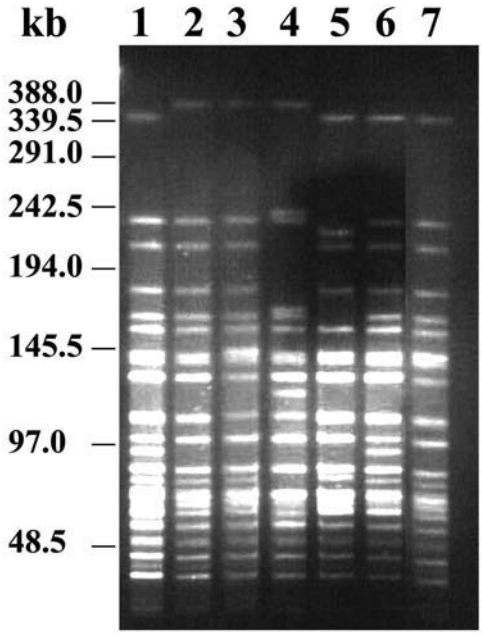

Fig. 5. *Not*I digested PFGE profiles of *V. cholerae* strains (lanes 1–7). *V. cholerae* strains used were: lanes1, CO366—H pattern; 2, CO370—I pattern; 3, CO388—J pattern; 4, CO392—K pattern; 5, PL33/2, 6, PG58; 7, PG218. The positions of bacteriophage λ ladder molecular size markers run on the same gel are indicated.

used by default (electrophoresis buffer: 0.5X TBE; run temperature 14°C; agarose PFC grade and concentration 1%; electrical parameters: 6 V/cm; run time 40.24 h; included angle 120°C; initial switch time 2.98 s and final switch time 26.95 s with linear ramping factor).

3.4.3.5. Documentation and Interpreting Results

1. After electrophoresis, the machine is turned off and the gel is placed into aqueous solution of ethidium bromide (0.5 µg/mL) for staining.
2. Electrophoresis buffer is drained; the chamber is washed with 2 L of water and kept dry for the next run. The gel is stained for 30 min at room temperature.
3. Ethidium bromide solution is discarded according to the respective institution's guidelines for hazardous waste disposal and the gel is destained with an equal volume of water for 20 min at room temperature.
4. The pulsotype pattern of the strains is documented using the Gel Doc system.
5. Results obtained with *V. cholerae* strains are presented in **Fig. 5**.

The *V. cholerae* O1 reference strains isolated from October 1993 to March 1994 in Calcutta were used in the assay ***(22)***. It is evident that pulsotype pat-

terns of these strains can be distinguished into 4 types, namely H, I, J, and K, for *V. cholerae* strains CO366, CO370, CO388, and CO392, respectively (lanes 1–4). Clinical rough *V. cholerae* strains PL33/2, PG58, and PG218 isolated during 1998 were also included in the figure. It is evident that PG58 (lane 6) showed an identical profile to that of the CO366 showing H pulsotype. Therefore, it may be considered that these strains probably evolved from the same ancestral lineage. The strain PG218 (lane 7) exhibited a pattern similar to that of the H pulsotype but with the absence of a single band in the region of 97 kb. The profile was designated as type H_a; *V. cholerae* O1 strains from clinical cases having this profile have been appearing since the end of the year 2000 in Kolkata (Nandy et al., unpublished data). The rough *V. cholerae* strain PL33/2 showed a profile different from those reported earlier ***(22)***.

4. Notes

1. The multitest medium can be used for a quick presumptive diagnosis of *V. cholerae.* The pH of the medium is 6.7 and enough care should be taken to adjust the pH to 6.7.
2. In a standard buffer, the optimal concentration of Mg^{2+} is quite low (1.5 m*M*). It is important that the preparation of template DNA does not contain a high concentration of chelating agents such as EDTA or negatively charged ionic groups such as phosphates. PCR buffers are generally supplied in 10X concentrations with or without $MgCl_2$ along with enzyme *Taq* polymerase.
3. Addition of excess enzyme may lead to amplification of nontarget sequences. To ensure high specificity of the synthesized products, the addition of *Taq* DNA polymerase is withheld until the reaction temperature is 80°C. This method is known as "hot start."
4. The size of the DNA is not a critical factor; however, target sequences are amplified slightly less efficiently when they are carried in closed circular DNAs rather than as liner DNAs. The concentration of target sequences in the template DNA varies from 0.001 ng to 1.0 ng/µL.
5. Alternatively, for increased DNA concentration, add 30 µL elution buffer to the center of the QIAquick column, let stand for 1 min, and then centrifuge for 1 min to collect eluted DNA.
6. An alternative for Saran Wrap is Cling Film (although any brand will work), which can be purchased in the food store. It is used in the household to cover food for refrigeration or for microwave cooking.
7. As a consequence of improvements in the PFGE technique, resolution of DNA molecules larger than 5000 kb can now be achieved. The recent PGFE apparatus version is known as contour-clamped homogeneous field electrophoresis (CHEF). In this system, the electric field is generated from multiple electrodes that are arranged in a hexagonal contour around the horizontal gel and are clamped to predetermined potentials. A square contour generates electric fields that are oriented at right angles to each other; a hexagonal array of electrodes generates fields at

angles of 120°. By using a combination of low field strengths, low concentrations of agarose, long switching intervals, and extended periods of electrophoresis, it is possible to resolve higher molecular weight DNA.

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11

HPLC Measurement of Aflatoxin B_1 and Metabolites in Isolated Rat Hepatocytes

Jennifer Colford

Summary

This chapter describes a high-pressure liquid chromatography (HPLC) method for measuring aflatoxin B_1 and its metabolites isolated from rat hepatocytes. AFB_1, AFQ_1, AFM_1, and AFP_1 concentrations are determined using reverse-phase HPLC. The isocratic mobile phase is 18% dimethylformamide in 0.01% phosphoric acid at a flow rate of 2.0 mL/min at 69°C, with ultraviolet (UV) detection at 360 nm. AFB-GSH conjugate levels are also measured. An isocratic mobile phase consisting of 69.05% H_2O, 30% methanol, and 0.05% acetic acid is used and the sample is eluted at ambient temperature with a flow rate of 1.0 mL/min and UV detection at 365 nm.

Key Words: Aflatoxin, AFB_1, AFM_1, AFP_1, and AFQ_1, AFB-GSH conjugate, HPLC, isolated hepatocytes.

1. Introduction

Aflatoxins are a major class of mycotoxins produced primarily by the molds *Aspergillus flavus* and *Aspergillus parasiticus.* They contaminate various grains and grain byproducts under conditions of high temperature and humidity. Aflatoxins are commonly found in food and animal feedstuffs; their presence can have significant health and economic consequences. Many countries have set limits regarding permissible levels ***(1)***.

Aflatoxin B_1 (AFB_1) is considered the most toxic of these compounds and has been classified as a human carcinogen by the International Agency of Research for Cancer ***(2)***, although there is disagreement whether it acts alone or in combination with the hepatitis B virus ***(3)***. AFB_1 has acute and chronic effects on humans and animals with the primary site of action being the liver. Like many other chemical carcinogens, AFB_1 must be metabolically activated before it can exert its carcinogenic effects. The major route of AFB_1 detoxification

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is the binding of the active AFB_1-epoxide with glutathione ***(4)***. Freshly isolated intact hepatocytes are commonly used to investigate procarcinogen metabolism in vitro ***(5,6)***. The following high-pressure liquid chromatography (HPLC) method allows measurement of AFB_1 and its metabolites from isolated rat hepatocytes.

2. Materials

2.1. Chemicals and Reagents

1. AFB_1 (Calbiochem-Behring Corp., La Jolla, CA) (*see* **Note 1**).
2. HEPES buffer, reduced glutathione (GSH), bovine serum albumin (BSA Type V), AFM_1, AFP_1, and AFQ_1 (Sigma Chemical Co., St. Louis, MO).
3. HPLC-grade *N, N*-dimethylformamide and trypan blue dye (Aldrich Chemical Co., Milwaukee, WI).
4. HPLC-grade methanol and water (Fisher Scientific, Springfield, NJ).
5. Heparin (0.9% in NaCl solution) and collagenase (Type II) (Worthington Biochem. Corp., Freehold, NJ).
6. Krebs-Henseleit buffer (pH 7.4) containing 0.2 m*M* EGTA and 0.22 m*M* methionine (*see* **Note 2**).
7. Krebs-Henseleit buffer (pH 7.4) containing 2% BSA.
8. 50 m*M* Potassium acetate buffer, pH 5.0, containing 10% MeOH.
9. Demineralized water.

All reagents should be of analytical grade.

2.2. Equipment

1. Hemocytometer.
2. Thermostatic oxygenator.
3. Medicut cannula.
4. Nylon mesh (64 µm pore size).
5. Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA).
6. HPLC unit consisting of a Waters model 510 pump, an Altex model 210 injector, a Waters Model 480 variable wavelength ultraviolet (UV) detector, and a Hewlett Packard 3390A reporting integrator. The column used is a Beckman ultrasphere ODS (25 cm × 4.6 mm; 5 µm sphere particle size) with a Waters HPLC column water jacket used to control temperature.

3. Methods

3.1. Hepatocyte Isolation

1. Hepatocytes from rats (215–270 g) are isolated according to a modified method of Berry and Friend ***(7)*** and Seglen ***(8)***.
2. The rat is anesthetized with diethyl ether, a longitudinal abdominal incision is made, and the hepatic portal vein is cannulated using an 18-g Medicut cannula containing 1500 U of heparin (0.6 mL, 0.9% NaCl).

3. The inferior vena cava is then severed to permit drainage and the liver is immediately perfused *in situ* with Ca^{2+}-free Krebs-Henseleit buffer (pH 7.4) containing 0.2 m*M* EGTA and 0.22 m*M* methionine at 37°C (*see* **Note 2)** without recirculation for 3 to 5 min. The inclusion of EGTA and methionine in the buffer aids in the maintenance of GSH levels during the isolation procedure.
4. The liver is then excised and placed on a platform above a beaker so that the perfusate is recirculated and aerated with 95% O_2/5% CO_2 to maintain the pH at 7.4.
5. The perfusate is maintained at 37°C and constantly gassed using a thermostatic oxygenator.
6. Collagenase (40 mg, Type II) is added to the perfusate and the liver is perfused for another 15 to 20 min with recirculation.
7. The liver is then removed and, using a glass rod, mechanically disaggregated in Krebs-Henseleit buffer containing 2% BSA.
8. The resulting cell suspension is filtered through 64-µm pore size nylon mesh, centrifuged for 1 min at 29*g,* 0°C, then washed and centrifuged twice before being resuspended in incubation medium.
9. The incubation medium contains Krebs-Henseleit buffer (pH 7.4) supplemented with 40 m*M* HEPES, 1% BSA, and an amino acid mixture used by Seglen ***(8)***.
10. Cells are counted and viability estimated with a hemocytometer, after addition of 0.2% trypan blue (in incubation buffer).

3.2. Hepatocyte Incubations

1. Freshly isolated hepatocytes (30×10^6 cells in 12 mL of incubation medium per flask) are incubated with 1.1 µ*M* AFB_1 for 3, 7, 11, or 15 min at 37°C under 95% O_2/5% CO_2.
2. The final concentration of ethanol added to cells should not exceed 0.2%; this is well below the level reported to perturb AFB_1 metabolism in isolated hepatocytes ***(9)***.
3. No effect of AFB_1 on hepatocyte viability has been observed at this level. The cell concentration used is within the range for optimal activation of AFB_1 by rat hepatocytes as reported by Gayda and Pariza ***(10)***.
4. Incubations are conducted under subdued light to avoid photochemical decomposition of AFB_1.
5. Levels of AF-DNA binding, AF-GSH conjugation, and AF metabolites are determined for each time point.
6. Hepatocyte viability is initially assessed by trypan blue dye exclusion (*see* **Note 3**) and only those cell preparations with greater than 90% viability are used for incubations.
7. Viability is also determined at the end of incubations by measuring the percentage of LDH leakage into the extracellular medium (*see* **Note 4**).

3.3. Analysis of AFB_1 and Metabolites

1. At the end of the incubation, reaction is stopped by addition of ice-cold buffer.
2. Cells are then transferred to ice-cold centrifuge tubes, pelleted by brief centrifugation (1 min at 30*g,* 0°C), and the supernatant and one wash from each hepatocyte incubation are used for determination of AFB_1 and its metabolites.

3. The combined supernatant and wash are twice passed slowly (0.5 mL/min) through a Sep-Pak C_{18} cartridge that has been prewashed with 10 mL of 100% MeOH and 10 mL of 50 m*M* potassium acetate buffer, pH 5.0, containing 10% MeOH.
4. The cartridge is then washed with 10 mL of the buffered 10% MeOH to remove salts, protein, and any exchanged tritium.
5. The cartridge is next washed with 60% buffered MeOH to elute AFB_1, its metabolites, and the AFB_1-GSH conjugate.
6. The eluate is then extracted twice with two volumes of chloroform:ethyl acetate (1:1).
7. Phases are separated by centrifugation; the lower lipophilic phase is dried down under N_2, redissolved in 0.3 mL of MeOH:H_2O (40:60) and used for HPLC analysis of AFB_1 metabolites.
8. For determination of the AFB_1-GSH conjugate, a modified method of Raj et al. ***(11)*** is used. The aqueous (upper) phase containing the AFB_1-GSH conjugate is passed through a Sep-Pak C_{18} cartridge.
9. Unadsorbed material is removed by washing the cartridge twice with 5.0 mL of H_2O and the AFB_1-GSH conjugate is then eluted with 2.0 mL 100% methanol.
10. The methanol is removed under nitrogen and the residue redissolved in 250 µL of MeOH:H_2O (1:1).

3.4. Preparation of AFB_1-GSH Conjugate Standard In Vitro

1. The AFB_1-GSH conjugate standard is prepared according to a modified method of Chang and Bjeldanes ***(12)***.
2. A microsomal sample is suspended in 0.1 *M* Tris-HCl buffer (pH 7.4). The incubation mixture contains 40 µ*M* AFB_1, 2.0 mg microsomal protein (approx 2 n*M* cytochrome P-450), 2 m*M* NADPH, 5 m*M* GSH, 5.0 mg cytosolic protein from rat liver, and 1.0 mL of 0.1 *M* phosphate buffer (pH 7.4).
3. After incubation in air for 30 min, the mixture is extracted as described above.

3.5. High-Pressure Liquid Chromatography Analysis

1. AFB_1, AFQ_1, AFM_1, and AFP_1 concentrations are determined using reverse-phase HPLC.
2. The system consists of a Waters model 510 pump, an Altex model 210 injector, a Waters Model 480 variable wavelength UV detector, and a Hewlett Packard 3390A reporting integrator.
3. The column used is a Beckman ultrasphere ODS (25 cm × 4.6 mm; 5 µm sphere particle size) with a Waters HPLC column water jacket used to control temperature.
4. The isocratic mobile phase is 18% dimethylformamide in 0.01% phosphoric acid at a flow rate of 2.0 mL/min at 69°C, with UV detection at 360 nm.
5. Quantitation of metabolites is determined from standard curves derived from injections of known standards (*see* **Note 5**). Average retention times for AFQ_1, AFM_1, AFP_1, and AFB_1 are 11.5, 12.5, 16.5, and 24 min, respectively (*see* **Fig. 1** for sample chromatogram).
6. The AFB_1-GSH conjugate is also quantitated using reverse-phase HPLC according to the method of Raj et al. ***(11)***.

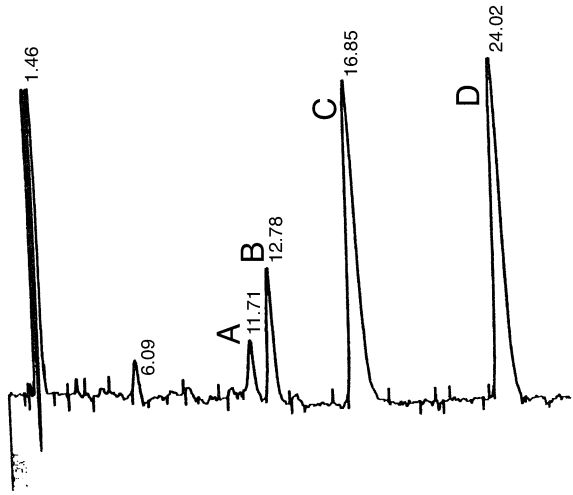

Fig. 1. HPLC chromatogram of chloroform:ethyl acetate extractable AFB metabolites. Numbers at peak denote retention time in minutes.
Peak A = AFQ_1; Peak B = AFM_1; Peak C = AFP_1; Peak D = AFB_1.

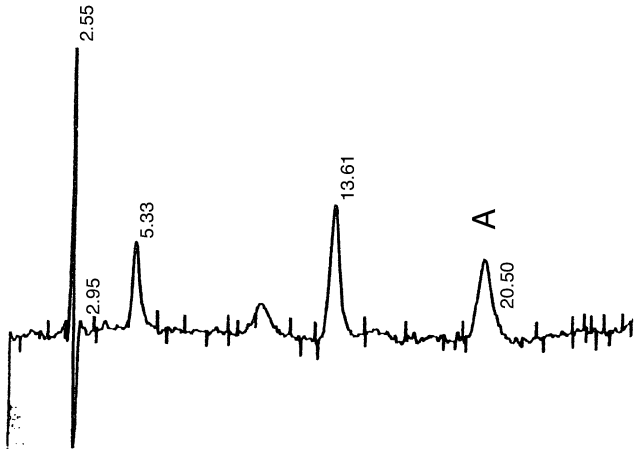

Fig. 2. HPLC chromatogram of aqueous phase AFB_1 metabolites. Numbers at peaks denote retention time in minutes. Peak A is the AFB–GSH conjugate.

7. An aliquot of the concentrate is injected onto a Beckman Ultrasphere ODS column (25 cm × 4.6 mm; 5 μm sphere particle size).
8. An isocratic mobile phase consisting of 69.05% H_2O, 30% methanol, and 0.05% acetic acid is used; the sample is eluted at ambient temperature with a flow rate of

1.0 mL/min and UV detection at 365 nm. The peak representing the AFB_1-GSH conjugate elutes at 20 min (*see* **Fig. 2** for sample chromatogram).

4. Notes

1. Aflatoxins are classified as human carcinogens and as such should be handled with extreme care. The International Agency for Research on Cancer (IARC) has issued decontamination procedures for laboratory wastes containing aflatoxins ***(13)***.
2. Ca^{2+}-free Krebs-Henseleit buffer, pH 7.4 (0.12 *M* NaCl, 4.83 m*M* KCl, 0.94 m*M* KH_2PO_4, 1.22 m*M* $MgSO_47H_2O$, 23.8 m*M* $NaHCO_3$, 40 m*M* HEPES, and 20 m*M* glucose).
3. The trypan blue dye exclusion test is used to estimate initial hepatocyte viability. This assay is based on the fact that cells with an intact membrane exclude the dye, whereas damaged cells are stained blue, particularly in the nucleus. Cell aliquots (0.1 mL) are diluted with 0.5 mL of wash buffer containing 0.2% trypan blue dye and 1% BSA and the total number of cells is determined using a Burker chamber (hemocytometer). Trypan blue viability is expressed as the number of unstained cells as a percentage of the total number of cells visualized. Only those cell preparations with greater than 90% dye exclusion are used for incubations.
4. The percent of LDH (lactate: NAD^+-oxidoreductase, EC 1.1.1.27) activity in the extracellular medium is another criterion that can be used to evaluate cell membrane integrity. The activity of LDH is assayed using the method of Bergemeyer and Bernt *(14)*. Cell aliquots are incubated with 0.01 *M* Na-pyruvate, 11 m*M* NADH in 50 m*M* potassium phosphate buffer (pH 7.5). The LDH catalyzed conversion of pyruvate to lactate at 25°C is measured by determining the disappearance of NADH over time (change of UV absorbance at 340 nm). Duplicate 0.25-mL aliquots of hepatocyte suspension are used. One aliquot is spun down for 10s at 15,600*g* in a Brinkman 5414 Eppendorf Centrifuge (Brinkman Instruments, Westbury, NY). The supernatent is assayed and used to estimate extracellular LDH activity (i.e., LDH leakage). In order to ensure complete release of LDH from the hepatocytes, 10 µL of Triton X-100 is added to the other aliquot, which is then sonicated for 2 min in a water bath sonicator at 10°C. Following this treatment, no intact hepatocytes are detected under a light microscope. The actvity of LDH in each fraction is determined and leakage expressed as the percent of the total activity that is present in the extracellular fraction.
5. Aflatoxin standards are prepared daily from stock solutions kept protected from light (in a dark bottle) and air and refrigerated (0–4°C). Stock solutions should be stable for up to 1 yr.

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II

The Viruses

12

Detection of Noroviruses of Genogroups I and II in Drinking Water by Real-Time One-Step RT-PCR

Christian M. Beuret

Summary

Noroviruses (NVs) are a genus belonging to the virus family Caliciviridae and are transmitted by the fecal–oral and the aerosol routes. NVs are the most common cause of nonbacterial gastroenteritis, accounting for two-thirds of all illnesses caused by known food-borne pathogens and for more than 90% of nonbacterial gastroenteritis in the United States. Whether viral outbreaks are initiated by infected food handlers or by contaminated food such as seafood, fruit, or vegetables, the main source of most norovirus outbreaks is water—by either direct or indirect ingestion. Therefore, either drinking or bottled water (as food) or water in relation with fishery or food processing (irrigation) must be regularly controlled for the presence of viral contaminants. A simple method for the isolation and detection of noroviruses in water is described, where both norovirus genogroups (NV gg I and NV gg II) are detected separately. The isolation of the viruses is performed by filtration of a 1-L water sample through a positively charged membrane, where the negatively charged noroviruses (protein envelopes or capsids) adsorb. Extraction of viral RNA is performed directly on the same membrane. Detection of noroviruses is made by amplifying a defined viral genome-region by using a real-time one-step reverse transcription polymerase chain reaction (RT-PCR). Two detection formats, SYBR Green-based and probe-based, are described. Noroviruses of genogroups I and II are detected separately.

Key Words: Norovirus; water; food; isolation; filtration; extraction; detection; confirmation; one-step RT-PCR; real-time RT-PCR.

1. Introduction

Noroviruses (NVs), former known as Norwalk-like viruses (NLVs), small round structured viruses (SRSV), or human caliciviruses (HuCVs), are single-stranded (+ss) RNA viruses of the family Caliciviridae. The genus NV is divided into two genogroups (I and II) and further into several species ***(1)*** (*see* **Note 1**). The original "Norwalk agent" was first discovered in fecal specimens collected

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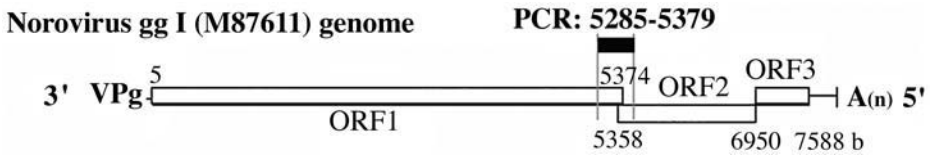

Fig. 1. Schematic drawing of a norovirus genogroup I genome (GenBank: M87611) showing three open reading frames (ORFs). The target region amplified by real-time one-step RT-PCR is labeled between RNA bases 5285 and 5379.

from an outbreak of nonbacterial gastroenteritis at a school in Norwalk, Ohio, in 1972 ***(2)***. The Norwalk virus genome was first sequenced in 1993 and contains three open reading frames (ORFs) (*see* **Fig. 1**).

ORF1 encodes for nonstructural proteins like an RNA-dependent RNA polymerase. ORF2 encodes a major capsid protein (VP1) and ORF3 a minor structural protein (VP2) ***(3)***. Since the first genome sequencing in 1972, molecular detection methods could be developed ***(4)*** and the epidemiology of noroviruses assessed. Unfortunately, NV cultivating methods to support molecular detection have not been developed yet ***(5)***. NVs seem to be responsible for more than 90% of all nonbacterial epidemic gastroenteritis in the United States (more than 20 million illnesses a year) and have an estimated food-borne transmission of over 40% ***(6–9)***. Thus, NV transmission via food has become evident ***(10–12)*** and NV contamination has been documented for bottled or drinking water ***(13–21)***, shellfish ***(22–27)***, frozen berries ***(28)***, salads ***(29)***, and sandwiches ***(30)***. Although many food-borne NV outbreaks are attributed to transmission by infected food handlers, the main source of outbreaks is water, by either direct or indirect contact. Direct contact means contamination by ingestion of drinking, tap, or recreational water ***(31,32)***; indirect contact is initiated by seafood fishing in, or irrigation of fruit and vegetables with, contaminated water. Therefore, analysis of water samples combined with good agriculture practice (GAP), good manufacturing practice (GMP), and hazard analysis of critical control points (HACCP) on raw food and the manufacturing environment are crucial factors to set up food quality control and limit unnecessary NV outbreaks ***(33–35)***. A method for the detection of noroviruses in water samples is outlined and consists of filtration-adsorption to a membrane, RNA-extraction, and a combined detection–confirmation step by real-time one-step reverse-transcription polymerase chain reaction (RT-PCR) ***(36–46)***. Two protocols are described, one for detection by SYBR Green (with subsequent melting curve analysis) and one for detection by using specific probes.

2. Materials

2.1. Concentration by Filtration

1. Water filtration equipment: (multiple) vacuum filtration apparatus (made of stainless steel and sterilizable) for the adaptation of 47-mm membranes and an optimal flow rate of 20 L/min for vacuum-filtration; filtration funnels (0.1–1 L, stainless steel or disposable).
2. Zetapor filter membrane, 47 mm, 0.45 µm pore size (CUNO Inc, Meriden, CT).
3. Prefilter (for foul water samples); Glass fiber disk with binder AP25, 47 mm (Millipore, Billerica, MA).
4. Disposable forceps (minimal length of 8 in./20 cm).
5. 12-mL culture tubes (12-mL reaction tube 17 × 77 mm, sterile, PPN, Greiner Bio-One, Inc, Longwood, FL).
6. Positive controls for noroviruses of genogroups I and II for water spiking. While noroviruses cannot be cultivated yet, positive controls must to be isolated from positive human stool specimens. Because noroviruses are not commercially available, medical diagnostic laboratories must be asked for bacterial-negative gastroenteritis stool specimens, and the contamination rate is at least 30%. If positive stool specimens cannot be obtained, another laboratory performing analysis must be asked for controls.
7. Negative control consisting of 1 L of sterile water (best would be the same water as the one to be analyzed, but without viral contamination).

2.2. RNA Extraction

RNA extraction using the indicated kit (QIAmp HCV mini kit) can be performed in two ways (indicated in the manufacturer's handbook): either by a centrifugation-procedure or by a vacuum-filtration procedure. For both protocols a centrifuge for 2-mL tubes processing at 16,000*g* is required. As the method describes processes with large extraction volumes, we strongly recommend using the second (vacuum) protocol. The centrifugation protocol can also be used, but larger extraction volumes extend the processing time. If RNA and DNA must be extracted in the same run, another commercial kit—for example, the QIAmp Ultra Sense kit (Qiagen Inc., Valencia, CA)—can be used with equal sensitivities. The following material is needed for the vacuum-filtration procedure:

1. QIAmp viral RNA mini kit (Qiagen) for RNA extraction, containing spin columns, collection tubes, AVL buffer, AVE buffer, carrier RNA and washing buffers AW1 and AW2; stored at room temperature. The kit must be prepared first before using; please check the manufacturer's handbook.
2. Additional buffer AVL, 155 mL (Qiagen), stored at room temperature.
3. Ethanol (96–100%).
4. QIAvac 24-vacuum manifold (Qiagen).
5. Vacuum pump or water jet pump with a flow rate of 20 L/min.

Table 1
Forward, Reverse Primers and TaqMan Probes for Noroviruses of Genogroups I (NV gg I) and II (NV gg II)

Primers	Sequence 5′–3′	Polarity	Localization[a]
NV gg I			
JJV1F[b]	GCCATGTTCCGITGGATG	sense	5282–5299
JJV1R[b]	TCCTTAGACGCCATCATCAT	antisense	5377–5358
JJV1P[b]	FAM–TGTGGACAGGAGATCGCAATCTC-BHQ	sense	5319–5341
NV gg II			
JJV2F[b]	CAAGAGTCAATGTTTAGGTGGATGAG	sense	5003–5028
COG2R[c]	TCGACGCCATCTTCATTCACA	antisense	5100–5080
RING2-TP[d]	FAM–TGGGAGGGCGATCGCAATCT-BHQ	sense	5048–5067

[a]Localizations are in reference to: Norovirus gg I (NV/8FiiA/68/US: M87661) and Norovirus gg II (Lordsdale virus: X86557).
[b]See ref. **58**.
[c]See ref. **59**.
[d]In duplex assays a 5_-FAM-labeled GI probe was combined with a 5_-JOE- or 5_-TET-labeled GII probe. BHQ, Black Hole quencher.
Nucleotide positions based on Norwalk (GI) (accession no. M87661) and Lordsdale (GII) (accession no. X86557) sequences.
Mixed base in degenerate primer: I=Inosine.

6. VacConnectors (Qiagen).
7. Extension tubes (Qiagen).

2.3. Detection and Confirmation

One-step RT-PCR can be performed either by "conventional" RT-PCR followed by confirmation by agarose gel electrophoresis, or by real-time RT-PCR. Similar forward and reverse primers can be used for conventional and real-time PCR. Evaluation of real-time one-step RT-PCR reagent is fastidious work. Several commercial kits have been evaluated. Material for the one-step real-time RT-PCR detection is described.

1. Real-time PCR equipment and PCR laboratory environment (3 work areas required: master mix-pipetting, master mix-RNA-mixing, and RT-PCR-performing area; each with separate pipets, filter tips, and cooling blocks for 1.5-mL tubes (4 and –20°C).
2. QuantiTect SYBR Green RT-PCR kit (Qiagen), stored at least at –20°C; for real-time one-step RT-PCR using the DNA-binding fluorophore SYBR Green.
3. QuantiTect Probe RT-PCR kit (QIAGEN), stored at least at –20°; for real-time one-step RT-PCR using an additional probe.
4. PCR-grade water, stored at 4°C.
5. Bovine serum albumin, acetylated, 1 µL/µg (Promega, Madison, WI).
6. Forward, reverse primer and probes (Applied Biosystems, Foster City, CA) for both genogroups (NV gg I and NV gg II) **(Table 1)**; store stock solutions (100 µ*M*) at

least at –20°C (avoid repeated thawing and freezing), ready-to-use solution (10 μ*M*) at 4°C. Dilutions are made with sterile buffers such as phosphate-buffered saline (PBS) or Tris-HCl and should be renewed if not used within 6 mo (at 4°C).

3. Methods

The methods outline the isolation (*see* **Notes 2** and **3**) and concentration (*see* **Notes 4–6**) of noroviruses out of water samples by filtration through a positively charged membrane ***(1)***, the extraction of viral RNA by using an evaluated commercial kit ***(2)*** (*see* **Note 7**), and the detection and confirmation by one-step real-time RT-PCR ***(3)*** (*see* **Notes 8** and **9**). Other food-borne viruses (enteroviruses or hepatitis A viruses) can be isolated and concentrated using the same procedure, but primers and probes for the detection by (RT-PCR must be changed, as a matter of course. Alternative detection methods to RT-PCR exist (*see* **Note 10**). Keep in mind that every experiment requires a negative and a positive control. Therefore, at least two additional water samples must be prepared for both genogroup-specific protocols: one negative control consisting of 1 L of sterile water (best would be the same water as the one to be analyzed, but without viral contamination), one positive control consisting of 1 L of water spiked with a sufficient virus concentration; sufficient means giving a clear RT-PCR signal, while being as close as possible to the detection limit of the protocol (about 50–100 PCR units). Although many protocols describe difficulties in isolating viruses from stool samples, no problems were encountered by applying a simple dilution step (of 1:10 with sterile water) followed by centrifugation for 60 s at 16,000*g* and room temperature. The supernant can be used for RNA extraction. As a clear RT-PCR signal being as close as possible to the detection limit of the protocol is expected, 100 viruses (PCR units) are spiked into 1 L of water (the same water as the one to be analyzed, but without viral contamination) just before starting the analysis. As the virus concentration can hardly be estimated, serial dilutions must be performed to determine the detection limit. Stool specimens are diluted with sterile buffers such as Tris-HCl or PBS and stored once (in 100-μL aliquots) at least at –20°C. Repeated thawing and freezing should be avoided.

3.1. Concentration by Filtration (see Notes 2 and 4)

1. Prepare reagents of the QIAmp viral RNA mini kit and the "additional buffer AVL" following the manufacturer's handbook, with one exception: according to the manufacturer, carrier RNA must be added to the buffer AVL. As a larger volume of buffer AVL (3 mL instead of 0.56 mL) is used in the following protocol, the concentration of carrier RNA in the buffer AVL must be reduced by approximately five.
2. Prepare the water filtration equipment by sterilizing (Bunsen burner for 5 s) the (multiple) vacuum filtration apparatus and the filtration funnels (1–5 dL, stainless steel or disposable).

3. Prepare the positive and the negative controls.
4. Place Zetapor filter membranes on the filtration apparatus by using sterileforceps; for foul water samples, place the prefilter above the Zetapor filter membrane within the filtration funnel.
5. Transfer 3 mL of buffer AVL (lysis buffer) to each 12-mL culture tube. AVL buffer contains guanidine thiocyanate, which is a powerful protein denaturant. Both the guanidine cation and thiocyanate anion are strong chaotropic agents, disrupting the structure of water and thereby promoting the solubility of nucleic acids.
6. Filter water samples and both controls through the membrane (and the prefilter, if required); take care that the membranes never dry up.
7. Discard the prefilters with sterile forceps. Roll up or fold membranes with sterile forceps and press them down the prepared 12-mL tube into the buffer AVL.

3.2. Extraction of Viral RNA

Before development of newer extraction reagents and commercial kits, working with RNA was quite delicate, as special care had to be taken to avoid degradation of the single-stranded RNA by RNases or high temperatures. For more details about the principles of nucleic acid extraction, please read **Notes 2** and **7**.

1. Shake the 12-mL culture tubes vigorously (on a vortex) for 20 s and store them for 10 min at room temperature (15–25°C) for complete lysis of viruses (as indicated in the manufacturer's handbook). Longer lysis times do not improve the protein denaturation, but decompose the membrane and hamper the filtration step by clogging the spin column.
2. During the 10-min "break," prepare the QIAvac 24-vacuum filtration unit: Place sterile VacConnectors, spin columns, and extension tubes on the filtration units.
3. Discard membrane by using sterile forceps.
4. Add an equal volume (3 mL) of ethanol (96–100%) to the 12-mL culture tubes and shake vigorously for 20 s to improve the precipitation of RNA (alcohol precipitation at room temperature).
5. Apply the buffer AVL-ethanol-mixture (approx 6 mL; 3 × 2 mL) to the spin columns (through the extension tubes); precipitated RNA is bound to the membrane within the spin column.
6. Discard the extension tubes and apply 700 µL of washing buffer AW1 to the spin columns; protein residues are denaturated by guanidinium chloride and washed through the spin column.
7. Apply 700 µL of washing buffer AW2 to the spin columns; this final washing step guaranties the elimination of most of the rest of the PCR inhibitors.
8. Place the spin columns into the provided 2-mL tubes and centrifuge for 60 s at 16,000*g* to eliminate any AW1 and AW2 traces.
9. Discard the 2-mL tubes and place the spin columns within sterile 1.5-mL tubes (final tubes for storage of RNA at least at –20°C).
10. Add 60 µL of elution buffer AVE to the spin columns.

11. After 60 s (allow buffer AVE to impregnate the hole membrane within the spin column), centrifuge spin columns (within the 1.5-mL tube) for 60 s at 6000*g* to collect pure viral RNA.
12. Discard spin columns and transfer RNA (nearly 60 µL AVE-RNA-mix) to other sterile 1.5-mL tubes and store at least at –20°C; buffer AVE stabilizes RNA for several hours at room temperature, but freezing is recommended.

3.3. Detection and Confirmation

Detection and confirmation are performed by two genogroup-specific one-step real-time RT-PCRs. If the second genus of the virus family Caliciviridae, called sapoviruses, must be detected, please *see* **Note 1**. One- and two-step protocols were compared using different reagents and commercial kits on different real-time PCR engines (*see* **Note 9**). QuantiTect kits (Qiagen) were chosen for one-step RT-PCR, as the sensitivity was similar to a two-step protocol ***(14)*** and no optimization is required. Two real-time protocols (1 and 2) are described, one for a SYBR Green-based and one for a probe-based detection.

As consensus genome sequences among noroviruses are rather rare, minor groove-binder (MGB) probes with short sequences were chosen to target both genogroups (I and II) separately. Both protocols are described in the manufacturer's handbook of the QuantiTect kits and require no optimization. Both protocols were tested on the following real-time PCR machines: TaqMan 5700 and 7000, Rotor-Gene 3000, SmartCycler and LightCycler I. Different reaction volumes can be used for real-time RT-PCR; 25 µL (20 µL master mix and 5 µL RNA) are used and fit within all available PCR tubes or capillaries. The preparation of the master mix, the mixing of the master mix and RNA within a PCR tube, and the real-time RT-PCR should be performed in three separate areas (a, b, and c) using distinct pipets and filter tips. The real-time PCR engine is configured for SYBR Green or probe detection before starting the protocol. Although both QuantiTect kits are laid out for working at room temperature, cooling blocks are used to avoid any surprises in case the room temperature exceeds 25°C.

3.3.1. Real-Time One-Step RT-PCR Using DNA-Binding Fluorophore SYBR Green

Area 1: Master mix preparation (20 µL master mix and 5 µL RNA per sample).

1. QuantiTect SYBR kit reagents (mix and PCR-grade water) are thawed and placed together with both ready-to-use primer solutions (10 µ*M* each) and two sterile 1.5-mL tubes into a cooling block (4°C).
2. 20 µL of master mix for each sample and both NV genogroups are prepared within two sterile 1.5-mL tubes. Take care to add approx 5% of master mix to every run to compensate for pipetting errors. The master mix is prepared as outlined in **Table 2**.

Table 2
Master Mix Protocol (Per Sample) for Real-Time One-Step RT-PCR Detection Using SYBR Green

	Final concentrations	µL
Water, PCR-grade		4.5
JJV1F or JJV2F (10 µ*M*)	0.5 µ*M*	1.3
JJV1R or COG2R (10 µ*M*)	0.5 µ*M*	1.3
QuantiTect RT mix	0.5 µL/reaction	0.5
QuantiTect SYBR mix 2X	1X	12.5
Total volume per sample		20.0

Final concentrations are adjusted to a reaction volume of 25 µL.

3. Mixing is done by pipetting the reagents five times up and down (using the vortex is not recommended, as the enzymes within the QuantiTect kit could be damaged). The 1.5-mL tube is centrifuged for 5 s at 500*g* ("short-spin") to collect any droplets on the bottom of the tube.
4. The master mix is transferred to area 2 and immediately placed into another cooling block (4°C).

Area 2: Master mix and RNA mixing.

1. PCR tubes are placed within a cooling block (4°C).
2. Extracted RNA (stored at least at –20°C) is placed within another cooling block (4°C).
3. 20 µL of master mix per sample are pipetted within each PCR tube.
4. 5 µL of RNA (samples and controls) are added.
5. PCR tubes are closed and transferred to area 3.

Area 3: RT-PCR area.

1. PCR tubes are immediately placed within the preconfigured real-time PCR cycler and the one-step real-time RT-PCR is started following this protocol:

2. Reverse transcription:	30 min at 50°C
Initial activation step	15 min at 95°C (Hot-Start PCR)
Three-step PCR for 40 cycles consisting of:	
Denaturation	15 s at 94°C
Annealing	30 s at 60°C
Extension	20 s at 72°C (data collection, SYBR Green mode)
Melting analysis	real-time cycler-dependent

Confirmation of results is made by melting curve analysis in reference to the positive and the negative controls. Melting peaks for noroviruses of genogroup I are located between 81 and 83°C; those for Noroviruses of genogroup II

Table 3
Master Mix Protocol (Per Sample) for Real-Time One-Step RT-PCR Detection Using MGB-TaqMan Probes

	Final concentrations	µL
Water, PCR-grade	–	4.5
JJV1F or JJV2F (10 µ*M*)	0.4 µ*M*	1.0
JJV1R or COG2R (10 µ*M*)	0.4 µ*M*	1.0
JJV1P or RING2-TP (10 µ*M*)	0.2 µ*M*	0.5
QuantiTect RT Mix	0.5 µL/reaction	0.5
QuantiTect Probe 2X	1X	12.5
Total		20.0

Final concentrations are adjusted to a reaction volume of 25 µL.

between 82 and 84°C. A subsequent sequencing reaction can be used as a second confirmation and enables epidemiological studies by GeneBank comparisons and phylogenetic analysis.

3.3.2. Real-Time One-Step RT-PCR Using an Additional Specific Probe (MGB-TaqMan)

Area 1: Master mix preparation (20 µL master mix and 5 µL RNA per sample).

1. QuantiTect Probe kit reagents (mixes and PCR-grade water) are thawed and placed together with both ready-to-use primer solutions (10 µ*M* each), the specific probe solution (10 µ*M*), and a sterile 1.5-mL tube within a cooling block (4°C).
2. 20 µL of master mix for each sample and both NV genogroups are prepared within two sterile 1.5-mL. Protocols are listed in Table 3.
3. Mixing is done by pipetting the reagents five times up and down (using the vortex is not recommended, as the enzymes within the QuantiTect kit could be damaged). The 1.5-mL tube is centrifuged for 5 s at 500*g* (short-spin) to collect any droplets on the bottom of the tube.
4. The master mix is transferred to area 2 and immediately placed within a cooling block (4°C).

Area 2: Master mix and RNA mixing.

1. PCR tubes are placed within a cooling block (4°C).
2. Extracted RNA (stored at least –20°C) is placed within another cooling block (4°C).
3. 20 µL of master mix are pipetted within each PCR tube.
4. 5 µL RNA (sample and both controls) are added.
5. PCR tubes are closed and transferred to area 3.

Area 3: PCR area.

1. PCR tubes are immediately placed within the preconfigured real-time machine and the one-step real-time RT-PCR is started following this protocol (protocol within the manufacturer's handbook of the QuantiTect kits):
2. Reverse transcription: 30 min at 50°C
   Initial activation step 15 min at 95°C (HotStart PCR)
   Two-step (TaqMan-specific protocol) PCR for 40 cycles consisting of:
   Denaturation 15 s at 94°C
   Annealing/Extension 50 s at 60°C (data collection, FAM and TET)

Additional confirmation is not required by using an additional specific probe. A subsequent sequencing reaction can be used as a second confirmation and enables epidemiological studies by GeneBank comparisons and phylogenetic analysis.

4. Notes

1. Caliciviridae: Within the family of Caliciviridae, besides the noroviruses, there is a second genus that can be harmful to men, called sapoviruses (former known as "Sapporo-like" viruses, as the first strain was found in Sapporo, Japan). Sapoviruses are divided into six species with important sequence variations. Therefore, primer designing for RT-PCR is rather difficult ***(49–51)***.
2. Virus detection methods in food: Regardless of the food sample to be analyzed for the presence of viruses, the methods prior to detection and confirmation by RT-PCR are divided into distinct steps. Figure 2 represents a compilation of possible analysis steps, showing that most popular methods are quite similar to each other and still composed of a sample preparation, an isolation and concentration (occasionally an elution and a reduction), and an extraction step prior to RT-PCR. Food is classified within three categories for analysis, depending on the consistency: Liquid like water, soft like oysters or berries, or hard like salad, prunes, or sandwiches.

   However, every method must fulfill two criteria before extraction: first, the separation and isolation of viruses from possible PCR-inhibitors and food components and second, the concentration of viruses to a volume suitable (less than 0.5 mL) for the RNA extraction. Therefore, the separation and isolation of viruses are still based on two principles: either the separation by ultracentrifugation ("weight-dependent") or the separation by the natural charge of viruses ("charge-dependent") ***(47)***. At pH values between 5.0 and 9.0, viruses have a negative charge, allowing their binding to any positive charged surface (such as silica-based membranes or beads, or reagents such as polyethylene glycol [PEG]).

   Virus detection methods in food are basically composed of methodic steps outlined in **Notes 3** to **8**.
3. Sample preparation: Food samples can be either homogenized in or rinsed with a buffer (glycine, PBS, or Tris-HCl buffer) at a pH value of approx 9.5, depending on whether viruses are "coated" at the surface (rinsing of salad leaves) or within food (homogenization of digestive tissue of seafood). Rinsing can also be replaced

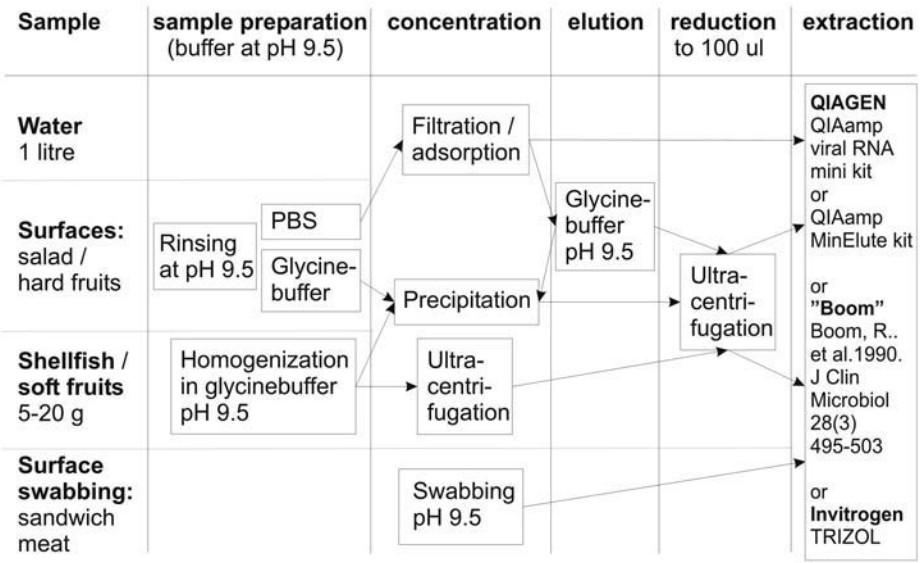

Fig. 2. Compilation of most-used methods for virus isolation and extraction in food, indicating similarities between different steps. Water, shellfish, vegetables, and fruit represent the main sources of food-borne norovirus outbreaks.

by surface swabbing. The high pH guaranties the elution of viruses from most surfaces, because the natural negative charge of viruses becomes positive and the binding is disrupted. Drinking water samples do not require any sample preparation step. Polluted groundwater samples should first be centrifuged to eliminate substances and particles, which could clog filtration membranes.

4. Concentration: Water samples (1 L) are filtrated through a positively charged membrane, whereby negatively charged viruses adsorb. Although the pore size of Zetapor filter membranes is 0.45 µm and NV are up to 40 nm in size, viruses are restrained by a charge interaction between the positively charged membrane and the negatively charged viruses. If food samples have been rinsed with large buffer volumes, viruses must be concentrated into a smaller volume suitable for RNA extraction. There are two possibilities, depending on the buffer used: (a) If buffers without PCR inhibitors (such as PBS or Tris-HCl) were used for rinsing, they could be filtrated like a water sample and viruses are adsorbed again to the positively charged membrane. Viruses bound to these membranes are either eluted with small buffer volumes (next step) or directly lysed (with lysing buffers) on it, followed by the extraction of the viral RNA. (b) If a rinsing buffer containing PCR inhibitors such as beef extract was used, viruses must be isolated by precipitation. Thereby, viruses are bound (at 4°C) over several hours to positively charged reagents such as PEG and concentrated in a pellet by precipitation and centrifugation.

Alternative concentration methods: Viruses on contaminated food surfaces can also be concentrated by surface swabbing, whereby the swab has been previously wetted in an elution buffer with high pH. Contaminated swabs and filtration membranes can be used directly for the RNA-extraction step. Another method uses immunomagnetic beads to concentrate viruses in a homogenized food sample ***(48)***.

5. Elution: Viruses bound to a positively charged membrane can be either directly lysed on it followed by the extraction of viral RNA, or eluted from the membrane again with a (glycine) buffer of high pH (9.5). If the buffer volume applied is too large, it has to be reduced by an ultimate ultracentrifugation step.
6. Reduction: The only function of this final step prior to the extraction of viral RNA is to reduce the buffer volume to a volume affordable for the reagent kit used for the extraction step. This step is performed using a separation column called a microconcentrator in a conventional centrifuge (no ultra-speed is needed).
7. Extraction: The extraction of viral RNA is a crucial step for the subsequent molecular detection by RT-PCR. As RNA has a very unstable structure compared to double-stranded DNA, it necessitates special care and handling to ensure an intact template for molecular detection. Several years ago, the extraction of RNA was a tricky method performed on ice to avoid degradation of the nucleic acid (by temperature and RNases). Today, many companies have developed ready-to-use reagent kits based on the capacity of nucleic acid to bind to silica-coated membranes or magnetic beads. Although these kits ensure a standardized methodology and guarantee a constant RNA quality, they may have different RNA recovery and purification rates. Therefore, comparison tests must be performed. Home-brew protocols can definitely be as sensitive as commercial kits, if well optimized, but using phenol and chloroform to perform extraction is rather unhealthy. If RNA or DNA extraction is performed regularly in large quantities, it could be simplified considerably by using an extraction robot. More and more companies are selling different systems to automate the extraction step, but sensitivities also vary between these engines and reagents. The principle of every kit is almost the same: Viral capsids are lysed using a protein denaturant such as guanidinium thiocyanate, while enough salt (chaotropic agent) must be in solution to neutralize the repulsion among the negatively charged strands of RNA by disrupting the molecular water complex enveloping nucleic acids. Once the water complex is disrupted, alcohol is added to precipitate the RNA by pulling water molecules out of the nucleic acid. Precipitated RNA is then centrifuged to a pellet or bound to positively charged membranes, beads, or other agents (most frequently silica with a positive charge). RNA is finally purified from different protein impurities by several washing steps using protein denaturants such as guanidinium chloride (by adjusting salt and pH conditions to ensure that digested proteins and other inhibitors are not retained). For water samples, we obtained best and equal results with the QIAmp viral RNA mini kit and the QIAmp MinElute kit (Qiagen), the latter kit enables the simultaneous extraction of RNA and DNA. Plant samples and food samples such as seafood or soft fruits (berries, etc.) are known to contain plenty of RT-PCR inhibitors such as phenyls or fatty acids. To ensure the removal of these inhibitors during the extraction step, the use of opti-

mized reagents for plant samples is indicated. Extraction techniques based on magnetic beads are very helpful, as inhibitors are not precipitated on a membrane by centrifugation or vacuum filtration.

8. Detection and confirmation: Detection and confirmation can be performed in different ways, either by conventional RT-PCR and a separate agarose gel electrophoresis or by real-time RT-PCR. There are also two possibilities to perform RT-PCR, either in a two-step reaction (RT and PCR in two subsequent reactions), or in a one-step reaction (RT and PCR in the same tube in one reaction). A one-step real-time RT-PCR was chosen to reduce processing time and practical efforts.

   Finally, for real-time RT-PCR two possibilities exist, either probe-based (two primers and a probe), or fluorophore-based (two primers and a DNA-binding fluorophore such as SYBR Green).

   For probe-based RT-PCR, besides two conventional primers (forward and reverse), an additional specific probe labeled with fluorophores is used. Different probe formats exist, for example TaqMan, hybridization, scorpions, molecular beacons, or minor groove-binder (MGB) probes. Every format has some positive and negative qualities; MGB-TaqMan probes were chosen for this protocol. The specificity given by both primers and an additional probe(s) combine detection and confirmation in one step. A second confirmation by sequencing can be performed, but it is optional.

   In fluorophore-based RT-PCR, besides two conventional primers (forward and reverse), a DNA-binding fluorophore is added to the master mix. Specificity is given by an additional melting curve analysis step after PCR, which allows comparing melting temperatures of different amplification products. However, because DNA-binding fluorophores (such as SYBR Green) intercalate with every kind of double-stranded DNA, additional melting peaks (MP) for primer-dimer and other unspecific products are possible. An additional acquisition step of 15 s at a temperature higher than primer-dimer MP, but lower than PCR-specific MP, can be performed, but the result is dubious. Unspecific products are not "seen," but are still present.

9. Reagents for real-time RT-PCR: Different real-time one-step RT-PCR kits were evaluated on different real-time PCR engines (TaqMan 5700 and 7000, Rotor-Gene 3000, SmartCycler and LightCycler I) and best results were obtained by using the RT-PCR QuantiTect kits (Qiagen). Similar sensitivities could be obtained by using home-brew reagents, but ready-to-use kits simplify the quality control and guarantee constant conditions. Although some reagents were evaluated on different real-time PCR engines (SmartCycler, LightCycler 1, Rotor-Gene 3000, TaqMan 5700 and 7000), it cannot be excluded that other real-time engines would perform better with other reagents. The most efficient combination of RT and PCR enzyme must be evaluated carefully; the detection limits vary considerably.
10. Alternative methods to RT-PCR: Different methods besides RT-PCR exist to detect and confirm the presence of noroviruses in different samples:
    - **Immunological methods:** An enzyme-linked immunoassay (EIA) was developed and compared to real-time PCR ***(52)***, but with poor results. Another study

compared the sensitivities of transmission electron microscopy (TEM), antigen ELISA, and PCR for the detection of noroviruses in stool samples ***(53)***. PCR (94%) was definitely more sensitive compared with both other methods (58% for TEM and 31% for ELISA). Detection by immunological methods (commercial ELISA kits are available) could be very useful for screening studies, but low sensitivity is a critical point, particularly for food analysis.

- **NASBA:** A good alternative to RT-PCR is nucleic acid sequence-based amplification (NASBA). Different NASBAs were compared to RT-PCR ***(54–56)*** for the detection of noroviruses in stool specimens. NASBA revealed detection rates as good as those of RT-PCR-based methods. NASBA is an isothermal gene amplification method that can be applied to both RNA and DNA targets. The reaction process for RNA is initiated by the annealing of an oligonucleotide primer (designated P1) to the RNA target present in the nucleic acid extract obtained from the test sample. The 3′ end of the P1 primer is complementary to the target analyte; the 5′ end encodes the T7 RNA polymerase promoter. After annealing, the reverse transcriptase is engaged and a cDNA copy of the RNA target is produced. The RNA strand of the resulting hybrid molecule is hydrolyzed by RNase H. Then, the second primer (P2; sense), which is complementary to an upstream region of the RNA target, anneals to the cDNA strand. The DNA-dependent DNA polymerase produces a double-stranded cDNA copy of the original RNA analyte, including a fully functional T7 RNA polymerase promoter at one end. This promoter is then recognized by the T7 RNA polymerase, which amplifies a large amount (up to 1000) of antisense, single-stranded RNA transcripts corresponding to the original RNA target. These antisense RNA transcripts can then serve as templates for the amplification process; however, the primers anneal in the reverse order. The entire NASBA process is conducted at 41°C. For DNA, the process is the same except that an initial heat-denaturing step (100°C for 5 min) is required before the addition of the enzymes to the reaction mix.
- **CIEF-WCID:** Isoelectric point determination of norovirus-like particles by capillary isoelectric focusing-whole column imaging detection (CIEF-WCID) is a recent development and shows great promise for norovirus detection in public health, clinical, and food samples ***(57)***. CIEF-WCID is a completely different approach for analysis, because there is no genetic or immunological detection, as viral capsid proteins are separated by the specific isoelectric point using CIEF.

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13

Detection of Enteroviruses

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Summary

Enteroviruses are members of the Picornaviridae family and represent one of the most important water-transmitted pathogens. Detection of enteroviruses in water sources, or water-contaminated food, is a very valuable tool not only to prevent waterborne diseases but also to track down animal or human environmental viral pollution. Nowadays, molecular biology techniques allow the use of very sensitive and specific reverse-transcription polymerase chain reaction (RT-PCR) procedures to detect enteroviruses. In this chapter, using bovine enterovirus as a model, we describe procedures for enterovirus detection. Detailed descriptions of proper sample collection, storage, and processing, including methods for water concentration and solid sample extraction to obtain viral RNA, are outlined. Next, we describe methods for enterovirus detection based on virus isolation in appropriate cell culture. Finally, protocols for molecular detection of enterovirus are described, including procedures for conventional, nested, and real-time RT-PCR.

Key Words: Environmental contamination, water concentration, RNA extraction, cell culture, virus isolation, molecular detection, reverse transcription (RT), polymerase chain reaction (PCR), real-time RT-PCR.

1. Introduction

More than 100 virus species have been identified so far as contaminants of water, although not all of them cause illness in humans or animals. Significant pathogens, such as poliovirus, hepatitis A and E viruses, coxsackieviruses, and coronaviruses, may be detected in sewage-polluted water and food (especially in shellfish), making them a very important water-related health problem worldwide ***(1)***.

Enteric viruses are shed in high concentrations in feces of infected individuals (10^5 to 10^{11} particles/g of stool) and are potential contaminants of water in its different uses: water supply, irrigation, and recreation ***(1)***. Therefore, detection of

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viruses in water sources, or water-contaminated food, is a valuable tool to prevent waterborne diseases; it can also be useful to indicate animal or human environmental viral contamination.

Enteroviruses are one of the most important water-transmitted viruses. They are very stable and may remain infectious for long periods of time under a wide range of environmental conditions. Enteroviruses belong to the Picornaviridae family (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/), thus being small RNA viruses, and include the most common virus infecting mammals.

Advances in molecular biology techniques have provided highly sensitive and specific reverse-transcription polymerase chain reaction (RT-PCR) procedures to detect enteroviruses. Here we describe our experience in the detection of bovine enteroviruses (BEV) as a model of water-contaminant enteroviruses ***(2)***. We have successfully applied a similar approach for other enteric viruses, such as porcine teschoviruses ***(3)***, as did others for detection of human enteroviruses, such as poliovirus, echovirus, and coxsackievirus ***(4–19)***.

2. Materials

1. Filters and filtration system: electropositive filters (Virosorb filters, 1 MDS, size 47 mm, CUNO Inc, Meriden, CT), prefilters (AMF CUNO, size 47 mm), Whatman filter paper no. 1 (Merck, Whatman 3MM, Darmstadt, Germany), peristaltic pump (Watson-Marlow, model IP55, Falmouth, England), silicone tubing, filter holders (AMF CUNO, size 47 mm, 60 PSI MAX), and 0.20- and 0.45-µm syringe filters (Pall Corp., Ann Arbor, MI)
2. Centrifuge Heraeus Megafuge 1.0 R (Kendro Laboratory Products GmbH, Hanau, Germany), microfuge (Hermle Z 160 M, Wehingen, Germany), ultracentrifuge Optima L-90K ultracentrifuge, (Beckman Coulter Fullerton, CA) with rotors SW28, SW41, or equivalent), high-speed centrifuge (Beckman Avanti J25 I with rotor JA-14 or equivalent). Ultracentrifuge and high-speed centrifuge tubes for various volumes.
3. Elution buffer: 0.1 *M* glycine, pH 9.5, with 3% beef extract (Sigma, St. Louis, MO)
4. Extraction buffer: phosphate-buffered saline (PBS) with antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL, Biowhittaker, Verviers, Belgium).
5. RNA extraction reagents (phenol-chloroform-isoamylalcohol) and equipment and/or commercial kits (QIAmp Viral RNA, Qiagen, Valencia, CA).
6. Cell and tissue culture equipment: laminar flow hood, water bath, phase contrast microscope, CO_2 incubator, plastic or glass ware, micropipets and tips, syringes.
7. Culture medium: Eagle's minimal essential medium (EMEM) (Biowhittaker), or similar medium, e.g., minimal essential medium (MEM), fetal calf serum (FCS) (Biowhittaker) and antibiotics (penicillin 10,000 U/mL, streptomycin 10,000 µg/mL, Biowhittaker).
8. RT-PCR 10X loading buffer: 0.025% Orange G (Sigma), 20% Ficoll 400 (Calbiochem Inc., La Jolla, CA) 0.1 *M* EDTA, pH 8.0.
9. Agarose (Promega, Madison, WI) and electrophoresis equipment.

10. Vortex, shaker, pH meter and storage equipment (refrigerator, –20°C and –70°C)
11. Thermocycler (Perkin Elmer Applied Biosystems, PEAB, Branchburg, NJ), optionally, real-time equipment (ABI Prism 7700, PEAB) and 0.2-mL optical PCR tubes and optical caps (PEAB).
12. Superscript one-step RT-PCR (Gibco BRL, Life Technol., Grand Island, NY)
13. 100-bp ladder (Roche Molecular Biochemicals, Mannheim, Germany).
14. RT-PCR reagents: primers, probes, enzymes, dNTPs.

3. Methods

3.1. Samples

Collection of samples is always the initial and crucial point to investigate a suspected case of water- and food-borne viral disease. Samples should be taken from the affected individuals and their contacts (serum, swabs, spinal fluid, tissue, etc.) and from the environment (water, feces, food [especially seafood and fish], etc.). Implementation of systematic procedures and databases, including integration of sample labeling, registration of essential information (such as date and place of collection, nature of the sample, and any other relevant data), conservation, and storage are always critical points to track back every result obtained during the investigation ***(20)***. Samples should ideally be split into aliquots and immediately processed as soon as they are received at the laboratory, but adequate means for conservation and storage of samples should be available in case further analysis will be required. In this regard, it should be noted that enteric viruses are usually relatively stable and persist in normal environmental conditions for long periods; therefore, liquid samples are adequately stored at 4°C for several days and at –70°C for years, but freeze–thaw of samples should be avoided. Solid samples (feces, seafood, etc.) can be stored at –70°C for years (*see* **Note 1**).

3.2. Sample Processing

3.2.1. Water Concentration

Viruses are usually at low concentration in water samples; therefore, it is necessary to concentrate the samples for proper virus detection. Several concentration methods have been described in the literature aimed at this purpose. These methods are based on organic flocculation, filtration-elution, ultrafiltration, lyophilization, ultracentrifugation, and combinations of two or more of these systems ***(8,12,21–24)***. Among them, the filtration-elution method using electronegative ***(23)*** or, more commonly, electropositive filters has gained acceptance, the latter one being the most used either alone or combined with other methods ***(8,12)***. As a consequence, the American Public Health Association has chosen it as the standard method for water-virus examination ***(24)***. Our experience is that this simple concentration method provides enough

concentration power (up to 100 times) for most water samples tested but if further concentration is needed, an additional ultracentrifugation step may be used (*see* **Subheading 3.2.1.2.**).

3.2.1.1. Concentration of Water Samples by Filtration and Elution Throughout Electropositive Filters

This method assumes that the net electrostatic charge of most viruses at neutral pH is negative and thus, filters with positive charges in their surface can retain them. Viral particles are then eluted from the filters by simply changing pH conditions. Here we provide a simple protocol using membrane electropositive filters for volumes up to 5 L. For higher volumes, cartridges that filtrate up to 1000 L of water are also commercially available (CUNO).

1. Before filtration, adjust pH of sample to 6.0 to 7.0.
2. Clarification: Environmental water samples contain variable amounts of materials in suspension; thus, to avoid filter clogging, a clarifying step is often required before filtration (*see* **step 3**). Coarse material might be decanted for at least 2 h at room temperature or, preferably, overnight at 4°C. If the decanted water still contains too many fine particles in suspension, it can be prefiltered through a Whatman filter paper, or further cleaned up by centrifugation at 9800*g* for 20 min in a Beckman (Avanti J25 I) centrifuge using a JA-14 rotor. This procedure yields a clarified supernatant already suitable for filtration.
3. Filtration: We use the filtration system outlined in **Fig. 1.** Basically, a peristaltic pump drives the liquid sample through a prefilter and an electropositive filter (*see* **Section 2**) placed consecutively down the flow. Optimal flow rate is dependent on the size of the filter. For CUNO 47-mm filters, the maximum flow rate is 70 mL/min.
4. Elution: Once the sample has been filtered, the filter must be removed from its cassette and incubated for 5 to 10 min, with shaking, in 10 mL elution buffer (0.1 *M* glycine, pH 9.5, with 3% beef extract [*see* **Notes 2** and **3**]).
5. Neutralization: Because excessive exposure to alkaline pH may produce loss of virus viability, the pH of the eluate must be neutralized by the addition of 0.1 *M* HCl immediately after incubation (*see* **Note 4**). After neutralization, and before storage, we find that filter sterilization of eluates through 0.2-µm pore-diameter syringe filters is useful to have them ready for further analysis that require sterile conditions, such as virus isolation in cell culture.

3.2.1.2. Virus Concentration by Ultracentrifugation

This concentration method may be useful in two instances:

1. When no virus is detected in the sample after a first filtration-elution step, this second concentration step may be applied before it is convincingly concluded that the sample is free of virus.

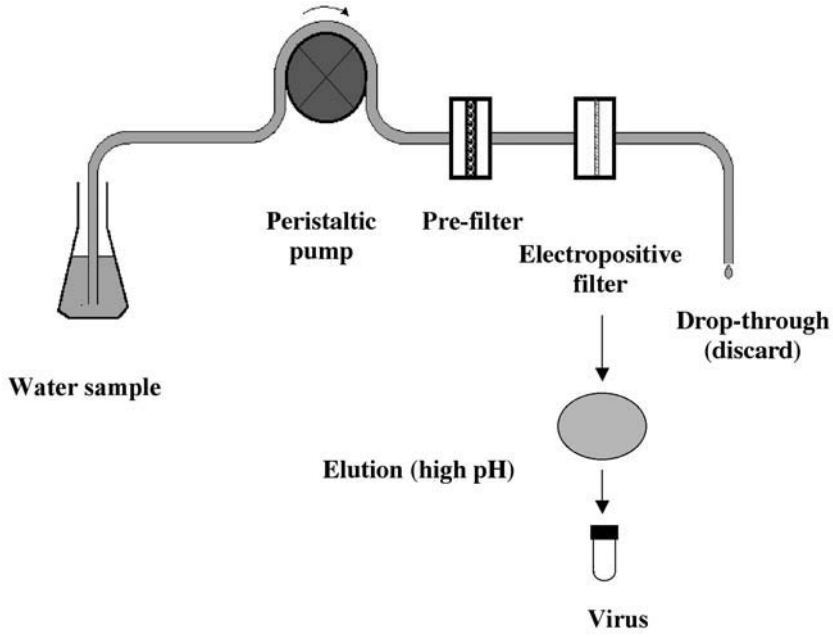

Fig. 1. Schematic representation of the devices for concentration of water samples by filtration and elution through electropositive filters.

2. When the filtration-elution method used could presumably prevent virus detectability, e.g., in infectivity tests (*see* **Note 4**).
   In these cases the following protocol is used:
   a. Clarify the samples by centrifugation at 9800*g* 20 min at 4 to 8°C and discard the pellet or, alternatively, filter the samples through 0.2-µm filters.
   b. Ultracentrifuge the supernatant at 120,000*g* using a SW-28 rotor (Optima L-90K ultracentrifuge, Beckman Coulter) for 3 h at 4 to 8°C (*see* **Note 5**).
   c. Discard the supernatant and resuspend the pellet in 0.5 to 1 mL of RNase-free water (*see* **Note 6**).

3.2.2. Feces Extraction

1. Mix fecal samples (1–15 g) with extraction buffer (PBS with antibiotics, penicillin 100 U/mL, streptomycin 100 µg/mL) at a 1:2 ratio (w/v).
2. Homogenize the mixture by vortex and/or other means (*see* **Note 7**).
3. Centrifuge the mixture at 1200*g* for 10 min and transfer the supernatant to a clean centrifuge tube. Repeat this step once more and, finally, transfer the supernatant to clean microfuge tubes and microfuge at maximum speed (around 16,000*g*) for 10 to 15 min.

4. Take the clarified supernatant and filter it through a 0.2-µm pore-diameter sterile syringe filter. Alternatively, treat it with chloroform to eliminate bacteria and enveloped viruses. Carefully, add chloroform to a final concentration of 10% and vortex vigorously for 1 min, then microfuge at maximum speed and transfer the aqueous phase (sample) to a clean tube. The clarified supernatant, either filtered or chloroform-treated, is suitable for both cell culture procedures and molecular detection methods (after RNA extraction; *see* **Subheading 3.3**) (*see* **Note 8**).

3.2.3. Solid Food Sample Extraction

In addition to the protocols describing the extraction of clinical specimens, such as serum and cerebrospinal fluid ***(16,18,19)***, several procedures have been described for solid sample extraction, including food ***(7,11,15)***.

Here we describe an example of a simple method for RNA extraction from oyster *(Crassotrea virgínica)* tissue.

1. At the place of sampling, using a syringe, aspirate the hemolymph from the adductor muscle and place it in clean tubes. Aspirates from 10 oysters can be mixed at this point or later on, just before extraction. Dissect the stomach and gills and place them in separate clean tubes. Store at 4°C for transportation.
2. Within 24 h of collection, cut individual tissues in small pieces, place them in clean tubes, and suspend them in 5 mL of MEM (1 mL/g of tissue) for 1 h with occasional vortexing.
3. Centrifuge at 1200*g* for 10 min.
4. Filter supernatants through a 0.4-µm filter into clean tubes, add antibiotics (penicillin 100U/mL, streptomycin 100 µg/mL) to the filtrates, and store at –20°C until use.

3.3. RNA Extraction

Enteroviruses are RNA viruses; therefore, as RNA is highly labile, during nucleic acid extraction and handling special care is needed afterward to maintain its integrity (use of sterile gloves; filter tips; specific pipets for RNA handling only; RNase-free tubes, buffers, and media; disposable labware). To avoid possible contamination during further PCR amplification, strict adherence to guidelines should be maintained ***(25)***, including the availability of a separate ("clean") area to perform RNA manipulations (ideally under a laminar flow hood) physically apart from the place where amplified cDNA is handled.

RNA extraction can be manual or automated. Manual methods are homemade or supplied as commercial kits. The former are based mostly in guanidinium isothiocyanate denaturation followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation ***(26)***, while the latter are usually based on virus denaturation and RNA adsorption to RNA-binding matrices, followed by a final elution step. Kits are convenient, easy to use, efficient, and fast, and avoid the use of harmful and environmentally hazardous reagents (such as

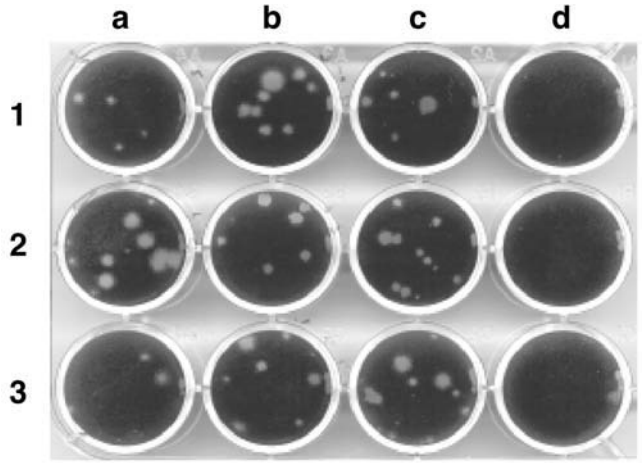

Fig. 2. Representative example of BEV CPE on MDBK cell monolayers. Calves (white spots) on the crystal violet stained cell monolayers are individual plaque-forming units (PFU) of BEV. Lanes 1–3 are triplicates (rows a, b, and c) of 3 BEV field samples. Wells of row d are control mock-infected cells.

phenol), but they are expensive and present important drawbacks, particularly when a high number of samples is to be processed (*see* **Note 9**). In this case, diverse automated systems for nucleic acid extraction are commercially available (such as ABI 6100 Nucleic Acid PrepStation, Applied Biosystems, www.appliedbiosystems.com; Biorobot 9604, Qiagen, www.qiagen.com).

3.4. Detection

3.4.1. Virus Isolation in Cell Culture

Detection of viral infectivity in cell culture or animal models is the only way to determine the presence of infectious viral particles, as molecular techniques do not establish whether the pathogen is active. Virus infection in cell culture is the "classic" method for virus detection, and it is still considered the "gold standard" in virus detection techniques. In addition, its combination with serological methods (virus neutralization with specific antisera) and/or molecular techniques (*see* **Subheading 3.5**) can lead to the characterization of the isolated virus.

Isolation of viruses in cell culture relies on the ability to detect a particular effect caused by in vitro virus propagation in the target cells. Cytopathic viruses, as most of the enteroviruses, cause a characteristic cell lysis known as cytopathic effect (CPE) **(Fig. 2).** For noncytopathic viruses, detection of virus propagation in cell culture is achieved by means of more sophisticated methodologies,

such as immunomicroscopy (immunofluorescence or immunohistochemistry) or *in situ* hybridization techniques, provided that virus-specific molecular probes and/or antibodies are available.

3.4.1.1. Cell Lines for Enterovirus Isolation

A wide variety of cell lines of different origin, available at the American Type Culture Collection, (ATCC, www.atcc.org), are commonly used for enterovirus isolation: HeLa, CaCo2, rhabdomyosarcoma, buffalo green monkey (BGM), baby hamster kidney (BHK), bovine epithelial cells (MDBK), porcine cell lines (IB-RS-2 [not available at ATCC] and PK15). In any case, it is usually better to use cell lines from the species where the virus was originally isolated.

3.4.1.2. Protocol for Virus Isolation

1. Inoculation: Carefully remove supernatant from semiconfluent (70–80%) cell monolayers in 25-cm^2 cell culture flasks (with screw cap) and overlay the cells with the inocula, consisting of a filter-sterilized virus suspension in physiologic media, i.e., neutral pH and isotonic salts (*see* **Note 10**).
2. Adsorption: Gently swing the flask to ensure spread of the inoculum over all the cell monolayer and close the cap of the flask. Incubate at 37°C in a CO_2 incubator for 30 to 60 min, swinging the flask gently every 10 to 15 min to prevent the monolayer from drying, as well as to allow interaction between remaining free virus and cells.
3. Incubation: Remove the remaining inocula and add fresh cell culture medium to the flasks (*see* **Note 11**). Incubate at 37°C in a CO_2 incubator and observe the cells daily for CPE (*see* **Note 12**), under the microscope if needed. Usually, 2 to 3 d are enough to detect CPE, but sometimes incubation for up to 5 to 6 d is recommended to assess infectivity signs. To conclude absence of cytopathic effect due to virus infection in a given sample, at least three "blind" passages are required, that is, absence of CPE after three successive rounds of infection using the supernatant of the former round of infection to infect a new monolayer.
4. Virus recovery: Clarify the supernatant of infection by centrifugation at 1200*g* for 10 min to remove cell debris (*see* **Note 13**).

This supernatant of infection constitutes the "isolate" and is the source of virus for further characterization, including biological, antigenic, and molecular analyses (*see* **Subheading 3.6**). Virus isolates are best conserved in aliquots frozen at –70°C, and freeze–thaw cycles should be avoided. Given that enteroviruses are highly stable, once thawed, each aliquot is better maintained at 4°C up to several weeks. Stored viruses must be labeled and registered in a way that allows easy identification. Registration data should include at least: name of sample, origin, date, and cells used for isolation and number of passages. Care must be taken to maintain virus isolates within a low number of passages, as RNA viruses are highly variable and can drift to cell-culture adapta-

tions as the number of passages grow, making cell-adapted viruses often quite different from those originally isolated.

3.5. Molecular Detection: RT-PCR Methods

RNA extracted from water concentrates, fecal extracts, sera, spinal fluid, supernatants of infection, and so on is assayed for the presence of enterovirus sequences by RT-PCR. Based on increasing sensitivity criteria, RT-PCR methods are classified as *conventional, nested,* and *real-time* modes. We find it convenient to use conventional RT-PCR methods when assaying samples with an expected high concentration of viral RNA, such as feces, gills, and supernatants of infection, whereas samples with an expected low viral RNA content, such as waters and water concentrates, require much more sensitive methods, such as nested or real-time RT-PCR. On the other hand, RT-PCR methods can be of *wide* or *narrow* range specificity, that is, respectively, those aimed at detecting "as many enterovirus types as possible" (generic methods) and those aimed at detecting a particular enterovirus species or strain (specific methods). The latter can be combined in the so-called multiplex methods to detect several different enterovirus species in a single determination ***(5,9)***. Technically, the main difference between generic and specific methods relies on the relative evolutionary conservation of the target viral RNA sequence selected for amplification (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/). The 5′-noncoding and polymerase regions of enterovirus genomes are highly conserved and thus are frequently chosen for generic methods ***(2,7,12,27)***, whereas specific methods rely on a primer design targeted at sequences found only in a particular enterovirus type, and that do not cross-react with other types of enterovirus. For this purpose, appropriate sequences are often found in genomic regions containing the highest variability, such as the domains encoding for the structural proteins ***(10,11,14,17,27–31)***. Primer design must thus take into account both the mode (conventional, nested, or real-time) and the range of specificity of the RT-PCR to be applied ***(30)***. Quantitative real-time RT-PCR ***(32)*** requires additional probe design, as those for the diverse available commercial devices (TaqMan, LightCycler, iCycler), but this issue is beyond the scope of this chapter.

Variation among the different procedures relies mainly on the primers chosen and therefore in the adjusted annealing temperature better fitted for them. Here we provide the protocols for conventional, nested, and *real-time* RT-PCR for an enterovirus model, the bovine enterovirus (BEV). A similar approach was applied for us by other enteric viruses, such as teschovirus, after setting the protocol conditions to those better fitted for the porcine teschovirus specific designed primers. By this approach we were able to track down a pig slurry spillage, and demonstrate that the developed methodology is similar to current

methods for determining unspecific organic matter and better than many other conventional chemical analyses applied to evaluate water contamination ***(3)***.

3.5.1. Protocol for Bovine Enterovirus Conventional RT-PCR

Viral RNA is extracted from 140 µL of sample (water, water eluates, oyster washes, fecal extracts, or culture supernatant) with a commercial kit (QIAamp viral RNA kit) following manufacturer's instructions, and eluted in 60 µL of the kit's elution buffer. Due to the ability of the PCR to amplify a single molecule, special care should be taken to avoid RNA contamination and false-positive results owing to trace amounts of DNA contaminants, particularly if positive controls are included in the extraction or RT-PCR procedures. To check for possible contamination, negative control (tubes with virus-free water, instead of sample) must be included in the reactions.

Six µL of RNA (1/10 of the total eluted volume) is used to carried out the RT-PCR with a commercial kit (such as Superscript one-step RT-PCR, Gibco BRL), or the like, following the kit's protocol. Additionally, RT and Taq polymerase can be purchased separately. In this case, manufacturer-recommended buffers should be used and the appropriate amount of dNTPs (around 200 m*M*) must be added to the mixture. RT-PCR conditions must be adapted to the specific target, so that selection of primers and best-fitted annealing temperature are critical points.

For bovine enterovirus, amplification with outer forward (5′ GGG GAG TAG TCC GAC TCC GC, nt 124 to143) and reverse (5′ CGA GCC CCA TCT TCC AGA G, nt 391 to 409) primers give rise to a 272-bp amplified fragment. The working concentration of primers is 0.2 to 0.5 µ*M* in a 25-µL final reaction volume. Positions of the primers correspond to the 5′ noncoding region of bovine enterovirus genome (PS87, GenBank accession no. X79368).

1. Briefly centrifuge all reagents before beginning the procedure.
2. Add 6 µL of eluted RNA (sample) in 0.2 mL sterilized PCR tubes (on ice).
3. Add 19 µL of the following premix to each tube (volumes are for 1 reaction/tube):
   a. 15 µL 2X kit buffer.
   b. 0.6 µL RT/Taq.
   c. 1.2 µL each primer.
   d. 4 µL RNase-free water.

Mix gently to produce a homogeneous mixture and centrifuge briefly to collect the sample at the bottom of the tube. Place tubes in the thermal cycler (*see* **Note 14**) and proceed with the amplification using the following conditions:

1.	RT:	30 min at 48°C	
2.	PCR (hot start):	2 min at 92°C	
3.	Cycles:	Denaturation:	30s at 94°C
		Annealing:	60s at 57°C
		Elongation:	60s at 72°C

Proceed for 40 cycles with a final elongation step for 10 min at 72°C.

Keep tubes at 4°C until analysis.

Assess correct size of amplified products by electrophoresis of 5 to 10 µL of the RT-PCR mixed with 1 µL of 10X loading buffer, through a 1.2% agarose gel stained with ethidium bromide (0.5 µg/mL) (*see* **Note 15**). Run the gel at 100 mA for 2 h. Include molecular weight markers of appropriate size for the amplified products, i.e., 5 µL (200 ng, 20 ng/band) of a 100-bp ladder, 0.2 mg/mL (Roche Molecular Biochemicals, Mannheim, Germany).

3.5.2. Protocol for Bovine Enterovirus "Nested" RT-PCR

Nested RT-PCR is usually applied when RNA sample concentration is low; it is based on the use of the first PCR product as starting material for the second PCR round. In some instances, a hemi-nested RT-PCR is used, where one of the second-round primers, usually the forward one, is the same as in the first-round RT-PCR. For the bovine enterovirus, first step of the nested RT-PCR is carried out as described above. The second step (nested) is carried out with internal forward (5′ ACT GGT ACG CTA GTA CCT TT, nt 166 to 185) and reverse (5′ CAG AGC TAC CAC TGG GGT TGT GG, nt 373 to 395) primers, yielding a 230-bp amplified fragment **(Fig. 3)**.

1. Prepare the following mixture (volumes are for 1 reaction/tube) and add 15 µL mix/tube:
   a. 0.6 µL *Taq* polymerase (Perkin-Elmer).
   b. 1.2 µL forward inner primer.
   c. 1.2 µL reverse inner primer.
   d. 12.5 µL 2X PCR buffer: for 5 mL of 2X buffer, mix 1 mL de (10X) PCR Buffer II (Perkin-Elmer), 40 µL of each dNTP (stocks at 50 m*M*), 0.6 mL of 25 m*M* $MgCl_2$, and 3.2 mL RNase-free water.
2. Add 8.5 µL RNase-free water/tube.
3. Add 1.5 µL of first-round RT-PCR product.

Mix gently, centrifuge briefly, place tubes on thermal cycler, proceed under the same conditions described above for the conventional PCR without the RT step, and assess correct size of amplified products by electrophoresis through agarose gels.

3.5.3. Protocol for Bovine Enterovirus Real-Time RT-PCR

In recent years several commercially available methodologies have been developed to carry out real-time PCR procedures. Real-time is a fluorescence-based RT-PCR that is easy to perform, capable of high throughput, and can combine high sensitivity with reliable specificity. Although real-time RT-PCR is a rapidly evolving methodology, it also engenders associated problems, however, these should be resolved in the coming years ***(32)***.

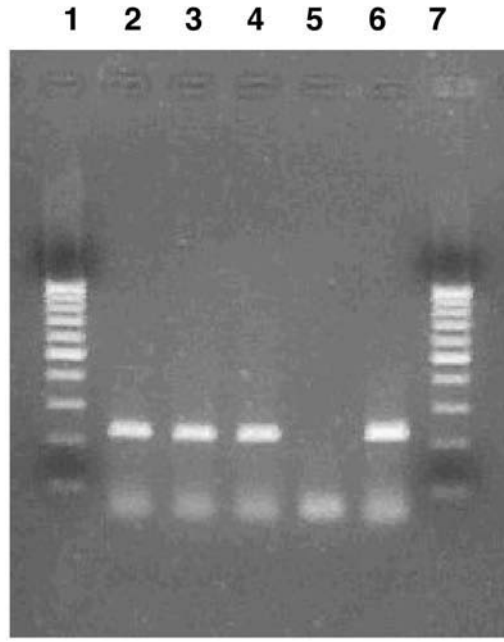

Fig. 3. Representative example of RT-nested-PCR amplified products of BEV resolved by electrophoresis through an ethidium bromide-stained 1.5% agarose gel. Lanes 1 and 7, molecular weight markers (100-bp ladder); lanes 2–4, BEV-positive field samples; lane 5, BEV-negative field sample; and lane 6, BEV-positive control.

At present a variety of real-time procedures has already been applied for enterovirus detection ***(3,16,17)***. The following procedure has been optimized for bovine enterovirus detection by the TaqMan technology, using the TaqMan One-step RT-PCR Master Mix Reagents kit (P-E AB). Viral RNA extraction is carried out as for conventional RT-PCR.

1. First, bovine enterovirus specific primers for the TaqMan procedure are diluted to a stock solution of 500 µ*M* (working final concentration 0.5 µ*M*). Primers are BEV5fl (5′ GCC GTG AAT GCT GCT AAT CC, nt 533 to 552) and BEV3fl (5′ GTA GTC TGT TCC GCC CCT GAC T, nt 604 to 625). Working concentration of the probe is 25 µ*M* (BEVprobe-FAM 5′ CGC ACA ATC CAG TGT TGC TAC GTC GTA AC, nt. 570 to 598). Nucleotide positions correspond to that of PS87 strain (GenBank accession no. X79368).
2. Prepare clean, RNase-free 0.2-mL optical PCR tubes for a 25-µL final volume of reaction.
3. Add 37 µL/tube of the following mixture (volumes are for 1 reaction):
   a. 28 µL reaction buffer (2X).
   b. 1.4 µL MS 40x (enzymes mix).

c. 5.6 µL each primer (5 µ*M*).
d. 0.56 µL probe (BEVprobe-FAM) 25 µ*M*.

4. Add up to 13 µL sample RNA/tube (complete with DEPC-treated water up to 13 µL, if needed). Close tubes with optical caps, place them on real-time thermal cycler, and proceed under the following conditions:

a. RT:	30 min at 48°C	
b. PCR (hot start)	10 min at 95°C	
c. 50 Cycles:	Denaturation	15s at 95°C
	Annealing/Elongation	60 s at 60°C

3.6. Virus Characterization

3.6.1. Antigenic Characterization

Enteroviruses are members of the *Picornaviridae* family and are characterized by their capacity to multiply in the gastrointestinal tract ***(33)***. Enteroviruses had been classically grouped by serological criteria based on neutralization of viral infectivity in cell culture, complement fixation, immunoprecipitation, and hemagglutinating activity ***(34)***. Later on, panels of antisera against different enteroviruses were made available to the scientific community to facilitate enterovirus identification ***(33)***. However, sometimes significant cross-reaction of serotype specific antibodies led to ambiguous serotyping ***(29)***. Nowadays, molecular techniques, particularly nucleotide sequence determination, are frequently applied for viral classification, so that under certain circumstances, molecular characterization is overtaking old serological procedures for enterovirus classification.

3.6.2. Molecular Characterization

Advances in molecular biology techniques have allowed the classification of enteroviruses on the basis of their nucleotide sequences and phylogenetic analyses and, as a consequence, in some instances, classical classification has been modified (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/). RT-PCR-amplified fragments are sequenced with commercial kits (such as the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 2.0, PEAB) following manufacturer's instructions and sequence reactions are further run in an automated sequencer (such as the ABI Prism 3100 Genetic Analyzer, PEAB). Due to the cost of sequencing equipment, not too many laboratories are equipped with such apparatus, but a variety of worldwide companies offer sequencing services. Today RT-PCR amplification and further sequencing of detected enteroviruses can be easily applied to molecular epidemiological studies, allowing surveillance, control, and eradication of waterborne disease outbreaks and tracking of viral contaminants ***(2,10,14,17,27–29)***. However, phylogenetic analyses need a skilled worker able to apply the appropriate methodology to

analyze the results. In any case, a variety of software for sequence analysis and phylogenetic and evolutionary studies is available (http://evolution.genetics.washington.edu/phylip/software.html), including some that can be obtained free of charge.

4. Notes

1. We find it convenient to store water samples after a first concentration step, as this greatly reduces the need for storage space. Similarly, solid samples (food, feces) are better stored after the extraction step. In the case of seafood, filtering organs (gills) accumulate virus filtered from the water; thus, a minimal processing step consisting of dissection and separation of gills will facilitate their storage.
2. The volume of elution buffer necessary for an adequate virus recovery is determined by the surface of the filter.
3. To prevent microbial growth, it is convenient to filter-sterilize the elution buffer and store it at 4°C, being careful to open it under sterile (laminar flow hood) conditions.
4. Some viruses completely lose their viability upon alkaline treatment during filtration through electropositive filters. On the other hand, at high concentrations, beef extract is directly toxic for most cell cultures, thus being necessary to "dilute the concentrates" to avoid this effect (this drawback can be partially overcome by reducing the beef extract content of the elution buffer to 1%). Consequently, the filtration-elution method is less effective when virus infectivity is to be tested. However, detection of virus by molecular methods is not affected by these drawbacks.
5. For this purpose we use ultracentrifuge rotor SW28 (Beckman). The capacity of the tubes for this rotor is approximately 38 mL. For higher volumes of sample one should fill as many tubes as needed, whereas for volumes lower than 38 mL, one should dilute it with distilled water up to 38 mL, or, alternatively, use tubes and rotors suited for lower volumes, i.e., SW41 (12 mL).
6. Virus recovery is increased when, before resuspension, pellets are kept overnight at 4°C with a RNase-free water overlay.
7. The homogenization is more efficient after an overnight incubation at 4°C with extraction buffer.
8. One aliquot can be treated with chloroform, and another one can be filter-sterilized. Comparison of the results of infectivity in cell culture obtained in each case indicates whether the cytopathic effect is due to enveloped or nonenveloped viruses.
9. Many commercial kits for RNA extraction are optimized for tissue or cell extractions, and thus are not well suited for liquid samples. We have found that those labeled as "viral RNA extraction kit" are better suited for the purposes discussed in this chapter.
10. The volume of the inoculum should ideally be high enough to overlay all the surface of the cell monolayer, but as low as possible to increase virus concentration to facilitate virus-cell contact. As a general rule, 15 to 20% of the volume of medium used for cell growth is adequate for inoculation.
11. Low fetal calf serum (FCS) concentrations during the infection (1–2%) are recommended in most cases, as the growth of many enteroviruses is prevented by FCS components, and remarkably by bovine serum albumin.

12. It is helpful to "mock-infect" one flask in parallel as a control for null CPE.
13. To increase virus content in the supernatant, before clarification, freeze–thaw the centrifuge tubes containing the infection supernatant three times successively.
14. When the thermal cycler does not have a top heater, then overlay the reaction mixture with 30 µL of mineral oil.
15. Extreme care should be taken when manipulating ethidium bromide, as it is a powerful mutagen. Gloves should be worn when working with solutions containing the mutagen and all reactive and gels in contact with it should be carefully discharged in appropriate containers.

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14

Detection of Hepatitis A Virus and Rotavirus Using Nucleic Acid Sequence-Based Amplification

Julie Jean and Ismaïl Fliss

Summary

Viral food-borne illnesses have become very common in humans worldwide. Three viruses—noroviruses, rotavirus, and hepatitis A virus (HAV)—are implicated frequently in food-borne illness and have been ranked among the top 10 causes of food-borne disease over the past 10 years. The most common food vehicles for the transmission of enteric viruses to humans are shellfish, fruits, and vegetables. Foods may be contaminated by water tainted with untreated sewage or by contact with infected human food handlers. Virus concentrations in foods are usually low, as they are not able to multiply *in situ.* Therefore, the ability to detect traces of viruses in foods is essential in the development of tools for the investigation and possible prevention of viral disease outbreaks. Molecular approaches based on the amplification of viral RNA have been proposed for the specific and ultrasensitive detection of enteric viruses in foods. Nucleic acid sequence-based amplification (NASBA) is one of these molecular techniques showing great promise in viral detection. In this chapter, we describe two applications using NASBA techniques for the detection of hepatitis A virus and rotavirus.

Key Words: NASBA; detection; microplate hybridization; hepatitis A virus; rotavirus.

1. Introduction

Enteric viruses have been identified as the agent responsible for the majority of food-borne diseases, accounting for at least 67% of estimated illnesses ***(1)***. In recent years, many researchers have focused their work on detection and characterization of this previously undetectable group of pathogens. With the development of molecular biology and epidemiology tools, our ability to study viruses has undoubtedly progressed faster than the emergence of the viruses themselves ***(2)***. In fact, the first cloning and sequencing of the Norwalk virus in 1990 ***(3)*** and subsequent development of diagnostic tools such as reverse-transcription polymerase chain reaction (RT-PCR) revealed the prevalence of noncultivable

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noroviruses and enteroviruses in shellfish ***(4)***. Those developments have considerably reduced the number of idiopathic cases of unknown etiology. In an effort to address the problems of poor detection sensitivity and specificity for enteric viruses in food and environmental samples, recent research has explored several approaches.

Hepatitis A virus (HAV) and noroviruses are the most epidemiologically significant viruses in food ***(5)***. In the United States, about 80,000 estimated illnesses resulting from HAV occur every year ***(1)***, including large outbreaks. Approximately 300,000 people in Shanghai, China, were infected by HAV after consumption of contaminated clams in 1988 ***(6)***. HAV is commonly implicated in illnesses traced to shellfish ***(7–9)*** and fresh produce ***(10,11)***.

Rotavirus is the leading cause of severe diarrhea among children worldwide ***(12)*** and leads to severe morbidity in developed countries, as well as frequent deaths (≥500,000 per year) in less developed countries. Although the role of rotavirus in diarrheal outbreaks in adults has not been well studied, it has been documented as a cause of adult diarrheal outbreaks in hospitals ***(13)***, nursing homes ***(14)***, isolated communities ***(15)***, and travelers ***(16)***. The oral-fecal transmission of rotavirus is mostly either through person-to-person contact or waterborne. However, food-borne infections involving sandwiches ***(17)*** and prepared foods in restaurants ***(18)*** have been reported, and rotavirus has been detected in lettuce in Costa Rican markets ***(19)***.

Numerous methods have been developed for the detection and the diagnosis of enteric viruses, including culturing, immunological, microscopic, and molecular methods. Molecular methods, particularly of the amplification type, appear to offer the most promising technology for the routine detection of enteric viruses, especially in food and environmental samples. The high sensitivity and specificity of these methods meet the essential requirements for detecting extremely low infectious doses in media of complex composition. Of the molecular detection techniques, RT-PCR has been the most widely used for the detection of enteric viruses in foods ***(20,21)***. More recently, nucleic acid sequence-based amplification (NASBA) has been developed for the detection of various enteric viruses ***(22–28)***. NASBA is an isothermic technique based on RNA amplification and is particularly suited to the detection of food-borne viruses of which the genome is formed essentially of RNA. It uses three enzymes—RNase H, T7 RNA polymerase, and reverse transcriptase—as well as two primers, one of which bears the T7 bacteriophage promoter sequence ***(29)***. It is particularly suited for the detection of RNA viruses, because there is no need for a separate reverse-transcription step. Furthermore, the amplification power of NASBA has been reported to be comparable to, or sometimes even higher than, that of PCR ***(22,30)***. NASBA techniques have also been developed for microbial pathogens in food and environmental samples, specifically for *Escherichia coli* ***(31)***, *Salmonella* ***(32)***, *Campylobacter* ***(33)***, and *Listeria monocytogenes* ***(34)***.

2. Materials

2.1. Cell Culture

1. Biosafety level II cell culture room.
2. Viral strains: HAV HM-175 (biosafety level 2), human rotavirus Wa (biosafety level 2) may be purchased at American Type Culture Collection (ATCC).
3. Cells lines: FRhK-4 cells and MA-104 cells may be purchased at ATCC.
4. Plasticware: 75-cm^2 flask, 6-well microtiter plates, 96-well microtiter plates.
5. Growth medium: 1X Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 0.1 m*M* nonessential amino acids, 0.113% sodium bicarbonate, 0.015 *M* HEPES buffer, and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) (Life Technologies, Burlington, ON).
6. Maintenance medium: same components as growth medium but with 2% FBS.
7. 2X overlay medium: 2X MEM, 4% FBS, 4 m*M* L-glutamine, 0.2 m*M* nonessential amino acid, 0.226% sodium bicarbonate, 0.03 *M* HEPES buffer, 0.1 *M* $MgCl_2$, and antibiotics (200 U/mL penicillin G and 200 µg/mL streptomycin).
8. Agarose type II (Sigma, Oakville, ON).
9. End-curve spatula.
10. Trypsin (Sigma).
11. Fixing solution: 3.7% formaldehyde solution in 0.85% NaCl (saline).
12. Staining solution: 0.1% crystal violet prepared in saline.
13. Acetone.
14. Anti-rotavirus antibody (Accurate Chemical, Westbury, NY).
15. Fluorescein isothiocyanate (FITC)-labeled anti-sheep IgG (H+L) antibody (Sigma).
16. Phosphate-buffered saline (PBS): 0.01 m*M* phosphate buffer, pH 7.2, 0.85% NaCl.
17. Glycerol.
18. Fluorescent microscope.

2.2. NASBA (see *Note 1)*

1. NASBA premixture (final concentration in 25 µL): 40 m*M* Tris-HCl (pH 8.5), 50 m*M* KCl, 12 m*M* $MgCl_2$, 1 m*M* of each deoxyribonucleoside triphosphate, 2 m*M* of each ribonucleoside-5′-triphosphate, 10 m*M* dithiothreitol (DTT), 15% (v/v) dimethylsulfoxide (DMSO), and 5 pmol of each gel-purified oligonucleotide primer.
2. Enzyme mixture: 2.6 µg of bovine serum albumin (in 50% glycerol; Roche Diagnostics, Laval, QC), 40 U T7 RNA polymerase (Pharmacia Biotech, Baie d'Urfé, QC), 8 U avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku America, Falmouth, MA), 0.2 U RNase H (Pharmacia Biotech), and 12.5 U RNasin (Promega, Madison, WI).
3. Circulating water bath.
4. Biotin-16-UTP (Roche Diagnostics).

2.3. Gel Electrophoresis

1. Agarose (Sigma).
2. Electrophoresis gel apparatus (Owl, Portsmouth, NH).

3. UV transilluminator (UVP, San Gabriel, CA).
4. RNA molecular weight marker (Roche Diagnostics).
5. 1X running buffer: 0.02 *M* borate buffer, pH 8.3; 0.2 m*M* EDTA.
6. Loading buffer (final concentration in 20µL): 1X running buffer, 6% formaldehyde, and 50% formamide.
7. Tracking dye: bromophenol blue and xylene cyanol in 50% glycerol.
8. Ethidium bromide.

2.4. Membrane Hybridization

1. Oligonucleotide-labeled (digoxigenin [DIG] or biotin) probes.
2. Positively charged nylon membrane (Roche Diagnostics).
3. Nucleic acid transfer apparatus.
4. 1X SSC: 15 m*M* sodium citrate and 150 m*M* NaCl, pH 7.0.
5. Hybridization solution: 5X SSC, 0.1% (w/v) *N*-laurylsarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS), and 1% (w/v) protein-blocking reagent (BR) (Roche Diagnostics).
6. Washing buffer: Maleic acid buffer (0.1 *M* maleic acid and 0.15 *M* NaCl, pH 7.5) and 0.3% (v/v) Tween-20.
7. Blocking solution: 1% BR in maleic acid buffer.
8. Anti-DIG-peroxidase conjugate (Roche Diagnostics).
9. PBS-T: 1X PBS and 0.05% Tween-20.
10. 3,3′,5,5′-tetramethylbenzidine (TMB) for membrane (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

2.5. Microtiter Plate Hybridization

1. Oligonucleotide amino-linked probes.
2. Coating buffer: 500 m*M* NaH_2PO_4 and 1 m*M* EDTA, pH 8.5.
3. 96-well DNA-binding microtiter plates (Corning, Acton, MA).
4. 1X TBS: 50 m*M* Tris-HCl and 150 m*M* NaCl, pH 7.6.
5. TBS-T: 1X TBS and 0.05% (w/v) Tween-20.
6. Blocking solution: TBS-T and 1% (w/v) BR.
7. Hybridization solution: 5X SSC and 0.1% SDS.
8. Streptavidin-peroxidase (Roche Diagnostics).
9. 3,3′,5,5′-tetramethylbenzidine (TMB) for microplate (Kirkgaard and Perry Laboratories).
10. Sulfuric acid (H_2SO_4).
11. Microtiter plate reader.

3. Methods

The procedures below describe the various steps required to detect two enteric viruses, hepatitis A virus and rotavirus, by NASBA. First, the viruses are propagated in cell culture and viral stock titer is determined. The RNA of the viral sample is then released and amplified by NASBA. Amplicons produced are analyzed by gel electrophoresis and solid-phase hybridizations.

3.1. Virus and Cell Propagation

The methods used for virus proliferation have been described previously by Mbithi et al. ***(35,36)*** for HAV and by Sattar et al. ***(37)*** for rotavirus. Hepatitis A virus strain HM-175, kindly provided by S. Bidawid, Bureau of Microbial Hazards, Health Canada, Ottawa, and human rotavirus Wa, which may be provided by American Type Culture Collection, are propagated, respectively, in FRhK-4 (fetal rhesus monkey kidney) and MA-104 (rhesus monkey embryonic kidney) confluent cell monolayers. All cell cultures are grown in 75-cm^2 flasks containing growth medium at 37°C under a humidified 5% CO_2 atmosphere with biweekly splits. For virus propagation and isolation, flasks are drained of medium, inoculated with small volumes of virus, and gently agitated periodically for 90 min at 37°C to allow viral adsorption. Rotavirus inoculum is first preactivated for 30 min at room temperature in a solution containing 20 U/mL trypsin. Cultures are then supplied with maintenance medium and incubated at 37°C under 5% CO_2 for viral propagation. The virus culture is harvested after 10 d and 24-h incubations for HAV and rotavirus, respectively, by means of three freeze–thaw cycles followed by low-speed centrifugation to remove cellular debris. The resulting supernatant is used as the virus stock suspension, stored in 1-mL aliquots at –80°C until needed. Titer determination of the viral stock suspension is performed as described in the following section.

3.2. Viral Titration

3.2.1. HAV Titer Determination by Plaque Assay

HAV titers are determined by the plaque formation assay of Mbithi et al. ***(35,36)***.

1. Grow in growth medium an overnight culture of 2×10^5 FRhK-4 cells/mL (2 mL/well) to confluence in 6-well microtiter plates at 37°C with 5% CO_2.
2. Discard the medium and inoculate with 250 µL of 10-fold serial dilutions of HAV suspension in maintenance medium.
3. Incubate for 90 min at 37°C under 5% CO_2 with periodic rocking by hand to allow viral infection.
4. Add 2 mL 1X overlay medium prepared from 1:1 (v/v) 2X overlay medium: 1.5% agarose and tempered at 42°C to each well. **Note:** *The agarose medium solidifies quickly.*
5. Allow to solidify at room temperature and incubate for 8 d at 37°C with 5% CO_2.
6. Add 2 mL of fixing solution to each well and incubate the microplate overnight at room temperature.
7. Discard the formaldehyde and, with an end-curve spatula, remove the remaining agarose overlay without scratching the surface of the well.
8. Air-dry the surface of each well and stain for 20 min at room temperature with 2 mL staining solution ***(38)***.

9. Remove the staining solution and air-dry.
10. Count the clear zones (lysis plaques), which correspond to PFU (plaque-forming units), and determine the HAV suspension titer by multiplying by the dilution factor to obtain PFU/mL.

3.2.2. Rotavirus Titer Determination by Immunofluorescence

Rotavirus titers are determined by fluorescent focus immunoassay with revelation of viral infection and multiplication by indirect immunofluorescence.

1. Grow in growth medium an overnight culture of 1.5×10^5 MA-104 cells/mL (100 µL/well) to confluence in 96-well microtiter plates at 37°C under 5% CO_2.
2. Preactivate the rotavirus suspension for 30 min at room temperature with 20 U trypsin/mL.
3. Inoculate each well with 25 µL of 10-fold serial dilutions of preactivated rotavirus in growth medium. **Note:** To be done in quadruplicate.
4. Add 200 µL of growth medium per well and incubate for 24 h at 37°C under 5% CO_2.
5. Drain the medium and add 100 µL 80% (v/v) acetone to each well.
6. Incubate for 30 min at 4°C, discard the solvent, and air-dry. **Note:** Microplates may be stored at 4°C until immunofluorescent detection.
7. Rehydrate cell monolayer by adding 100 µL of PBS per well and temper at room temperature for 10 min.
8. Discard buffer by absorption onto paper towel and destroy using appropriate means.
9. Incubate for 30 min at 37°C after adding 50 µL/well of anti-rotavirus antibody diluted 1:300 in PBS.
10. Wash five times with 200 µL/well of PBS.
11. Incubate 30 min at 37°C after adding 50 µL/well of anti-sheep IgG (H+L) FITC conjugate diluted 1:3000 in PBS.
12. Wash five times with 200 µL/well of PBS.
13. Add 50 µL of glycerol/PBS (3:1) to each well and seal the microplate.
14. Observe by epifluorescent microscopy with a suitable filter at a magnification of ×40. Titration of rotavirus stock is then calculated and expressed as 50% tissue culture infective dose ($TCID_{50}$) using the method of Reed and Muench ***(39)***.

3.4. Amplification Procedure

3.4.1. Primers

Suitable primers and probes are synthesized, gel-purified, and designed in the conserved region of the viral genome in order to maximize specificity and sensitivity and to minimize theoretical primer and probe self dimers, pair dimers (*see* **Note 2**). The oligonucleotide sequences used for the detection of HAV and rotavirus by NASBA are presented in **Table 1**. In designing, the forward primer included the bacteriophage T7 RNA polymerase promoter at the 5′ end (underscored in **Table 1**). HAV oligonucleotide sequences are located in the capsid pro-

Table 1
Nucleotide Sequences of Oligonucleotide Primers and Probes Used in Monoplex and Biplex NASBA Reactions

Primer/ probe	Sequence	Position	Size
BB1	5′-CAGATTGGCTTACTACACA-3′	1000–1018	474
BB2 + T7	5′AATTCTAATACGACTCACTATAGGGAGA CATGCAACTCCAAATCTGT-3′	1428–1446	
BB-probe	5′-GATTGATCTGTGCTATGGTTCCTGGTGACC-3′	1171–1200	
Rota-1	5′-GTAAGAAATTAGGTCCAAGAG-3′	794–814	286
Rota-2 + T7	5′-AATTCTAATACGACTCACTATAGGGAGA GGTCACATCGAACAATTC-3′	1045–1062	
Rota-probe	5′-CAAACTGAGAGAATGATGAGAGTGAATTGG-3′	886–915	

tein VP2 (GenBank accession no. M14707) and for rotavirus, primers and probes are selected in the gene 9 encoding a serotype-specific antigen VP7 (GenBank accession no. K02033).

3.4.2. Monoplex and Biplex NASBA

The NASBA reactions are performed as described by Blais et al. ***(40)*** with modifications. The principle of the NASBA reaction is shown in **Fig. 1** (*see* **Note 3**). NASBA reactions are carried out in a final volume of 25 µL as follows:

1. Prepare 18 µL NASBA premixture solution in a 0.6-mL sterile microfuge tube (per tube). **Note:** For monoplex NASBA, 5 pmol each of primers BB1 and BB2+T7 is added for HAV and 5 pmol each of primers Rota-1 and Rota2+T7 for rotavirus. For the biplex version, the same quantity of each primer is added to the prereaction mixture.
2. Add 5 µL of viral RNA from samples (*see* **Note 4**) released by heating at 100°C for 10 min.
3. Incubate at 65°C for 5 min to destabilize secondary RNA structures.
4. Temperate in 40°C water bath for 5 min for primer annealing.
5. Add 2 µL enzyme mixture to each tube (*see* **Note 5**) and incubate at 40 ± 1°C for 180 min.
6. Analyze the amplified products immediately by gel electrophoresis, Northern blot, dot blot, or microtiter plate hybridizations as described below or store at –20°C.

For microtiter plate hybridization detection, NASBA products are biotinylated. The same NASBA protocol is used except that 0.4 m*M* of biotin-16-UTP is incorporated into the NABSA reaction mixture.

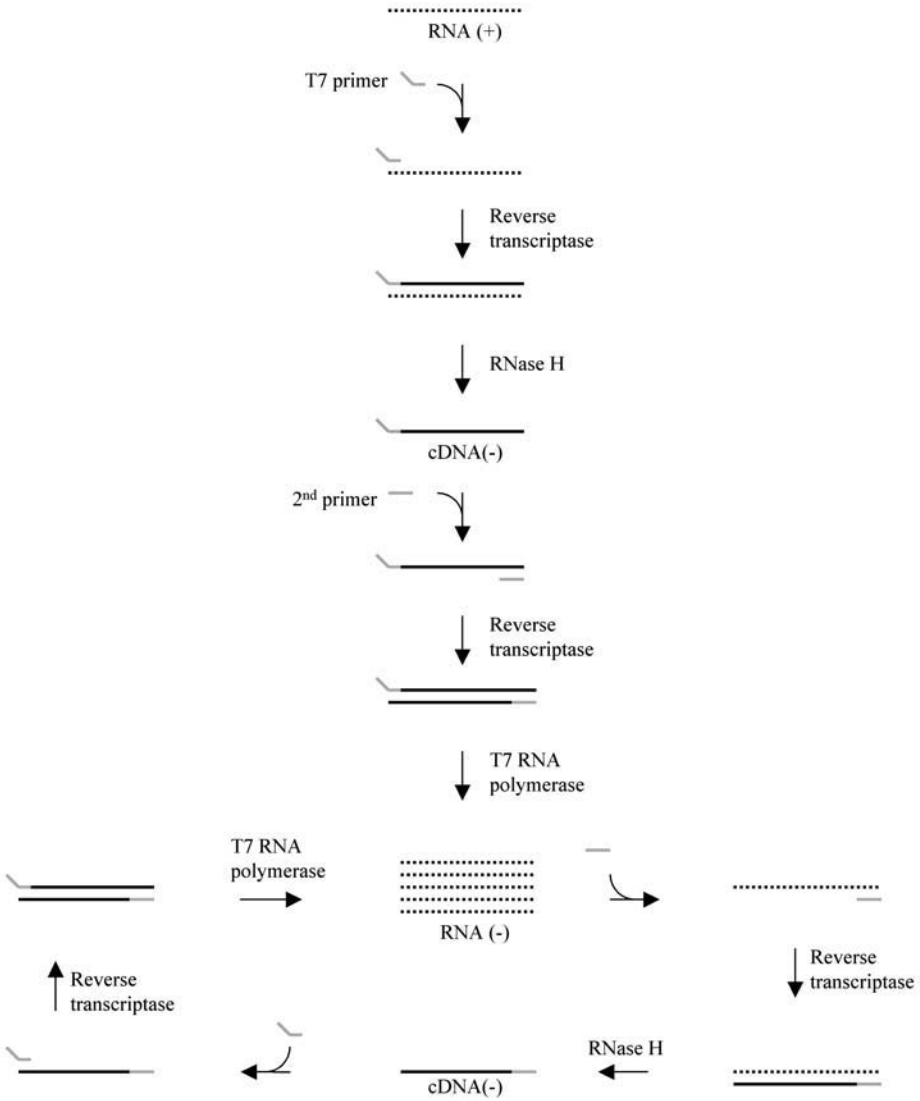

Fig. 1. Scheme for the amplification of RNA by the NASBA reaction.

3.5. Amplicon Analysis

3.5.1. Agarose Gel Electrophoresis

Amplified RNA (*see* **Note 6**) is analyzed by gel electrophoresis under denaturing conditions in agarose-formaldehyde using standard molecular biology methods with modifications ***(41)***.

1. Prepare 50 mL of 1.2% agarose gel in 1X running buffer containing 6% formaldehyde.
2. Pour the agarose gel solution and allow it to set.
3. Adjust the volume of NASBA product and RNA molecular weight marker to 4.5 µL and mix with 16.5 µL of loading buffer.
4. Heat the samples at 65°C for 2 min, cool down on ice, and add 2 µL of tracking dye.
5. Pour 400 mL of 1X running buffer containing 6% formaldehyde onto the gel.
6. Load NASBA product and marker into sample wells.
7. Run the gel at 120 V in 1X running buffer with 6% formaldehyde until the bromophenol blue is approx 2 cm from the bottom of the gel.
8. Stain the gel with ethidium bromide for 15 min and destain overnight in water.
9. Visualize by UV transillumination and photograph the gel if a permanent record is desired. The characteristic bands correspond to 474 nucleotides for HAV (**Fig. 2, panels A** and **E**) and 286 nucleotides for rotavirus (**Fig. 2, panels C** and **E**).

3.5.2. Nucleic Acid Immobilization for Northern and Dot Blots

For confirmation of the NASBA-amplified product, Northern blot analysis is performed as follows: unstained agarose-formaldehyde denaturing gel is rinsed with several changes of deionized water sufficient to cover the gel in order to reduce hindrance when transferring the amplified RNA to the positively charged nylon membrane (Roche Diagnostics). Transfer is completed in 1 h using a vacuum transfer apparatus (PosiBlot pressure blotter, Stratagene, La Jolla, CA) in the presence of 10X SSC.

As a qualitative assay, dot blot analysis is performed for a more rapid confirmation and to determine the detection limit of the test. Three microliters of a 1:1 dilution of NASBA-amplified product in 20X SSC are spotted manually using a micropipet onto a strip of dry nylon membrane presoaked in 20X SSC.

After transfer and/or spotting, RNA is immobilized to the dry membrane by a 2-min exposure to ultraviolet (UV) light (254 nm).

3.5.3. Hybridization on Nylon Membrane

RNA transferred from the gel or applied by dot blot onto nylon membrane is hybridized and detected as follows:

1. Prehybridize the membrane for 30 min at 55°C with RNase-free hybridization solution using gentle rotation in a hybridization oven.
2. Hybridize with 50 n*M* specific biotinylated or DIG-labeled probe(s) in hybridization solution at 55°C for 2 h using gentle rotation in a hybridization oven.
3. Wash for 5 min twice with 2X SSC and 0.1% SDS at room temperature.
4. Wash for 15 min twice with 0.1X SSC and 0.1% SDS at the hybridization temperature.
5. Wash quickly the membrane in washing buffer.

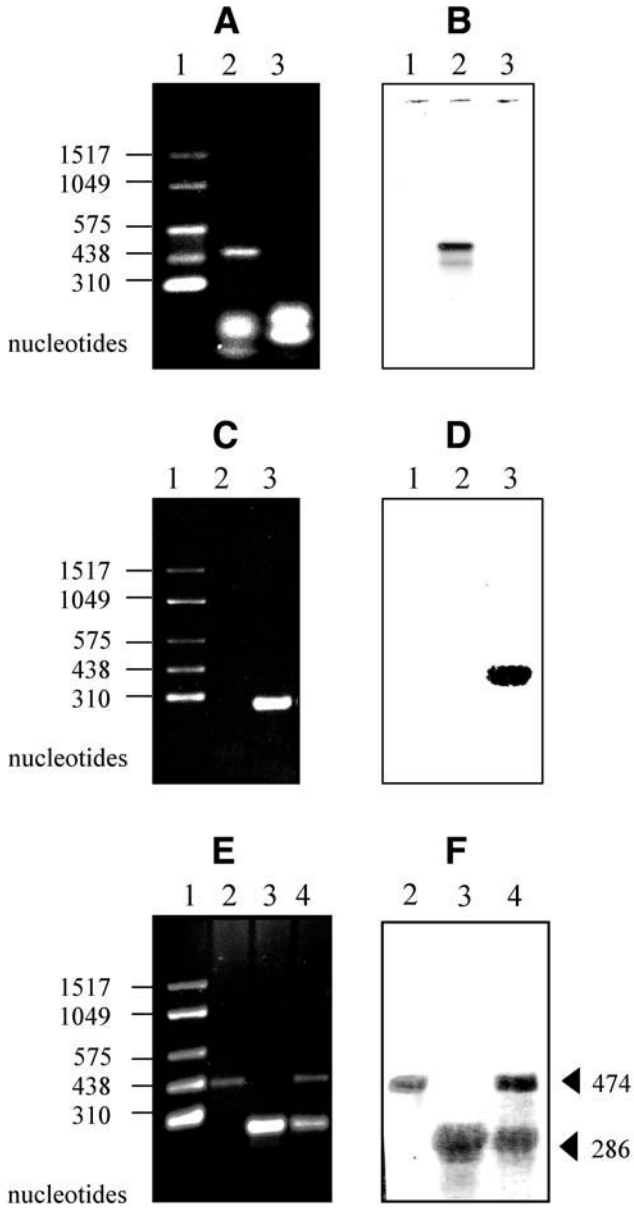

Fig. 2. Analysis of multiplex NASBA products by denaturing agarose gel electrophoresis (Panels A, C, and E) and Northern blot using DIG-labeled BB-probe (Panel B), DIG-labeled Rota-probe (Panel D) and both DIG-labeled probes simultaneously (Panel F). Lane 1, RNA molecular marker; lane 2, multiplex NASBA product using HAV as template; lane 3, multiplex NASBA negative control; lane 4, multiplex NASBA product using rotavirus as template; lane 5, multiplex NASBA product using both HAV and rotavirus as templates.

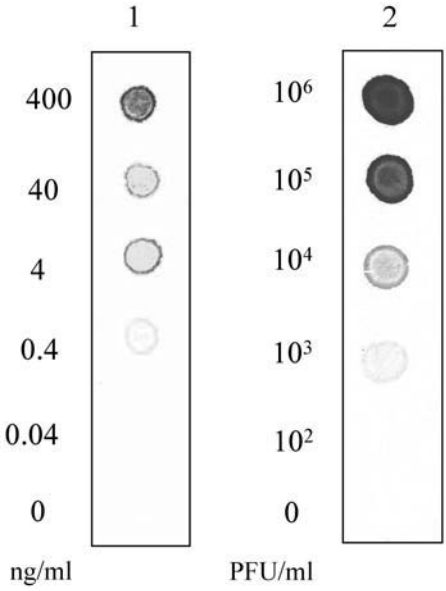

Fig. 3. Sensitivity of the NASBA system. Samples containing 10-fold serially diluted HAV RNA (lane 1) and HAV (lane 2) were amplified by NASBA and detected by dot blot hybridization. The detection limit is the minimum concentration of HAV giving a detectable signal.

6. Incubate for 30 min in blocking solution at room temperature.
7. Detect the hybrid formed between RNA and biotinylated or DIG-labeled probe with 0.25 µg/mL streptavidin-peroxidase or 75 mU/mL anti-DIG-peroxidase conjugate respectively in blocking solution for 30 min at room temperature.
8. Wash five times with PBS-T to remove unbound conjugate.
9. Add colorimetric peroxidase substrate, TMB solution (for membrane) to the RNA side of the membrane for few minutes to visualize the positive results.
10. Rinse the membrane to stop the reaction with water and air-dry.

Membrane coloration may thus be visualized. Amplification by NASBA is thus confirmed using Northern analysis (**Fig. 2, panels B, D,** and **F**). Detection limit, as determined for HAV by dot blot, is shown in **Fig. 3**.

3.5.4. Microtiter Plate Hybridization

Biotinylated NASBA-amplified products are detectable in a semiquantitative microtiter plate hybridization assay.

1. Add 100 µL of 0.2 µ*M* specific amino-linked probe diluted in coating buffer to each well of a 96-well DNA-binding microtiter plate and incubate for 30 min at 37°C.
2. Wash each well three times with 250 µL 1X TBS.

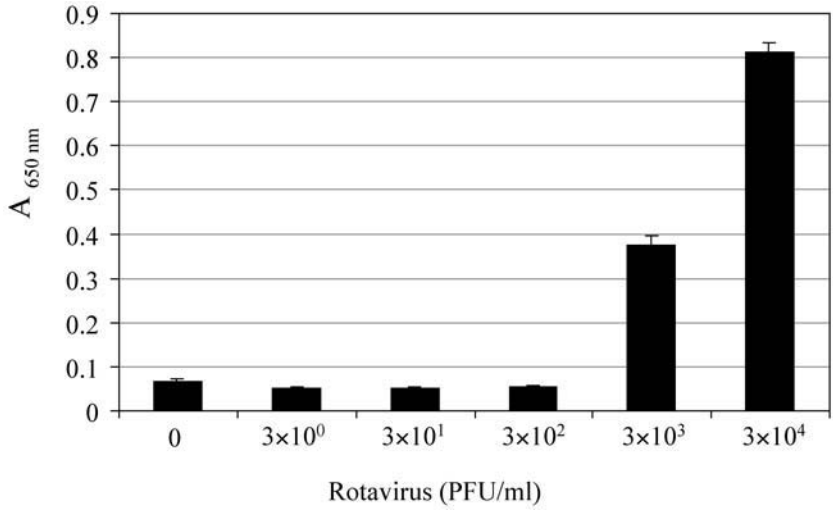

Fig. 4. Sensitivity of the rotavirus NASBA-ELISA in sewage treatment effluent. Ten-fold serial dilutions of rotavirus in sewage treatment effluent were amplified by NASBA and detected using microtiter plate hybridization. Detection limit is defined as the highest dilution giving an absorbance above A+3σ, where A is mean absorbance generated by the negative control and σ is standard deviation. Results are means of triplicate analyses.

3. Block with 200 µL of blocking solution for 30 min at 37°C.
4. Denature the RNase H in the biotinylated NASBA product for 5 min at 100°C and cool down the mixture on ice.
5. Add 100 µL of NASBA product diluted 1:50 in hybridization solution to each well coated with specific probe and hybridize for 1 h at 55°C with gentle horizontal agitation.
6. Wash three times with 250 µL of TBS-T to remove unbound amplified RNA.
7. Block with 200 µL of blocking solution in each well for 30 min at room temperature.
8. Add 100 µL of 0.25 µg/mL streptavidin-peroxidase diluted 1:4000 in blocking solution and incubate the microtiter plate for 30 min at room temperature.
9. Wash five times with 250 µL of TBS-T.
10. Add 100 µL TMB substrate (for microplate) to each well.
11. Read absorbance at 650 nm in a microtiter plate reader and stop reaction with 0.18 *M* H_2SO_4. After stopping the reaction, absorbance must be read at 450 nm.

The detection limit is determined when absorbance is in the range of blank absorbance plus three times the standard deviation. The detection limit of NASBA for rotavirus as determined by microtiter plate hybridization is shown in **Fig. 4**. The sensitivity of monoplex and biplex systems is also compared by microtiter plate hybridization **(Fig. 5)**.

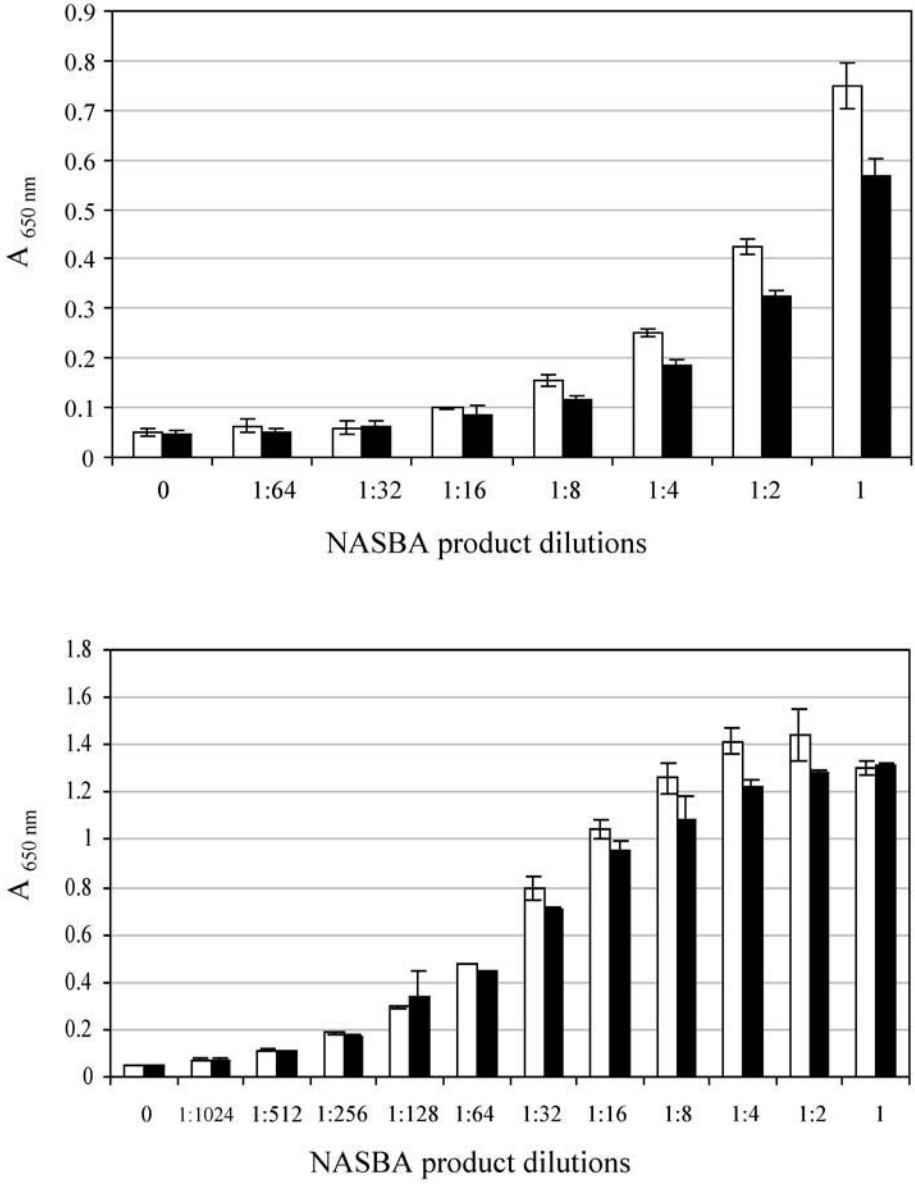

Fig. 5. Detection of biotinylated monoplex (□) and biplex (■) NASBA products of HAV (panel A) and rotavirus (panel B) using microtiter plate hybridization. NASBA was done with 5×10^6 PFU/mL of HAV or 4×10^7 PFU/mL of rotavirus. Biotinylated amplified RNAs were twofold serially diluted. Results are means of triplicate analyses.

4. Notes

1. The difficulty of working with RNA is that most ribonucleases are very stable and active enzymes that require no cofactors to function. Utmost care should be taken to avoid any RNase contamination of buffers and enzymes. Only water free of nuclease may be used in any experiments involving RNA. It is not recommended to use diethylpyrocarbonate (DEPC)-treated water or plasticware unless the DEPC is completely inactivated, or else the NASBA reaction may be inhibited ***(42)***.
2. Primers used for RT-PCR are not necessary good candidates for NASBA. As with PCR, it may be necessary to design and test more than one primer pair for each target in order to find the one that gives the desired performance ***(42)***. Synthesized oligonucleotide primers in the NASBA reaction must be gel-purified before use.
3. In the NASBA reaction, single-stranded RNA acts as a template. Double-stranded RNA (the case for rotavirus) needs to be denatured before amplification. Single- and double-stranded DNA need to be primed and extended using T7 promoter-containing P1 and DNA polymerase ***(29)***.
4. Preliminary steps should be performed on food samples prior to NASBA amplification depending on the composition and nature of the analyzed food. Usually, those steps include extraction and concentration of the viruses ***(43)***. Food extracts often contain extraneous materials such as acidic polysaccharides, glycogen, and lipids, which may inhibit the enzymatic amplification reaction ***(44)***. Viral nucleic acid purification steps may also be necessary to reduce the levels of inhibitory compounds present in the sample.
5. The enzyme cocktail is the key to consistent amplification. The origin and the source (supplier) are very important, as enzymes from different suppliers may not function similarly in NASBA.
6. The risk of amplicon contamination in the NASBA method may be higher than for PCR, since the number of copies produced by the amplification is higher. In fact, an optimized NASBA system can produce 10^9 copies from a single target RNA molecule ***(45)***. This high sensitivity means that the operator must be extremely careful not to contaminate a sample about to undergo amplification with either target RNA or previously amplified products remaining in the laboratory environment ***(42)***. Special care must also be taken to avoid amplicon contamination.

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III

The Parasites

15

Isolation and Characterization of Cathepsin-L1 Protease From *Fasciola hepatica* Excretory-Secretory Products for Serodiagnosis of Human Fasciolosis

Sandra M. O'Neill, Grace Mulcahy, and John P. Dalton

Summary

The major antigens secreted by the parasite *Fasciola hepatica* are cathepsin-L cysteine proteases. These enzymes can be isolated from the parasite excretory-secretory products in sufficient quantities for use as an antigen for the serodiagnosis of human fasciolosis. The methods illustrated in this chapter will explain the isolation of cysteine proteases from *F. hepatica* excretory-secretory products by gel filtration and anion exchange chromatography, and their subsequent characterization and employment in an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-fasciola antibodies in the serum of infected humans.

Key Words: Cathepsin-L proteases; gel filtration; anion exchange chromatography; ELISA; in vitro culture; parasites.

1. Introduction

Fasciola hepatica is a helminth parasite that causes liver fluke disease in cattle and sheep worldwide, and has recently emerged as an important pathogen of humans ***(1)***, particularly in countries such as Bolivia ***(2,3)***, Peru ***(4,5)***, Iran ***(6,7)***, and Egypt ***(8,9)***. Infection is acquired when watercress or aquatic plants contaminated with dormant metacercaria are ingested. The parasite emerges in the intestine and migrates through the gut and then the liver to gain access to the bile ducts, where it sexually matures. The parasite secretes proteolytic enzymes that are crucial for its survival within the host, as they perform such important functions as facilitating parasite entry into the host ***(10)***, acquisition of nutrients from host cells ***(11,12)*** and modulation of host immune responses that are important to host protection against the parasite ***(13–16)***. These proteolytic enzymes are excellent diagnostic candidates because they

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are secreted at all stages of development within the definitive host, making diagnosis of the acute and chronic stages of infection possible ***(17)***. In addition, because it is the major protein secreted in the parasites excretory-secretory products, it can be isolated in sufficient quantities to facilitate diagnosis of a large number of humans and animals. We have developed an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of human fasciolosis based on the detection of IgG4 antibodies to *Fasciola hepatica,* cathepsin-L1 cysteine proteases (CLs). This single purified antigen can be isolated in high concentrations from parasite excretory-secretory products by gel filtration and anion exchange chromatography, and was shown to be more specific and sensitive when compared to assays where crude parasite antigen or excretory-secretory products are employed ***(18)***. The methods illustrated in this chapter will explain the isolation and characterization of CL from *F. hepatica* excretory-secretory products and their application in the serological detection of human fasciolosis.

2. Materials

2.1. Culture of Adult Fluke

1. Mature adult liver flukes can be obtained from the bile ducts of infected livers of condemned cattle or sheep at a local abattoir.
2. Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, and 8.1 m*M* Na_2PO_4H, pH 7.3 (Sigma-Aldrich, Leicester, England).
3. RPMI-1640, pH 7.3, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), glutamine (2 n*M*) and 2% glucose (Gibco BRL, Life Technologies, Paisley, England).
4. 2 µm Amicon 8400 Ultrafiltration unit (Millipore, Billerica, MA).
5. Ym3 filtration membrane (3-kDa molecular mass cut-off) (Millipore).
6. BCA protein assay reagent kit (Pierce and Warriner, Chester, England) or Bradford Protein kit (Bio-Rad Laboratories, Hercules, CA).
7. Allegra 25R Refrigerated Benchtop centrifuge (Beckman Coulter, Buckinghamshire, UK).

2.2. Purification of Cathepsin L

1. Sephadex S200 gel filtration column (2.6 cm × 74.5 cm) (Amersham Biosciences, Uppsala, Sweden).
2. 0.1 *M* Tris-HCl, pH 7.0. (Sigma-Aldrich).
3. 400 m*M* NaCl in 1.0 *M* Tris-HCl, pH 7.0 (Sigma-Aldrich).
4. 1.0 *M* Tris-HCl, pH 7.0 (Sigma-Aldrich).
5. Flow-through LKB Uvicord spectrophotometer (Bio-Rad).
6. Z-phe-arg-NHMec (Bachem UK Ltd, Merseyside, UK).
7. 50 mL QAE-Sephadex column (2.5 cm × 10.0 cm) (Amersham Biosciences) equilibrated in 1 *M* Tris-HCl, pH 7.0 (Sigma-Aldrich).

2.3. Measurement of Cathepsin-L Activity Using Fluorogenic Substrate Z-phe-arg-NHMec

1. Z-phe-arg-NHMec (Bachem UK Ltd, Merseyside, UK).
2. 0.1 *M* Tris-HCl, pH 7.0, containing 0.5 m*M* dithiothreitol (Sigma-Aldrich).
3. 96-Well microtiter plate (Nuclon, Kamstrup, Roskilde, Denmark).
4. 1.7 *M* acetic acid (Sigma-Aldrich).
5. Perkin-Elmer LAMBDA 650 fluorescence spectrophotometer with excitation set at 370 nm and emission at 440 nm (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

2.4. Analysis of Purified Cathepsin L by Zymography and SDS-PAGE

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running gel: 12% (w/v) acrylamide, 0.27% (w/v) bisacrylamide, 0.373 *M* Tris-HCl, pH 8.0, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulfate, and 0.08% TEMED (Sigma-Aldrich).
2. Stacking gel: 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide, 0.125 *M* Tris-HCl, pH 6.8, 0.075% (w/v) ammonium persulfate, 0.1% (w/v) SDS, and 0.023% (w/v) TEMED (Sigma-Aldrich).
3. Nonreducing preparation buffer: 0.12 *M* Tris-HCl, pH 6.8, containing 5% (w/v) SDS (Sigma-Aldrich), 10% (w/v) glycerol, 0.01% (w/v) bromophenol (Riedal de Haen, Seelze, Germany).
4. Reducing sample buffer: As for nonreducing preparation buffer before, except 5% 2-mercapthoethanol (Sigma-Aldrich) is added and the sample boiled for 2 min.
5. Vertical slab gel apparatus with power source (Bio-Rad).
6. Electrode buffer: 25 m*M* Tris-HCl, 192 m*M* glycine, and 0.1% SDS, pH 8.3 (Sigma-Aldrich).
7. Gel soaking buffer: 0.1% (v/v) Coomassie™ brilliant blue R, 20% (w/v) methanol, and 10% acetic acid for 1 h at room temperature (Sigma-Aldrich).

2.5. Visualization of Protease Activity by Gelatin-Substrate Polyacrylamide Gel Electrophoresis

1. Gelatin-substrate polyacrylamide gel electrophoresis (GS-PAGE) running gel: the preparation of the gel is identical to that of SDS-PAGE except that 1% gelatin (Sigma-Aldrich) is added to the separating gel solution.
2. Washing buffer: 0.1 *M* sodium citrate, pH 4.5, containing 2.5% Triton X-100 (Sigma-Aldrich).
3. 0.1 *M* sodium citrate, pH 4.5, containing 10 μ*M* cysteine (Sigma-Aldrich).
4. Gel soaking buffer: Coomassie brilliant blue R solution as described in **Subheading 2.4.** (Sigma-Aldrich).

2.6. Immunoblot Studies

1. Reducing SDS-PAGE electrophoresis gel as described in **Subheading 2.4.**
2. Nitrocellulose paper (Schleicher and Schuell Biosciences, Dassel, Germany).

3. Blocking solution: 0.5% bovine serum albumin/0.1% Tween-20 (Sigma-Aldrich).
4. Anticathepsin L1 or nonimmune rabbit serum (gift from Professor John Dalton).
5. Alkaline-phosphate-conjugated anti-rabbit serum (Sigma-Aldrich).
6. Substrate for alkaline phosphatase: Nitro-blue tetrazolium (5 mg/mL) and 5-bromo-4-chloro-indolyl phosphate (10 mg/mL) prepared in 100% dimethylformamide (Sigma-Aldrich).

2.7. Diagnosis of Human Fasciolosis Using Purified Cathepsin-L1 Cysteine Proteases by ELISA

1. Flat-bottom 96-well microtiter plates (Kamstrup).
2. 100 µL of cathepsin L (5 µg/mL), isolated as outlined in **Subheading 3.2.**
3. PBS/0.1% Tween-20 (Sigma-Aldrich).
4. Blocking buffer: 2% bovine serum albumin (200 µL) diluted in PBS/0.1% Tween-20 (Sigma-Aldrich).
5. Biotin-conjugated anti-human IgG4 (1:1000 dilution) (Sigma-Aldrich).
6. Avidin-conjugated peroxidase (1:4000 dilution) (Sigma-Aldrich).
7. Azino/Bis phosphate citrate buffer: 2,2-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (25 mg/mL) in phosphate citrate buffer, pH 5.0 (0.2 *M* Na_2HPO_4, 0.1 *M* citric acid, mixed in a ratio of 25.7:24.30) (Sigma-Aldrich).
8. Anthos 2001 microtiter plate reader at 405 nm (Anthos Labtec Instruments GmbH, Salzburg, Austria).

3. Methods

The following methods are outlined: (1) culture of adult flukes in media to obtain *F. hepatica* excretory-secretory products; (2) Purification of cathepsin L1 from *Fasciola* excretory-secretory products by gel filtration and anion exchange chromatography; (3) Characterization of cathepsin L1; and (4) Diagnosis of human fasciolosis using purified CL as an antigen in ELISA.

3.1. Culture of Adult Flukes to Obtain F. hepatica Excretory-Secretory Products

1. Flukes should be washed six times in sterile PBS, pH 7.3, in order to remove debris and bile.
2. Adult flukes (8 g of flukes per 150 mL of media) should be cultured in vitro in RPMI-1640, pH 7.3. To ensure that all "host" molecules that were ingested and excreted by the liver fluke are removed from the media, the culture media should be discarded after 2 h and replenished every 8 h for a total of 24 h.
3. The culture media from all three incubations are pooled and centrifuged at 13,000*g* for 30 min to remove eggs and debris.
4. The supernatant (excretory-secretory [ES] products) is sterilized by passing through a 2-µm membrane and concentrated to 10 mL using an Amicon 8400 Ultrafiltration unit and a Ym3 filtration membrane (3-kDa molecular mass cutoff).

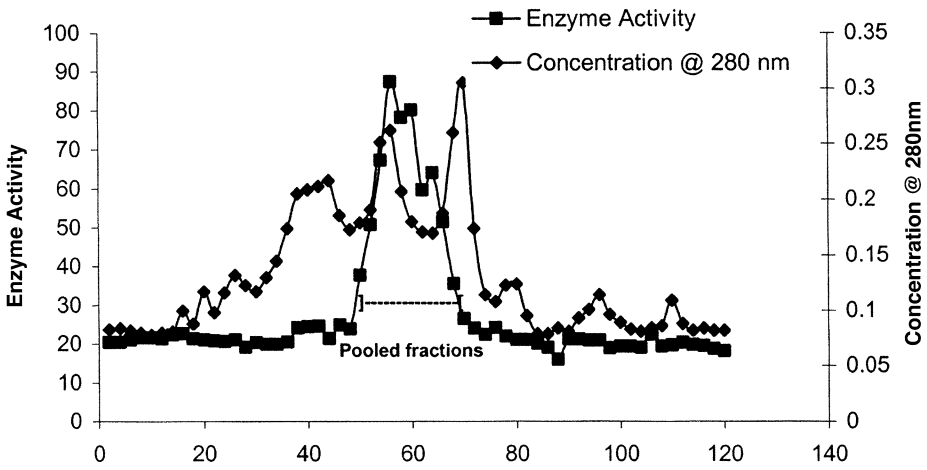

Fig. 1. Purification of *F. hepatica* cysteine proteases by Sephacryl S200HR chromatography. The culture medium in which mature *F. hepatica* were maintained was concentrated to 10 mL and applied to a Sephacryl S200HR column (19 × 42 cm). The mobile phase was 0.1 *M* Tris-HCl, pH 7.0. Protein elution from the column was monitored using a flow-through spectrophotometer. Cysteine proteinase activity in collected fractions is assayed using the fluorogenic substrate Z-phe-arg-NHMec. Fraction containing Z-phe-arg-NHMec cleaving activity were pooled and applied to a QAE-Sephadex column (25 × 10 cm) equilibrated in 0.1 *M* Tris-HCl, pH 7.0.

5. A second centrifugation at 13,000*g* for 30 min is carried out to remove any insoluble protein (*see* **Note 1**). Protein concentration of the concentrated ES was calculated using a BCA protein assay reagent kit per manufacturer's instructions. Using this method, an estimated 4 to 10 mg/mL of total protein is obtained from 2 L of culture medium ***(19)***.

3.2. Purification of Cathepsin L From ES Products

Cathepsin-L protease is purified from ES as described previously ***(20,21)***.

1. Concentrated ES products (4–10 mg/mL) is applied to a Sephadex S200 gel filtration column (2.6 cm × 74.5 cm) equilibrated in 0.1 *M* Tris-HCl, pH 7.0, at 4°C.
2. The column is eluted with 0.1 *M* Tris-HCl, pH 7.0, and after a void volume of 110 mL has passed, 5-mL fractions are collected.
3. Each fraction is monitored for protein concentration at 280 nm using a flow-through LKB Uvicord spectrophotometer and for cathepsin-L activity using the fluorogenic substrate, Z-phe-arg-NHMec, as described in **Subheading 3.2.3.**
4. The protein concentration and cathepsin-L activity from each fraction is plotted on a graph **(Fig. 1)**. A broad protein peak is observed around the peak of cysteine protease activity.

5. These fractions are pooled and concentrated to 10 mL using an Amicon 8400 ultrafiltration unit (*see* **Note 2**). The concentration of the pooled fractions is determined and usually is between 1.5 and 4 mg/mL.
6. The protease fractions pooled from the gel filtration column contain two proteases, termed cathepsin L1 and cathepsin L2 ***(20,21)***.
7. To obtain a pure fraction of cathepsin L1, the Sephacryl S200 concentrated fractions are applied to a 50-mL QAE-Sephadex column (2.5 cm × 10.0 cm) equilibrated in 1 *M* Tris-HCl, pH 7.0.
8. The QAE Sephadex column is washed with 300 mL 0.1 *M* Tris-HCl, pH 7.0, and the cathepsin L1, which does not bind to the column, is collected (*see* **Note 3**).
9. Cathepsin L2, which does bind to the column, is eluted with 400 m*M* NaCl in 0.1 *M* Tris-HCl ***(20)***. The cathepsin-L1 protease is concentrated to 10 mL as previously described and the protein concentration determined (approx 1–2 mg protein).

3.3. Characterization of Purified F. hepatica *Cathepsin-L Cysteine Proteinase*

3.3.1. Measurement of Cathepsin-L Activity Using Fluorogenic Substrate Z-phe-arg-NHMec

Cathepsin-L activity is measured fluorometrically using Z-phe-arg-NHMec as substrate ***(22)***.

1. Assays (210 µL volume) are performed with 1 µg of protein with substrate at a final concentration of 10 µM in 0.1 *M* Tris-HCl, pH 7.0, containing 0.5 m*M* dithiothreitol on a 96-well microtiter plate.
2. Plates are incubated at 37°C for 30 min and the reaction stopped by the addition of 50 µL of 1.7 *M* acetic acid.
3. The amount of 7-amino-4-methylcouramin (NHMec) released is measured using a Perkin-Elmer fluorescence spectrophotometer with excitation set at 370 nm and emission at 440 nm. One unit of enzyme activity is defined as the amount that catalyses 1 µ*M* of NHMec per minute at 37°C as determined using a standard curve of NHMec (concentrations 0–10 µmole) against enzyme activity.

3.3.2. Analysis of Purified Cathepsin L by Zymography and Reducing Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophesis

Cathepsin L1 is analyzed by one-dimensional, 12% denaturing SDS-PAGE, according to the method of Laemmli ***(23)***.

1. The running gel is prepared and the stacking gel applied and comb removed.
2. Samples are prepared in nonreducing buffer or reducing sample buffer.
3. Gels are run in a vertical slab gel apparatus in electrode buffer at 25 mA at room temperature. A voltage of 8 V/cm^2 is applied and the gel is run until the bromophenol blue dye reaches the bottom of the gel.
4. The gel is removed and the proteins are visualized by soaking the gel in Coomassie brilliant blue R solution for 1 h at room temperature.

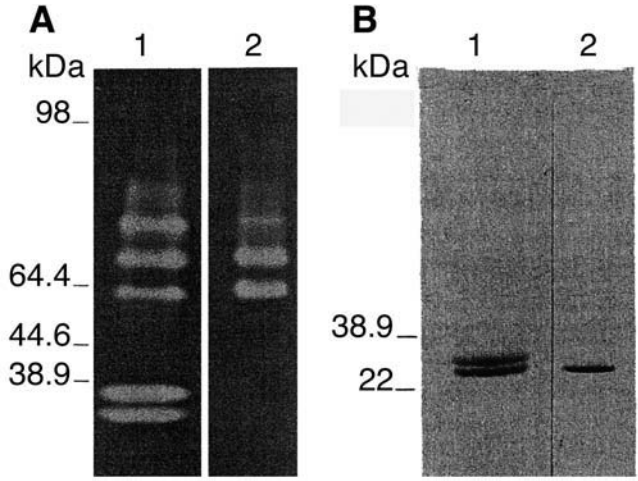

Fig. 2. SDS-PAGE and GS-PAGE analysis: **(A)** Zymogram analysis of ES products (lane 1) and purified cathepsin L1 (5 µg) (lane 2). **(B)** Reducing SDS-PAGE analysis of ES products (lane 1) and purified cathepsin L1 (5 µg) (lane 2).

5. SDS-PAGE analysis under these reducing condition shows that ES products contain two major proteins, which represent cathepsin L1 and cathepsin L1, while the homogenous cathepsin L1 and migrates as a single band at 27.5 kDa **(Fig. 2B).**

3.3.3. Visualization of Protease Activity by Gelatin-Substrate Polyacrylamide Gel Electrophoresis

GS-PAGE is performed as described by Dalton and Heffernan ***(19)***.

1. Prepare GS-PAGE separating gel.
2. Samples are applied in nonreducing sample buffer (and without boiling) to maintain biological activity.
3. After electrophoresis the gels are washed for 60 min in washing buffer.
4. The gels are subsequently incubated in 0.1 *M* sodium citrate, pH 4.5, containing 10 µ*M* cysteine for 12 h at 37°C.
5. Stain gels in Coomassie brilliant blue R solution. Zymography shows the presence of multiple gelatinolytic bands in ES products and that homogenous cathepsin L1 migrates as two major and several minor bands **(Fig. 2A)**.

3.3.4. Immunoblot Studies

1. Purified cathepsin L1 is run on a reducing SDS-PAGE.
2. The protein is electrophorectically transferred to nitrocellulose paper using a semi-dry electroblotting system as previously described ***(20,21)***.

3. Following blocking in 0.5% bovine serum albumin and 0.1% Tween-20, the nitrocellulose membrane is incubated in anticathepsin L1 or nonimmune rabbit serum.
4. Bound immunoglobulin is visualized using alkaline-phosphate-conjugated anti-rabbit serum.
5. Nitro-blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate prepared in dimethylformamide were used as a substrate for alkaline phosphatase. The blot demonstrates that the anticathepsin L1 sera are reactive with the single protein 27.5-kDa band that corresponds to cathepsin L1.

3.4. Diagnosis of Human Fasciolosis Using Purified Cathepsin-L1 Cysteine Proteases by ELISA

The optimal dilutions of antigen, serum, and secondary antibodies for the ELISA method described below were determined by a checkerboard titration procedure *(17)*.

1. Microtiter plates are coated with 100 µL of cathepsin L1 (5 µg/mL) and incubated overnight at 37°C.
2. The plates are washed six times with PBS/0.1% Tween-20 and excess protein binding sites blocked using blocking buffer added to each well for 2 h at room temperature (*see* **Note 4**).
3. After a further wash step, serum samples are tested at a range of dilutions between 1:50 and 1:218,700, and left to incubate for 1 h at 37°C.
4. The wash step is repeated and biotin-conjugated anti-human IgG4 (1:1000 dilution) is added and the plates incubated at 37°C for 1 h (*see* **Note 5**).
5. Following a further wash step, bounded biotin-conjugated antibodies are detected by the addition of 100 µL of avidin-conjugated peroxidase (1:4000 dilution). After a final washing, 100 µL of Axino/Bis-phosphate citrate buffer is added.
6. After an incubation period of 10 min the plates are read on an Anthos 2001 microtiter plate reader at 405 nm. The antibody titer is expressed as a log titer and all samples performed in triplicate.

The titers illustrated in **Fig. 3** are from volunteers residing in the Bolivian Altiplano. Fecal samples were also obtained for coprological analysis from individuals *(17)*. Individuals were divided into groups based on coprological analysis and clinical symptoms. Those who were coprologically negative but serologically positive are in the acute stages of infection compared with those who were coprologically positive and serologically positive. As negative controls, sera from volunteers in the laboratory were employed **(Fig. 3)**.

4. Notes

1. The method describing the culturing of *F. hepatica* adult liver flukes to obtain ES products can be utilized to obtain ES products from all species of liver fluke,

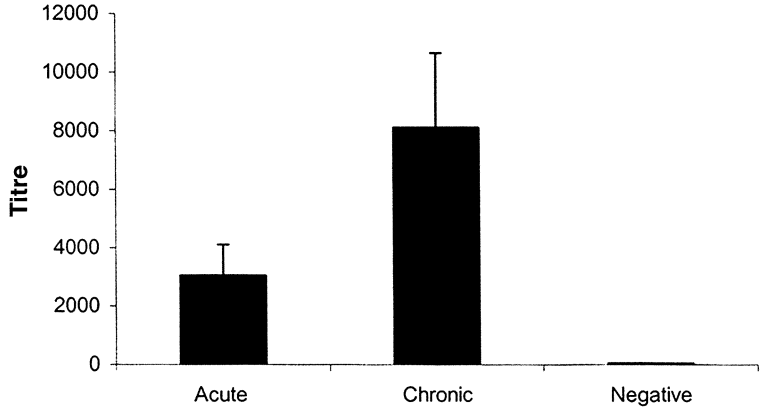

Fig. 3. Analysis of sera from individuals in the acute or chronic stages of *Fasciola* infection by IgG4-ELISA using cathepsin L1 as antigen. Each bar represents the mean titer for a total of 10 individuals per group. Negative samples were obtained from volunteers at Dublin City University.

including *F. gigantica* and *F. buski.* Similar to *F. hepatica,* these species of *Fasciola* secrete significant quantities of cysteine proteases in their excretory-secretory products.

2. The protein concentration of eluted fractions can also be measured using a Bradford assay or BCA protein assay reagent kit, rather than using a flow-through LKB Uvicord spectrophotometer. These assays can be run in parallel with the enzymatic assays so that enzyme units and specificity activity for each fraction can be determined.
3. If the cathepsin L1 is required for in vivo or in vitro cellular studies, the column can be eluted with PBS rather than Tris-HCl. This will also avoid the need for a dialysis procedure.
4. The ELISA assay can be performed using anti-human total IgG. However, since the predominant antibody isotype elicited by liver fluke in humans is IgG4, the sensitivity of this assay using this antibody isotype is superior.
5. The sensitivity of this ELISA is not significantly altered if the coating antigen is incubated at 4°C overnight, or if casein or milk is employed in the blocking buffer.

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16

Molecular Biology Methods for Detection and Identification of *Cryptosporidium* Species in Feces, Water, and Shellfish

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Summary

Techniques based on nucleic acid amplification have proven to be essential for the detection and epidemiological tracking of members of the genus *Cryptosporidium.* This gastrointestinal protozoan parasite cannot be routinely cultivated and it has an extremely low infectious dose, possibly below 100 oocysts. As *Cryptosporidium* is an important pathogen, particularly in immunocompromised hosts, there is a pressing need to employ sensitive and discriminatory systems to monitor the organism. A number of fairly standard target genes have been assessed as detection targets, including 18*S* rRNA, microsatellites, and heat-shock (stress) proteins. As our knowledge of the biology of the organism increases, and as the full genome information becomes available, the choice of target may change. Genes encoding parasite-specific surface proteins (gp60, TRAP-C2, COWP) have already been examined. Much of the effort expended in molecular diagnostics of *Cryptosporidium* has been directed toward developing robust nucleic acid extraction methods. These are vital in order to recover amplifiable DNA from environments where small numbers of oocysts, often fewer than 100, may exist. Methodology based on adaptation of commercial kits has been developed and successfully employed to recover amplifiable DNA directly from water, food (particularly seafood), and fecal samples.

Key Words: *Cryptosporidium;* DNA; feces; environmental; shellfish; PCR; IFA; sequencing; phylogenetic.

1. Introduction

Members of the genus *Cryptosporidium* are protozoan parasites that are increasingly associated with both human and animal gastrointestinal infections. In immunocompetent humans, *Cryptosporidium* parasites cause acute infections of the digestive system, but in immunocompromised patients they cause a chronic,

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life-threatening disease ***(1)***. Infections result from oral ingestion of oocysts contaminating food or water and through direct contact with infected animals or humans ***(2)***. Human cryptosporidiosis, caused by either *Cryptosporidium parvum* or the recently named *C. hominis,* emerged as an important gastrointestinal infection in the 1990s ***(3)***. The organism cannot be routinely cultivated in the laboratory; the infectious dose is extremely low, likely <100; and oocysts are resistant to chemical inactivation, allowing long-term environmental persistence ***(4)***. These factors combine to make detection and epidemiological monitoring of pathogenic *Cryptosporidium* a technically difficult task.

In the absence of cultivation as a detection method, direct detection is the only option. Direct microscopic observation, using a modified Ziel Neilson staining, suffers from a lack of both sensitivity and specificity. Immunofluorescence partially addresses these deficiencies, but the methods to date do not allow species identification and are prone to interference in environmental samples ***(5–7)***. It was therefore inevitable that molecular methods would increasingly be used to detect the organism. The first use of PCR with *C. parvum* was published in 1991 and was concerned with identification of a thymidylate synthase gene in the organism ***(8)***. This was quickly followed by publications outlining the use of this technology to detect the organism in a variety of matrices; the systems have been refined over the intervening years based on changes to both the choice of target gene and also to the system for nucleic acid extraction. Information has been steadily accumulating considering the choice of different genes for different applications. Genes have been considered for the identification of species (18*S* rRNA), typing to genotype and subgenotype levels (Hsp70, gp60, COWP, and microsatellites), and most recently gene targets for determination of viability by mRNA detection have begun to be evaluated (Hsp70, β Tubulin) ***(9–15)***. With the recent availability of the whole *C. parvum* genome it is expected that many new sequences will be evaluated in the next few years ***(16)***.

Detection of *Cryptosporidium* is required for environmental surveillance and diagnostic applications. In practical terms this primarily equates to detection in animal and human feces, raw and treated water supplies, and foodstuffs, principally seafood and vegetables. These differing matrices bring many challenges for recovery of enough oocysts and extraction of amplifiable nucleic acids. It has been increasingly attractive to researchers to adapt existing nucleic acid extraction kits for use with *C. parvum* and *C. hominis* in an effort to introduce greater efficiency and reproducibility into assays. The inclusion of an efficient immunomagnetic separation system has improved the sensitivity of many assays, but is too expensive for routine use ***(9)***.

Initially a conventional single-step PCR was the system of choice but it has been increasingly found that nested assays and real-time systems are more

appealing. These offer greater sensitivity and, particularly with real-time systems, are more attractive to routine use, having automated detection built into the assay ***(17)***. The use of molecular tools has also led to the identification of geographic and temporal differences in the transmission of *C. parvum* and *C. hominis,* and better appreciation of the public health importance of other *Cryptosporidium* species/genotypes and the frequency of infections with mixed genotypes or subtypes ***(19)***.

Water-borne outbreaks of cryptosporidiosis have been well documented ***(20,21)***. As water supplies directly affect the food industry, the implications of the organism contaminating food and beverage products through this route must be considered. Cryptosporidiosis has also been associated with the increasing popularity of drinking unpasteurized milk; eating raw fish and shellfish; consuming undercooked pork, poultry, and eggs; and having contact with pets ***(22–25)***.

This chapter focuses on systems in routine use in our laboratories, with an emphasis on methods for processing nucleic acids.

2. Materials

2.1. Cryptosporidium *DNA Isolation From Fecal and Environmental Water Samples and Shellfish Using FastDNA SPIN Kit*

1. 2.5% Potassium dichromate solution (Sigma).
2. Distilled water.
3. FastDNA SPIN Kit (cat. no. 6560-200, Bio 101 Systems).

2.2. Detection and Differentiation of Cryptosporidium *Oocysts by Nested PCR of 18S rRNA Followed by Endonuclease Restriction*

1. Primary PCR primers:
   Forward (F1): 5′-TTCTAGAGCTAATACATGCG-3′
   Reverse (R1): 5′-CCCATTTCCTTCGAAACAGGA-3′
2. Secondary PCR primers:
   Forward (F2): 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′
   Reverse (R2): 5′-CTC ATA AGG TGC TGA AGG AGT A-3′
3. 10X PCR buffer with 15 m*M* Mg^{2+} (cat. no. N808-0129, PE Applied Biosystems, Foster City, CA).
4. 100 m*M* dNTP (cat. no. U1240 Promega, Madison, WI). To make a 1.25 m*M* working solution, add 12.5 µL of each dNTP to 950 µL of distilled water. Store the working solution at –20°C before use.
5. *Taq* polymerase (Promega).
6. 25 m*M* $MgCl_2$ (Promega).
7. *Ssp*I and appropriate *Ssp*I buffer (New England BioLabs, Beverly, MA).
8. *Dde*I and Buffer 3 (New England BioLabs).

9. *VspI* and Buffer D (Promega).
10. Agarose (Sigma).
11. Horizontal gel electrophoresis apparatus (Horizon 11.14 Life Technologies).
12. Power supply (Model 200/2.0, Bio-Rad).
13. PCR machine (Biometra TRIO–THERMOBLOCK).
14. Gel DNA 100-bp ladder (Promega).
15. Ethidium bromide solution.

2.3. Detection and Differentiation of Subgenotypes of Cryptosporidium parvum *Oocysts by gp60–Polymorphism Analysis*

1. Primary PCR primers:
   Forward (F1): 5′-ATAGTCTCCGCTGTATTC-3′
   Reverse (R1): 5′-GGAAGGAACGATGTATCT-3′
2. Secondary PCR primers:
   Forward (F2): 5′-TCCGCTGTATTCTCAGCC-3′
   Reverse (R2): 5′-GCAGAGGAACCAGCATC-3′
3. 10X PCR buffer with 15 m*M* Mg^{2+} (Product no. N808-0129, PE Applied Biosystems).
4. 100 m*M* dNTP (cat. no. U1240, Promega). To make a 1.25 m*M* working solution, add 12.5 µL of each dNTP to 950 µL of distilled water. Store the working solution at –20°C before use.
5. *Taq* polymerase (cat. no. M2665, Promega).
6. 25 m*M* $MgCl_2$ (cat no. A351F, Promega).
7. BigDye® Terminator V3.1 Cycle Sequencing Kit. (cat. no. 4336917, Applied Biosystems).

3. Methods

3.1. Cryptosporidium *DNA Isolation From Fecal and Environmental Water Samples and Shellfish Using FastDNA SPIN Kit*

The FastDNA spin kit (Q-BIOgene) has proven to be an extremely versatile DNA extraction system able to handle a variety of matrices that differ greatly in the amount and composition of organic material. The kit works, in conjunction with sets of dedicated extraction buffers, on the FastPrep cell lysis instrument. The latter employs homogenization of the sample in the presence of small pellets and appropriate lysis buffers to generate material that is suitable for a variety of postextraction analyses.

3.1.1. Preparation of Human Fecal Samples for DNA Extraction

1. Fecal samples containing *Cryptosporidium* oocysts should be stored from fresh in 2.5% potassium dichromate solution at 4°C in a ratio of 1:2 (v/v).
2. Prior to use resuspend the fecal material in 2.5% potassium dichromate solution by vigorously shaking the suspension and transferring approx 500 µL of the suspension to a sterile 1-mL Eppendorf tube.

3. Wash the samples in distilled water to remove the potassium dichromate. Briefly centrifuge the 500-µL sample at 13,000*g* for 10 min, discard the supernatant, and resuspend in 500 µL of distilled water.
4. Repeat wash step 3 until the potassium dichromate solution is removed (i.e., until the yellow coloration of the solution turns clear).
5. Add up to 250 to 500 mg of the pellet to the Lysing Matrix E Tube from the FastDNA SPIN Kit (*see* **Note 1**) and follow the DNA extraction procedure supplied by the manufacturer.

3.1.2. Preparation of Shellfish Samples for DNA Extraction

The following method was developed for the common mussel *(Mytilus edilus):*

1. Open shell, excise the gills with a sterile scissors, and place in 5 mL of Hank's Balanced Salt Solution (HBSS) in a 15-mL centrifuge tube (*see* **Note 2**).
2. Cap tube and agitate for 15 s with a vortex mixer.
3. Centrifuge (1500*g* for 10 min).
4. Aspirate the supernatant and resuspend the pellet in 10 mL of dH_2O (*see* **Note 3**).
5. Wash the pellet at least two times with distilled water.
6. Centrifuge at 1500*g* for 10 min, aspirate the supernatant, and subject the pellet to five freeze–thaw cycles (*see* **Note 4**).
7. Add up to 250 to 500 mg of the pellet to Lysing Matrix E Tube from the FastDNA SPIN Kit and follow the DNA extraction procedure supplied by the manufacturer.

3.1.3. Preparation of Fish Samples for DNA Extraction

1. Weigh and measure the fish tissue and remove the intestine and stomach.
2. Wash the gastrointestinal tract and scrape the mucosa from the stomach and intestine using a scalpel blade onto a clean glass slide.
3. Wash the mucosa into a tube using 400 µL to 5 mL of dH_2O depending on the size of the fish and amount of mucosal scrapings.
4. Cap tube and vortex for 20 s.
5. Centrifuge at 1500*g* for 10 min.
6. Aspirate the supernatant and subject the pellet to five freeze-thaw cycles.
7. Add up to 250 to 500mg of the pellet to Lysing Matrix E Tube from the FastDNA SPIN Kit.

3.1.4. Preparation of Water Samples for DNA Extraction

For the concentration and recovery of *Cryptosporidium* oocysts from environmental water samples, up to 1000 L of water were filtered through Gelman Envirochek sampling capsules (standard and high-volume [HV] filters).

1. Pellet water concentrate in 2.0-mL tube by centrifuge at 10,000*g* for 5 min.
2. Add up to 250 to 500 mg of the pellet to Lysing Matrix E Tube from the FastDNA SPIN Kit and follow the DNA extraction procedure supplied by the manufacturer.

3.2. PCR and Endonuclease Restriction Analysis of SSU 18 rRNA gene

3.2.1. Using Primary PCR Primers

Preparation of master mix: for each PCR reaction, prepare the following:

10X Perkin-Elmer PCR buffer	10 µL
dNTP (1.25 m*M*)	16 µL
F1 primer (40 ng/µL)	2.5 µL
R1 primer (40 ng/µL)	2.5 µL
$MgCl_2$ (25 m*M*)	6 µL
Bovine serum albumin (10 mg/mL)	4 µL
Distilled water	57.5 µL
Taq polymerase	0.5 µL
Total	99 µL

1. Add 99 µL of the master mix to each PCR tube.
2. Add 1 µL of DNA sample to each tube.
3. Run the following PCR program: 94°C, 3 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; then 72°C for 7 min and 4°C soaking.

3.2.2. Using Secondary PCR Primers

Preparation of master mix: For each PCR reaction, prepare the following:

10X Perkin-Elmer PCR buffer	10 µL
dNTP (1.25 mM)	16 µL
F2 primer (40 ng/µL)	5 µL
R2 primer (40 ng/µL)	5 µL
$MgCl_2$ (25 m*M*)	6 µL
Distilled water	55.5 µL
Taq polymerase	0.5 µL
Total	98 µL

1. Add 98 µL of the master mixture to each PCR tube.
2. Add 2 µL of the primary PCR reaction to each tube.
3. Run the following PCR program: 94°C, 3 min; 35 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min; then 72°C for 7 min and 4°C soaking.

3.2.3. Endonuclease Restriction

1. Prepare master mixture using the following formula, which is for one restriction digestion reaction:

	Buffer	Water	Enzyme
*Ssp*I	4 µL of New England BioLabs Buffer *Ssp*I	22 µL	4 µL
*Vsp*I	4 µL of Promega Buffer D	24 µL	2 µL
*Dde*I	4 µL of New England BioLabs Buffer 3	24 µL	2 µL

Table 1
Restriction Fragment Length[a] Polymorphism in SSU rRNA Gene of Common *Cryptosporidium* spp. and genotypes

Species	PCR fragment	*Ssp* I digestion[b]	*Vsp* I digestion[b]
C. muris/C. andersoni	833	385, 448	102, 731
C. serpentis	831	370, 414	102, 729
C. baileyi	826	254, 572	102/104, 620
C. felis	864	390, 426	102/104, 182, 476
C. meleagridis	833	108, 254, 449	102/104, 171, 456
C. wrairi	834	109, 254, 449	102/104, 628
C. saurophilum	834	109, 255, 418	102/104, 628
C. canis	829	105, 254, 417	94/102, 633
Cryptosporidium ferret genotype	837	111, 254, 449	102/104, 174, 457
C. suis	838	365, 453	102/104, 632
Cryptosporidium marsupial genotype	837	109, 254, 441[b]	102/104, 631
C. hominis	837	111, 254, 449	70, 102/104, 561
C. parvum A gene	834	108, 254, 449	102/104, 628
C. parvum B gene	831	119, 254, 449	102/104, 625
Cryptosporidium mouse genotype	838	112, 254, 449	102/104, 175, 457

[a]In basepairs; only sizes of visible bands are shown.
[b]An additional upper band (about 583 bp) from the heterogeneous copy of the gene is usually present.

2. Transfer 30 µL of the master mixture to each tube, add 10 µL of secondary PCR reaction to the tube, and mix well.
3. Incubate in a 37°C water bath for 2 h or overnight.

3.2.4. Gel Electrophoresis of Restriction Endonuclease Fragments

Load 40 µL of restriction digestion reaction on 1.2% agarose gel (Sigma). Identify *Cryptosporidium* species and genotypes based on restriction fragment length pattern (RFLP) banding patterns. **Table 1** shows the restriction fragment length (in basepairs; only sizes of visible bands are shown) polymorphism in the SSU rRNA gene of common *Cryptosporidium* spp. and genotypes. **Figure 1** illustrates the differentiation of common *Cryptosporidium* species and genotypes by a nested PCR-RFLP procedure based on the SSU rRNA gene. **Figure 2** illustrates the differentiation of *C. andersoni* and *C. muris* by RFLP analysis of SSU rRNA gene PCR products using *Dde*I. **Figure 3** shows the sequence diversity among *Cryptosporidium* species and genotypes in the polymorphic region of the SSU rRNA gene.

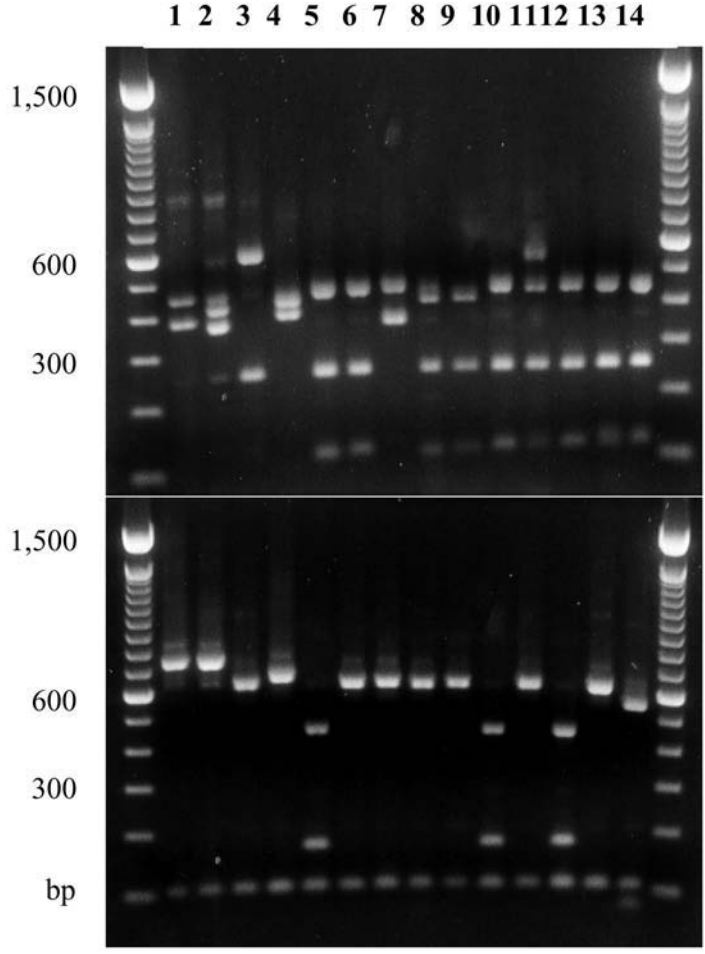


Fig. 1. Differentiation of common *Cryptosporidium* species and genotypes by a nested PCR-RFLP procedure based on the SSU rRNA gene. Lane 1, *C. muris* or *C. andersoni;* lane 2, *C. serpentis;* lane 3, *C. baileyi;* lane 4, *C. felis;* lane 5, *C. meleagridis;* lane 6, *C. wrairi;* lane 7, *C. suis*; lane 8, *C. canis;* lane 9, *C. saurophilum;* lane 10, *Cryptosporidium* ferret genotype; lane 11, *Cryptosporidium* marsupial genotype; lane 12, *Cryptosporidium* mouse genotype; lane 13, *C. parvum*; and lane 14, *C. hominis*. The upper panel are *Ssp*I digestion products, and the lower panel are *Vsp*I digestion products. Molecular markers are 100-bp ladders.

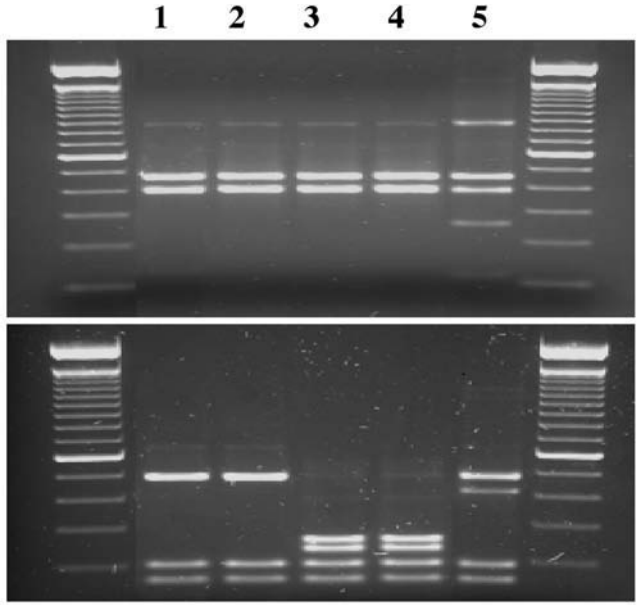


Fig. 2. Differentiation of *C. andersoni* and *C. muris* by RFLP analysis of SSU rRNA gene PCR products using *Dde*I. Lanes 1 and 2, *C. andersoni;* lanes 3 and 4, *C. muris;* and lane 5, *C. andersoni* and *C. hominis*. The upper panel are *Ssp*I digestion products, and the lower panel are *Dde*I digestion products. The top band in lane 5 of the *Ssp*I products was due to partial digestion. Molecular markers are 100-bp ladders.

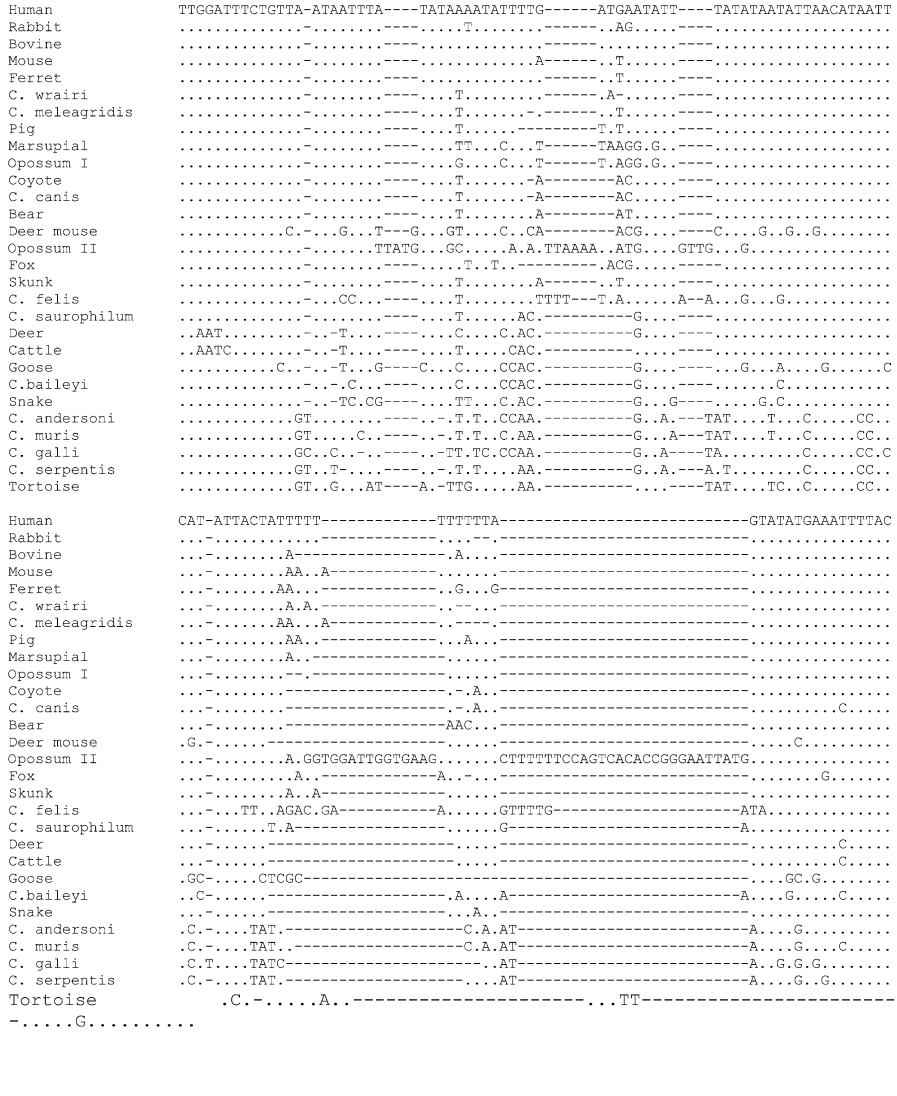
3.3. Detection and Differentiation of Subgenotypes of Cryptosporidium parvum *Oocysts by gp60–Polymorphism Analysis*

3.3.1. PCR of GP60 Gene

3.3.1.1. Primary PCR

Preparation of master mix: for each PCR reaction, prepare the following:

10X Perkin-Elmer PCR buffer	10 µL
dNTP (1.5 m*M*)	16 µL
F1 primer (40 ng/µL)	5 µL
R1 primer (40 ng/µL)	5 µL
$MgCl_2$ (25 m*M*)	6 µL
Bovine serum albumin (10 mg/mL)	4 µL
Distilled water	52.5 µL
Taq polymerase	0.5 µL
Total	99 µL



1. Add 99 μL of the master mix to each PCR tube.
2. Add 1 μL of DNA sample to each tube.
3. Run the following PCR program: 94°C, 3 min; 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; then 72°C for 7 min and 4°C soaking.

3.3.1.2. Secondary PCR

Preparation of master mix: for each PCR reaction, prepare the following:

Fig. 3. Sequence diversity among *Cryptosporidium* species and genotypes in the polymorphic region of the SSU rRNA gene. Dots denote sequence identity to the *C. parvum* human genotype (top sequence) and dashes denote deletions. Human: *C. hominis*; rabbit: *Cryptosporidium* rabbit genotype; bovine: *C. parvum*; mouse: *Cryptosporidium* mouse genotype; ferret: *Cryptosporidium* ferret genotype; pig: *C. suis*; marsupial: *Cryptosporidium* marsupial genotype; opossum I: *Cryptosporidium* opossum genotype I (related to the *Cryptosporidium* marsupial genotype; coyote: *C. canis* coyote genotype; bear: *Cryptosporidium* bear genotype; deer mouse: *Cryptosporidium* deer mouse genotype; opossum II: *Cryptosporidium* opossum genotype II; fox: an unnamed *Cryptosporidium* sp. in foxes; deer: an unnamed *Cryptosporidium* sp. in deer; cattle: *C. bovis* in cattle (rare in prevalence); goose: an unnamed *Cryptosporidium* sp. in geese; snake: an unnamed intestinal *Cryptosporidium* sp. in snakes; and tortoise: an unnamed gastric *Cryptosporidium* sp. in tortoises.

10X Perkin-Elmer PCR buffer	10 µL
dNTP (1.5 m*M*)	16 µL
F2 primer (40 ng/µL)	5 µL
R2 primer (40 ng/µL)	5 µL
$MgCl_2$ (25 m*M*)	6 µL
Distilled water	54.5 µL
Taq polymerase	0.5 µL
Total	97.5µL

1. Add 97.5 µL of the master mix to each PCR tube.
2. Add 2.5 µL of the primary PCR reaction to each tube.
3. Run the following PCR program: 94°C, 3 min; 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; then 72°C for 7 min and 4°C soaking.

3.3.1.3. Gel Electrophoresis of PCR Fragments

1. Load 14 µL of the PCR product on 1.5% agarose gel (Sigma), and run on a horizontal gel electrophoresis apparatus (Horizon 11.14 Life Technologies) at 100 V for 60 min.
2. Visualize under UV transilluminator.

4. Notes

1. Because of the vigorous motion of the FastPrep® instrument, a significant pressure buildup is observed in the tube. For this reason the total volume of the sample and the Lysing Matrix should not exceed 7/8 of the volume of tube. Leaving space in the tube also improves homogenization of the sample.

2. This protocol is not suitable for siphon feeders such as clams. In this case, the protocol for *Cryptosporidium* oocyst recovery/DNA extraction from fish is more suitable.
3. When large numbers of shellfish are required to be processed, pool the gill washings from 5 to 6 shellfish, centrifuge (1500*g* for 10 min), resuspend the pellet in 5 mL dH_2O, and proceed to **step 4**.
4. Oocysts can also be recovered from the hemolymph but much higher numbers are usually recovered from the gills (Dr. Ron Fayer, pers. comm.).

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17

Molecular Identification of Nematode Worms From Seafood (*Anisakis* spp. and *Pseudoterranova* spp.) and Meat (*Trichinella* spp.)

Giuseppe La Rosa, Stefano D'Amelio, and Edoardo Pozio

Summary

Fish-borne and meat-borne parasitic infections represent an important public health concern, given the increasing risk of acquiring these pathogens and related allergies through the consumption of raw or undercooked seafood and meat. This can, in part, be attributed to the increased globalization of both the food industry and eating habits. For the analysis of food-borne pathogens and for molecular epidemiology, in vitro amplification of nucleic acids using polymerase chain reaction (PCR) has become a powerful diagnostic tool. Each parasite species has a specific distribution area and range of hosts. Because the infecting larval stages of the species belonging to the genera *Anisakis, Pseudoterranova,* and *Trichinella* are morphologically indistinguishable, the only chance of identifying these pathogens at the species or genotype level is through PCR-derived methods. PCR amplification of ITS1 and ITS2 regions, followed by restriction fragment length polymorphism (RFLP), allows for the distinction among species of the genera *Anisakis* and *Pseudoterranova.* For *Trichinella* worms, a multiplex PCR analysis can be used to distinguish among the eight recognized species and four genotypes (*Trichinella* T6 and three populations of *T. pseudospiralis*), whereas to distinguish the genotypes *Trichinella* T8 and T9 from *Trichinella britovi,* PCR-RFLP can be performed.

Key Words: Anisakiasis; trichinellosis; *Trichinella; Anisakis; Pseudoterranova;* molecular analysis; molecular epidemiology; PCR; RFLP; ITS.

1. Introduction

The risk of acquiring parasitic food-borne infections through the consumption of raw or undercooked seafood and meat has, in recent years, increased as a result of the growing globalization of both the food industry and eating habits ***(1,2)***. Identifying the etiological agents of these infections at the species or genotype level is quite important in aiding physicians in diagnosis and treatment, in

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Table 1
Principal Features of the *Anisakis* and *Pseudoterranova* Species Characterized by PCR-RFLP

Species	Distribution	Main paratenic hosts[a]	Definitive hosts[a]
A. simplex s. s.	North Atlantic and North Pacific	Herring, cod, salmon, cephalopods	Whales, dolphins
A. pegreffii	Mediterranean, Southern Atlantic, Southern Pacific	Hake, horse mackerel, tuna, blue whiting, scabbardfish,	Sperm whale, ziphiids, bottlenose dolphins
A. simplex C	Southern Pacific, Southern Atlantic, Pacific coasts of Canada	Orange roughy, snoek	Ziphiids, pilot whale, pigmy sperm whale, false killer whale
A. ziphidarum	Mediterranean, Southern Atlantic	Mackerel, hake	Mainly in ziphiids
A. physeteris	Mediterranean, Central Atlantic	Hake, swordfish, blue whiting	Mainly in sperm whale
A. typica	Mediterranean, Central Atlantic	Hake, horse mackerel, several tuna species,	Striped dolphin, *Sotalia* spp.
A. schupakovi	Caspian Sea	Unknown	Caspian seal
P. decipiens s.s.	North Atlantic	Cod, haddock, pollock	Mainly in harp seal, also in gray seal
P. krabbei	Northeastern Atlantic	Cod, haddock, pollock	Mainly in gray seal
P. bulbosa	North Atlantic	Mainly flatfish	Mainly in bearded seal

[a]The list of paratenic (or intermediate) and definitive hosts is only indicative and it is incomplete, considering the wide array of fish and mammalian hosts for this group of parasites.

tracing the source of the infection, in determining the area of origin of the infected food, and in developing the most appropriate measures for controlling infection at all phases of food production, from fishing, hunting, or breeding to processing and postprocessing. In this chapter, we describe the means of identifying at the molecular level nematode worms that have been implicated in food-borne infections, specifically those belonging to the genera *Anisakis* spp., *Pseudoterranova* spp., and *Trichinella* spp.

The species belonging to the genera *Anisakis* and *Pseudoterranova,* grouped as complexes of morphologically indistinguishable (or "cryptic") species,

infect, at the larval stage, fish, cephalopods, and shrimp, and, at the adult stage, fish, fish-eating birds, and marine mammals ***(1)***. However, each species has a specific distribution area and range of hosts **(Table 1)**; thus identifying these parasites at the species level is crucial in reducing the risk for the consumer, which may include such measures as avoiding particular fishing areas, sizes of fish, or even particular species of fish ***(1)***. Moreover, whether or not a fish is infected with these parasites can depend on the methods of capturing, handling, and storage, which can also affect the number of parasites present ***(1)***. All marine fish, mollusks, and probably crustaceans are potential reservoirs of infective larvae (i.e., third-stage larvae, L_3), which, although unable to develop to the adult stage in humans, can induce a variety of symptoms, depending on where they are located in the human body (i.e., gastric, intestinal, or extragastric-intestinal symptoms). Furthermore, in previously sensitized persons, severe allergic reactions can occur ***(3)***. Given that morphological traits are not sufficient for definitively identifying the larvae of *Anisakis* spp. and *Pseudoterranova* spp. at the species level, it is necessary to use molecular methods based on polymerase chain reaction (PCR). The molecular identification of single *Anisakis* and *Pseudoterranova* larvae is carried out using PCR-restriction fragment length polymorphism (RFLP). However, the PCR-RFLP protocol has been published only for *Anisakis* ***(4–6)***; for *Pseudoterranova,* we developed the protocol presented herein on the basis of previously published sequences ***(7)***.

Nematode worms of the genus *Trichinella* are a complex of species **(Table 2)** that are transmitted by two cycles, in particular, the sylvatic cycle and the domestic cycle. In the sylvatic cycle, the main reservoirs are carnivorous and omnivorous mammals, although these parasites have also been detected in birds and reptiles ***(2,8)***. Most of these reservoirs have cannibalistic and/or scavenger behavior. In the domestic cycle, the main reservoirs are pigs and horses. Humans are infected mainly through the consumption of raw or undercooked pork ***(2)***; game meat has also been implicated (e.g., wild boar, bear, and walrus). The clinical picture and prognosis of human infection depend on several factors: the number of infective larvae ingested, the specific *Trichinella* species, and the allergic reaction of the host. The lack of morphological markers does not allow the species to be easily or rapidly identified; thus methods based on PCR are used. Species identification is of great importance in tracing the source of infection, in determining and predicting the clinical course of infection, in estimating the potential risk for pigs, in establishing appropriate strategies for control and eradication, and in better understanding the epidemiology of the infection. The use of PCR-derived methods also allows the species to be identified based on a single larva, which is important because, frequently, only one larva is detected in human biopsies and in muscle samples of animal hosts. Furthermore, the identification of single larvae allows more than one species of

Table 2
Principal Features of *Trichinella* Species and Genotypes *(2,8)*

Trichinella species Genotype	Distribution	Cycle	Hosts	Collagen capsule
T. spiralis	Cosmopolitan[a]	domestic and sylvatic	swine, rats, carnivores	yes
T. nativa	Arctic and subarctic areas of Holoarctic region[b]	sylvatic	terrestrial and marine carnivores	yes
Trichinella T6	Canada, USA[c]	sylvatic	carnivores	yes
T. britovi	temperate areas of Palearctic region,[d] West Africa	sylvatic, seldom domestic	carnivores, seldom swine	yes
Trichinella T8	South Africa	sylvatic	carnivores	yes
Trichinella T9	Japan	sylvatic	carnivores	yes
T. pseudospiralis	Cosmopolitan[e]	sylvatic, seldom domestic	mammals and birds	no
T. murrelli	temperate areas of Nearctic region	sylvatic	carnivores	yes
T. nelsoni	Ethiopic region	sylvatic	carnivores, seldom swine	yes
T. papuae	Papua New Guinea	sylvatic, seldom domestic	mammals and reptiles	no
T. zimbabwensis	Zimbabwe	sylvatic	mammals and reptiles	no

[a]This species has not been detected in Arctic regions.
[b]The isotherm –5°C in January is the southern limit of distribution.
[c]Alaska, Idaho, and Montana.
[d]The isotherm –6°C in January is the northern limit of distribution.
[e]Three different populations have been identified in the Nearctic region (Alabama and Texas), the Palearctic region (many foci), and the Australian region (Tasmania).

Trichinella to be detected in the same host (mixed infections). The molecular identification of single larvae of *Trichinella* is carried out with a multiplex-PCR analysis ***(9)***, which allows all known species and genotypes to be identified **(Table 2)**, with the exception of the genotypes *Trichinella* T8 and *Trichinella* T9, which can be distinguished from *Trichinella britovi* only using a PCR-RFLP analysis of the gene encoding for a 43-kDa protein ***(10)***.

2. Materials

2.1. Hosts of Anisakis *spp. and* Pseudoterranova *spp. Larvae*

The presence and the number of L_3 is related to the host species, the season, and the geographical region **(Table 1)**. L_3 are present in the celomatic cavity, yet they migrate to muscles when the animals dies. To avoid this migration, seafood should be maintained in ice or at 0°C immediately after fishing. Freezing at –20°C for at least 52 h kills the larvae ***(1)***.

2.2. Hosts and Preferential Muscles of Trichinella

The animals most commonly infected with *Trichinella* are those with carnivorous and/or omnivorous behavior and at the top of the food chain, specifically mammals (e.g., wolf, fox, mustelid, bear, raccoon dog, raccoon, hyena, lion, walrus, pig, wild boar, rat, and horse), birds (e.g., crow, eagle, and hawk), and equatorial reptiles (e.g., crocodile and varan) **(Table 2)**. The identification of *Trichinella* worms is most commonly based on muscle larvae, which are very easy to collect from both animals and humans. The preferential muscles (i.e., those with the highest density of larvae) vary according to the specific host species, but as a general rule, the tongue can be considered the preferential muscle. Other important muscles are the pillar of the diaphragm for swine, the anterior tibial for foxes and wolves, and the masseter for carnivores and horses. Human biopsies are generally taken from the deltoid muscle.

2.3. Isolation and Preservation of Larvae (L_3) of Anisakis *spp. and* Pseudoterranova *spp.*

1. A plexiglass surface lit from underneath by fluorescent lights (375–540 lux) placed 13 cm below the working surface.
2. Scalpels, forceps, and small brushes.
3. Conical vials (0.5 mL) and racks.
4. Disposable gloves.
5. Petri dishes (5–6 cm diameter).
6. 70% ethyl alcohol.

2.4. Isolation and Preservation of Trichinella *Larvae*

1. Commercial blender with a volume of at least 500 mL.
2. Suction pump (e.g., water pump).
3. Incubator (37–45°C) with a capacity of at least 100 L and with an inner electrical socket.
4. Magnetic stirrer and magnets.
5. Precision scale.
6. Dissection microscope (×20–40).
7. Thermometer.

8. Two automatic pipets (range of 1–20 µL and 10–200 µL).
9. Beakers (capacity of at least 1 L).
10. Scissors and forceps.
11. Conical vials (0.5 mL and 50 mL) and racks.
12. Cooler.
13. Disposable gloves.
14. Petri dishes (5–6 cm in diameter).
15. Pepsin 1:10,000 (*see* **Note 1**).
16. Hydrochloric acid.
17. Ethyl alcohol, anhydrous.
18. Tap water (37–45°C).
19. Sterile H_2O (4°C)
20. Phosphate-buffered saline (PBS) at 37–45°C: 137 m*M* NaCl (8 g/L), 7 m*M* K_2HPO_4 (1.21 g/L), KH_2PO_4 (0.34 g/L).
21. Digestion fluid: 1% pepsin (w), 1% HCl (v), tap water (37–45°C).

2.5. Primer Sets for PCR and for PCR-RFLP

2.5.1. PCR Amplification of Entire ITS Regions for Anisakis spp.

Primer pair:

– NC5, 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′
– NC2, 5′- TTAGTTTCTTCCTCCGCT-3′

Amplicon size: 1 kb. Each primer must be diluted at 50 pmol/µL in sterile H_2O.

2.5.2. PCR Amplification of ITS2 Region for Pseudoterranova decipiens Complex

Primer pair:

– XZ1, 5′-ATTGCGCCATCGGGTTCATTCC-3′
– NC2, 5′-TTAGTTTCTTTTCCTCCGCT-3′

Amplicon size: 300 bp. Each primer must be diluted at 50 pmol/µL in sterile H_2O.

2.5.3. Multiplex-PCR and PCR-RFLP for Trichinella

2.5.3.1. Multiplex-PCR (for Amplicon Sizes *see* **Table 3**)

Primer pair I: 5′-GTTCCATGTGAACAGCAGT-3′
5′-CGAAAACATACGACAACTGC-3′
Primer pair II: 5′-GCTACATCCTTTTGATCTGTT-3′
5′-AGACACAATATCAACCACAGTACA-3′
Primer pair III: 5′-GCGGAAGGATCATTATCGTGTA-3′
5′-TGGATTACAAAGAAAACCATCACT-3′
Primer pair IV: 5′-GTGAGCGTAATAAAGGTGCAG-3′
5′-TTCATCACACATCTTCCACTA-3′

Table 3
Multiplex-PCR Amplicon Sizes (in Basepairs) of Primer Sets of 11 *Trichinella* Genotypes

Primer pair	Ts	Tn	Tb[a]	Tps-Ne	Tps-Pa	Tps-Au	Tm	T6	Tne	Tpa	Tz
I	173	127	127	310	340	360	127	127	155	240	264
II			253								
III								210			
IV							316				
V									404		

Abbreviations: *T. spiralis* (Ts); *T. nativa* (Tna); *T. britovi* (Tb); *T. pseudospiralis* (Tps) of Palearctic (Pa), Nearctic (Ne) and Australian (Au) regions; *T. murrelli* (Tm); *Trichinella* T6 (T6); *T. nelsoni* (Tne); *T. papuae* (Tpa); and *T. zimbabwensis* (Tz).

[a]*Trichinella* T8 and *Trichinella* T9 genotypes show the same PCR pattern as *T. britovi.*

Primer pair V: 5′-CAATTGAAAACCGCTTAGCGTGTTT-3′
5′-TGATCTGAGGTCGACATTTCC-3′

Each primer is diluted at 100 pmol/µL in sterile H_2O.

Multiplex primer set concentration: combine the same volume of each primer; final concentration: 10 pmol/µL of each primer (*see* **Note 2**).

2.5.3.2. PCR-RFLP, 43 kDa

Ts43CAF: 5′-ATGCGAATATACATTTTTCTTA-3′
Ts43CAR: 5′-TTAGCTGTATGGGCAAGG-3′

Each primer is diluted at 100 pmol/µL in sterile H_2O.

2.6. Preparation and Amplification of Anisakis *and* Pseudoterranova *Larva DNA*

1. Holmes-Bonner solution: 7 *M* urea, 100 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 350 m*M* NaCl, 2% SDS. For 50 mL of solution, combine 21.02 g of urea, 5 mL of 20% SDS, 3.5 mL of 5 *M* NaCl, 1 mL 0.5 *M* EDTA, and 5 mL 1 *M* Tris-HCl, pH 8.0.
2. TE buffer: 10 m*M* Tris-HCl, pH 8.0 (10 mL/L), 1 m*M* EDTA, pH 8.0 (2 mL/L).
3. TE containing RNase: dissolve 2 mg of crude RNase I in 2 mL of TE.
4. TE containing sodium acetate (NaAc): dilute 1 volume of 3 *M* NaAc, pH 5.2, in 10 vol of TE.
5. *Taq* DNA polymerase (Amplitaq Gold, Applied Biosystems, CA) 5 U/µL (*see* **Note 3**).
6. Amplitaq Gold 10X buffer containing 25 m*M* $MgCl_2$.
7. Pestles for 1.5 mL tube.
8. Phenol-chloroform-isoamyl alcohol (50:49:1).
9. Chloroform.

10. Anhydrous ethyl alcohol.
11. dNTP solution: final concentration of 2.5 m*M* of each dNTP (Applied Biosystems).
12. Restriction endonucleases: HhaI, HinfI, MaeI, and TaqI with 10X restriction buffers (New England BioLabs, Beverly, MA).
13. Dry bath.
14. PCR device: Perkin-Elmer 2400 (Perkin-Elmer Corp., Norwalk, CT), Perkin-Elmer 9600, MJ Minicycler (MJ Research inc., Watertown, MA) (*see* **Note 4**).
15. TBE buffer: Tris base (10.8 g/L), boric acid (5.5 g/L), 0.5 *M* EDTA (4 mL/L); pH 8.0.
16. TAE buffer: Tris base (4.85 g/L), glacial acetic acid (1.15 mL/L), 0.5 *M* EDTA (2 mL/L); pH 8.0.
17. 0.5 M EDTA solution: 18.6 g/70mL $Na_2EDTA2H_2O$, pH 8.0, with 10 *N* NaOH, H_2O to 100 mL.
18. Agarose: molecular biology standard grade (Fisher, Cincinnati, OH).

2.7. Preparation and Amplification of Trichinella *Larva DNA*

1. PBS washing buffer: 137 m*M* NaCl (8 g/L), 7 m*M* K_2HPO_4 (1.21 g/L), KH_2PO_4 (0.34 g/L).
2. Sterile Tris-HCl buffer: 1 m*M* Tris-HCl, pH 7.6.
3. Proteinase K: 20 mg/mL in sterile H_2O; store 0.5-mL aliquots at –20°C.
4. Taq DNA polymerase: 5 U/µL of Ex Taq™ from Takara (Otsu, Shiga, Japan) (*see* **Note 3**).
5. 10X Ex Taq™ buffer containing 20 m *M* $MgCl_2$.
6. dNTPs solution: final concentration of 2.5 m*M* of each dNTP (Takara).
7. Restriction endonucleases: SspI and DdeI with 10X restriction buffers (New England BioLabs).
8. Primer mix: 1 µL of multiplex primer set. Store in aliquots of 200 µL at –20°C.
9. Mineral oil: sterile, PCR grade.
10. Dry bath.
11. PCR device: Perkin-Elmer 2400 (Perkin-Elmer Corp.), Perkin-Elmer 9600, MJ Minicycler (MJ Research) (*see* **Note 4**).
12. TBE buffer: Tris base (10.8 g/L), boric acid (5.5 g/L), 0.5 *M* EDTA (4 mL/L), pH 8.0.
13. TAE buffer: Tris base (4.85 g/L), glacial acetic acid (1.15 mL/L), 0.5 *M* EDTA (2 mL/L), pH 8.0.
14. 0.5 *M* EDTA solution: 18.6 g/70 mL $Na_2EDTA{\cdot}2H_2O$, pH 8.0, with 10 *N* NaOH, H_2O to 100 mL.
15. Agarose: molecular biology standard grade (Fisher).

3. Methods

3.1. Isolation of Anisakis *spp. and* Pseudoterranova *spp. Larvae From Fish*

1. Anisakid larvae are relatively large (20–50 mm in length, 0.3–1.22 mm in width) and can be seen with the naked eye in the body cavity, muscle, or fillet of fish and

cephalopods. L_3 may still be in their cuticle (*see* **Note 5**), coiled, or partially embedded in the surface of the liver or gonad.
2. L_3 can be easily collected from the celomatic cavity using flat-nose pliers.
3. To locate larvae in the muscle, it is necessary to perform candling (i.e., placing the muscle on a lit plexiglass surface and collecting the larvae with the help of a scalpel and pliers).
4. Larvae can be stored either frozen (at –20°C; if they are to be stored for more than 3 mo, they should be frozen at –80°C) or in 70% ethyl alcohol.

3.2. Isolation of Trichinella *spp. Larvae From Muscles (*see *Notes 6–8)*

1. Preparation of the digestion fluid: The ratio of muscle (w) and digestion fluid (v) should be 1:20 to 1:40. Using a blender, dissolve pepsin in a small amount of tap water (*see* **Subheading 2.4, step 18**). Add additional tap water until reaching the final volume, then add 1% HCl (final concentration). The digestion fluid should be maintained at around 37 to 45°C for all steps.
2. Cut the muscle sample into small pieces (1–3 g), removing all nonmuscle tissue (tendons, fat, etc.). The most infected muscle tissue is that near the muscle insertion. Place the muscle sample and a small amount of digestion fluid in a blender and blend for 20 to 30 s. Add additional digestion fluid and blend again for about 10 s. Place the fluid in a beaker containing a magnet. To collect residual fluid from the blender, add additional digestion fluid and blend. Place residual fluid in the same beaker.
3. Place the beaker on the magnetic stirrer in the incubator at 37 to 45°C and stir for 20 min. Switch off the magnetic stirrer, collect 4 to 5 mL of digestion fluid from the bottom of the beaker, place the fluid in a Petri dish, and observe under a dissection microscope. If the larvae are free of muscle debris and are out of the capsule, stop the digestion. If larvae are still in the muscle and/or in the capsule, continue the digestion for another 5 to 10 min.
4. Allow the digestion fluid to sediment for 10 to 15 min, according to the height of the beaker (about 1 min for each centimeter of height). Remove the supernatant by placing the suction pump at about 2 cm from the bottom of the beaker, being careful not to remove the sediment, which contains the larvae. Add PBS (37–45°C) (same quantity as supernatant removed) and then allow to sediment. Remove the supernatant and place the sediment in 50-mL conical vials (5 mL for each vial). Add PBS (37–45°C) and allow to sediment. Repeat this procedure until the supernatant is fairly transparent (i.e., you should be able to read newspaper text through the glass). Remove the supernatant and place the sediment in a Petri dish and place under a dissection microscope.
5. If the larvae are dead (C-shaped or comma-shaped) (*see* **Note 6**), the following procedures should be carried out as rapidly as possible to avoid DNA destruction: Collect larvae with a 5-µL pipet and place them in a Petri dish containing cold sterile H_2O. Then collect the single larvae with 5 µL of the cold sterile H_2O and place each of them in a separate 0.5-mL conical vial. Freeze at –30°C or, if the larvae need to be shipped, store them in absolute ethyl alcohol at 4°C (the latter method

allows for shipping without dry ice, although the larvae must be rehydrated through a graded alcohol series before molecular identification) or in 0.5% merthiolate solution.

3.3. Preparation of Crude DNA From Single Larvae of Anisakis spp. and Pseudoterranova spp.

1. A 1.5-mL plastic tube containing the larva should be repeatedly frozen (in liquid nitrogen) and thawed (at room temperature).
2. Pulverize the frozen larva using a sterile pestle.
3. Add 100 µL of Holmes-Bonner solution and continue to pestle until achieving complete homogenization.
4. Add 100 µL of phenol-chloroform-isoamyl alcohol (50:49:1) and stir at room temperature for 10 min.
5. Add 100 µL of TE containing NaAc.
6. Centrifuge at 12,000*g* at 4°C for 10 min.
7. Transfer the supernatant to a new sterile 1.5-mL tube.
8. Add 100 µL of chloroform and centrifuge at 12,000*g* at 4°C for 5 min; then transfer the supernatant to a new 1.5-mL sterile tube.
9. Repeat **step 7**.
10. Add 200 µL of absolute ethanol; store at –20°C for 1 h.
11. Centrifuge at 12,000*g* for 20 min, rinse the pellet with 200 µL of 70% ethanol, discharge the ethanol, and then dry using a vacuum pump.
12. Resuspend the pellet in 100 µL of TE containing RNase and store at room temperature for 30 min.
13. Run 5 µL on agarose gel to test.

3.4. Preparation of Crude DNA From Single Trichinella Larvae

1. Wash single larvae 10 times in PBS. Place each larva, with 5 µL of PBS, in a 0.5 mL tube; store at –20°C until use (*see* **Note 9**).
2. Add 2 µL Tris-HCl, pH 7.6.
3. Add one drop of sterile mineral oil.
4. Heat sample at 90°C for 10 min and then cool on ice.
5. Add 3 µL of proteinase K solution (final concentration 100 µg/mL); spin sample.
6. Incubate sample at 48°C for 3 h.
7. Heat sample at 90°C for 10 min and cool on ice.
8. Store sample at –20°C until use.

3.5. PCR Protocol for Larvae of Anisakis spp. and Pseudoterranova spp.

Prepare a PCR mix multiplying each reagent for the number of the individuals to be examined plus two sets for a positive and a negative control:

1. Add sequentially for each sample 5 µL 10X PCR buffer, 5 µL $MgCl_2$, 4 µL dNTPs, 0.5 µL of each primer, 0.3 µL *Taq* DNA polymerase, and sterile H_2O up to 48 µL in a sterile 1.5-mL tube (*see* **Note 10**).

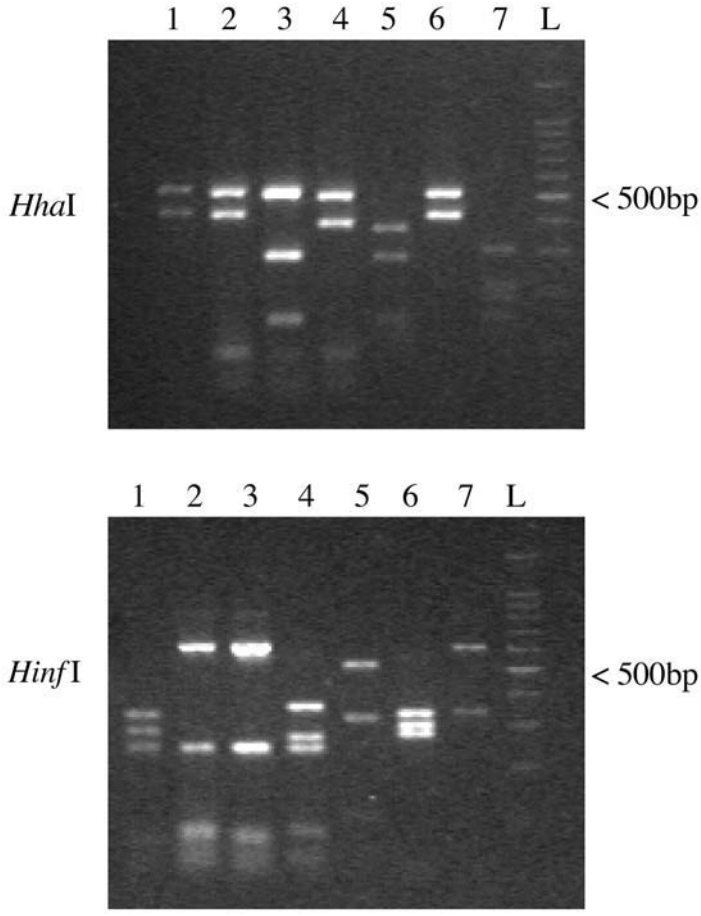

Fig. 1. PCR-RFLP identification **(Subheading 3.7.)** of single larvae of the genus *Anisakis.* Photograph of an ethidium bromide-stained 2% agarose gel under ultraviolet light illumination. Lane: 1, *A. pegreffii;* lane 2, *A. simplex sensu stricto;* lane 3, *A. simplex* C; lane 4, *A. physeteris;* lane 5, *A. schupakovi;* lane 6, *A. ziphidarum;* lane 7, *A. typica.* L (ladder 100) sizes are in basepairs.

2. Add 2 µL of genomic DNA to each 0.2-mL thin-walled tube.
3. Add 48 µL of PCR mix to each 0.2-mL thin-walled tube.
4. Place tubes on ice.
5. PCR cycle: pre-amplification cycle at 95°C for 10 min, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 75 s at 72°C, followed by a final elongation of 7 min at 72°C.
6. Place tubes on ice.
7. Electrophoresis: load 5 µL of each amplification **(Fig. 1)**.

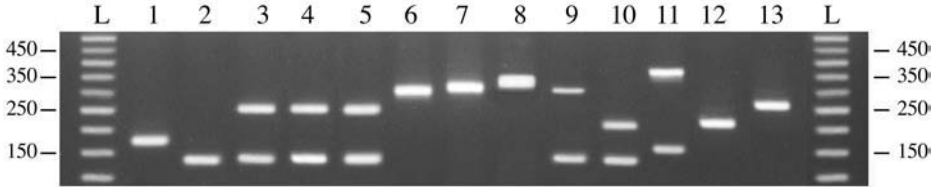

Fig. 2. Multiplex PCR amplification **(Subheading 3.6.)** of single larvae of 13 genotypes of *Trichinella.* Photograph of an ethidium bromide-stained 2.5% agarose gel under ultraviolet light illumination. The samples are as follows: L (ladder 50) sizes are in base pairs; lane 1, *T. spiralis;* lane 2, *T. nativa;* lane 3, *T. britovi;* lane 4, *Trichinella* T8; lane 5, *Trichinella* T9; lane 6, *T. pseudospiralis* (Palearctic isolate); lane 7, *T. pseudospiralis* (Nearctic isolate); lane 8, *T. pseudospiralis* (Tasmanian isolate); lane 9, *T. murrelli;* line 10, *Trichinella* T6; lane 11, *T. nelsoni;* lane 12, *T. papuae;* lane 13, *T. zimbabwensis.*

3.6. Multiplex-PCR Protocol for Trichinella *spp. Larvae*

1. Thaw sample of crude DNA extraction on ice (at this point, each tube should contain 10 µL of the larva preparation).
2. To set up PCR (*see* **Note 9**), add sequentially 5 µL 10X PCR buffer, 4 µL dNTPs, 2 µL set of primers, 0.1 µL *Taq* DNA polymerase (*see* **Note 3**), 4 µL of crude DNA extraction (*see* **Note 11**), and H_2O up to 50 µL in a 0.2 mL thin-walled tube.
3. Place tubes on ice.
4. PCR cycle: pre-amplification cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 58°C for 30 s, and 72°C for 1 min; extension cycle at 72°C for 4 min; place on ice.
5. Hot start at 94°C: Wait until the thermocycler reaches 94°C and then place the tubes on the hot plate.
6. Electrophoresis: use 20 µL of each amplification reaction (*see also* **Table 3; Fig. 2**).

3.7. RFLP Protocol for Larvae of Anisakis *spp. and* Pseudoterranova *spp.*

1. Add sequentially 10 µL of PCR-amplified DNA, 3 µL of distilled water, 0.5 µL of restriction enzyme, 1.5 µL of enzyme buffer, and 0.2 µL of BSA up to a final volume of 15.2 µL.
2. Incubate at 37°C for 90 min (with the exception of the endonuclease *Taq*I, to be incubated at 65°C).
3. Electrophoresis: load all the reaction on a 2% ethidium bromide-stained agarose gel in TBE buffer and run at 10 V/cm (*see also* **Tables 4** and **5; Fig. 1**).

3.8. RFLP Protocol for Identification of Trichinella *T8 and* Trichinella *T9 Genotypes*

1. Thaw sample of crude DNA extraction on ice (at this point, each tube should contain 10 µL of the larva preparation).

Table 4
PCR-RFLP Amplicon Sizes of Principal Bands (in Basepairs) of Species of the Genus *Anisakis*

	Restriction enzymes	
Anisakis species	*Hinf*I	*Hha*I
A. pegreffii	370–300–250	–
A. physeteris	380–290–270	–
A. schupakovi	520–340–120	–
A. typica	620–350	–
A. ziphidarum	370–320–290	–
A. simplex sensu stricto or *A. simplex* C	620–250–80	–
A. simplex sensu stricto	–	550–430
A. simplex C	–	550–300–130

Table 5
PCR-RFLP Amplicon Sizes of Principal Bands (in Basepairs) of Three Species of the *Pseudoterranova decipiens* Complex

	Restriction enzymes	
Pseudoterranova species	*Taq*I	*Mae*I
P. krabbei	200–100	–
P. decipiens s.s. or *P. bulbosa*	300	–
P. decipiens s.s.	–	140–160
P. bulbosa	–	300

2. To set up PCR, add sequentially 5 µL 10X PCR buffer, 4 µL dNTPs, 2 µL set of primers, 0.2 µL *Taq* DNA polymerase, 10 µL of crude DNA extraction, and H_2O up to 50 µL in a 0.2-mL thin-walled tube.
3. Place tubes on ice.
4. PCR cycle: preamplification cycle at 94°C for 5 min, followed by 30 cycles at 98°C for 20 s and 60°C for 15 min; extension cycle at 72°C for 4 min; place on ice.
5. Hot start at 94°C: Wait until the thermocycler reaches 94°C and then place the tubes on the hot plate.
6. Electrophoresis: use 10 µL of the amplification reaction. Select samples showing good single-band amplification for restriction analysis.
7. Restriction analysis: transfer 20 µL of the amplification reaction into a 1.5-mL conical tube; add 5 µL of the respective 10X restriction buffer, 10 units of the selected enzyme, and H_2O up to 50 µL.

Table 6
PCR-RFLP Amplicon Sizes of Principal Bands (in Basepairs) of *Trichinella britovi* Genotypes

	Restriction enzymes	
Trichinella Genotype	*Dde*I	*Ssp*I
T. britovi	700, 680, 560, 520	2100
Trichinella T8	700, 650, 560, 330	2300, 2100
Trichinella T9	700, 650, 560, 520	1500, 1200, 650

8. Incubate at 37°C for 2 h.
9. Transfer on ice; stop the reaction with 5 µL of 0.5 *M* EDTA.
10. Electrophoresis: Load all of the reaction onto the agarose gel (*see also* **Table 6**).

3.9. Electrophoresis Conditions

1. Standard agarose gel: follow standard procedures to prepare 1 to 1.5% agarose gel in TBE or TAE buffer; run at 10 V/cm.
2. High-resolution agarose gel: to have an adequate resolution of *T. pseudospiralis* isolates, run the amplification products on 3% metaphor agarose gel at 10 V/cm.

4. Notes

1. Pepsin should be stored in the dark at room temperature (20°C or less, but not below +4°C); avoid exposure to humidity. Pepsin should be no more than 6 mo old.
2. Balancing of primers: The primer-set mix prepared with equimolar concentrations of all oligonucleotides generally provides good results; if results are not optimal, and the presence of *T. murrelli* is suspected, the concentration of the primer set IV can be doubled.
3. If using *Taq* DNA polymerases other than those suggested, it is important to perform specific tests to evaluate their effectiveness.
4. For the automatic amplification of DNA, other thermocyclers could be used, but it could be necessary to first determine their efficiency in amplifying DNA.
5. When collecting the larvae, it is strongly suggested to remove their cuticle simply using a small brush and a forceps.
6. Collection of worms from frozen samples: Larvae from frozen muscle samples should be collected according to the protocol of **Subheading 3.1**; to avoid DNA destruction, however, all procedures after digestion should be carried out very quickly and sedimentation should be carried out on ice.
7. Collection of worms from formalin-fixed samples: Formalin-fixed host tissues cannot be used to collect larvae because formalin destroys the DNA.
8. Collection of worms from ethyl alcohol-fixed muscle samples: Worms can be collected as follows: using a scalpel, cut the muscle sample into grain-sized pieces.

Crush the pieces between two trichinoscope slides (8 mm thick) and check for the presence of larvae among the muscle fibers under a dissection microscope at ×20–40. Mark the position of the larva on the bottom slide. Gently remove the upper slide and cut away the muscle surrounding the larva using a scalpel and one or two small needles (if the larva is encapsulated, remove it from the capsule with the scalpel and needles) under a dissection microscope at ×20–40. Place the larva in a 0.5-mL conical vial with 400 µL of cold H_2O. Wash the larva 3 to 4 times with cold H_2O, then store in 5 µL of H_2O at –20°C.

9. Pooled larvae: The protocol for the preparation of crude DNA of a single larva can also be used for pooled larvae by simply increasing the quantity of solution (for example, for 10 larvae, it is sufficient to double the quantity of solution used for a single larva). When using pooled larvae, it should be kept in mind that the presence of larvae belonging to two or more genotypes (mixed infections) could affect the interpretation of the results.
10. Precautions for PCR: Use tip with barrier and gloves.
11. Pipetting the sample for PCR amplification: A sufficient quantity of DNA is critical for a successful amplification; thus, be sure to pipet the sample at the bottom of the tube and to avoid collecting the mineral oil. To remove the oil from the tube, it is best to use pipetting, in that chloroform or other organic solutions could remove part of the DNA sample.

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IV

Parallel Studies to the Analysis of Food-Borne Pathogens

18

Approaches to Developing Quantitative Risk Assessment Models

Enda J. Cummins

Summary

Risk assessment has become increasingly important as a tool in assessing risks from food-borne pathogens. There are many methodologies that may be used when constructing a risk assessment model, and there are many methodological issues, which are left to the risk assessor's choice. A number of different approaches to developing a risk assessment model are detailed in this chapter, including the use of deterministic and stochastic variables. A step-by-step approach to creating a quantitative risk assessment model is given. The approach is illustrated with a worked example focusing on potential human exposure to bovine spongiform encephalopathy via meat products. A general framework and guiding principles for constructing a quantitative risk assessment are given, in addition to an outline of the use of Monte Carlo simulation, event tree/fault tree analysis, and sensitivity analysis. This chapter presents a procedure that will enable readers to familiarize themselves with the risk assessment process and equip them with the procedures necessary to construct risk assessment models for food-borne pathogens.

Key Words: Risk assessment; BSE; Monte Carlo; simulation.

1. Introduction

Risk assessment can be defined as the qualitative or quantitative estimation of the likelihood of the occurrence of an adverse effect ***(1)***. Risk assessment has increasingly been used as a tool in microbiology for assessing risks posed by various food-borne pathogens ***(2–5)***. In certain circumstances there is a regulatory requirement for a risk assessment to be performed. International trade agreements such as the General Agreement on Tariffs and Trade (GATT) ***(6)*** and the North American Free Trade Agreement (NAFTA) ***(7)*** have requirements for risk assessment in their sanitary and phytosanitary (S&P) clauses, highlighting the growing need for risk assessment methodologies in trade situations. In addition to having a regulatory role, risk assessment techniques are beneficial in

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identifying areas in which risks can be reduced and also in comparing the costs and benefits of alternative control strategies. A risk assessment can be applied not only to predict what could happen, but also to quantify how likely or unlikely are the consequences. There are many methodologies that can be used when creating a risk assessment ***(8)***; these largely depend on its aims and scope, the available data, and the end user. This chapter details a step-by-step approach to creating a quantitative risk assessment model. A quantitative risk assessment is one in which numerical values are used to define risk. Inputs to the model are defined by a probability of occurrence; hence some quantitative data are required for this type of risk assessment.

2. Materials

There are many software packages that have been developed for the purposes of carrying out quantitative risk assessments, probably the two most common being the Excel add-on packages, @Risk (Palisade, NY) and Crystal Ball (Decisioneering Inc., Denver, CO). The minimum platform required is IBM PC-compatible Pentium-equivalent or higher, 16 MB RAM, Windows 95/98, NT 4.0, Windows 2000, Windows XP. The recommended platform is 32 MB RAM or greater. The spreadsheet can be Windows Excel 97, Excel 2000, or Excel XP. The software used for carrying out the sample risk assessment in this chapter is @Risk (version 4.05).

3. Method

A number of broad steps to be included in a risk assessment are outlined by the Codex Alimentarius Commission (CAC) ***(9)***, these are: hazard identification, exposure assessment, hazard characterization, and risk characterization (*see* **Note 1**). There are many methodological issues left to the assessor's choice when constructing a risk assessment model; however, it is useful to have a general framework from which to work ***(10,11)***. To assist in this role, a number of substeps have been included within the broad framework outlined by CAC and are detailed here.

1. Hazard identification
   - Define the scope: outline the hazard to be assessed and the focus of the risk assessment; this includes brief information on size and nature of the risk assessment. Possible information would include whether the risk assessment is a production model, a risk ranking, a dose–response, or an exposure model (*see* **Note 2**).
2. Exposure assessment
   - Develop scenarios using event tree/fault tree analysis (*see* **Note 3**).
   - Decide on a modeling approach.
   - Collect data.
   - Build a probabilistic model to account for uncertainty/variability (*see* **Note 4**).
   - Validate the model.

3. Hazard characterization
   - Estimate the dose–response relationship between the hazard and the host.
4. Risk characterization
   - Run Monte Carlo model.
   - Calculate the likelihood and severity of the hazard.
   - Perform sensitivity analysis.

The use of this template in creating a risk assessment model has previously been shown ***(12,13)***. The methodology is illustrated in this chapter, including two types of analysis (event tree and fault tree analysis), with an example looking at the risks associated with bovine spongiform encephalopathy (BSE) and the potential infection of humans via the food chain. Each of the steps outlined in the framework above is taken in turn.

3.1. Hazard Identification

The hazard of interest is BSE. The focus of the risk assessment given here is very specific, focusing only on potential exposure of humans to BSE infectivity via meat products.

3.2. Exposure Assessment: Developing Scenarios

This stage assesses the extent of exposure to a hazard and an analysis of the pathways through which a hazard can result in harm (*see* **Note 5**). Common techniques employed in carrying out this stage include fault tree analysis (FTA) and event tree analysis (ETA).

3.2.1. Fault Tree Analysis

This is a graphic technique that provides a systematic description of the combinations of possible failures in a system, which can result in an undesirable outcome. This method should combine all failure events that could trigger an undesirable result. Take the risk assessment example as detailed in **Fig. 1**, where the most serious outcome (or top event) is human exposure to BSE infectivity via a meat product. The fault tree is constructed by relating the sequences of events that, individually or in combination could lead to the top event. This may be illustrated by considering the probability of infectivity in a meat product and constructing a tree with AND and OR logic gates. Fault trees are constructed using inductive or backward logic. In other words, the process starts with a hypothesized system or subsystem failure, and works backward to identify which combinations of component failures could give rise to that top event. The tree is constructed by deducing in turn the preconditions for the top event and then successively for the next levels of events, until the basic causes are identified. By ascribing probabilities to each event, the probability of a top event can be calculated. This requires knowledge of probable failure rates. At

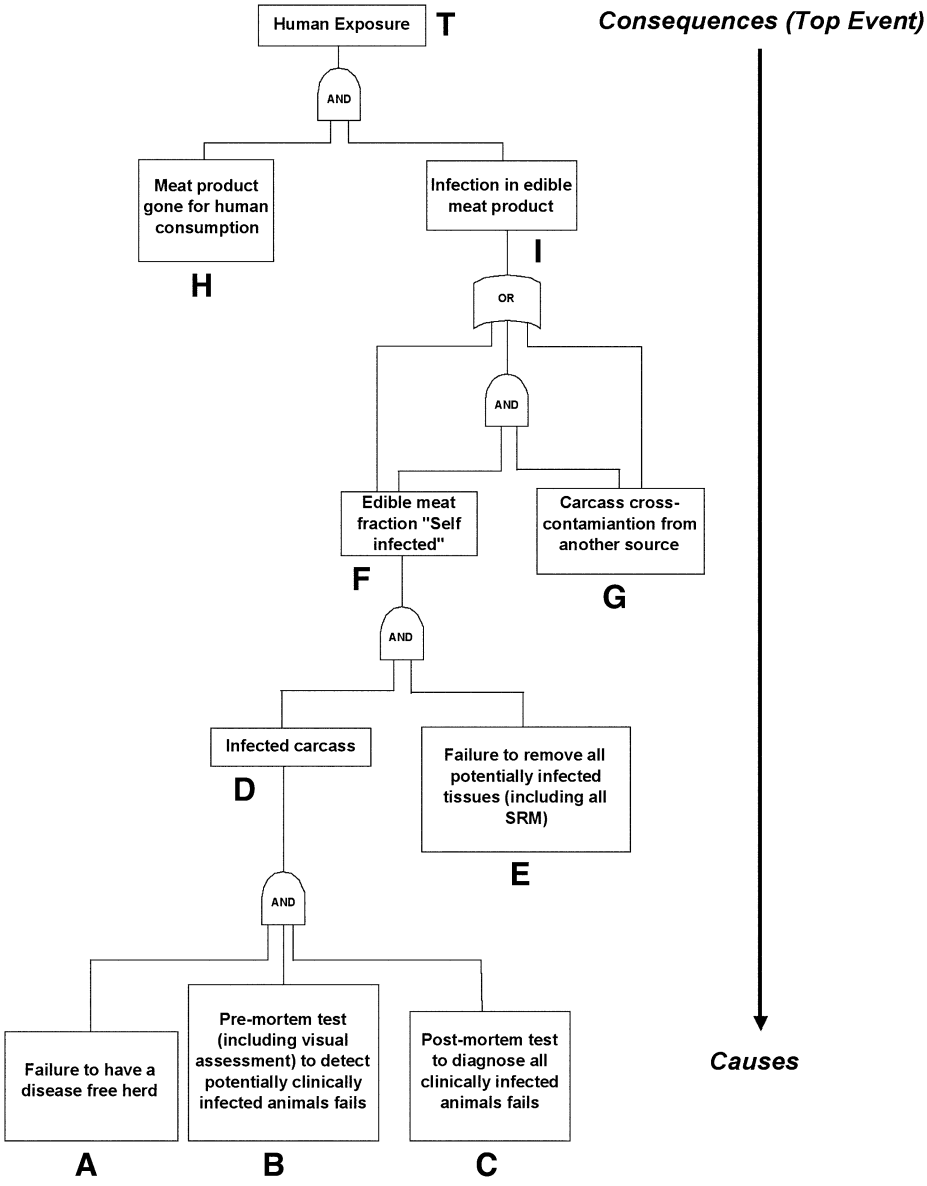

Fig. 1. Fault tree for human exposure to BSE via meat products.

an OR gate the probabilities must be added to give the probability of the next event, whereas at an AND gate, the probabilities are multiplied. FTA provides a powerful technique for identifying the failures that have the greatest influence on bringing about the top event.

Figure 1 shows a simplified fault tree for a system looking at human exposure to BSE infectivity via a meat product (includes all edible meat). The fault tree implies that the top event (i.e., human exposure) occurs if, and only if, both subsystems H *and* I fail. Subsystem I fails if subsystem F *or* G fails or F *and* G fail simultaneously; subsystem F fails if subsystems D *and* E fail; while subsystem D fails if systems A *and* B *and* C fail. Assuming that all components are independent, the relationships and probability (P) of the top event (T) occurring are:

$$P(T) = P(H)\,P(I)$$

$$P(I) = P(F) + P(G) + (P(F)\,P(G))$$

$$P(F) = P(D)\,P(E)$$

$$P(D) = P(A)\,P(B)\,P(C)$$

3.2.2. Event Tree Analysis

Event tree analysis is based on binary logic, in which an event either has or has not happened or a component has or has not failed. It works forward from an initiating event (frequency represented by *i*), by identifying all possible combinations of subsequent events (i.e., successes or failures of particular components or subsystems) and determines which sequences of events could cause failure of a system as a whole ***(14)***. The consequences of the event are followed through a series of possible paths. Each path is assigned a probability of occurrence and the probability of the various possible outcomes can be calculated.

Using the same example as used in the FTA, an event tree was constructed to calculate the risk of BSE infectivity reaching a consumer. An animal that is brought to a slaughter plant will have a certain probability, P(A), that it is infected and a certain probability that it is not infected [1 – P(A) or $P(\bar{A})$]. (Failure of an event in a given sequence is indicated by a $^{-}$ over the failed event.) Following the subsequent events, the animal is subjected to a premortem test for BSE (this may be as simple as a visual assessment), which will yield a positive [with probability P(B)] or negative result [with probability $P(\bar{B})$]. If the result is positive, and hence the animal is a suspect BSE case, the animal is slaughtered and sent for a postmortem test; the meat will not be sent for human consumption. If the animal passes the premortem test, it is subsequently tested using a highly sensitive postmortem test. If the animal tests positive for the disease, P(C), it is then sent for destruction. In the European Union there is a statutory requirement for the removal (from animals destined for human consumption)

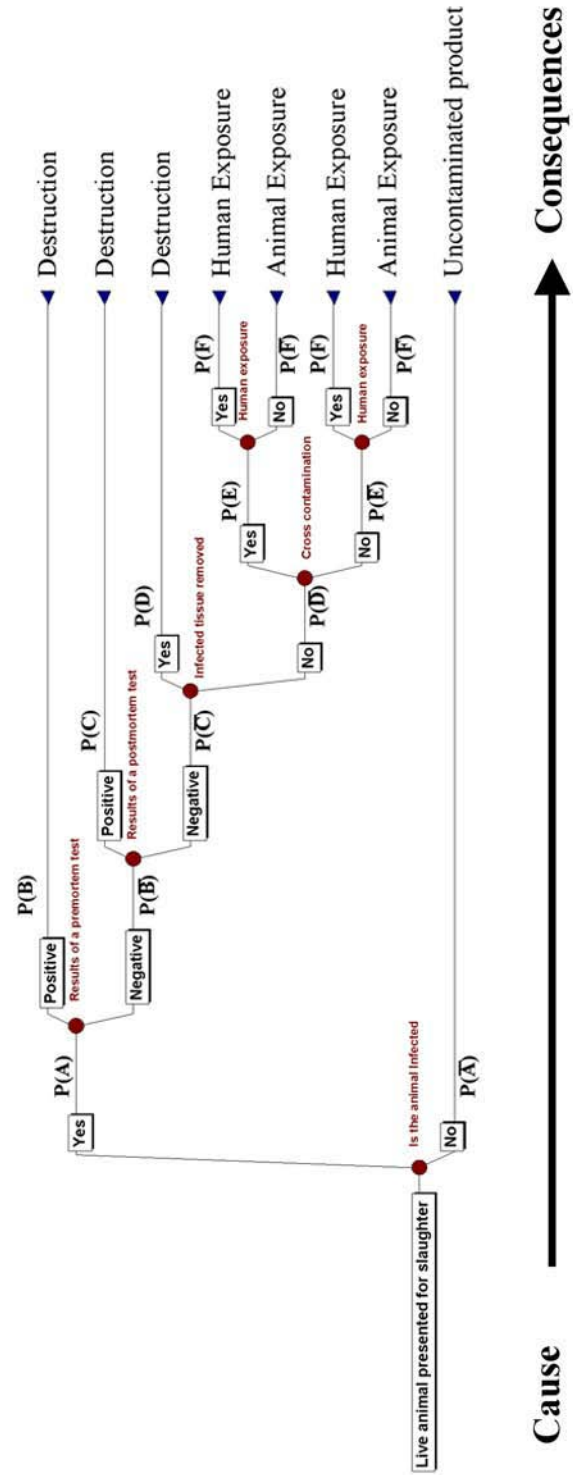

Fig. 2. Event tree of potential human exposure to BSE via meat products.

of animal tissues that could potentially contain high concentrations of the BSE causative agent (such tissues are termed specified risk material [SRM]). There is a probability, P(E), that all the infectivity is removed, and a corresponding probability, P($\overline{E}$), that a portion of infectivity remains with the carcass. There is also a probability, P(G), that there is crosscontamination from another animal and a probability, P(H), that the meat product goes for human consumption. Finally the event tree leads to the final outcome, human exposure with a probability P(T).

Figure 2 illustrates an event tree representing an initiating event (animal presented for slaughter) and the subsequent response of subsystems (A, B, C, D, and E). For each system the upper branch represents success and the lower branch represents failure. Starting at the initiating event, there are seven possible scenarios, which can occur depending on success or failure of each of the subsequent events.

The frequency of each sequence *(S)* can be quantified based on the probability (P) allocated to each success or failure branch. For example the frequency *(f)* of the sequence AB can be quantified as:

$$f(S) = f(i)\ \mathrm{P(A)}\ \mathrm{P(B)}$$

or the probability of any sequence can be quantified by the product of the probabilities that make up that sequence e.g. the probability that the sequence A $\overline{B}$ C D will occur is given by: $\mathrm{P(A)} \times \mathrm{P}(\overline{B}) \times \mathrm{P(C)} \times \mathrm{P(D)}$.

3.2.3. Modeling Approaches

There are two types of quantitative risk assessment: (1) deterministic (or single point estimate) and (2) stochastic (or probabilistic).

3.2.3.1. Deterministic Risk Assessment

In a deterministic risk assessment, point estimates are used for each input into the model, i.e., point estimates are used for each of the probabilities in **Figs. 1** and **2**. The value used should be a conservative best estimate of the input parameter. The output of a deterministic risk assessment will also be a point risk estimate, e.g., the number of *v*CJD cases per year. A numerical estimate of risk makes it more easily comparable with everyday risks. Deterministic risk assessment is limited, however, as it does not take into account the uncertainty inherent in the system being assessed.

3.2.3.2. Stochastic Risk Assessment

Stochastic risk assessments apply probability distributions to take account of the uncertainty around model input parameters (unlike the point estimates used in the deterministic modeling approach). Because of its use of probabilities it is also more commonly known as probabilistic risk assessment (PRA).

By using probability distributions to represent uncertainties around the model input parameters, the resultant output of a PRA is also a probability distribution that should identify and quantify the risks for all possible scenarios. As pointed out by Kaplan and Garrick ***(15)***, the PRA should answer three basic questions: (1) What can go wrong? (2) How likely is it to go wrong? (3) What will be the consequences if it does go wrong?

In order to answer these questions, all possible risk scenarios need to be detailed and quantified in terms of the likelihood or probability of the occurrence of each risk, in addition to an assessment of the consequences associated with the occurrence of a risk (e.g., number of illnesses per year).

3.2.3.3. Collecting Data and Accounting for Data Uncertainty

Data gathering is probably the most difficult stage involved in creating a quantitative risk assessment model. A review of all scientific literature is required to estimate the probabilities associated with each event (and their uncertainty, in the case of a PRA). The data can be based on experimental results, expert opinion, a best-guess estimate, or a combination of two or more of these data sources. Techniques in creating distributions from available data are given in the literature ***(16,17)***; it is recommended that such a reference be consulted to ensure correct use of probability distributions (*see* **Note 6**). For transparency the source of the data should always be indicated. The inputs and sources used in the model in the fault tree analysis detailed in **Fig. 1** are shown in **Table 1**.

P(A) is the probability that an animal is infected and can be represented by the prevalence in a herd. The Office International des Épizooties (OIE) ***(18)*** indicated that for a country to be designated as having a low BSE incidence rate (as calculated over a 12-mo period), the number of indigenous BSE cases must be greater than or equal to one case per million and less than or equal to one hundred cases per million within the animal population older than 24 mo of age. To model this scenario a uniform distribution was used with a minimum of 1 and a maximum of 100, i.e., the number of BSE cases (N) can be anywhere between 1 and 100 with each scenario having equal probability. The uniform distribution is used where there are very few or no data available ***(16)***. This distribution was incorporated into a beta distribution, which is used to model the disease prevalence.

The probability P(B) represents the probability that an animal will turn out to be positive following a premortem BSE test. The premortem test can be as simple as a visual assessment; more recently developed tests analyze blood or urine for evidence of the disease. Depending on the stage of progression of the disease in the animal a premortem test (such as visual assessment) is unlikely to be successful in diagnosing BSE cases. There are few data available on the failure rate of the premortem test; hence, as a worst-case estimate, the model

Table 1
Model Inputs As Used in Fault Tree Analysis

Variable	Description	Units	Model/distribution	Source
S	Unit herd size	animals	Fixed value (1,000,000)	
N	Number of BSE cases per S	animals	Uniform(1100)	***18***
P(A)	Disease prevalence		Beta (N+1, S-N+1)	***16***
P(B)	Premortem detection failure rate		Uniform (0.9, 0.95)	Worst-case value
P(C)	Postmortem detection failure rate		Uniform (0.05, 0.1)	***19***
P(D)	Probability of infected carcass at slaughter		$P(A) \times P(B) \times P(C)$	Calculation
P(E)	Infectivity removal failure rate		Uniform (0.9, 1)	Worst-case value
P(G)	Probability of crosscontamination to another carcass		Fixed value (1.0e6)	Worst-case value
P(F)	Probability of self-infection		$P(D) \times P(E)$-$[P(D) \times P(E) \times P(G)]$	Calculation
P(H)	Fraction of product consumed by humans		Fixed value (100%)	Worst-case value
P(I)	Probability of infected product		$P(F)+ [P(D) \times P(E) \times P(G)] + [P(F) \times P(D) \times P(E) \times P(G]$	Calculation
I	Fraction of infectivity remaining following processing		Uniform (0.01,0.05)	***22***
I_{sc}	Fraction of infectivity on self-infected carcass		Triangular (0,1)	
I_{cc}	Fraction of infectivity distributed on cross contaminated carcass		$1 - I_{sc}$	Calculation
I_{ca}	Infectivity in clinical animal	ID_{50}	Log-normal (9.305, 1.0329)	***23***
S_b	Species barrier		Triangular (1,1000,10000)	***23***
T	Top event (probability of societal human exposure)		$P(I) \times P(H)$	Calculation
E	Human exposure	ID_{50}	$I_{ca} \times I/S_b$	Calculation

incorporates a uniform distribution with a minimum value of 90% and a maximum value of 95%, i.e., there is a 90 to 95% chance that a BSE-infected animal will not be detected at this stage. The probability P(C) represents the probability that an animal will not be detected following a postmortem test (such as the Enfer chemiluminescent immunoassay test, employed in many cattle slaughtering factories). The sensitivity and specificity of these tests are very high—results show that some of these tests give 100% sensitivity and 100% specificity, i.e., if the animal is infected the postmortem test will detect it with a 100% success rate ***(19,20)***. P(C) is modeled with a triangular distribution with 0% as the minimum and most likely and 10% as the maximum value. The triangular density distribution is used as a modeling tool when the range and the most likely value within that range can be estimated. The triangular distribution offers considerable flexibility in its shape while accounting for the uncertainty within the given range ***(16)*** and hence is used in this study to take account of the large uncertainly surrounding the true value.

P(D), the probability of an infected carcass, is obtained by the product of P(A), P(B), and P(C). P(E) is the probability that there is a failure to remove all infectivity in an infected animal. Indications are that not all infectivity will be removed if an animal is infected; the probability of this was modeled using a uniform distribution with a minimum of 90% and a maximum of 100%, i.e., 90 to 100% of the time some infectivity will remain with the animal carcass. The probability that an infected animal retains some infectivity and hence contaminates the edible meat, P(F), is thus calculated. P(G) represents the probability of cross-contamination, e.g., via aerosols from an infected carcass. Research has indicated that crosscontamination from aerosols is unlikely ***(21)***; however a fixed probability of one in a million was used in the model as a 'worst-case' scenario. The probability of infection in a meat product P(I) can thus be calculated. P(H) is the probability that a meat product goes for human consumption; this was pessimistically modeled using a single point estimate of 100%, i.e., all products go for human consumption. Multiplying P(I) and P(H) results in the calculation of the probability of human exposure, P(T) (i.e., the top event). This answers two of the questions posed by Kaplan and Garrick ***(15)***, namely, what can go wrong and how likely is it to go wrong. The model still needs to address the issue of the consequence of these events, i.e., if a human is exposed to infectivity, at what level does exposure occur and is this level sufficient to cause disease?

Additional quantitative terms are used in the model in order to determine potential levels of human exposure. *"I"* represents the fraction of infectivity remaining following processing. With the removal of all potentially highly infected tissues (termed SRM), between 95 and 99% of the infectivity in an animal is estimated to be removed ***(22)***. Hence *I* is modeled using a uniform distribution with a minimum of 1% and a maximum of 5% of the infectivity remain-

ing. If a carcass crosscontaminates another carcass it will distribute some of its infectivity to that carcass; the fraction of infectivity that remains with the carcass *(I_{sc})* is modeled using a triangular distribution with the minimum 0, most likely and maximum value of *I,* i.e., this distribution takes into account the fact that the carcass is more likely to retain the infectivity rather than crosscontaminate another carcass. The fraction of infectivity distributed to a carcass *(I_{cc})* that is crosscontaminated is thus given as $1 - I_{sc}$. Infectivity in a clinical animal was modeled using a log-normal distribution with a mean of 9.305 and a standard deviation of 1.0329. This distribution is based on the distribution of infectivity in animal tissues as indicated by the European Commission ***(23)***. The sum of all the tissue infectivity was combined and modeled as a single distribution. Research suggests that infectivity is confined mainly to the end of the incubation period, with a peak when clinical signs appear ***(24,25)***. This would suggest that subclinical animals have substantially less infectivity in their tissues than animals exhibiting clinical symptoms. This suggests that a factor could be introduced to BSE risk assessments dealing with undiagnosed cases to reflect the lower infectiousness associated with earlier stages of the disease. As a "worst-case" assumption, this subclinical factor was set to a value of one, i.e., that all infected animals carry the full clinical infective load.

The species barrier *(S_b)* is a term used to describe the natural resistance to transmission when a particular species is exposed to a transmissible degenerative encephalopathy (TDE) of another species. There is considerable uncertainty about this term and this is accounted for in the wide distributions recommended by the European Commission's Scientific Steering Committee (SSC) ***(23)***. The distribution used is an adjusted triangular density distribution on an arithmetic scale with a mode value of 10^3 and within the range 10^0 to 10^4. Model data should always be validated where possible.

3.3. Hazard Characterization

This model considers the infectious dose associated with infectious material as measured in ID_{50} units, where the ID_{50} value represents the level of infectivity required to induce disease in 50% of the exposed population. This is used as the dose-response relationship. No further attempt is used to quantify the dose-response relationship. Exposure is calculated in terms of ID_{50}.

3.4. Risk Characterization

3.4.1. Run the Model

The Monte Carlo model was run with 100,000 iterations of the model (*see* **Note 7**) using the Excel add-on package @Risk (version 4.05). The number of iterations should be sufficiently large that further iterations of the model will not significantly change the mean value of the model output(s).

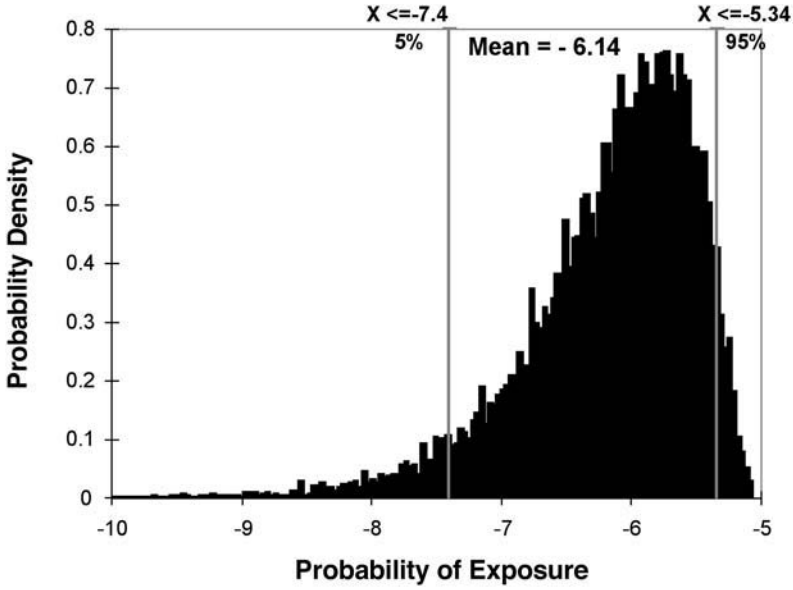

Fig. 3. Simulated probability of human exposure to BSE.

3.4.2. Calculate Likelihood and Severity of Hazard

The model outputs in the BSE model were identified as the probability of exposure to the BSE agent and the level of exposure to the BSE agent. **Figure 3** gives a distribution (with percentiles) for the probability of human exposure to BSE on a log scale. The mean value is –6.14, indicating that there is less than 1 in a million chance that the top event (i.e., human exposure) would occur given the underlying assumptions of the model. **Figure 4** gives a distribution for the level of infectivity, including percentile values. The mean value is –1.19 log ID_{50}, indicating that if the top event does occur the mean societal exposure to infectivity would be –1.19 log ID_{50}. The resulting distribution reflects the uncertainty about the input parameters.

3.4.3. Sensitivity Analysis

A sensitivity analysis provides a measure of the sensitivity of the risk calculations to variations in input factors (*see* **Note 8**). A sensitivity analysis (measured by the rank correlation) was performed for each simulation. The correlation values can vary from –1 to +1. Negative correlation values indicate varying degrees of inverse correlation, whereas positive correlation values indicate varying degrees of direct correlation. **Figure 5** shows the sensitivity analysis performed for the probability of human exposure to BSE infectivity. The fail-

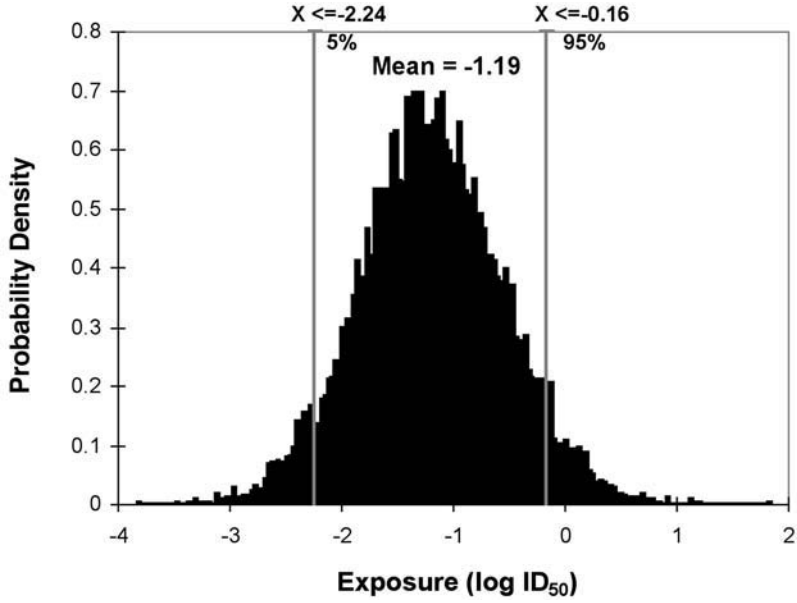

Fig. 4. Simulated level of human exposure to BSE infectivity.

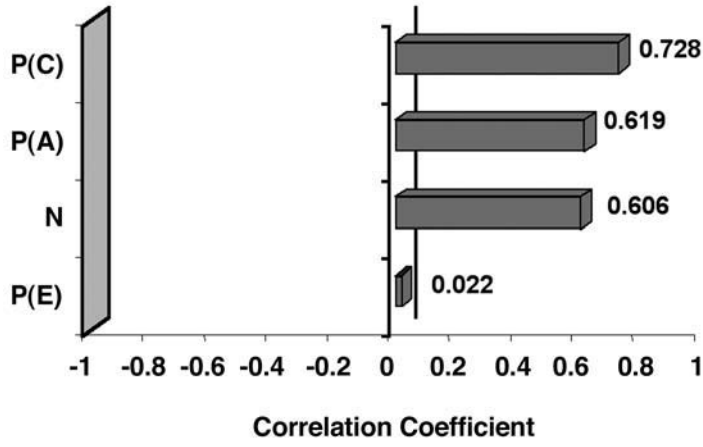

Fig. 5. Sensitivity analysis for the probability of human exposure.

ure probability having the greatest effect was postmortem failure rate P(C). This highlights the importance of a reliable postmortem test in reducing the probability of human exposure, a fact of interest to risk managers. **Figure 6** shows the sensitivity analysis performed for the level of human societal exposure to

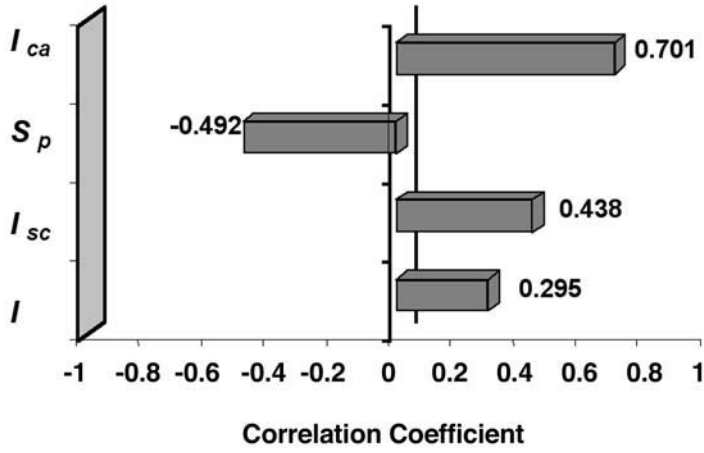

Fig. 6. Sensitivity analysis for the level of human exposure to BSE.

infectivity. The analysis shows that the level of infectivity in an infected animal (I_{ca}) is the parameter having greatest impact on the risk calculations. The species barrier is also having a significant impact, implying that further research is needed to reduce the uncertainty about these parameters.

4. Notes

1. Risk assessment is a component of risk analysis and should not be done in isolation. A risk assessment should be integrated with the other components of the risk analysis process, namely risk management and risk communication.
2. Decide on the model scope. Using too wide a scope for the top event can result in a large, complex, and unfocused risk assessment model.
3. Use consistent nomenclature for the same events; failure to do so prevents one from finding events that occur in multiple branches of the fault tree.
4. Take precautions to err on the side of safety.
5. Ensure that all outcomes are modeled; failure to do so may result in an oversight of a potential risk pathway.
6. Distribution type, size, and uncertainty will vary depending on available data. A good guide to applying distributions to available data, including expert opinion, is given by Vose ***(16)***. A guide to selecting the correct type of distribution for the available data is also usually given with the risk assessment software.
7. The number of model iterations should be sufficient that further iterations of the model will not affect the output to the required level of accuracy (e.g., up to two decimal points). This may necessitate the running of the model for various iteration values.
8. Sensitivity analysis can be performed automatically by the risk assessment software.
9. "Models should be as simple as possible, but no simpler" *(A. Einstein).*

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A Review of Surveillance Networks of Food-Borne Diseases

Camelia Molnar, Rita Wels, and Catherine C. Adley

Summary

Food-borne diseases include infections caused by bacteria, parasites, and viruses. Each year, millions of persons experience food-borne illness, although only a fraction seek medical care, and an even smaller number submit laboratory specimens. To monitor the impact of these food-borne pathogens on human health, systems giving further information are required; a food-borne disease surveillance program is an essential part of a food safety program. Food-borne surveillance should be able to issue early alerts about contaminated food to which a large population is exposed, report food-borne disease incidents on a regular basis, and use sentinel and specific epidemiological studies as required. This chapter is a short overview of various surveillance networks specializing in food-borne diseases.

Key Words: Food-borne disease; public health surveillance; control.

1. Introduction

Food-borne diseases pose a considerable threat to human health and the economy of individuals, families, and nations. Their control requires a concerted effort on the part of the three principal partners, namely governments, the food industry, and consumers ***(1,2)***. Food-borne diseases include infections caused by bacteria, such as *Salmonella, Shigella*, *Escherichia coli* 0157, *Campylobacter* spp. *Vibrio* spp., and *Listeria monocitogenes;* parasites, such as *Cryptosporidium* and *Cyclospora;* and viruses, such as the enteroviruses and noroviruses. To monitor the impact of these food-borne pathogens on human health, systems giving further information can be used ***(3)***.

Public health surveillance drives a number of disease prevention programs, including tuberculosis control, polio eradication, and food-borne disease prevention. Surveillance is the systematic collection of reports of specific health

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events as they occur in a population. This monitoring is linked to action. Surveillance defines the current magnitude and burden of a disease for which prevention measures are planned or in place. It identifies unusual clusters, or outbreaks of the disease, so that the action can be taken. Surveillance also measures the impact of control and prevention efforts, and it serves to reassure the public that this critical part of public safety is in place.

Surveillance of food-borne disease is a fundamental component of any food safety system. Surveillance data are used for planning, implementing, and evaluating public health policies. Worldwide, food-borne diseases, and more especially diarrheal diseases, are an important cause of morbidity and mortality.

Control of food-borne illness is an exceptionally challenging task for physicians and public health organizations. A number of factors conspire to make efforts at disease prevention difficult and make obstacles to performing disease surveillance quite formidable. A major obstacle to surveillance is that it is difficult to detect diseases that one is not specifically seeking. The emergence of new pathogens is inevitable ***(4)***.

2. Materials

2.1. WHO Global Food-Borne Disease Surveillance Network

There is a need to develop and coordinate a global approach to strengthen surveillance at national, regional, and international levels. Food-borne disease reporting should be integrated into the revision of the International Health Regulations. The World Health Organization (WHO)'s Department of Communicable Disease Surveillance and Response (CSR) assists countries to strengthen their national and regional food-borne disease and pathogen surveillance systems ***(5)***. Food safety is one of WHO's top 11 priorities; the organization calls for more systematic and aggressive steps to be taken to significantly reduce the risk of microbiological food-borne diseases ***(6)***. CSR coordinates WHO Global Salm-Surv (www.who.int/salmsurv), a global surveillance network on *Salmonella* set up in January 2000. The network comprises institutions and individuals who work on the isolation, characterization, and surveillance of food-borne pathogens. Activities have consisted of regional training for microbiologists, external quality assurance and reference testing, an electronic discussion group, and a Web-based databank containing an annual summary of laboratories. Currently the isolation of *Campylobacter* is included in the training courses' program. Over the following one to five years, Global Salm-Surv plans to improve regional coverage, introduce epidemiology training, expand to other food-borne pathogens, produce training manuals in microbiology and epidemiology, and establish regional centers. The WHO Surveillance Program for Control of Food-Borne Infections and Intoxications in Europe was launched in

1980 as a result of the international awareness of the socioeconomic impacts of the increase of food-borne diseases. The Food and Agricultural Organization (FAO) of the United Nations/WHO Collaborating Center manages this program for Research and Training in Food Hygiene and Zoonoses under the responsibility of the WHO European Centre for Environment and Health in Rome. The main objective of the program has been to provide information for the prevention and control of food-borne diseases in the region. Particular objectives include:

1. Identification of the causes and epidemiology of food-borne diseases in Europe.
2. Distribution of relevant information on surveillance.
3. Collaboration with national authorities in the identification of priorities in the establishment or reinforcement of their systems of prevention and control of food-borne diseases.

Since its establishment in 1980, interest in the program has grown continually, reaching 51 countries at the end of 1998. The program is particularly interested in inviting all the countries in the WHO-EURO region to provide them with support in their efforts to reinforce their surveillance system. The system includes the following countries: Albania, Andorra, Armenia, Austria, Azerbaijan, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Malta, Monaco, the Netherlands, Norway, Poland, Portugal, Republic of Moldova, Romania, Russian Federation, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, the former Yugoslav Republic of Macedonia, Turkey, Turkmenistan, Ukraine, United Kingdom, Uzbekistan, and Yugoslavia. Each participating country designates a national contact point, usually at the health ministry, who collects and reports official data on food-borne outbreaks and other relevant information. The program compiles and reports the data. Country reports include:

- General information on the surveillance systems in each country.
- Data from statutory notification.
- Information on epidemiologic investigated outbreaks.
- Additional information.

The general information section includes a description of the official surveillance and reporting system in the corresponding country.

Statutory notification presents data from the official notification system in the countries. In a number of countries these data refer only to the number of cases notified to the health agencies with or without laboratory confirmation and without any further epidemiologic background information.

The section on epidemiologically investigated outbreaks includes information on:

- Number of affected people.
- Causal agents.
- Incriminated foods.
- Place where food was contaminated, acquired, or consumed.
- Factors contributing to the outbreak.

This information is frequently based on the reports of laboratories involved in the investigation of food-borne incidents.

Finally, the section of additional information may include comments from the national contact points and, when available, links to the participating countries' related Web sites with information on actual figures or trends in food-borne diseases.

2.2. FoodNet

Estimates of the magnitude of food-borne illness in the United States have been imprecise. To quantify, better understand, and more precisely monitor food-borne illness, since 1996 the Food-Borne Diseases Active Surveillance Network (FoodNet) has collected data to monitor nine food-borne diseases in selected US sites *(7)*. The Centers for Disease Control and Prevention (CDC) is actively involved in preventing food-borne disease. The CDC's principal role in the interagency national Food Safety Initiative has been to enhance surveillance for and investigation of infections that are often food-borne. FoodNet is the principal food-borne disease component of the CDC's Emerging Infections Program (EIP). FoodNet is a collaborative project among participating EIP sites, the US Department of Agriculture (USDA), and the US Food and Drug Administration (FDA) *(8)*.

The objectives of FoodNet are: (1) to describe the epidemiology of new and emerging bacterial, parasitic, and viral food-borne diseases of national importance; (2) to more precisely determine the frequency and severity of food-borne diseases in the United States; and (3) to determine the proportion of food-borne disease caused by eating specific foods.

FoodNet provides a precise measure of the laboratory-diagnosed cases of food-borne illness and performs additional surveys and studies to interpret trends over time. The FoodNet data indicate a decline in several of the major bacterial and parasitic causes of food-borne illnesses and temporal variations in diagnostic practices. The trends also may reflect implementation of disease prevention efforts.

Laboratory-based surveillance programs such as FoodNet have significant limitations. The criterion for inclusion in the study is simply the physician's decision to send a stool culture. Many enrolled cases may have had mild diarrheal illnesses that did not truly merit obtaining stool cultures; conversely, it is highly likely that the stool cultures were never sent on the majority of cases with bacterial enteropathogens. It is impossible in studies such as these to determine the rate of

missed or inappropriate cases. Because microbiology laboratories report the culture results, cases are usually not linked to clinical information. Most surveillance programs attempt to obtain data retrospectively only on patients with positive culture results, leading to clinical information that is incomplete and of questionable accuracy. Despite these limitations, laboratory-based disease surveillance programs are relatively inexpensive and provide a wealth of information about trends in food-borne illnesses ***(9)***. One such program of particular relevance to emergency physicians is the EMERGEncy IDNet.

2.2.1. EMERGEncy IDNet

This program has collected data on patients with acute diarrheal illnesses, as well as other infections disease presentations ***(10,11)***. Although providing a supplement to programs such as FoodNet, these efforts are more labor-intensive and more expensive on a case-by-case basis and are more limited in scope than county- and state-based disease surveillance efforts ***(4)***.

2.3. PulseNet

In 1995, the CDC, with the assistance of the Association of Public Health Laboratories (APHL), selected the state public health laboratories in Massachusetts, Minnesota, Texas, and Washington as area laboratories for a national molecular subtyping network for food-borne bacterial disease surveillance. This network later became known as PulseNet. PulseNet, which began in 1996 with 10 laboratories typing a single pathogen (*Escherichia coli* O157:H7), now includes 46 state and 2 local public health laboratories and the food safety laboratories of the FDA and the USDA. Four food-borne pathogens (*E. coli* O157:H7, nontyphoidal *Salmonella* serotypes, *Listeria monocytogenes,* and *Shigella*) are being subtyped, and other bacterial, viral, and parasitic organisms will be added.

A national database of pulsed-field gel electrophoresis (PFGE) patterns is being assembled for food-borne bacterial pathogens. These databases reside on the PulseNet server at the CDC. For each bacterial pathogen, the normalized PFGE pattern is associated with a pattern database and the database of epidemiological and clinical information for isolates. Standardized protocols for food-borne bacterial pathogens were developed in priority order based on the ability of PFGE to discriminate among strains of the organism and the epidemiological utility of the resulting data. Standardized PFGE protocols have been developed for *E. coli* O157:H7, *Salmonella enterica* serotype Typhimurium, *L. monocytogenes,* and *Shigella* species.

2.3.1. Role of PulseNet in Outbreak Investigations

PulseNet plays several roles in detecting, investigating, and controlling outbreaks. Identification by PulseNet of an increase in a specific subtype of a

pathogen may be an early indication of an outbreak. PFGE patterns submitted to the national database by participating laboratories may link apparently unrelated cases that are geographically dispersed. Rapid sharing of PFGE subtyping data through PulseNet plays a critical role in linking apparently unrelated outbreaks and identifying a common vehicle. Although PulseNet has proven invaluable in detecting food-borne disease outbreaks and facilitating their investigation, molecular subtyping is an adjunct to epidemiologic investigation and not a replacement for it ***(12)***.

2.4. Enter-net

Funded by the European Commission, Enter-net (formerly Salm-net) is an international surveillance system for *Salmonella* infections (including data on antibiotic resistance) and *E. coli* O157 infections. Microbiologists and epidemiologists responsible for national laboratory-based surveillance of these pathogens in 15 European countries form the Enter-net network ***(13)***. Enter-net participants are working toward a common set of laboratory protocols, including procedures for serotyping, phage typing, and toxin typing. They report disease cases to the international Enter-net database on a regular basis, through the Internet, by using standardized data fields. Every year, the participants from each member country attend a workshop to discuss technical issues and principles of collaboration. Potential conflicts addressed at workshops include ownership of data, confidentiality, outbreak control measures, and liability concerns (e.g., what happens when a food product is implicated by Enter-net as a vehicle of disease transmission) ***(14)***.

2.5. OzFoodNet

In Australia, the Commonwealth Department of Health and Ageing established OzFoodNet in 2000 as a collaborative project with state and territory health authorities to provide better understanding of the causes and incidence of food-borne disease. OzFoodNet provides a network for responding to nationally important new and emerging food-borne diseases, monitoring the burden of these illnesses, and identifying the sources of specific food-borne outbreaks. OzFoodNet reports surveillance data for several bacterial pathogens and summary information from outbreaks potentially related to food and water ***(15)***.

2.6. Surveillance of Food-Borne Diseases in Other Countries

Except for a few countries such as Japan, China, and Indonesia, relatively little in the way of surveillance of food-borne disease is carried out in Asia. In 2001 the National Food-Borne Monitoring and Surveillance System was established in China. Data on acute food-borne diseases were collected from 1992 to 2001. It was found that the microbial food-borne diseases had higher inci-

dences, followed by chemical food-borne diseases. Infectious diseases such as cholera, viral hepatitis, bacterial and amoebic diarrhea, typhoid and paratyphoid, and other infectious diarrhea must be reported. Food poisoning events, with more than 30 cases, or more than one death in an outbreak, or one that happened in a school, must be reported directly to the Ministry of Health ***(16)***.

The National Institute of Infectious Diseases in Japan under the Ministry of Health, Labor, and Welfare established the Infectious Disease Surveillance Center (IDSC) as the national center for infectious disease surveillance and for exchanging information on infectious diseases with other nations' surveillance centers. As of January 1999, all patients who visit designated clinics or hospitals are reported to health centers (about 600 in the nation), which are then electronically reported to the prefectural/municipal health departments and the IDSC.

Most Latin American countries now consider food-borne disease important enough to justify some kind of surveillance scheme and are trying to develop better ways of determining numbers of cases and their causes.

3. Conclusions

Twenty years ago, most food-borne outbreaks were local problems that typically resulted from improper food handling practices. Outbreaks were often associated with individual restaurants or social events and often came to the attention of local public health officials through calls from affected persons. These persons, who may have known others who had become ill after eating a shared meal or visiting the same restaurant, provided health officials with much of the information needed to begin an investigation.

Today food-borne disease outbreaks involve widely distributed food products that are contaminated before distribution, resulting in cases that are spread over several states or countries. It is less common for ill persons to know others who were ill or to be able to identify a likely source of their infection. For these reasons, it is becoming increasingly important to be able to identify potential common exposures through DNA fingerprinting of patient isolates. Health, food safety, industry, and agricultural agencies should develop closer links to share information about the occurrence of food-borne pathogens. State and territory health departments should continue to conduct rigorous checks on the quality of surveillance data maintained on surveillance databases. There is a need to establish the global food-borne disease surveillance network, and to share the information. Improvement of surveillance on a worldwide basis is essential to show the extent of the problem of food-borne disease in the many countries and regions with no existing system.

Many of the weaknesses of laboratory surveillance programs are overcome by performing prospective, multicenter epidemiological investigations. Such

studies allow investigators to clearly define enrollment criteria and obtain detailed clinical data from all patients at selected study centers suspected of having bacterial enteropathogens.

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