# DEVELOPMENT OF CLOTH-BASED HYBRIDIZATION SYSTEMS FOR THE DETECTION AND CHARACTERIZATION OF FOODBORNE PATHOGENIC BACTERIA

by

Martine E. Gauthier, B.Sc., (Biology)

A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Master of Science

College of Natural Sciences (CNS)
Department of Biology
Carleton University
Ottawa, Ontario

January 21, 2005

*Your file Votre référence*
*ISBN: 0-494-00780-X*
*Our file Notre référence*
*ISBN: 0-494-00780-X*

For Kevin

"The important thing in science
is not so much to obtain new facts as to
discover new ways of thinking about them."

~William Lawrence Bragg

## ABSTRACT

A cloth-based hybridization array system (CHAS) approach was developed in which a multiplex polymerase chain reaction (PCR) incorporating digoxigenin-dUTP was used for simultaneous amplification of multiple target sequences, with subsequent rapid detection of the amplicons by hybridization with an array of probes immobilized on polyester cloth, and immunoenzymatic assay of the bound digoxigenin label. Three separate CHAS were developed: (1) a CHAS for detection of multiple antibiotic resistance and other marker genes in the characterization of *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 isolates; (2) a CHAS for the detection of various toxin genes associated with major foodborne pathogenic bacteria; and (3) a CHAS for the detection and identification of different *Clostridium botulinum* toxin gene types. In addition, a cloth-based dot blot hybridization system (C-DBHS) was developed in which the PCR products are assayed by spotting on a polyester cloth sheet capable of accomodating multiple samples, with subsequent detection of the amplicons by hybridization with a digoxigenin-labelled target-specific DNA probe and immunoenzymatic assay of the bound label.

## ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to Dr. Burton W. Blais, my thesis supervisor, for offering me such a wonderful research opportunity, and more importantly for his excellent guidance and encouragement throughout the course of my degree. I would also like to thank Dr. Sharon Regan, my co-supervisor, for her constant support and availability when I needed it most. Many thanks to Dr. Douglas Johnson and Dr. Franco Pagotto, my thesis advisors, who have taken much time from their busy schedules to attend my M.Sc advisory committee meetings and for providing me with constructive suggestions. I would also like to extend my gratitude to Jessica Bosley and to Brigitte Cadieux for providing me with excellent technical assistance throughout certain phases of this project. I am also grateful to everyone at the Ottawa Laboratory (Carling) of the Canadian Food Inspection Agency, especially the Technology Development Section "team" for helping make my stay a productive and pleasant experience. Finally, I thank my family and my friends for their support, encouragement, and understanding throughout this busy time, and I reserve a very special thank you to Kevin Exner for his unfailing moral support throughout the entire course of my degree.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ABBREVIATIONS

| | |
|---|---|
| $A_{450}$ | absorbance at 450 nm |
| ACh | acetylcholine |
| AChR | acetylcholine receptor |
| ATCC | American Type Culture Collection |
| BoNT | botulinum neurotoxin |
| BSA | bovine serum albumin |
| °C | degrees Celcius |
| cAMP | cyclic adenosine monophosphate |
| cfu | colony forming unit |
| cGMP | cyclic guanosine monophosphate |
| CCP | critical control point |
| C-DBHS | cloth-based dot blot hybridization system |
| CHAS | cloth-based hybridization array system |
| CHS | cloth-based hybridization system |
| cm | centimeter |
| CT | cholera toxin |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| dUTP | deoxyuridine triphosphate |

| | |
|---|---|
| EHEC | enterohemorrhagic *E. coli* |
| EIEC | enteroinvasive *E. coli* |
| ELISA | enzyme-linked immunosorbent assay |
| EPEC | enteropathogenic *E. coli* |
| ETEC | enterotoxigenic *E. coli* |
| $Gb_3$ | globotriaosylceramide |
| h | hour(s) |
| HACCP | Hazard Analysis Critical Control Point |
| HC | heavy chain |
| HRP | horseradish peroxidase |
| HUS | aemolytic uremic syndrome |
| LA | latex agglutination |
| LC | light chain |
| LT | heat-labile enterotoxin |
| M | molar |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| ng | nanogram |
| NCBI | National Center for Biotechnology Information |
| PBS | phosphate buffered saline |
| PBST | PBS containing Tween 20 |

| | |
|---|---|
| PBST-B | PBST containing blocking reagent |
| PCR | polymerase chain reaction |
| pg | picogram |
| rRNA | ribosomal RNA |
| s | second(s) |
| SNAP-25 | synaptosome-associated membrane protein of 25 kDa |
| SNARE | soluble NSF-attachment protein receptors |
| ST | heat-stable enterotoxin |
| TMB | tetramethylbenzidine |
| TSA | tryptone soya agar |
| TSB | tryptone soya broth |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| VAMP | vesicle-associated membrane protein |
| VT | verotoxin |
| VTEC | verotoxin-producing *E. coli* |

# CHAPTER 1

## General Introduction

Foodborne illness is a significant and growing health problem worldwide, with more than 200 different diseases currently known to be transmitted through food. In the United States, foodborne diseases have been estimated to cause approximately 76 million illnesses, 325 000 hospitalizations, and 5 000 deaths each year. Illnesses, with a known aetiology, account for an estimated 14 million cases, 60 000 hospitalizations, and 1 800 deaths. In Canada, statistics on the incidence of foodborne illness are widely accepted to be 1/10 of the figures reported for the United States. It has been estimated that 2.2 million people contract foodborne illness per year in Canada. Among all illnesses attributable to foodborne transmission, 30% are caused by bacteria (Mead *et al.* 1999). Bacterial foodborne diseases are illnesses acquired by the consumption of foods containing pathogenic bacteria and/or their toxins. Bacterial foodborne diseases are grouped into three categories: (1) infections, (2) intoxications, and (3) toxicoinfections. Foodborne infections (e.g. salmonellosis) occur when live pathogenic microorganisms present in the food are ingested, colonize the intestine, and sometimes invade the mucosa or other tissues. Foodborne microbial intoxications (e.g. botulism) occur by consuming food containing harmful toxins released during the growth stages of the microorganism. Foodborne toxicoinfections (e.g. cholera) arise when a microorganism from ingested food grows in the intestinal tract and elaborates a toxin(s) that damages tissues or interferes with normal tissue/organ function.

## 1. Pathogenic bacteria involved in foodborne illness

Listed in Table 1.1 are some of the common pathogenic bacteria known to cause foodborne disease, some of which will be presented in more detail.

**Table 1.1** Characteristics of common foodborne pathogenic bacteria: annual cases of human illness, incubation period, symptoms, infectious dose, and associated foods

| Pathogen | Estimated #cases/year[a] | Incubation period[b,c] | Symptoms[b,c] | Infectious dose[b] | Associated foods[b,c] |
|---|---|---|---|---|---|
| *Bacillus cereus* | 27,360 | 6-16 hrs | Abdominal cramps, watery diarrhea, nausea | $> 10^6$ cells/g of food | Meats, stews, gravies, vanilla sauce |
| *Clostridium botulinum* | 58 | 12-72 hrs | Nausea, vomiting, fatigue, dizziness, headache, dryness of skin, mouth and throat, blurred vision, double vision, trouble breathing, and descending muscle paralysis (foodborne) Constipation, poor feeding, lethargy, weakness, pooled oral secretions, and weak cry (infant) | A few μg of toxin (oral ingestion) | Home-canned foods with low acid content, improperly canned commercial foods, home-canned or fermented fish, herb-infused oils, baked potatoes in aluminum foil, cheese sauce, bottled garlic, foods held warm for extended periods of time, honey and home-canned vegetables and fruits (infants) |
| *Campylobacter* spp. | 2,453,926 | 2-5 days | Diarrhea, cramps, fever, and vomiting; diarrhea may be bloody | ~ 500 cells | Raw and undercooked poultry, unpasteurized milk, contaminated water |
| *Clostridium perfringens* | 248,520 | 8-22 hrs | Watery diarrhea, nausea, abdominal cramps; fever is rare | $> 10^8$ cells | Meats, poultry, gravy, dried or precooked foods |
| *Escherichia coli* O157:H7 | 73,480 | 1-8 days | Severe diarrhea that is often bloody, abdominal pain, vomiting, low-grade fever | < 10 cells | Undercooked beef, unpasteurized milk and juice, raw fruits and vegetables, salami, salad dressing, and contaminated water |
| *E. coli*, non-O157:H7 EHEC | 36,740 | 1-8 days | Bloody diarrhea with pus in stools and low-grade fever | 10 cells | Raw beef and chicken |

**Table 1.1** Continued

| Pathogen | Estimated #cases/year[a] | Incubation period[b,c] | Symptoms[b,c] | Infectious dose[b] | Associated foods[b,c] |
|---|---|---|---|---|---|
| *E. coli*, enterotoxigenic | 79,420 | 1-3 days | Watery diarrhea, abdominal cramps, some vomiting, low-grade fever | 100 million to 10 billion cells | Water and food contaminated with human feces |
| *E. coli*, other diarrheogenic | 79,420 | 1-6 days | Vomiting, diarrhea, abdominal pain, and fever | | Water and food contaminated with human feces |
| *Listeria monocytogenes* | 2,518 | 1 day – 6 weeks | Fever, muscle aches, and nausea or diarrhea. Pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth. Elderly or immunocompromised patients may have bacteremia or meningitis. Infants infected from mother at risk for sepsis or meningitis | < 1000 cells | Fresh soft cheeses, unpasteurized milk, inadequately pasteurized milk, ready-to-eat deli meats, hot dogs |
| *Salmonella* Typhi | 824 | 1-3 days | Fever, headache, constipation, malaise, chills, and myalgia. Diarrhea rare, vomiting not severe | 15 to 20 cells | Fecal contamination of water supplies or street-vended food |
| *Salmonella*, non-typhoidal | 1,412,498 | 1-3 days | Diarrhea, fever, abdominal cramps, vomiting | 15 to 20 cells | Contaminated eggs, poultry, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables |
| *Shigella* spp. | 448,240 | 12-48 hours | Abdominal cramps, fever, and diarrhea. Stools may contain blood and mucus | 10 cells | Food or water contaminated with fecal material. |

**Table 1.1** Continued

| Pathogen | Estimated #cases/year[a] | Incubation period[b,c] | Symptoms[b,c] | Infectious dose[b] | Associated foods[b,c] |
|---|---|---|---|---|---|
| Staphylococcus food poisoning | 185,060 | 1-6 hours | Severe nausea and vomiting. Abdominal cramps. Diarrhea and fever may be present | < 1 μg of toxin | Unrefrigerated or improperly refrigerated meats, potato and egg salads, cream pastries |
| Streptococcus foodborne | 50,920 | Group A: 1-3 days | Group A: sore throat, pain in swallowing, high fever, headache, nausea, vomiting, malaise | Group A: < 1000 cells | Group A: milk, ice cream, eggs, steamed lobster, ground ham, potato salad, egg salad, custard, rice pudding, and shrimp salad |
| | | Group D: 2-36 hours | Group D: diarrhea, vomiting, abdominal cramps, fever, and dizziness | Group D: > $10^7$ cells | Group D: sausage, evaporated milk, cheese, meat products, pudding, raw milk, and pasteurized milk |
| *Vibrio cholerae*, toxigenic | 54 | 24-72 hrs | Profuse watery diarrhea and vomiting, which can lead to severe dehydration and death within hours if untreated. | $10^8$ cells | Contaminated water, fish, shellfish, and other contaminated foods. |
| *V. vulnificus* | 94 | 1-7 days | Vomiting, diarrhea, abdominal pain, bacteremia, and wound infections. | Less than 100 cells | Undercooked or raw shellfish, especially oysters; other contaminated seafood, and open wounds exposed to sea water |
| *Vibrio*, other | 7,880 | 4-96 hours | Diarrhea, abdominal pain, nausea, vomiting, headache, fever, and chills. | > $10^6$ cells | Undercooked or raw seafood, such as fish, shellfish |
| *Yersinia enterocolitica* | 96,368 | 24-48 hrs | Gastroenteritis with diarrhea, fever, and abdominal pain | Unknown | Undercooked pork, unpasteurized milk, contaminated water |

[a] From Mead *et al.* 1999
[b] From U.S. Food & Drug Administration, 1992
[c] From Anderson *et al.* 2001

## 1.1 *Salmonella*

Members of the genus *Salmonella* constitute an important group of foodborne pathogens that are widely distributed in the food supply and are the leading cause of bacterial foodborne disease. Currently, non-typhoidal *Salmonella* is estimated to be responsible for 1.4 million illnesses, of which 95% are foodborne, and salmonellosis accounts for approximately 30% of deaths resulting from foodborne illnesses in the United States (Mead *et al.* 1999).

### 1.1.1 Modes of transmission

There are three main routes by which *Salmonella* can enter the food supply and cause illness: (1) food-producing animals harboring *Salmonella* can implicate meats, poultry, eggs, and milk as important vehicles of transmission; (2) *Salmonella*, which are introduced into the environment, possibly through manure, can persist and contaminate fruits and vegatables on the farm; (3) *Salmonella* can also enter the food supply and cause salmonellosis through cross-contamination between raw poultry and ready-to-eat (RTE) products, such as raw vegetables (IFT, 2004).

The incubation period before the onset of symptoms varies from 1 to 3 days and is dependant on the size of the infecting dose (as few as 15 cells can cause illness), the virulence of the microorganism, the susceptibility of the host, and the physicochemical composition of the transmitting food (IFT, 2004). The principal symptoms of non-typhoid *Salmonella* infections include non-bloody diarrhea, abdominal pain, fever, nausea, and vomiting (Tietjen and Fung, 1995), and usually resolve in five to seven days without necessitating antibiotic treatment. However, bacteremia occurs in 3% to 10% of reported, culture-confirmed cases and is particularly common in immunocompromised

patients. For these patients, appropriate antimicrobial therapy can be lifesaving (Tauxe, 1991).

### 1.1.2 Antimicrobial resistance

The use of antimicrobial agents in any environment creates selection pressures that favor the survival of bacterial strains with resistance to the agent. The routine practice of giving antimicrobial agents to food-producing animals as a means of preventing and treating bacterial infections, as well as promoting growth, has been suggested as an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans via the food chain (Schwarz and Chaslus-Dancla, 2001). Multidrug-resistant phenotypes of *Salmonella* have been increasingly described around the world (Lee *et al.* 1994; Threlfall *et al.* 1993). In the United States, tetracycline resistance has increased in *Salmonella* species from 9% in 1980 to 24 % in 1990 and ampicillin resistance increased from 10% to 14% (Lee *et al.* 1994). Increased rates of resistance were reported in Great Britain where resistance in *Salmonella enterica* serotype Typhimurium more than doubled between 1981 and 1989 (Threlfall *et al.* 1993).

### 1.1.3 *Salmonella enterica* serotype Typhimurium DT104: a multidrug-resistant pathogen

The emergence of multidrug-resistant *S. enterica* serotype Typhimurium DT104 over the past decade has become a global health problem due to its involvement in diseases of both animals and humans (Poppe *et al.* 1998; Threlfall, 2000). These multidrug-resistant strains were first detected in the United Kingdom in cattle and humans in the late 1980s, but have become common in other animals species such as poultry, pigs, and sheep

(Threlfall, 2000). The consumption of chicken, beef, pork sausages, and meat paste all have been implicated in human infections with multidrug-resistant DT104 isolates (Threlfall, 2000). The DT104 epidemic has reached a global scale with a considerable number of outbreaks occuring in the United States and Canada since 1996 (Glynn *et al.* 1998; Poppe *et al.* 1998).

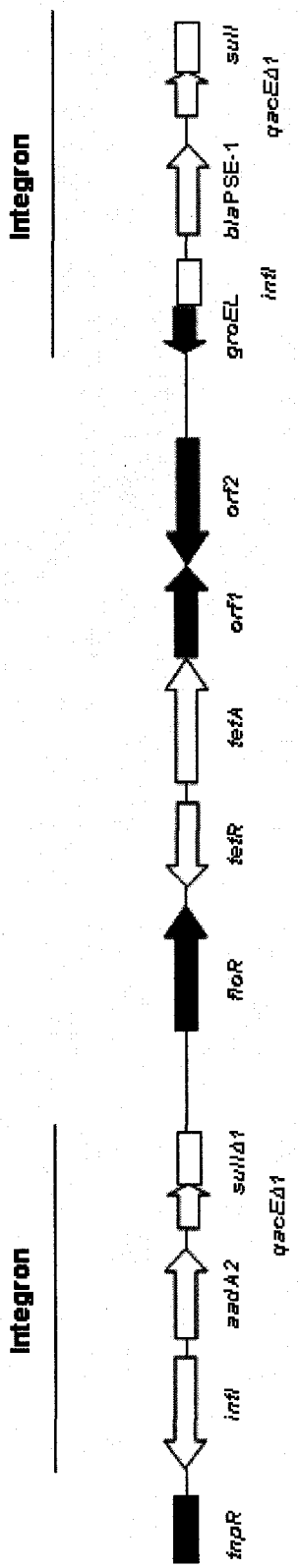
Of particular concern has been the resistance of the organism to a wide range of antimicrobial agents. Multidrug-resistant *S.* Typhimurium DT104 strains are commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT resistance type), and this spectrum of resistance is chromosomally encoded (Cloeckaert and Schwarz, 2001; Threlfall, 2000). Two different integrons with the genetic location of the antibiotic resistance genes were identified in 1998 using PCR and sequencing (Ridley and Threlfall, 1998; Sandvang *et al.* 1998). Most of the DT104 ACSSuT-type strains contain at least two class 1 integrons, one containing the *aadA2* gene, conferring resistance to streptomycin and spectinomycin, and one containing a β-lactamase gene, $bla_{PSE-1}$, encoding resistance to ampicillin. Genes encoding sulfonamide resistance (*sul1*) and resistance to disinfectants (*qacEΔ1)* are found at the 3' end of both integrons. Integrons are mobile DNA elements able to incorporate single or groups of antibiotic resistance genes (gene cassettes) by site-specific recombination systems and can be located either on the chromosome or on plasmids (Hall and Stokes, 1993). Integrons consist of two conserved segments, the 5' conserved segment (5'CS) and the 3' conserved segment (3'CS), and an internal variable region. The 3'CS is the target for the integration of gene cassettes that encode antibiotic resistance with a recombination site termed the 59-base element (Hall *et al.* 1991). The 5'CS contains the integrase gene (*intI*), the cassette integration site (*attI*) for the integron and promoters for the expression

of all downstream gene cassettes (Recchia and Hall, 1995). The locations of the genes encoding resistance to chloramphenicol and tetracyclines were identified in 1999 (Arcangioli *et al.* 1999; Bolton *et al.* 1999; Briggs and Fratamico, 1999; Ng *et al.* 1999) and are located between both class 1 integrons described above, clustering all antibiotic resistance genes on a chromosomal locus of approximately 12.5 kb. The organization of the antibiotic resistance gene cluster is shown in Figure 1.1 (Cloeckaert and Schwarz, 2001).

### 1.1.4 Speculations on the origin of multidrug-resistance in *S.* Typhimurium DT104

Several authors have speculated on the origin of the *S.* Typhimurium DT104 gene cluster (Angulo and Griffin, 2000; Collignon, 2000; Davis *et al.* 2000). The use of antimicrobial agents in agriculture may have contributed to the emergence of multidrug-resistant *S.* Typhimurium DT104 (Angulo and Griffin, 2000; Collignon, 2000). Chloramphenicol resistance in DT104 is due to *floR*, a florfenicol resistance gene (Bolton *et al.* 1999). Florfenicol is a veterinary antimicrobial agent that has been approved in the United States in 1996 for the treatment of bovine respiratory pathogens. However, florfenicol has been used in aquaculture in Asia since the early 1980s (Angulo and Griffin, 2000) and *floR* was first described in *Pasteurella piscicida*, a pathogen of fish (Bolton *et al.* 1999; Kim and Aoki, 1993). Another line of evidence that suggests the implication of antimicrobial use to the emergence of resistance in DT104 is the presence of a class G tetracycline resistance gene which was first identified in *Vibrio anguillarum*, another fish pathogen (Briggs and Fratamico, 1999; Zhao and Aoti, 1992). Furthermore, the chromosomal

**Figure 1.1** Gene organization of the chromosomal antibiotic resistance gene cluster of *Salmonella enterica* serotype Typhimurium DT104 (reproduced from Cloeckaert and Schwarz, 2001). The first integron contains the *aadA2* gene which confers resistance to streptomycin and spectinomycin. The second integron contains the β-lactamase gene, $bla_{PSE-1}$, which encodes resistance to ampicillin. Each integron carries the *sulI* and *qacEΔ1*genes which encode resistance to sulfonamides and disinfectants, respectively. Situated between both integrons are the resistance genes conferring resistance to chloramphenicols (*floR*) and tetracyclines (*tetR* and *tetA*).



locus where the class G tetracycline resistance and *floR* genes are located in DT104 is closely related to a plasmid found in *P. piscicida*. These data suggest that the resistance determinants found in *S.* Typhimurium DT104 may have emerged among bacteria in aquaculture and been subsequently transferred to *S.* Typhimurium DT104 via horizontal transfer. Once multidrug-resistant DT104 are introduced into food-producing animals in a region, the use of antibiotics in animals would offer a selective advantage to these organisms, therefore contributing to their dissemination (Angulo and Griffin, 2000). Another hypothesis on the origin of the *S.* Typhimurium DT104 gene cluster is the acquisition of resistance genes from nosocomial pseudomonads (Davis *et al.* 2000). The class G tetracycline resistance gene first described in *V. anguillarum* (Zhao and Aoti, 1992) also occurs in *Pseudomonas aeruginosa* (Ng *et al.* 1999). *FloR* is also closely related to the *P. aeruginosa* chloramphenicol resistance gene *cm1A* (Arcangioli *et al.* 1999), and the $bla_{PSE-1}$ gene encoding β-lactamase is a common feature of hospital *P. aeruginosa* isolates (Davis *et al.* 2000).

There has been speculation that *S.* Typhimurium DT104 appears to be more virulent in human beings than other salmonellas (Wall *et al.* 1994). A study was conducted to test out this hypothesis and found that invasive disease with multidrug-resistant DT104 is uncommon (Threlfall and Rowe, 1998). However, since the genes included in the multidrug-resistance gene cluster of DT104 strains confer resistances to drugs of four of the five classes of antimicrobials (β-lactams aminoglycosides, sulfonamides and tetracyclines), the choice of antimicrobial is very limited should antibiotic therapy be needed (Cloeckaert and Schwarz, 2001). Also, because the genes that confer the multidrug-resistance in DT104 are chromosomally encoded within a transferrable element (Briggs and Fratamico, 1999; Ridley and Threlfall, 1998; Sandvang

*et al.* 1998), there is a potential danger that these resistance genes can be transferred to other, perhaps more virulent pathogens.

## 1.2 *Escherichia coli*

*Escherichia coli* is a commensal of the gut microflora of all warm-blooded animals, including humans. However, among this species are fully pathogenic strains that have the ability to cause distinct syndromes of diarrheal disease. There are four main categories of diarrheagenic *E. coli* described on the basis of their clinical manifestations, their interactions with the intestinal mucosa, differences in epidemiology, and distinct virulence properties: (1) enterotoxigenic *E. coli* (ETEC), (2) enterohemorrhagic *E. coli* (EHEC), (3) enteropathogenic *E. coli* (EPEC), and (4) enteroinvasive *E. coli* (EIEC) (Levine, 1987). With regards to pathogenesis, however, these four distinct categories of diarrheagenic *E. coli* have underlying common features: (1) critical virulence properties are encoded on plasmids, (2) characteristic interaction with the intestinal mucosa, (3) production of enterotoxins or verotoxins (VTs), and (4) strains fall within certain O:H serotypes within each category (Levine, 1987).

### 1.2.1 Enterotoxigenic *E. coli* (ETEC)

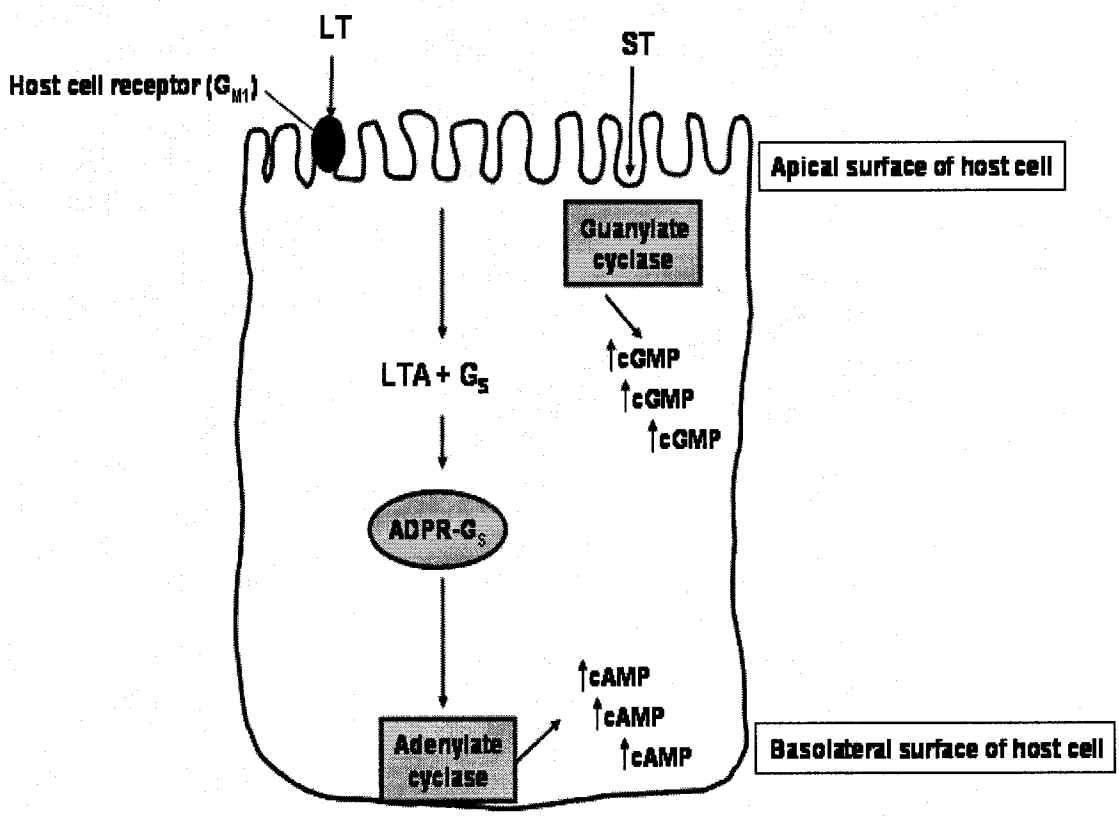
ETEC infections are the major cause of bacterial diarrhea in developing countries and of traveler's diarrhea and are acquired by ingesting contaminated food or water. Clinical features of this foodborne disease include watery diarrhea, nausea, abdominal cramps, and low-grade fever (Levine, 1987), and is an example of a toxicoinfection. ETEC are distinguished from other groups of *E. coli* by their ability to elaborate one or both of two

plasmid-encoded toxins: (1) a cholera-like toxin called the heat-labile (LT) enterotoxin, and (2) a diarrheal toxin called the heat-stable (ST) enterotoxin.

#### 1.2.1.1 Mechanism of pathogenesis of ETEC

The mechanism of pathogenesis involves the adherence of ETEC strains to the small intestinal mucosa by means of specific fimbriae, referred to as colonization factor antigens I and II. The diarrhea produced by these strains is caused by the action of LT, ST or both enterotoxins (Figure 1.2). The LT enterotoxin possesses an A-B type toxin structure composed of 5 B subunits linked to an A subunit, and has the same structure and mechanism of action as the cholera toxin. The LT toxin acts by first binding to the host cell receptor $G_{M1}$ via its B subunit. Once bound to the host cell, the enzymatically active A subunit is internalized and ADP-ribosylates a protein ($G_s$) responsible for the control of adenylate cyclase activity, an enzyme located on the basolateral surface of the host cell. The stimulation of adenylate cyclase results in an increase of cyclic adenosine monophosphate (cAMP) leading to an increase in chloride secretion and inhibition of sodium chloride uptake. This disruption in ion transport causes intestinal cells to lose control of the flow of water, leading to tissue water loss and diarrhea (Salyers and Whitt, 2003). ST has the same effect on the host cell, leading to watery diarrhea in the patient, but via a slightly different mechanism of action. The host cell receptor for ST is guanylate cyclase, located in the apical membrane of the host cell. Binding of ST activates guanylate cyclase, leading to an increase in cyclic guanosine monophosphate (cGMP) and subsequently, a disruption in ion transport resulting in water loss (Salyers and Whitt, 2003).

**Figure 1.2** Mechanism of action and cellular location of the targets of LT and ST (adapted from Salyers and Whitt, 2003). LT binds to host cell receptor ($G_{M1}$) via its B subunit, and the A subunit is internalized and ADP-ribosylates a G protein, $G_s$, that controls the activity of adenylate cyclase located on the basolateral surface of the host cell. The stimulation of adenylate cyclase results in an increase in intracellular cAMP leading to a disruption in ion transport and consequently in the loss of water from the tissues and diarrhea. ST activates guanylate cyclase by binding it on the apical surface of the host cell. Activation of guanylate cyclase results in a rise in host cell levels of cGMP.



### 1.2.2 Enterohemorrhagic *E. coli* (EHEC)

EHEC causes illness associated with a wide spectrum of symptoms, including watery diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) which can be life threatening (Karmali, 1989), and is another example of a toxicoinfection. HC is a distinct clinical syndrome characterized by abdominal cramping and bloody diarrhea, with evidence of colonic mucosal edema, erosion, or hemorrhage, in the absence of infection by recognized enteric pathogens in the stool (Riley, 1987). The incubation period for EHEC infections is 3 to 4 days and typically begins with severe abdominal cramping, followed by watery diarrhea within a few hours. Symptoms such as nausea and vomiting occur early, and the watery diarrhea progresses to bloody diarrhea within 1 to 2 days, with the amount of blood varying from a few small streaks to stools that are almost all blood (Mead and Griffin, 1998). In most cases, HC resolves in about 8 days without the need for specific therapy, but serious complications such as HUS and death can occur (Riley, 1987). HUS most commonly occurs in children and is characterized by diarrhea, hemolytic anemia, thrombocytopenia, and ultimately renal failure (Karmali, 1989). Treatment for patients with EHEC infections is mainly supportive. Patients should be monitored for dehydration and other signs of symptoms that might suggest HUS. Treatment of patients with EHEC infections with antimicrobials is controversial. There are conflicting data on the efficacy of treating patients with antimicrobials. Some studies have suggested that HUS is more likely to develop in patients treated with an antimicrobial agent than those not receiving treatment, and some studies report the opposite result (Boyce *et al.* 1995; Mead and Griffin, 1998).

The serotype most frequently implicated in human cases of bloody diarrhea and HUS in Canada and the United States is *E. coli* O157:H7, but other non-O157:H7 serotypes have also been reported to cause disease (Griffin and Tauxe, 1991). Currently, *E. coli* O157:H7 is estimated to be responsible for 73 000 cases of diarrheal disease, resulting in 2 100 hospitalizations and 60 deaths annually in the United States. The estimate for non-O157:H7 EHEC infections that occur annually is about 37 000 cases, with about 1 000 hospitalizations, and 30 deaths (Mead *et al.* 1999).

#### 1.2.2.1 Modes of transmission of EHEC

The infectious dose of EHEC is very low (Karch *et al.* 1999) and cattle are believed to be the major reservoir for the contamination of the food supply with *E. coli* O157:H7, although other animals such as deer, sheep, goats, and horses may also shed the microorganism in their feces (Mead and Griffin, 1998). Data collected from EHEC outbreaks involving *E. coli* O157:H7 identified three main routes of transmission: 1) contaminated food and contaminated drinking or swimming water, 2) person-to-person transmission, and 3) animal contacts (Karch *et al.* 1999). The first reported multistate outbreak of *E. coli* O157:H7 was associated with the consumption of undercooked ground beef at fast-food restaurants in 1982 (Riley *et al.* 1983). The largest reported outbreak in North America affected over 700 people of which 4 died, in the states of Washington, Idaho, Nevada, and California and the vehicle of transmission was traced to undercooked hamburgers from a single fast-food restaurant chain (Bell *et al.* 1994). Undercooked ground beef is of particular concern because the pathogens that may be present on the surface of the meat after slaughter are transferred to the interior of the meat

during the process of grinding. As a result, if the ground meat is undercooked, the bacteria can survive and be ingested. Since a lot of ground beef may include meat from many carcasses, a small number of infected animals can contaminate a large supply of ground beef (Boyce *et al.* 1995). Other vehicles of transmission implicated in foodborne infections include salami (Tilden *et al.* 1996), raw milk (Anonymous, 1996), cantaloupe (Feng, 1995), and unpasteurized apple cider (Feng, 1995). Several outbreaks of *E.* coli O157:H7 infections have also been associated with contaminated drinking or swimming water (Anonymous, 2000; Keene *et* al. 1994). The first and largest outbreak of *E. coli* O157:H7 infection associated with a treated municipal water supply in Canada occurred in May 2000 in the town of Walkerton, Ontario. The consumption of the contaminated water was responsible for 1 300 reported illnesses, resulting in 65 hospitalizations, and 6 deaths. This tragedy occurred as a result of a series of unfortunate circumstances including heavy rainfall accompanied by flooding, the presence of *E. coli* O157:H7 in the environment, and a well subject to surface contamination (Anonymous, 2000).

#### 1.2.2.2 Mechanism of pathogenesis of EHEC

Human isolates of EHEC are known to produce either one or both of two immunologically distinct, bacteriophage-encoded verotoxins (VTs) (VT1 and VT2) and are therefore also referred to as verotoxin-producing *E. coli* (VTEC) (Karmali, 1989). The verotoxins possess an A-B type toxin structure, composed of five B subunits noncovalently linked to a single A subunit (Schmitt *et al.* 1999). The A subunit is responsible for the enzymatic activity while the B subunit plays a crucial role in the binding of the toxin to the target cell. Target cells, such as epithelial enterocytes,

vascular endothelial cells, smooth muscle cells, renal endothelial cells, and erythrocytes possess specific glycolipid receptors called globotriaosylceramide ($Gb_3$) onto which the toxin binds via its B subunit. Once bound to $Gb_3$, the verotoxins enter the cells via endocytosis and are transported to the Golgi apparatus and endoplasmic reticulum (LeBlanc, 2003). In the meantime, the A subunit is cleaved into an enzymatically active $A_1$ portion and an $A_2$ portion linked by a disulphide bond which is later reduced. Once translocated to the cytoplasm, the $A_1$ interacts by rRNA-*N*-glycosidase activity to inactivate the 60S ribosomal subunit by cleaving a single adenine residue from the 28S ribosomal RNA, ultimately resulting in the inhibition of protein synthesis, leading to cell death (LeBlanc, 2003).

## 1.3 *Clostridium botulinum*

*Clostridium botulinum* is an anaerobic, gram positive, rod-shaped, spore-forming bacterium commonly found in soil samples, aquatic sediments, gills and viscera of shellfish, and the intestinal tracts of animals and fish and is the causative agent of the paralytic disease known as botulism (Hatheway, 1990; Rhodehamel *et al.* 1992). Seven types of *C. botulinum* (A, B, C, D, E, F, and G) are recognized based on the antigenic specificity of the neurotoxins they produce. Strains of *C. botulinum* are divided into four groups (designated Groups I to IV), based on physiological differences. Group I includes the proteolytic strains of toxin types A, B, and F. Group II contains all toxin type E strains and the nonproteolytic strains of toxin types B and F. Group III contains strains of toxin types C and D and are responsible for animal botulism. Group IV contains the strains that produce type G toxin. *C. botulinum* strains involved in human

illness fall into Groups I and II (Hatheway, 1990; Rhodehamel *et al.* 1992). Although serologically distinct, all types of botulinum neurotoxins (BoNT) have the same biological effect – inhibition of the release of the neurotransmitter acetylcholine (ACh) at the neuromuscular junction causing a flaccid paralysis (Rhodehamel *et al.* 1992). BoNTs are extremely toxic to both humans and animals. The lethal dose of crystalline BoNT type A for humans from primate data was estimated by extrapolation. For a 70 kg (154 lb) human, the lethal dose would be approximately 0.09-0.15 μg via intravenous or intramuscular injection, 0.70-0.90 μg via inhalation, and 70 μg via oral ingestion (Arnon *et al.* 2001).

### 1.3.1 Types of botulism

Four categories of botulism are recognized: (1) foodborne, (2) infant, (3) wound, and (4) undetermined. Foodborne botulism results from the consumption of food in which *C. botulinum* has grown and produced a toxin. Therefore, foodborne botulism is the result of an intoxication rather than an infection. The clinical signs and symptoms of botulism develop 12 to 72 hours after consumption of the toxin-containing food and are listed in Table 1 along with associated foods. The illness can last anywhere between 1 and 10 days and is dependent on various factors including host resistance, type and amount of toxin ingested, and type of food. Treatment options include administration of therapeutic antitoxin and appropriate supportive care, particularly respiratory assistance (Rhodehamel *et al.* 1992). Infant botulism is caused by the ingestion of *C. botulinum* spores that colonize and produce toxin in the intestinal tract of infants. This type of botulism is an example of a toxicoinfection. Honey has been implicated as a source of

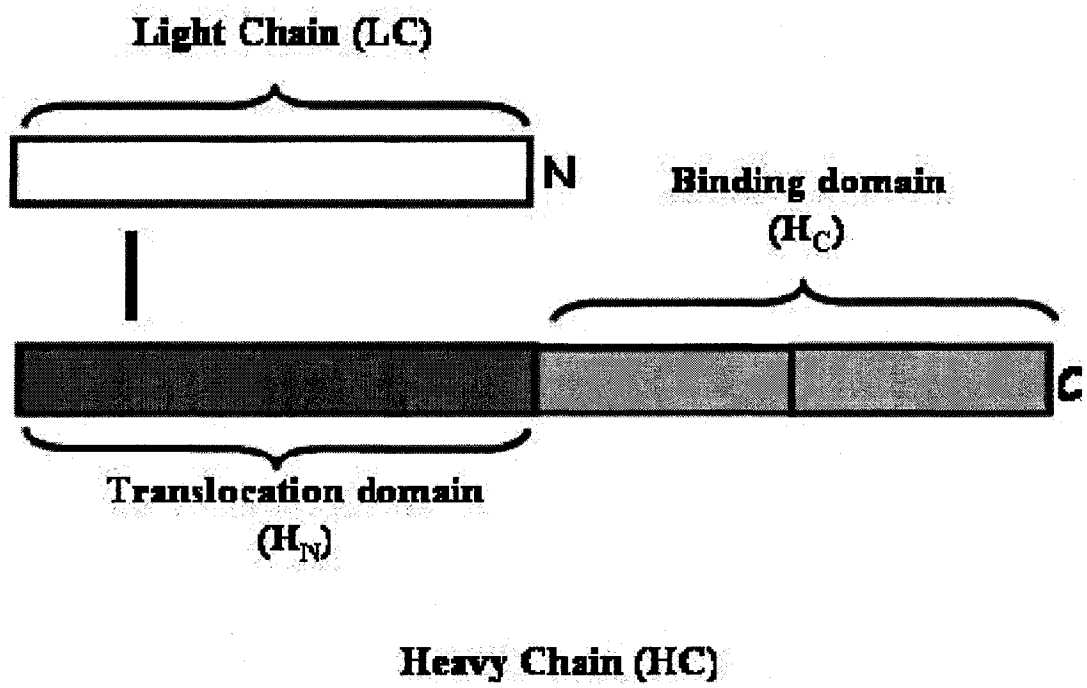
spores in outbreaks of infant botulism and clinical symptoms are given in Table 1.1. The rarest form of the disease is wound botulism and results when *C. botulinum* infect a wound and produce toxins which circulate to other parts of the body via the bloodstream. The fourth category of botulism, undetermined, involves individuals older than 12 months in which no food or wound source is implicated. The mechanism of paralysis is identical and the manifestations of the disease are generally the same, regardless of the manner in which the toxin gains access to the body (Hatheway, 1990, Rhodehamel *et al.* 1992).

### 1.3.2 BoNT structure and mechanism of action

BoNTs are synthesized as single-chain polypeptides of approximately 150 kDa that are cleaved by proteolytic enzymes to form active di-chain molecules (Figure 1.3). The active di-chain molecules consist of a light chain (LC) of approximately 50 kDa and a heavy chain (HC) of approximately 100 kDa. The LC acts as a zinc-dependent endopeptidase. The heavy chain comprises two fragments, each of approximately 50 kDa. The N-terminal fragment of the heavy chain is the translocation domain ($H_N$), which forms ion channels in lipid bilayers and translocates the LC into the neuron cytosol. The C-terminal half of the heavy chain is the binding domain ($H_C$), which plays an important role in the binding of the neurotoxin to the target cell membrane and internalization of the LC into cholinergic neurons (Montecucco *et al.* 1996; Turton *et al.* 2002).

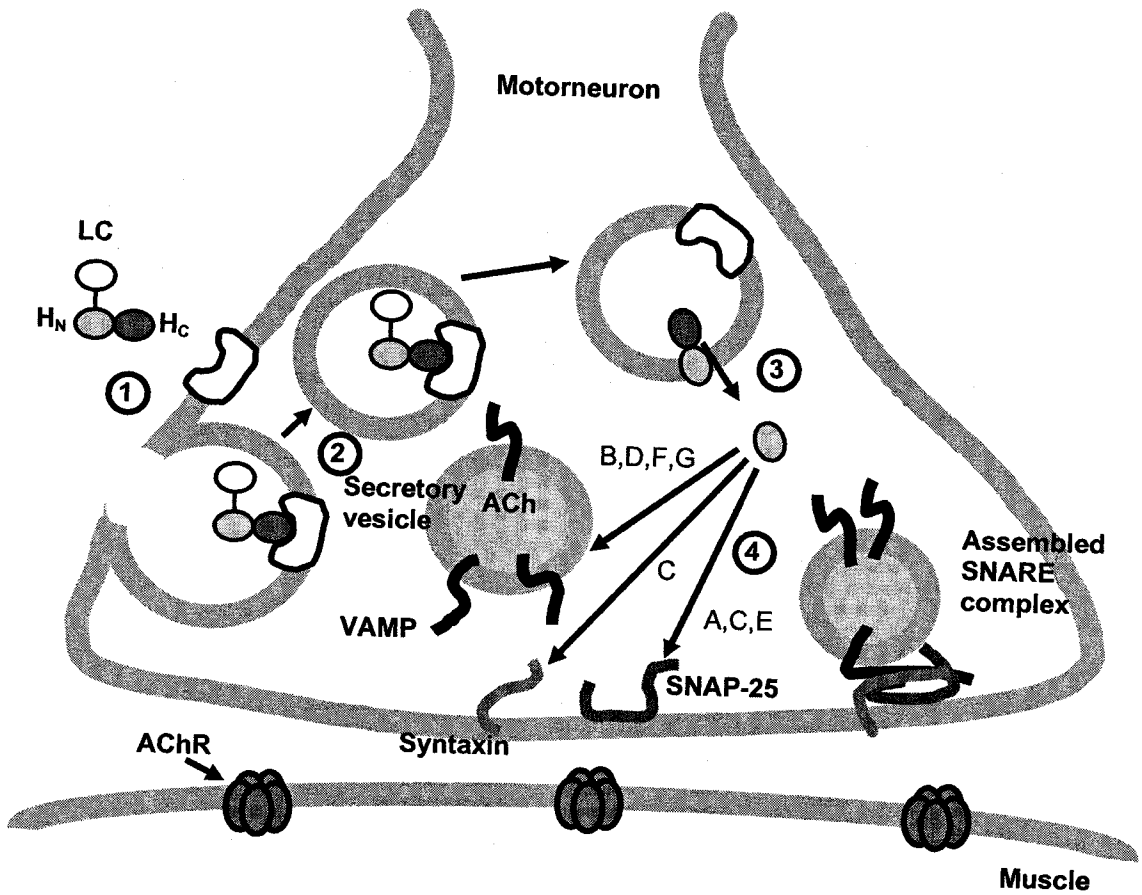
The mechanism of action by which BoNT intoxication causes paralysis is a multi-step process that involves each of the neurotoxin functional domains and can be

**Figure 1.3** Active di-chain structure of botulinum neurotoxins (BoNT) (reproduced from Turton *et al.* 2002). The active form consists of a light chain (LC) (*ca* 50 kDa) and a heavy chain (HC) (*ca* 100 kDa) linked together by a single disulfide bond. The LC (shown in white) acts as a zinc-dependent endopeptidase. The heavy chain contains two functional fragments, each of approximately 50 kDa. The N-terminal half of the heavy chain (shown in dark grey) is the translocation domain ($H_N$) which is responsible for ion channel formation through the endosomal membrane. The C-terminal half of the heavy chain (shown in light grey) is the binding domain ($H_C$).



described as the outcome of four distinct stages: (1) binding of neurotoxin to target cell, (2) neurotoxin internalization, (3) neurotoxin translocation, and (4) inhibition of neurotransmitter release (Figure 1.4). In the first step, BoNTs bind, via their $H_C$ domains, to ganglioside receptors on cholinergic nerve terminals. Once bound, the toxin-receptor complex is internalized by temperature and energy-dependent endocytosis. Once internalized in vesicles within the cell, the toxins can no longer be neutralized by antisera. The third step involves the translocation of the LC endopeptidase into the cytosol. This process is not fully understood, but is believed to involve pH-dependent conformational changes of the toxin within the acidic intracellular compartment, leading to greater hydrophobicity of the toxin molecule thus facilitating its penetration through the lipid bilayer. Once released from the intracellular compartment into the cytosol, the active LC performs the final stage in the intoxication process – highly specific proteolytic cleavage of one of the proteins of the SNARE (soluble NSF-attachment protein receptors) complex, leading to a block in the release of ACh to the extracellular environment resulting in an impairment of muscular contraction because acetylcholine receptors (AChR) remain inactive in the absence of ACh. The BoNT are highly specific and they only recognize and cleave three protein components of the SNARE complex that mediate neurotransmitter release at the synaptic terminals. This complex is composed of vesicle-associated membrane protein (VAMP, a protein of the ACh-containing small synaptic vesicles), and two proteins of the cytosolic surface of the presynaptic membrane: synaptosome-associated membrane protein of 25 kDa (SNAP-25) and syntaxin. In a normal muscle contraction, the influx of $Ca^{2+}$ at the nerve terminal triggers the fusion of the vesicle with the membrane of the presynaptic terminal, resulting in the release of

**Figure 1.4** Mechanism of action of botulinum neurotoxin (BoNT) (reproduced from Turton *et al.* 2002). Paralysis resulting from BoNT intoxication occurs as a multi-step process with four distinct phases: (1) binding of BoNT to target cell via its binding domain, (2) internalization of BoNT into target cell, (3) translocation of active LC into target cell cytosol, and (4) inhibition of neurotransmitter release. Abbreviations: $H_N$, heavy chain translocation domain; $H_C$, heavy chain binding domain; SNARE, soluble NSF-attachment protein receptors; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal-associated protein of 25 kDa; ACh, acetylcholine; AChR, acetylcholine receptor.



ACh. As mentioned, the BoNT serotypes are highly specific for the proteins they cleave (Figure 1.4). BoNTs A and E act on SNAP-25, whereas BoNTs B, D, F, and G cleave VAMP, and BoNT C cleaves both SNAP-25 and syntaxin. When individual SNARE proteins are cleaved, complex formation is not prevented, but $Ca^{2+}$ entry and membrane fusion is disrupted, thus blocking the release of ACh, and ultimately leading to muscle paralysis (Montecucco *et al.* 1996; Turton *et al.* 2002).

## 1.4 *Vibrio cholerae*

*Vibrio cholerae* is a Gram negative, motile, curved rod that belongs to the family Vibrionaceae. Over 200 recognized O serogroups are known, but only the O1 and O139 serogroups have been associated with severe disease and cholera pandemics. The O1 serogroup is divided into two biotypes, classical and El Tor, differentiated on the basis of physiological properties, and each of the O1 serogroups can be further subdivided into one of three different serotypes termed Inaba, Ogawa, and a minor serotype Hikojima (Reidl and Klose, 2002; Sack *et al.* 2004).

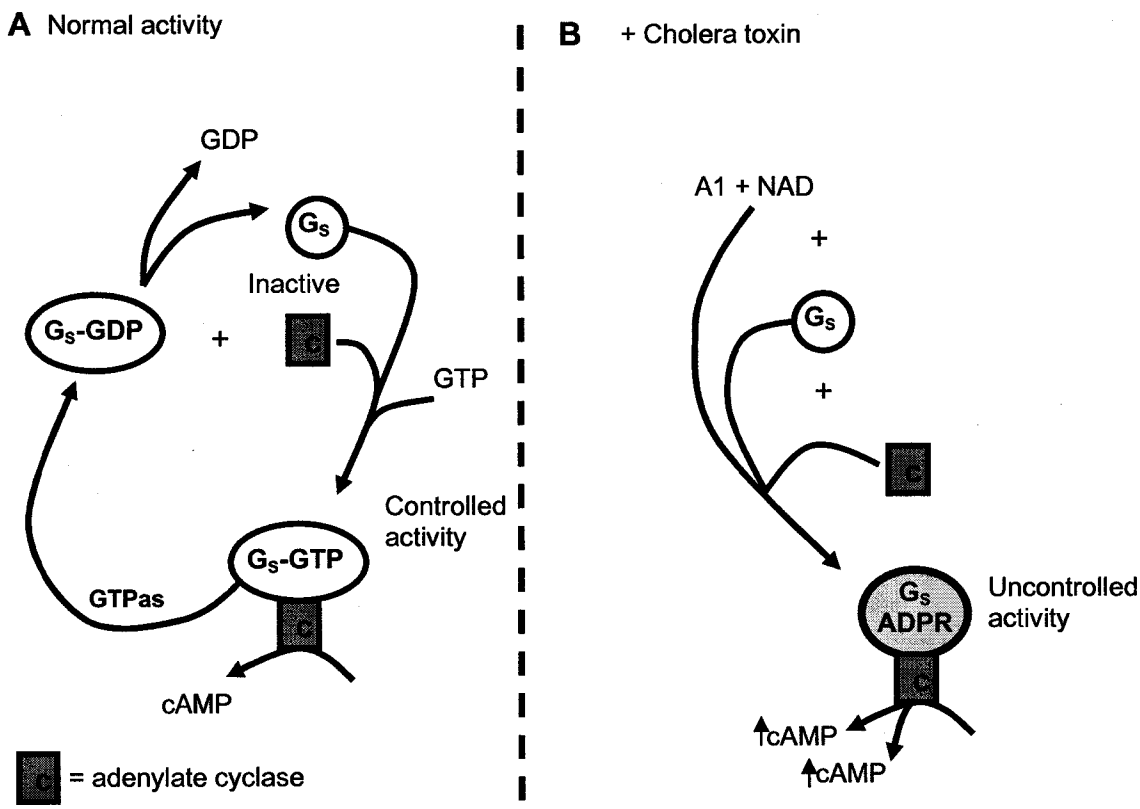
Infections by *V. cholerae* usually begin with the oral ingestion of the organism. Although epidemiological evidence indicates that cholera is mainly a water-borne disease, various foods, depending on their physico-characteristics, have also been recognized to be an important vehicle of transmission of the organism. Food characteristics that enhance the growth of *V. cholerae* include low temperature, high-organic content, neutral or alkaline pH, high-moisture content, and the absence of competing microorganisms in the food. Foodborne outbreaks of cholera have often occurred due to the consumption of contaminated seafoods (Rabbani and Greenough III,

1999). The infectious dose for *V. cholerae* infection is very high (Table 1.1) and is probably due to the acid sensitivity of the organism, which is exposed to low pH in the stomach (Kaper *et al.* 1995; Reidl and Klose, 2002). Cholera is characterized by painless massive watery diarrhea resembling rice-water, and vomiting. In the most severe cases, the rate of diarrhea can rapidly reach 500 to 1000 ml per hour, leading to severe dehydration, and death if not treated promptly (Sack *et al.* 2004).

### 1.4.1 Mechanism of action of cholera toxin (CT)

After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of a variety of factors including filamentous protein structures called toxin coregulated pili (TCP). TCP mediate colonization of the small intestine by facilitating microcolony formation via pilus-mediated bacteria interactions (Sack *et al.* 2004). The mechanism of action by which *V. cholerae* causes the symptoms of cholera is mediated by the action of the CT (Figure 1.5). The CT has an A-B type toxin structure consisting of one A subunit in association with five B subunits where each of the subunits has a specific function. The role of the B subunit is to bind the toxin to the epithelial cell receptors (ganglioside, $G_{M1}$), whereas the A subunit possesses enzymatic activity. The A subunit is not enzymatically active until it is nicked to produce fragments $A_1$ and $A_2$, which are linked by a disulfide bond. Once bound to the $G_{M1}$ ganglioside receptors, the enzymatically active $A_1$ subunit is released from the toxin and is translocated into the host cell cytosol where it ADP-ribosylates a host cell membrane protein called $G_s$, responsible for the regulation of the activity of adenylate cyclase. ADP-ribosylation of $G_s$ permanently activates adenylate activity, leading to

**Figure 1.5** Mechanism of action of CT. (A) Diagram illustrating the active and inactive forms of $G_s$, a GTP-hydrolyzing protein, which regulates the activity of host cell adenylate cyclase in a hormone-dependent manner, thus determining the level of cAMP in host cells. The active form of $G_s$ increases the activity of adenlyate cyclase, whereas the inactive GDP-bound form inactivates adenylate cyclase. (B) ADP-ribosylation (ADPR) of $G_s$, permanently activates adenylate cyclase so that cAMP continues to be produced and levels of cAMP rise in the host cell leading to a disruption in ion transport and resulting in severe water loss in the form of diarrhea (reproduced from Salyers and Whitt, 2003).



uncontrollably high levels of cAMP, resulting in the alteration of sodium and chloride transporter activity producing ion imbalances causing the severe water loss associated with cholera (Kaper *et al.* 1995; Reidl and Klose, 2002).

## 2. Enhancing food safety: Hazard Analysis Critical Control Point (HACCP) programs

The presence of potentially life-threatening bacterial pathogens in our environment, the ability of some of them to produce harmful toxins, and, for some pathogens, the ability to resist to a range of antimicrobial agents underscores the seriousness of the potential hazards with which we are faced. In order to enhance food safety, the food industry began instituting hazard analysis critical control point (HACCP) programs in food processing establishments to limit potential hazards. In Canada, federally registered fish and seafood processing establishments are required by law to have HACCP systems in place. In addition, meat slaughter establishments exporting to the U.S. are required to implement a HACCP system in order to maintain access to the U.S. market. Many other commodity sectors, including processed fruits and vegetables, shell and processed eggs, hatcheries, dairy, honey and maple syrup are also voluntarily implementing HACCP principles in their establishments. HACCP programs are designed to identify and prevent hazards, whether biological (e.g. bacteria, viruses, or parasites), chemical (e.g. pesticide residues, veterinary drugs), or physical (e.g. contaminated raw material), posed by contaminated food. The HACCP approach is based on seven principles aimed at identifying hazards in food production, controlling these hazards through monitoring at critical control points in the process, and finally verifying that the system is working

properly. First, food processing companies identify hazards that have the potential to cause illness or injury to the consumer and list preventive measures to control them. For the food safety hazards identified, companies are required to identify the critical control points (CCPs) in the food processing continuum, and establish critical limits for their control. Next, CCPs must be monitored to ensure that the critical limits are not violated and establish corrective actions in the event of deviations to the critical limits. Finally, companies must maintain effective record-keeping associated with the HACCP system. While the food industry is responsible for ensuring that adequate control measures are in place for any potential hazards identified in the HACCP system, government inspectors working in HACCP systems are responsible for auditing the establishment's records and procedures, assessing specified control measures and corrective actions taken, and observing on-line processing specifically at critical control points. It is impossible to create a risk-free food supply, which underscores the continued need for methods that will provide adequate information on the presence of foodborne pathogens, such as bacteria, in the finished food products in order to verify that the HACCP systems established are working properly.

## 3. Methods in food safety testing

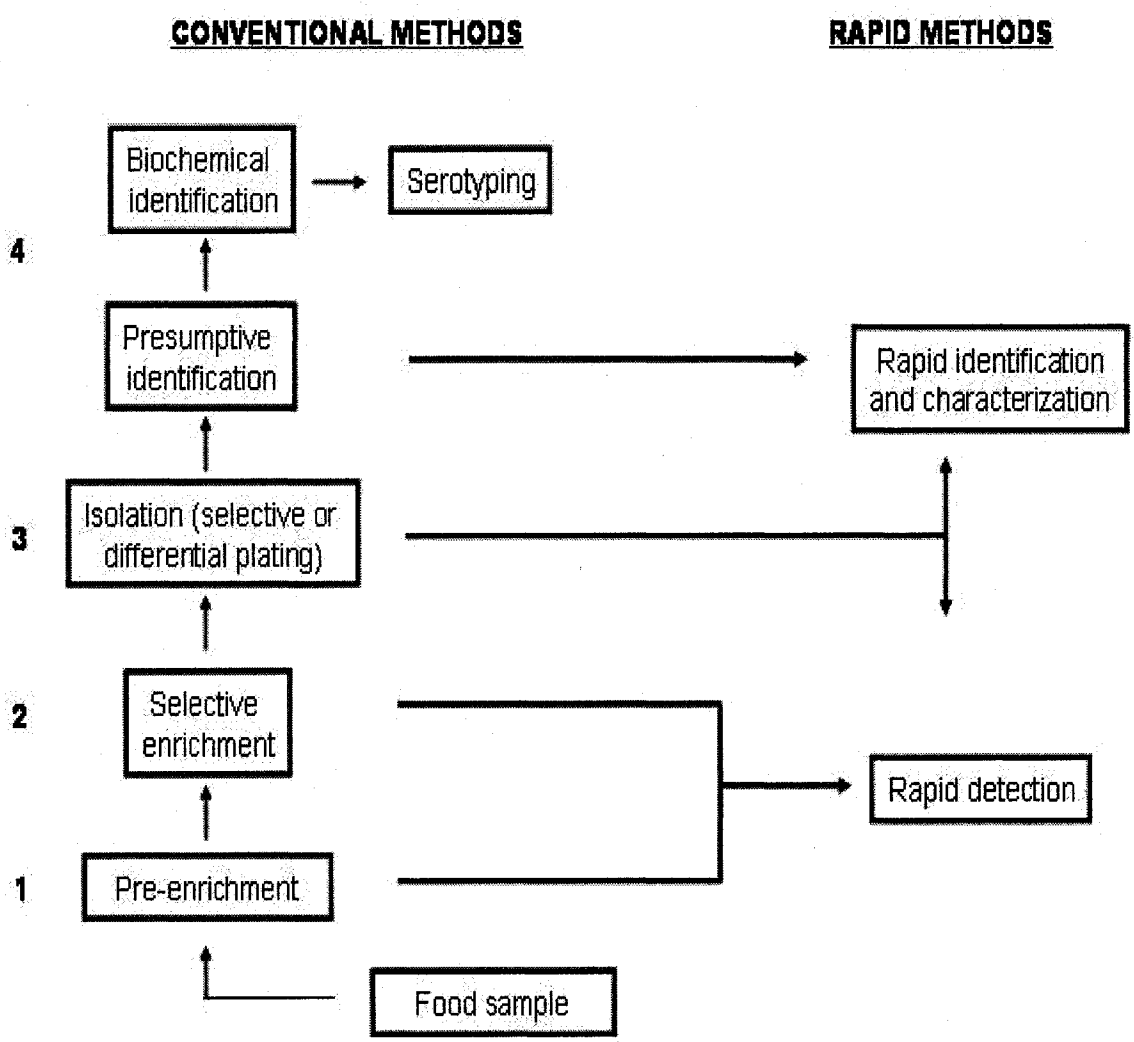
The analysis of foods for a specific pathogen or a toxin is a challenging task. Foods are composed of a wide array of ingredients, some of which can adversely affect bacterial cell viability, thus interfering with their recovery from the food sample. Secondly, some foods contain large populations of indigenous microbial flora which can mask the presence of pathogenic bacteria often found in very low numbers in the food sample. To

overcome these challenges, food microbiologists have relied on cultural methods for the detection and identification of pathogenic bacteria.

## 3.1 Conventional methods

The traditional approach for the detection and identification of pathogenic bacteria in food samples involves a process with four distinct phases (Figure 1.6): (1) pre-enrichment, to allow the recovery of any stressed or injured organisms that may be present in the food sample; (2) selective enrichment, to allow the proliferation of the target bacteria whilst suppressing the growth of non-target bacteria in the broth; (3) isolation, using selective agar plates to produce presumptive isolates of the target organism; (4) confirmation of presumptive isolates by a battery of tests of a morphological, immunological, or biochemical nature. Such conventional methods are time-consuming because they rely on the ability of bacteria to multiply to visible colonies. In addition, tasks such as culture medium preparation, inoculation of plates, colony counting, biochemical screening, and serological characterization make these procedures labour-intensive. For example, a typical isolation and identification scheme for detecting *Salmonella* from foods requires a minimum of 4 days for the confirmation of presumptive positive results, which includes 24 hours of pre-enrichment, 24 hours of selective enrichment, 24 hours of selective plating, followed by purification of presumptive positive isolates and their characterization using a variety of biochemical, serological and molecular tests (D'Aoust and Purvis, 1998). As a result, standard methods are inadequate in providing information within a timeframe enabling timely

**Figure 1.6** Steps involved in a conventional scheme for the isolation and characterization of pathogenic bacteria from foods. Also shown are the steps at which rapid methods could be applied in the interest of saving time.



assessments on the microbiological safety of foods. In a HACCP system, there is a need to screen final food products quickly for quality control purposes. The use of rapid methods for this purpose would allow food manufacturers to release lots of product for distribution without the need to wait several days for standard food microbiology isolation and identification.

## 3.2 Rapid methods

In response to the labour-intensive and time-consuming conventional microbiological tests, scientific advances in diagnostic technology have introduced a new generation of testing methods aimed at simplifying microbiological procedures and, more importantly, reducing assay times (Figure 1.6). These "rapid methods" can be divided into various categories including: (1) miniaturized biochemical tests; (2) antibody-based assays; and (3) nucleic acid-based technologies (de Boer and Beumer, 1999).

### 3.2.1 Miniaturized biochemical tests

Once bacteria are isolated from foods, they are usually identified based on their biochemical characteristics. The generation of biochemical profiles using standard methods is labour-intensive and media-consuming. In the interest of simplifying and automating the identification of individual microorganisms, several commercially available biochemical identification kits have been developed. [e.g. API® systems (BioMérieux), Enterotube II™ (BBL)]. Kit formats are similar, consisting of disposable strips containing 14 to 20 tests. The performance characteristics of these miniaturized

systems are accurate, efficient, labour and space saving, and cheaper than conventional procedures (Swaminathan and Feng, 1994).

### 3.2.2 Immunological methods

Immunological methods rely on the highly specific binding of an antibody to an antigen. These methods can be classified into different categories on the basis of their assay formats including latex or slide agglutination, immunoprecipitation and enzyme-linked immunosorbent assays (ELISA). Latex agglutination (LA) uses antibody-coated colored latex beads to agglutinate specific antigens (bacterial cells) to form a visible clump or precipitate. Commercially available kits using this assay format have been developed such as the *E. coli* O157:H7 LA test (Unipath) and the RAPIDTEST™ for *Salmonella* (Unipath). Although these assays are simple and specific, they lack in sensitivity requiring approximately $10^7$ bacterial cells to obtain positive reactions. A similar assay format, the reverse passive latex agglutination (RPLA), is used for the analysis of bacterial toxins in foods, and differs from the traditional LA in that it detects soluble antigens (toxins) rather than insoluble antigens (cells). The Verotox-F assay (Denka Seiken), was developed for the detection of VT in *E. coli* culture filtrates and for the characterization of toxin phenotypes and is commercially available (Feng, 1997). Several commercially available antibody assays, which employ the immunoprecipitation format, have been introduced for detecting foodborne pathogens. The format for these assays is the dipstick, which is a small disposable plastic device coated with detection and capture antibodies to capture cells (antigen) from pre-enrichment or selective enrichment media. As the enrichment sample is absorbed across the pads, the antigen, if present, will first

react with the labelled antibody and then with the second antibody which captures the complex to form a visible band of immunoprecipitation (Feng, 1997). These assays are simple and rapid, reducing the time required for detection. Commercial assays employing this format have been developed such as the Reveal™ lateral flow device (Neogen) for the detection of *E. coli* O157:H7 or the choleraSMART™ (New Horizon) for the detection of *Vibrio cholerae*. The ELISA is the most widely used assay format for the detection of bacteria in foods (Feng, 1997). Many commercially available kits are designed in the form of a "sandwich" assay format. This format involves the capture of the antigen of interest by an antibody that is previously immobilized on a solid matrix, followed by addition of a second antibody conjugated with an enzyme to form an antibody-antigen-antibody (sandwich) complex. The sandwich complex is then detected by addition of the enzyme substrate, and the results can be recorded visually or with a spectrophotometer (Swaminathan and Feng, 1994). The most common solid phase used in ELISA is the microtiter plate, but other supports such as microporous membranes (Feng, 1997), and polyester cloth can also be used (Blais and Yamazaki, 1997). Several commercially available kits have been developed to detect pathogenic bacteria and bacterial toxins in foods using the ELISA format. Examples are the Assurance™ EHEC ELISA (Biocontrol), and the ELCA™ *C. botulinum* toxin ELISA (Elcatech). Immunoassay techniques such as ELISA are simple to operate and offer the potential for high sample throughput, but are relatively insensitive, requiring high target cell densities ($10^5$ CFU/ml) to produce significant assay signals (Feng, 1997).

### 3.2.3 Nucleic acid-based methods

The past decade has seen a dramatic increase in the development of nucleic acid- based methods for the detection and characterization of foodborne pathogens. The essential principle of this technology is the specific formation of double stranded nucleic acid molecules from two complementary, single stranded molecules under defined physical and chemical conditions. When performed *in vitro*, this process is termed hybridization. DNA-based methods have become increasingly popular for the detection and identification of foodborne bacterial pathogens. Included in this category are gene probes and nucleic acid amplification techniques such as the polymerase chain reaction (PCR), which are available for most common foodborne bacteria.

#### 3.2.3.1 Gene probes

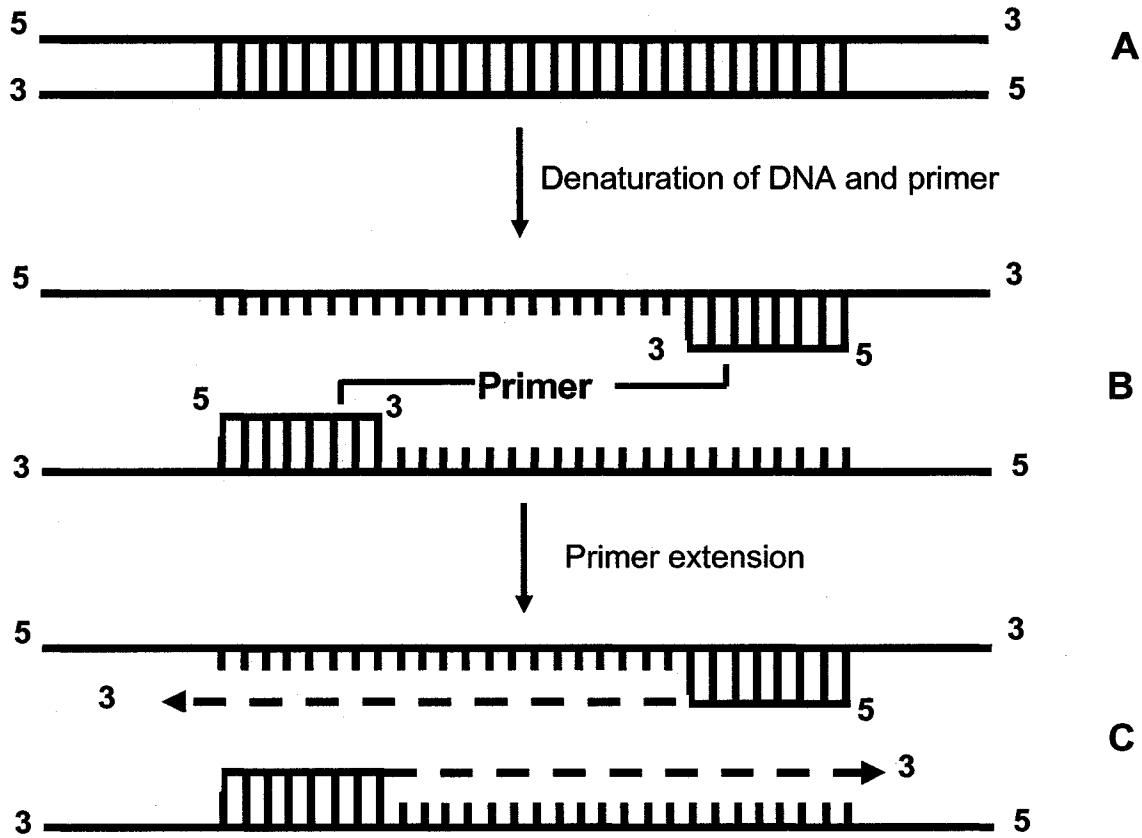
A gene probe is a short sequence of nucleotide bases (15 to 30 nucleotides) used to distinguish target bacteria from nontarget bacteria by binding of the probe to specific regions of a target sequence of nucleotides where the homology between target sequence and the DNA probe results in a stable hybridization (Tietjen and Fung, 1995). DNA probe assays use one of two solid-phase DNA hybridization formats. The dot-blot hybridization format uses nitrocellulose or nylon membranes as a solid phase for the immobilization of crude or purified DNA containing the target sequence. The colony hybridization format involves the transfer of bacterial cells from colonies on plating media to nitrocellulose or nylon membranes. Samples are prepared by lysing the bacterial cells (for colony hybridization tests) to release the double stranded DNA. Double-stranded DNA is converted to single-stranded DNA using heat or alkaline

treatment, followed by immobilization of the single-stranded DNA to the solid support. The probe is then added under specific conditions and allowed to hybridize with the single stranded DNA on the solid support (Swaminathan and Feng, 1994). The probes can be labelled using radioisotopes but the risks associated with using these labels has led to the use of alternative non-radioactive labels such as biotin or digoxigenin (Sambrook and Russell, 2001). Several hybridization assays for the detection and identification of foodborne bacteria have been commercialized. The Gene-Trak® system (Neogen Corporation) uses pathogen-specific DNA probes specific to ribosomal RNA targets in the bacteria and a colorimetric system for the detection of the specific probe-target hybrids. DNA hybridization assays are more specific than antibody-based assays because they directly target the nucleic acid of an organism. However, these assays require the selective enrichment of food samples to obtain the required sensitivity of $10^4$ – $10^5$ bacteria cells (Swaminathan and Feng, 1994).

#### 3.2.3.2 The polymerase chain reaction (PCR)

The PCR technique has revolutionized the field of molecular diagnostics through the provision of ultra-sensitive amplification and detection of specific nucleic acid sequences. The amplification of target nucleic acid sequences is achieved through a cyclic three step process of denaturation, primer annealing and primer extension resulting in an exponential amplification of the target sequence when the cycle is repeated many times (Figure 1.7). The first step in the PCR cycle involves the denaturation of the double-stranded DNA molecule into single-stranded DNA using heat (usually 94 °C). In the second step of the cycle, primers that are homologous to flanking segments of

**Figure 1.7** Steps in the basic PCR. (A) A target gene of interest is chosen for amplification. Short oligonucleotide sequences that serve as primers for DNA synthesis are designed so that they flank the region to be amplified. (B) The target DNA strands are denatured at high temperature (usually 94 °C) to form single strands. The temperature is then lowered to a level that allows the specific annealing of the primers to the target strands. (C) The temperature is then raised to a level optimum (usually 72 °C) for a thermostable DNA polymerase, which synthesizes new strands using the oligonucleotide sequences as primers and the target DNA as template, thus in theory, doubling the amount of target DNA after each cycle. When the PCR is repeated for many cycles using newly synthesized DNA as template in addition to the original target DNA, an exponential amplification of the target DNA is achieved (reproduced from Hill, 1996).



complementary strands of the target DNA are allowed to anneal to the target DNA under stringent conditions of temperature. The choice of temperature for the annealing step is a critical parameter. If the chosen annealing temperature is too high, primers will anneal poorly, resulting in a low yield of amplified DNA. On the other hand, if the annealing temperature is too low, non-specific annealing of the primers can occur, resulting in the amplification of unwanted DNA fragments. The third step involves the extension of the annealed primers by a thermostable DNA polymerase using target DNA sequences as template (Swaminathan and Feng, 1994). The most important advantages of PCR over traditional culture methods are its speed, sensitivity, and specificity. For example, a single target can generate $1.5 \times 10^6$ copies after 30 cycles of PCR (Swaminathan and Feng, 1994). In theory, this method can detect as low as one molecule of target DNA within a PCR reaction, and can therefore be used to indirectly detect extremely low concentrations of microbes based on a target gene sequence.

#### 3.2.3.2.1 Choice of a target for PCR sequence amplification

One of the first and most important parameters in assuring specific detection and identification of a bacterial pathogen in a PCR-based assay is the choice of the target sequence for amplification. The choice of target for primer design can include virulence genes, antibiotic resistance genes, or strain-specific genes. In designing PCR-based assays for this purpose, a good choice of target is a gene sequence that is unique to the target organism and that is absent in non-target organisms. Another wise choice for primer design is targeting a gene that distinguishes pathogenic bacteria from non-pathogenic bacteria within the same species. For example, while *E. coli* are commensal

members of the gut flora of warm-blooded animals, certain strains of *E. coli* (i.e. EHEC) are a major cause of diarrheal disease. Pathogenic and non-pathogenic strains of *E. coli* are difficult to distinguish from each other using selective or differential cultivation methods. Therefore, PCR could be used as an alternative method to the traditional cultivation techniques for the rapid characterization of isolates through the amplification of their toxin genes. A great number of PCR-based assays have been developed for the detection of several foodborne pathogenic bacteria including but not limited to *Salmonella*, *E. coli*, *Shigella*, *V. cholerae*, *Yersinia enterocolitica*, *Campylobacter*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *C. botulinum* (Olsen *et al.* 1995).

#### 3.2.3.2.2 Multiplex PCR

An increasingly popular approach that increases the efficiency of diagnostic PCR is to combine multiple primer pairs in one single PCR reaction allowing simultaneous amplification of multiple gene targets, a process called multiplex PCR. The co-amplification of several molecular markers in one PCR reaction increases the amount of information that can be rapidly generated from a sample in order to assist in investigations of contamination incidents. Several multiplex PCR methods have been developed for the specific detection and characterization of foodborne pathogens (Khan *et al.* 2000; Lindström *et al.* 2001; Nagano *et al.* 1998; Osek, 2001).

#### 3.2.3.2.3 PCR amplicon detection

In order to verify that the PCR assay is both specific and sensitive, it is necessary to detect the PCR amplicon. This can be achieved using size-dependant or sequence-dependant methods (Lantz *et al.* 2000). One of the most commonly used methods for detecting amplified nucleic acids on the basis of their molecular size consists of electrophoresis in agarose gels followed by ethidium bromide staining. Although very simple, this method of detection has low sensitivity, requiring approximately 10 ng of amplicon to produce a visible band on a gel (Sambrook and Russell, 2001), and is not suitable for processing large numbers of samples (Swaminathan and Feng, 1994). In addition to low sensitivity, results can sometimes be difficult to interpret: more than one band on the gel where only one was expected, a diffuse or faint band. Detection of PCR amplicons using this method could also yield false positive results in the event that non-specific amplification products of similar size are produced by chance (Altwegg, 1995). In the event that any of these scenarios present themselves, the results of PCR tests must always be confirmed using other techniques, such as restriction endonuclease analysis.

Assay specificity and sensitivity can be greatly improved by the incorporation of non-radioactive labels [e.g. digoxigenin (DIG)] into the PCR amplicons, followed by hybridization with a DNA probe and detection of the bound label using a suitable detector reagent (e.g. anti-DIG-peroxidase conjugate) (Monteiro *et al.* 1997). The use of a micro-well format in which the DNA capture probe is immobilized in the wells of a microtiter plate enables the processing of large numbers of samples. Another advantage of nucleic acid hybridization assays is that the specificity of the amplicons is confirmed

by hybridization with a target specific probe, obviating the need for time-consuming restriction endonuclease analysis.

More recently, DNA microarray technology has emerged as a useful endpoint of multiplex technology. Basically, DNA microarrays are a miniaturized form of a dot blot assay, only in a high-throughput format. Applications of this technology are usually directed at gene expression analysis or screening samples for single nucleotide polymorphisms (SNPs) (Heller, 2002), but a number of DNA microarray systems have recently been developed for use in the detection and identification of pathogenic bacteria (Chizhikov *et al.* 2001; Strizhkov *et al.* 2000). A typical DNA microarray experiment is a four-step process involving array fabrication, probe preparation, hybridization, and data analysis. The microarray technique is based on a sophisticated approach in which a large number of different molecular marker sequences (probes) are deposited on miniaturized arrays (typically on glass slides) using an arrayer. The probes are then immobilized onto the glass matrix and denatured using heat or alkali treatments. DNA samples for analysis are labelled with fluorescent dyes and hybridized with the probes on the microarrays and then scanned using a specialized (and costly) microarray scanner to record the fluorescence patterns (Gabig and Wegrzyn, 2001; Ye *et al.* 2001) and analysed using specialized software. In many instances, a rather large preponderance of marker sequences (>1000 of individual probes) are used, many of which correspond to DNA sequences of unknown function. The information obtained from such microarray systems is highly complex, and while useful to the epidemiologist and specialized reference laboratory, is of limited value to the food testing laboratorian simply wishing to ascertain the presence or absence of a pathogen, and perhaps, to identify some key markers such as

virulence, toxin and antibiotic resistance genes. These systems also rely on sophisticated and costly instruments to prepare and process the arrays, and are not generally within the scope of analytical capabilities of the typical food microbiology laboratory.

## 4. Need for simple, rapid, and inexpensive methods for pathogen detection and characterization

There is a need for simple, rapid, and inexpensive low density DNA arrays that have the ability to detect the presence of specific key molecular markers associated with foodborne pathogenic bacteria.

### 4.1 Choice of a solid phase for DNA hybridization

The choice of a solid phase matrix for nucleic acid hybridization assays is one of the most important considerations in the design of a diagnostic test. The nature of the solid phase will dictate the format of the test along with its operational characteristics. In the interest of designing a low density DNA array technology for the detection and characterization of pathogenic bacteria in finished food products as a requirement in a HACCP system, the test should have a rapid turnaround time, be easy to perform, and most importantly, incur minimal costs to generate results on the nature of the food product. Nucleic acid hybridization techniques have traditionally been performed using a variety of non-porous (e.g. microtiter plates), microporous (e.g. membranes), and particulate (e.g. beads) solid phases (Wolcott, 1992). While effective, these solid phases have some or all of the following drawbacks: slow reaction kinetics, limited accessibility of reactants to the solid

phase surface, complex handling and washing procedures at each step of the assay, and difficulty in simultaneously processing multiple samples.

## 4.2 Macroporous hydrophobic polyester cloth: a novel solid phase

To overcome some of the disadvantages inherent in the traditional solid phases, the concept of utilizing macroporous hydrophobic polyester cloth as a solid phase was originally developed for enzyme immunoassays (EIA) (Blais and Yamazaki, 1997).

### 4.2.1 Advantages of polyester cloth as a solid phase

The use of polyester cloth as a solid phase offers a number of different advantages. The hydrophobic surface of the cloth allows for the immobilization of a wide variety of reactants (e.g. protein, lipid, and nucleic acids) by passive adsorption to create a high affinity solid phase for the capture of specific analytes, and their subsequent assay by immunoenzymatic detection. The macroporosity of the polyester cloth presents a large surface for rapid and efficient reactions with the test sample. Its greater thickness, in comparison to conventional microporous membranes (e.g. nylon and nitrocellulose), enables the accommodation of relatively larger volumes of sample per unit of area. Furthermore, the ease with which liquids flow through the cloth matrix results in much simpler, and faster, washing procedures to efficiently remove unbound material after each step in the assay. Finally, polyester cloth is an inexpensive material and economical with respect to reagent consumption, particularly when simultaneously processing multiple samples (Blais and Yamazaki, 1997).

### 4.2.2 Various applications using polyester cloth as a solid phase

Various applications have been developed over the past decade, which rely on macroporous hydrophobic polyester cloth as a solid phase. One such application was the development of a cloth-ELISA test for the detection of *Brucella* antigens from broth enrichment cultures of blood or tissues in order to assist in the more rapid diagnosis of infectious diseases in clinical specimens (Blais *et al.* 1989). Another important application that exploits the advantages of polyester cloth as a solid phase was the development of a cloth-based enzyme immunoassay for the detection of peanut proteins as a simple and inexpensive test for peanut allergen detection in foods (Blais and Phillippe, 2000). Since then, a more efficient approach was developed using a reverse dot blot EIA format and polyester cloth as a solid phase for the simultaneous detection of multiple allergens in foods, including hazelnut, Brazil nut, and peanut (Blais *et al.* 2003). Another application was developed recently in response to consumer concerns about genetically modified crops. This application uses polyester cloth as solid phase for the detection of amplicons from a multiplex PCR, targeting a variety of genetic elements in transgenic crops, by hybridization with an array of immobilized probes (Blais *et al.* 2002).

## 5. Objectives

This thesis describes the development of cloth-based hybridization systems (CHS) as simple and cost-effective tools for the rapid detection and characterization of foodborne pathogenic bacteria. These systems would provide information to the food testing laboratory on the nature of foodborne isolates within a timeframe enabling a more timely

response by regulators and the food industry in order to minimize the spread of contamination of the food supply with bacterial pathogens.

The labelling and detection of nucleic acids, such as PCR amplicons, is a fundamental process in molecular biology. Non-radioactive methods for the labelling of nucleic acids have replaced traditional radioactive methods because they offer equal sensitivity, are much faster, more convenient and, most importantly, safer. The labelling of nucleic acids using biotin or digoxigenin as non-radioactive labels have been described (Morris *et al.* 1990; McQuaid *et al.* 1995). Biotin has been reported to work well, but high levels of background can occur during hybridization assays because biotin is a ubiquitous constituent of mammalian tissues, and biotinylated probes tend to stick non-specifically to solid phases such as nylon membranes (Sambrook and Russell, 2001). Digoxigenin, unlike biotin, has low affinity for membranes of all types and does not occur naturally in most biological sample material, only in Digitalis plants (Sambrook and Russell, 2001). In Chapter 2, different methods are compared for the non-radioactive labelling of polymerase chain reaction (PCR) products with either digoxigenin or biotin for their detection by hybridization with immobilized DNA probes. The most effective method for the incorporation of the chosen label into PCR amplicons will be applied in the development of the cloth-based hybridization systems described in the following chapters.

The primary objective of the food-testing laboratory is to provide the information required for judicious analyses of the hazards associated with the distribution and consumption of foods (and related agricultural commodities), and occasionally, to provide details on specific characteristics of the hazardous agent which might be useful in

trace back investigations. In food microbiology testing, the nature of the information can vary from merely assessing the presence or absence of specific pathogenic bacteria, to determining the serotype or the presence of key virulence markers and the toxigenic potential of the organism.

As mentioned earlier, the traditional approach for the detection and characterization of foodborne isolates is a laborious process involving the cultural enrichment of foods in various selective and non-selective media to favor the growth and the isolation of pathogenic bacteria of interest. Presumptive positive isolates are then subjected to characterization, using a variety of biochemical, serological, and molecular tests which can take several days to weeks before results can be generated. Chapter 3 describes the development of a simple cloth-based hybridization array system for the detection of antibiotic resistance and other marker genes associated with the multidrug-resistant foodborne pathogen *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104. This system will provide a reliable tool for the characterization of presumptive positive *Salmonella* colonies isolated from foods and related agricultural commodities, based on their antibiotic resistance profiles, using suitable enrichment culture techniques, therefore providing information to the food testing laboratorian on the nature of the isolates in a quicker timeframe to standard characterization procedures.

In addition to testing for many pathogens associated with foods, most food testing laboratories are required to have the capacity to process a large number of samples for the presence of specific pathogenic bacteria in foods. For example, the isolation of *Salmonella* from a raw retail chicken carcass should not come as a surprise since raw poultry is considered to be a major source of this foodborne pathogen (Jorgensen *et al.*

2002). Therefore, the isolation of this pathogen from a poultry carcass should not be the cause for alarm since we are encouraged to cook chicken prior to consumption. What is significant is the isolation of strains such as *S.* Typhimurium DT104 from food commodities such as poultry, whose multidrug-resistance to a core of antimicrobial agents can make treatment a challenge. In response to these needs, Chapter 4 describes an attempt to develop a method for the detection of *S.* Typhimurium DT104 directly from foods, a procedure combining a polymerase chain reaction targeting the integrase gene, and a simple cloth-based dot blot hybridization system as a means to screen a large number of food samples for the presence of this significant foodborne pathogen.

The presence of toxigenic bacteria in the food supply is a major public health risk, and their detection to prevent foodborne transmission to humans is a major challenge for regulatory agencies and the food industry. While enrichment culture techniques for the isolation of the major pathogenic bacteria from foods are generally well established, there is a need for methods that facilitate the characterization of the toxigenic potential of isolates in order to provide information for judicious risk assessments of the hazards associated with food consumption. Chapter 5 describes the development of a simple cloth-based hybridization array system for the identification of toxin genes associated with major foodborne pathogenic bacteria. This system will provide information on the nature of the isolates at a much earlier juncture than standard bioassay or cell culture techniques, enabling its use as a reliable tool for the rapid and specific characterization of colonies isolated from food samples or clinical specimens using suitable enrichment culture techniques.

*C. botulinum* is an important foodborne pathogen with the ability to produce highly potent neurotoxins, which are responsible for the paralytic disease known as botulism. The standard method for toxin detection and the characterization of bacterial isolates is the mouse bioassay, a procedure which requires specialized facilities, and is costly, labour-intensive, and time-consuming. There is a need for rapid and practical methods for the characterization of bacterial isolates with respect to botulinum toxin production. Chapter 6 describes the development of a cloth-based hybridization array system for the detection of *C. botulinum* based on neurotoxin serotypes A, B, E, and F. This system will provide information regarding the serotype of the isolates at an earlier juncture than the conventional mouse bioassay, enabling its use for the rapid and specific characterization of *C. botulinum* strains isolated from food samples or clinical specimens using suitable enrichment culture techniques.

# CHAPTER 2

## Comparison of Different Approaches for the Incorporation of Non-Radioactive Labels into Polymerase Chain Reaction Products

"This chapter has been published in a peer-reviewed scientific journal:

Gauthier, M., and Blais, B.W. (2003) *Biotechnology Letters* **25**: 1369-1374."

**Abstract**

Different methods for labelling polymerase chain reaction (PCR) products with non-radioactive labels for their detection by hybridization with immobilized DNA probes were compared. The use of digoxigenin (DIG) as a label provided greater sensitivity than biotin in a PCR system targeting the *invA* gene from *Salmonella enterica* serotype Typhimurium. Incorporation of digoxigenin into amplicons in the form of 5 -DIG-labelled oligonucleotide primers resulted in better assay signals and was more economical than DIG-labelled dUTP.

**Introduction**

The PCR technique has revolutionized the field of molecular diagnostics through the provision of ultra-sensitive amplification and detection of specific nucleic acid sequences. The traditional approach to PCR involves the amplification of target nucleic acids followed by the detection of amplicons on the basis of their molecular size by agarose gel electrophoresis. The specificity and sensitivity of amplicon detection can be improved by the incorporation of non-radioactive labels [e.g. digoxigenin (DIG) or biotin] into the amplicons, followed by hybridization with a DNA probe and detection of the bound label using a suitable detector reagent (e.g. anti-DIG antibody-peroxidase or streptavidin-peroxidase conjugates for DIG and biotin labels, respectively) (Monteiro *et al.* 1997). The micro-well format in which the DNA capture probe is immobilized in the wells of a microtiter plate enables the processing of large numbers of samples and may be automated.

Another advance in molecular diagnostic technology is the DNA array technique in which multiple molecular characteristics can be determined in a single assay procedure. An essential feature of the DNA array technique is the hybridization of labelled DNA fragments (such as PCR amplicons) with arrays of immobilized capture probes (Martin *et al.* 2000, Blais *et al.* 2002, Gauthier and Blais, 2004). For example, in a simple cloth-based hybridization array system (CHAS) for detection of a variety of genetic elements in transgenic crops, amplicons from a multiplex PCR incorporating DIG-dUTP were detected by hybridization with an array of probes immobilized on a strip of polyester cloth (Blais *et al.* 2002). The CHAS was also applied in the identification of antibiotic resistance and other genes associated with the multidrug-resistant food pathogen *S.* Typhimurium DT104 (Gauthier and Blais, 2004) (Chapter 3, this thesis).

In order to obtain optimal hybridization signals in the operation of these molecular diagnostic systems, while incurring minimal expense in terms of the cost of the labelling agent, it is important to consider the means used to incorporate label into PCR products. Two possible approaches can be employed for the incorporation of labels: (1) the label can be attached to a deoxyribonucleotide triphosphate (e.g. DIG-dUTP) and enzymatically incorporated in the amplicons by the DNA polymerase; or (2) the label can be attached to the 5 -end of the oligonucleotide primers during their synthesis. While the former approach might be expected to yield amplicons with a higher specific activity for the label (i.e. more molecules of label incorporated per amplicon) than the latter, the efficiency of enzymatic incorporation of labelled nucleotides may be impaired due to steric effects of the bulky label, such as happens with the incorporation of DIG-UTP by RNA polymerases (Heer *et al.* 1994). This chapter examines the most effective means of

incorporating labels into PCR amplicons for their assay by hybridization with immobilized capture probes, by conducting a comparative evaluation of both approaches. As a model system, I focused on the detection of amplicons generated in a PCR system targeting the *invA* gene (Rahn *et al.* 1992) of the bacterial foodborne pathogen *S.* Typhimurium using microtiter plate and cloth-based hybridization assay formats for detection of the labelled amplicons.

## Materials and Methods

### *DNA extraction and PCR primers*

Genomic DNA was extracted from a *S.* Typhimurium DT104 strain selected from the Ottawa Laboratory (Carling) culture collection. Genomic DNA was purified using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions, and stored at -20 °C until use. This was the source of template DNA for the PCR experiments.

PCR primers used in this study were designed to amplify the *invA* gene (Rahn *et al.* 1992) of *S.* Typhimurium, and are shown in Table 2.1. All PCR primers were synthesized by Sigma Genosys.

### *DNA probe preparation*

DNA probe was prepared by PCR using primer pairs specific for the *invA* gene (INVA-3, INVA-4) (Table 2.1). For the PCR, 10 $\mu$l template DNA (10 ng) was added to 90 $\mu$l PCR mixture containing: 2.5 units HotStarTaq DNA Polymerase and 1 $\times$ PCR buffer

**Table 2.1** Oligonucleotide primers used to amplify the *invA* gene of the foodborne pathogen *S.* Typhimurium

| Primer | Sequence | Amplicon size | Source |
|---|---|---|---|
| PCR: incorporation of a detectable label | | | |
| INVA-1 | GTG AAA TTA TCG CCA CGT TCG GGC A | 284 bp | NCBI[c], M90846 (Rahn *et al.* 1992) |
| INVA-2 | TCA TCG CAC CGT CAA AGG AAC CGT A | | |
| DIG-INVA-1[a] | DIG-GTG AAA TTA TCG CCA CGT TCG GGC A | 284 bp | NCBI, M90846 (Rahn *et al.* 1992) |
| DIG-INVA-2[b] | DIG-TCA TCG CAC CGT CAA AGG AAC CGT A | | |
| Probe preparation | | | |
| INVA-3 | TTA TTG GCG ATA GCC TGG CG | 219 bp | NCBI, M90846 (Rahn *et al.* 1992) |
| INVA-4 | TCC CTT TCC AGT ACG CTT CG | | |

[a] Forward oligonucleotide primer targeting *invA* gene sequence, labelled with digoxigenin at the 5' end.
[b] Reverse oligonucleotide primer targeting *invA* gene sequence, labelled with digoxigenin at the 5' end.
[c] National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

with 1.5 mM $MgCl_2$ (Qiagen), 200 $\mu$M each dNTP (Promega), 0.5 $\mu$M each primer (INVA-3, INVA-4) (Table 2.1), and 2 $\mu$g BSA/ml (Sigma). The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf) using the following program: initial heating at 94 °C for 15.5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 1.5 min, with an additional 2 min at 72 °C following the last cycle. PCR product was ethanol-precipitated and re-suspended in deionized distilled water.

***PCR incorporating digoxigenin and biotin labels***

Labels were incorporated into PCR amplicons for their subsequent detection by hybridization with an immobilized DNA probe, either through incorporation of labelled dUTP or 5 -labelled oligonucleotide primers. For the PCR incorporating labelled dUTP, 5 $\mu$l template DNA was added to 45 $\mu$l PCR mixture containing 1.25 units HotStarTaq DNA polymerase and 1.5 × PCR buffer with 2.25 mM $MgCl_2$, plus 200 $\mu$M each dNTP, 0.1 $\mu$M each primer (INVA-1, INVA-2) (Table 2.1), and 2 $\mu$g BSA/ml. In addition, reaction mixtures contained 10 $\mu$M of either DIG-11-dUTP (Roche), Biotin-11-dUTP (PerkinElmer), or Biotin-16-dUTP (Roche). For the PCR incorporating DIG-labelled oligonucleotide primers (DIG-INVA-1, DIG-INVA-2), 5 $\mu$l template DNA was added to 45 $\mu$l PCR mixture containing 1.25 units HotStarTaq DNA polymerase and 1.5 × PCR buffer with 2.25 mM $MgCl_2$, plus 200 $\mu$M each dNTP, 0.1 $\mu$M of each primer (DIG-INVA-1 and DIG-INVA-2) (Table 2.1) and 2 $\mu$g BSA/ml. For both PCR

approaches, cycling conditions were as above, with the exception that an annealing temperature of 52 °C was used.

***Cloth-based hybridization assay of PCR product***

Polyester cloth (DuPont, Sontara 8100) was cut into 1 x 1 cm segments, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The segments were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M $MgCl_2$ and 0.15 M NaCl] and lightly blotted immediately prior to use for DNA probe immobilization.

The *invA* DNA probe (10 ng/$\mu$l in coating buffer) was denatured by heating at 100°C for 10 min, then placed on ice. The probe was spotted (5 $\mu$l) in the centre of each cloth segment and incubated at 37 °C for 30 min. DNA probe was cross-linked to the cloth by exposing the segments to UV light for 1 min (254 nm, 100 mJ/cm$^2$) using a UVP cross-linker (Model CL-1000, VWR Scientific). The segments were pre-hybridized for 1 h at 37 °C with hybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they were washed with a total of about 5 × 1 ml of 0.01 M phosphate-buffered (pH 7.2)/0.15 M NaCl (PBS) containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth segments were stored at 4 °C until use.

For the assay of labelled PCR products, amplicons were denatured by heating at 100 °C for 10 min, then placed on ice. The PCR product (25 $\mu$l) was mixed with 225 $\mu$l

hybridization solution containing 50% (v/v) formamide. The entire mixture (250 $\mu$l) was pipetted onto a segment of probe-coated cloth and incubated for 30 min at 45 °C, followed by washing with PBST as described above. All subsequent incubations were carried out at room temperature. Segments were saturated with 250 $\mu$l of either anti-DIG-peroxidase conjugate (Roche) diluted 1/2000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) (for digoxigenin-labelled amplicons) or streptavidin-HRP (Sigma) diluted 1/1000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) (for biotin-labelled amplicons), and incubated for 10 min. After washing with PBST, the segments were saturated with 250 $\mu$l of tetramethylbenzidine (TMB) as a membrane peroxidase substrate (KPL), and incubated for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

***Microtiter plate hybridization assay of PCR product***

The *invA* DNA capture probe [1 ng/$\mu$l in high salt buffer; 0.3 M Tris/HCl (pH 8.0), 0.5 M $MgCl_2$, 1.5 M NaCl] was denatured by heating at 100 °C for 10 min, and 100 $\mu$l was added to each well of a 96-well microtitre plate, then incubated overnight at 37 °C. The wells were emptied and allowed to air-dry, and the *invA* probe was fixed to the wells by exposing the microtitre plate to UV light for 3 min (254 nm, 100 $mJ/cm^2$) using a UVP cross-linker. After three washes with 300 $\mu$l/well of washing buffer [0.1 M Tris/HCl (pH 8.0), 2 mM $MgCl_2$, 1 M NaCl, 0.1% (v/v) Tween 20], the wells were blocked for 1 h at 37 °C with 100 $\mu$l/well of hybridization solution [5 × SSC, 0.02% (w/v) SDS, 0.1% (w/v)

N-lauroyl sarcosine, and 1% (w/v) protein blocking reagent]. The wells were then washed 6 times with PBST.

For the assay of PCR products, amplicons were denatured by heating at 100 °C for 10 min, then placed on ice. PCR product (10 $\mu$l) was mixed with 100 $\mu$l hybridization solution containing 50% (v/v) formamide, and the mixture was pipetted into each well, followed by incubation for 1 h at 45 °C, and then 6 washes with PBST. All subsequent incubations were carried out at room temperature. The DIG-labelled PCR product was detected by successive addition of 100 $\mu$l/well of anti-DIG-peroxidase conjugate diluted 1/2000 in PBST containing 0.5% (w/v) protein blocking reagent for 20 min and 100 $\mu$l TMB peroxidase substrate solution/well (KPL), with PBST washes between each step. The reaction was stopped by addition of 100 $\mu$L 1 M $H_2SO_4$/well, and the absorbancy at 450 nm ($A_{450}$) was measured.

**Results**

There are a number of different possibilities for incorporating labels into PCR amplicons, such as the choice of labels (e.g. DIG or biotin), and the carrier for the labels (dNTPs or 5 -labelled oligonucleotide primers). This chapter compares the different approaches for incorporation of labels to detect amplicons either qualitatively by reverse dot blot hybridization (e.g. on polyester cloth) or quantitatively using a microtiter plate hybridization format. As a model system, I focused on the detection of the *invA* gene in the foodborne pathogen *S.* Typhimurium DT104.

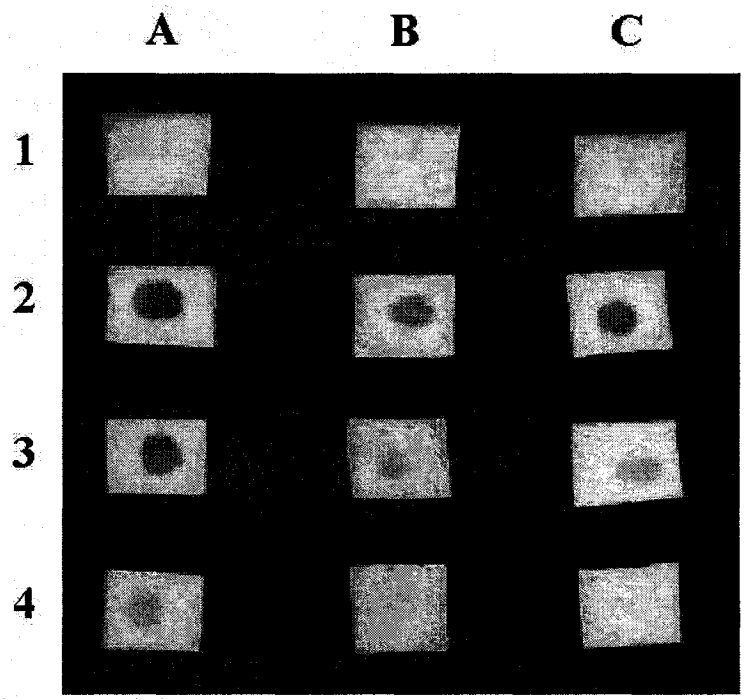
***Comparison of digoxigenin and biotin labels***

The incorporation of digoxigenin-labelled deoxyribonucleotides (DIG-11-dUTP) and biotin-labelled deoxyribonucleotides (Biotin-11-dUTP or Biotin-16-dUTP) into amplicons generated in a PCR targeting the *invA* gene was compared by hybridization of the PCR products with a capture probe immobilized on polyester cloth as described in Methods. Cloth hybridization results confirmed that all of the labels examined were successfully incorporated into the *invA* amplicon during PCR amplification (Figure 2.1). Positive hybridization results were obtained with all three labels using a minimum of 0.1 pg DNA template though, on a qualitative basis, the signals were strongest using DIG-11-dUTP. However, when using a minimum of 0.01 pg genomic DNA positive signals were obtained only for the amplicons labelled with DIG-11-dUTP. These experiments were repeated and the results were reproducible (data not shown).

***Comparison of digoxigenin incorporation in the form of DIG-11-dUTP and 5'-labelled primers***

The preceding experiments demonstrate the incorporation of a label attached to a dNTP molecule into a PCR product. It is also possible to incorporate a label attached to the 5 -end of one or both oligonucleotides used in the DNA amplification process. I compared the incorporation of the digoxigenin label into PCR products using both 5 -DIG-labelled oligonucleotide forward and reverse primers, or various concentrations of DIG-11-dUTP, followed by hybridization with a capture probe immobilized on polyester cloth as described in Methods. The cloth-hybridization assay results demonstrate that

**Figure 2.1** Comparison of digoxigenin and biotin incorporation into amplicons by PCR. Different quantities of template DNA (1, none; 2, 1 pg; 3, 0.1 pg; 4, 0.01 pg) were subjected to PCR containing different labelling reagents (A, DIG-11-dUTP; B, Biotin-11-dUTP; C, Biotin-16-dUTP) followed by hybridization of amplicons with a probe immobilized on polyester cloth segments and immunoenzymatic assay as described in Methods.



digoxigenin incorporation into PCR amplicon can be accomplished using both DIG-11-dUTP as well as DIG-labelled primers (Table 2.2). Positive results were obtained with all labelling approaches using a minimum of 0.01 pg template DNA. Increasing the concentration of DIG-11-dUTP in the reaction mixtures from 10-40 μM did not produce any significant improvement in the sensitivity of the assay or the quality of the spots, but did result in a dramatic increase in the cost of the assay due to the high cost of this labelling reagent. Sporadic positive signals (i.e. one replicate per duplicate sample set) were observed using 0.001 pg of DNA template with DIG-11-dUTP incorporation, but were not considered significant as this quantity of DNA template represents less than one bacterial genome equivalent, and can be attributed to the variability inherent in the sampling statistics for solutions containing low concentrations of DNA molecules.

***Comparison of digoxigenin incorporation by microtiter plate hybridization assay***

The preceding results were obtained using a qualitative, reverse dot blot hybridization format and provides useful information on the relative merits of different labelling approaches for researchers designing DNA array techniques. However, there are instances where a microtiter plate hybridization format for detection of PCR products is advantageous, especially in terms of quantitative analysis, sample throughput capability and the potential for automation. The incorporation of the digoxigenin label into PCR products using various regimens involving DIG-labelled primers and different concentrations of DIG-11-dUTP was examined on a quantitative basis by hybridization of the PCR products with a probe immobilized in the wells of a microtiter plate followed

**Table 2.2** Incorporation of DIG-11-dUTP and 5 -DIG-labelled primers into amplicons by PCR[a]

| Template (pg) | Positives/total[b] DIG-11-dUTP (10 $\mu$M) | DIG-11-dUTP (20 $\mu$M) | DIG-11-dUTP (40 $\mu$M) | DIG-INVA primers |
|---|---|---|---|---|
| 0.1 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.01 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.001 | 1/2 | 0/2 | 1/2 | 0/2 |
| 0 | 0/2 | 0/2 | 0/2 | 0/2 |

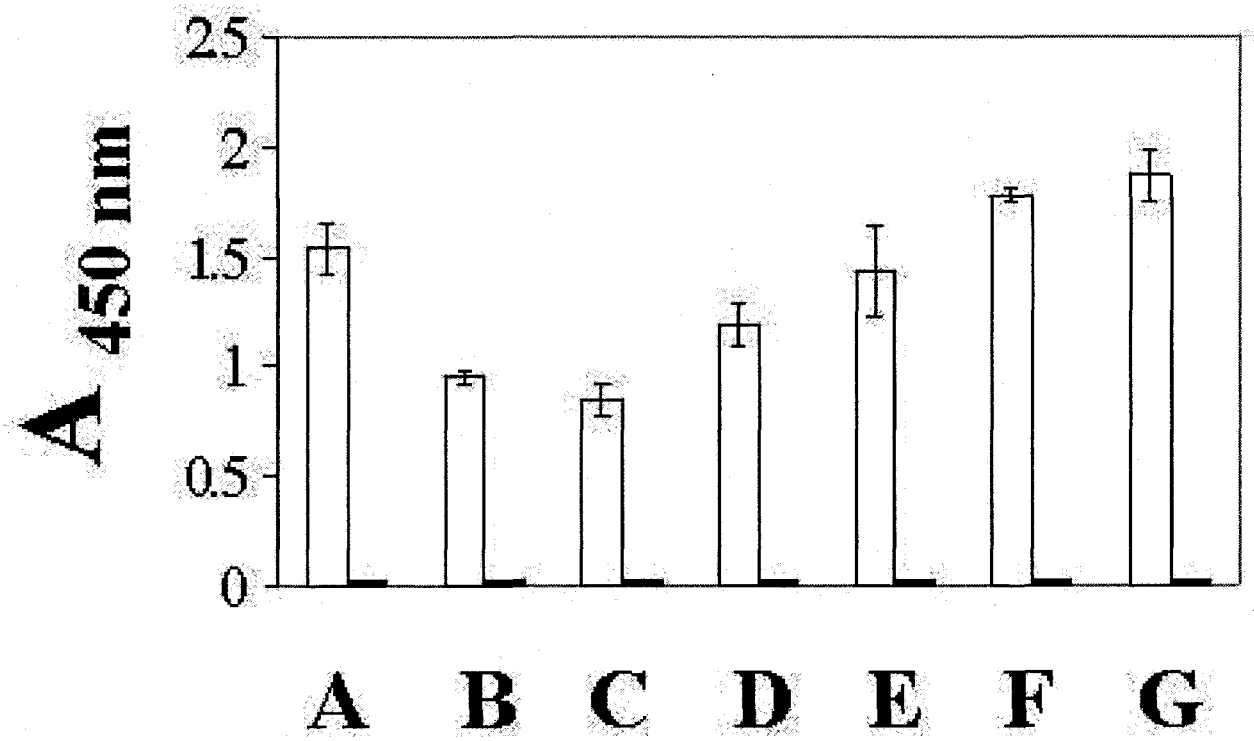
[a] Various quantities of template DNA were subjected to PCR incorporating digoxigenin by different approaches (DIG-11-dUTP or DIG-labelled primers), followed by hybridization of amplicons with a probe immobilized on polyester cloth segments and immunoenzymatic assay as described in Methods.
[b] Number of positives per total number of replicates tested. The concentrations of DIG-11-dUTP used in the reaction mixtures are indicated in brackets.

by immunoenzymatic assay as described in Methods. For the incorporation of DIG-labelled primers, I also examined the effect of having the label attached to either the forward, reverse or both primers. The microtiter plate hybridization assay results confirmed that digoxigenin was incorporated into PCR products by all of the approaches in a template dependent-manner (Figure 2.2). A significantly higher assay signal was obtained when amplicons were produced by incorporation of both DIG-labelled forward and reverse primers (Figure 2.2 A) than when only one primer (forward or reverse) was labelled (Figure 2.2 B,C). On the other hand, when amplicons were labelled by incorporation of DIG-11-dUTP the assay signal ($A_{450}$ value) increased with the concentration of labelling reagent in the reaction mixture (Figure 2.2 D-G). A minimum of 20 μM DIG-11-dUTP (Figure 2.2 E) was required to achieve the assay signal that was attained by incorporation of DIG-labelled primers (Figure 2.2 A). However, at this level, the use of DIG-11-dUTP for labelling the amplicons incurs a *ca* twenty-fold higher cost over the use of the labelled primers.

**Discussion**

These studies demonstrate that, when incorporated in the form of labelled dUTP, digoxigenin is superior to biotin as a label in enabling the sensitive detection of PCR products by hybridization with an immobilized probe. While both DIG-11-dUTP and 5 - DIG-labelled primers are equally satisfactory for qualitative reverse dot blot and quantitative microtiter plate hybridization assays, the latter was more cost-effective. A cost comparison (in Canadian funds) reveals that incorporating digoxigenin in the form of

**Figure 2.2** Comparison of digoxigenin incorporation by the microtiter plate hybridization assay system. Samples with (□) and without (■) template DNA (0.1 pg) were subjected to PCR using various digoxigenin labelling regimens followed by hybridization with a probe immobilized in a microtiter plate and immunoenzymatic assay as described in Methods. Amplicon was labelled with digoxigenin during PCR using the following approaches as described in methods: (A) 0.1 μM each of primers DIG-INVA-1 and DIG-INVA-2, (B) 0.1 μM each of primers DIG-INVA-1 and INVA-2, (C) 0.1 μM each of primers INVA-1 and DIG-INVA-2, (D) 10 μM DIG-11-dUTP and 0.1 μM each of primers INVA-1 and INVA-2, (E) 20 μM DIG-11-dUTP and 0.1 μM each of primers INVA-1 and INVA-2, (F) 40 μM DIG-11-dUTP and 0.1 μM each of primers INVA-1 and INVA-2, (G) 80 μM DIG-11-dUTP and 0.1 μM each of primers INVA-1 and INVA-2. Results are expressed as the mean $A_{450}$ value $\pm$ SD (n=3).



DIG-11-dUTP (at 10 μM) is approximately ten times more costly per reaction than using 5'-DIG-labelled oligonucleotide primers. Therefore, for the types of assay systems described in this chapter, I recommend the use of 5 -DIG-labelled primers as an effective means of labelling PCR products. In instances where large multiplex PCR systems are used (as described in Chapters 3, 5, and 6 of this thesis), in which multiple primer pairs are involved, the number of individual labelled primers and their collective cost becomes more significant. In these types of systems, I recommend the use of DIG-dUTP as an effective and more economical means of labelling PCR products.

# CHAPTER 3

## Cloth-Based Hybridization Array System for the Detection of Multiple Antibiotic Resistance Genes in *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104

"This chapter has been published in a peer-reviewed scientific journal:

Gauthier, M., and Blais, B.W. (2004) *Letters in Applied Microbiology* **38**: 265-270."

**Abstract**

A simple DNA macroarray system was developed for detection of antibiotic resistance and other marker genes associated with the multidrug-resistant food pathogen *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104. A multiplex polymerase chain reaction (PCR) incorporating digoxigenin-dUTP was used to simultaneously amplify seven marker sequences, with subsequent rapid detection of the amplicons by hybridization with an array of probes immobilized on polyester cloth and immunoenzymatic assay of the bound label. This system provided sensitive detection of the different genetic markers in the *S.* Typhimurium DT104 genome, giving positive reactions with as few as 10 cfu, and the hybridizations were highly specific, with no reactions of amplicons with heterologous probes on the array. This cloth-based hybridization array system (CHAS) provides a simple, cost-effective tool for monitoring *S.* Typhimurium DT104 in foods and their production environment, and the concept is broadly applicable to the detection and characterization of food pathogens.

**Introduction**

The emergence of multidrug-resistant food pathogens such as *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 has become a major public health issue for the food industry and regulatory agencies worldwide. Most DT104 strains commonly exhibit chromosomal resistance to the antibiotics ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su) and tetracycline (T), and are referred to as ACSSuT-type. Rapid detection methods specific for this pathogen are an important element in any

strategy to control its spread in the food supply, particularly in poultry products, where there is a high incidence of *Salmonella* contamination (Abouzeed *et al.* 2000).

The traditional approach for the identification of *S.* Typhimurium DT104 involves enrichment culture followed by serological, phage- and antibiotic-susceptibility testing (White *et al.* 2001), a process which can take more than two weeks to complete, and is therefore of limited value in assuring a timely response to contamination incidents involving this pathogen. In order to improve turnaround times in the detection of this pathogen, several investigators have developed polymerase chain reaction (PCR) methods targeting specific molecular markers for the characterization of isolates (Ng *et al.* 1999, Carlson *et al.* 1999, Khan *et al.* 2000). Of particular interest are multiplex PCR techniques in which several molecular markers are co-amplified in a single reaction, thus increasing the amount of information which can be rapidly collected from an isolate to assist in investigations of contamination incidents. The detection of amplicons in multiplex PCR can be achieved by agarose gel electrophoresis or with the use of fluorogenic probes (Carlson *et al.* 1999, Khan *et al.* 2000), though these procedures can be cumbersome, costly and the results difficult to interpret. High density microarray systems for detection and characterization of gene markers in bacteria have also been developed (Stritzhkov *et al.* 2000; Chizhikov *et al.* 2001), but these rely on the use of highly sophisticated instruments to prepare and process the arrays, and are not generally within the scope of analytical capabilities of the typical food microbiology laboratory.

This chapter describes the development of a simple low density array technique based on the use of a macroporous, hydrophobic polyester cloth as a solid phase for the detection of amplicons from a multiplex PCR, targeting key DT104 markers by their

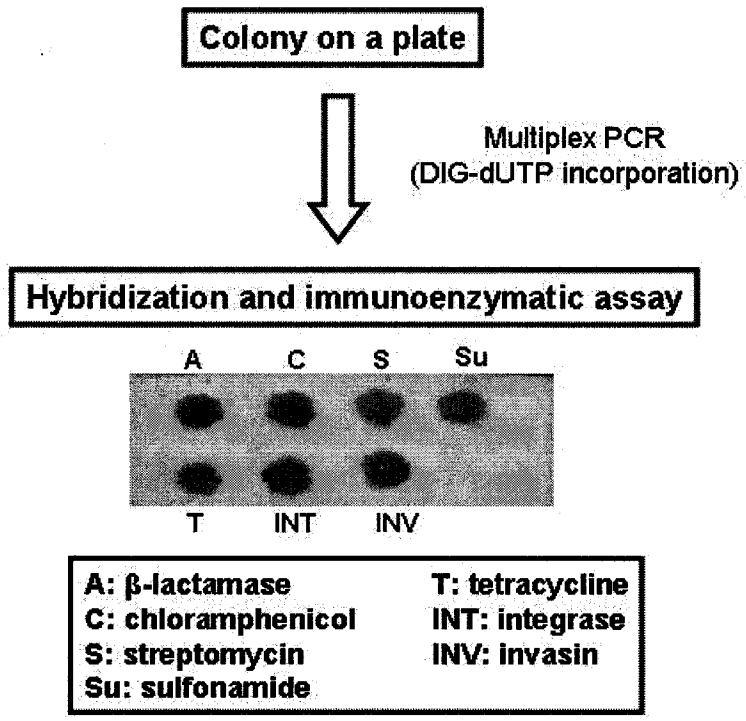
hybridization with immobilized DNA probes. The advantages of polyester cloth as a DNA adsorbent for nucleic acid hybridization assays have previously been demonstrated (Blais and Phillippe 1995, Blais *et al.* 2002). Polyester cloth is a cost-effective support yielding improved reaction kinetics due to a large and readily accessible surface, and is easy to wash between reaction steps to remove unbound reagents. In the present assay, amplicons incorporating digoxigenin-labelled dUTP generated in a multiplex PCR (targeting antibiotic resistance and the integrase gene associated with the DT104 integrons, as well as the *Salmonella invA* virulence gene) are detected by hybridization with an array of amplicon-specific DNA probes immobilized in discrete spots on a polyester cloth strip, followed by immunoenzymatic assay of the bound label using anti-digoxigenin antibody-peroxidase conjugate (Figure 3.1). Thus, the identity of isolates obtained by standard enrichment culture techniques is inferred by detection of the antibiotic resistance and integron marker genes most commonly associated with DT104 strains (Ng *et al.* 1999), as well as the *Salmonella* genus-specific *invA* gene (Rahn *et al.* 1992). The applicability of this cloth-based hybridization array system (CHAS) to the characterization of DT104 isolates is demonstrated.

## Materials and Methods

### *DNA extraction and PCR primers*

Genomic DNA was extracted from a *S.* Typhimurium DT104 strain exhibiting the pentaresistant phenotype ACSSuT (ampicillin, A; chloramphenicol, C; streptomycin, S; sulfonamides, Su; and tetracycline, T) selected from the Ottawa Laboratory (Carling) (OLC) culture collection of the Canadian Food Inspection Agency. Genomic DNA was

**Figure 3.1** Scheme for the combined multiplex PCR-CHAS as applied in the characterization of *S.* Typhimurium DT104 isolates.



extracted and purified using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20 °C until use. This was the source of template DNA for the production of probes by PCR.

PCR primer sequences targeting antibiotic resistance genes and marker genes (integrase, INT; and *invA*, INV) are given in Table 3.1. All primers were synthesized by Sigma Genosys.

***Bacteria***

The *Salmonella* and non-*Salmonella* bacteria examined in this study, including pure cultures from the American Type Culture Collection (ATCC) and the Ottawa Laboratory (Carling) (OLC) culture collection of the Canadian Food Inspection Agency are listed in Table 3.2. The *S.* Typhimurium DT104 strains were a kind gift from Dr. C. Poppe (Health Canada Laboratory, Guelph, Ontario). Growth conditions for the various bacteria were as previously described (Wang *et al.* 1995).

***Antibiotic susceptibility testing***

The *S.* Typhimurium DT104 strains used in this study (Table 3.2) were tested for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline by the disk agar diffusion method performed on Mueller Hinton (Difco) agar plates. The antibiotic disks used in this study were purchased from Becton, Dickinson and Company. Disks contained the following amounts of antibiotic: ampicillin 10 μg, chloramphenicol 30 μg, streptomycin 10 μg, sulfisoxazole 250 μg, and tetracyline 30 μg. The sensitivity

**Table 3.1** Oligonucleotide primer sequences targeting antibiotic resistance genes and marker genes used in multiplex PCR and probe production

| Primer | Sequence (5' to 3') | Amplicon size | Source / accession number |
|---|---|---|---|
| Multiplex PCR | | | |
| A-1 | CAA GTA GGG CAG GCA ATC ACA CTC G | 216 bp | NCBI[a], AF261825 |
| A-2 | GAG TTG TCG TAT CCC TCA AAT CAC C | | |
| C-1 | CAT TGA TCG GCG AGT TCT TGG GAT G | 236 bp | NCBI, AF261825 |
| C-2 | AGC CGT CGA GAA GAA GAC GAA GAA G | | |
| S-1 | TCA TTG AGC GCC ATC TGG AAT CAA C | 210 bp | NCBI, AF261825 |
| S-2 | GTG ACT TCT ATA GCG CGG AGC GTC T | | |
| Su-1 | CCG ATG AGA TCA GAC GTA TTG CGC C | 209 bp | NCBI, AF261825 |
| Su-2 | GCG CTG AGT GCA TAA CCA CCA GCC T | | |
| T-1 | CTC TAT ATC GGC CGA CTC GTG TCC G | 204 bp | NCBI, AF261825 |
| T-2 | CGG CGG CGA TAA ACG GGG CAT GAG C | | |
| INT-1 | CGC ACG ATG ATC GTG CCG TGA TCG A | 235 bp | NCBI, AF261825 |
| INT-2 | TAC GGC AAG GTG CTG TGC ACG GAT C | | |
| INV-1 | GTG AAA TTA TCG CCA CGT TCG GGC A | 284 bp | NCBI, M90846 [Rahn *et al.* 1992] |
| INV-2 | TCA TCG CAC CGT CAA AGG AAC CGT A | | |

**Table 3.1** Continued

| Primer | Sequence (5' to 3') | Amplicon size | Source/ accession number |
|---|---|---|---|
| Probe production | | | |
| A-3 | ACT ATG ACT ACA AGT GAT AA | 146 bp | NCBI[a], AF261825 |
| A-4 | GAG CTT ACC TTC ATT TAA AT | | |
| C-3 | GCA GGC GAT ATT CAT TAC TT | 177 bp | NCBI, AF261825 |
| C-4 | GTG CCC ATA CCG GCG CTA AA | | |
| S-3 | TTG CTG GCC GTG CAT TTG TA | 146 bp | NCBI, AF261825 |
| S-4 | GAA AGC CGA AGC CTC CAT AA | | |
| Su-3 | TCT TAG ACG CCC TGT CCG AT | 144 bp | NCBI, AF261825 |
| Su-4 | GCA ATA TCG GGA TAG AGC GC | | |
| T-3 | TCA CGG GCG CAA CCG GAG CT | 141 bp | NCBI, AF261825 |
| T-4 | CCG AGC ATG CCA CCA AGT GC | | |
| INT-3 | GAT CCT TGA CCC GCA GTT GC | 174 bp | NCBI, AF261825 |
| INT-4 | TGG CTT CAG GAG ATC GGA AG | | |
| INV-3 | TTA TTG GCG ATA GCC TGG CG | 219 bp | NCBI, M90846 [Rahn *et al.* 1992] |
| INV-4 | TCC CTT TCC AGT ACG CTT CG | | |

[a] NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

**Table 3.2** Strains, source, and specificity results of the multiplex PCR-CHAS in the assay of target and non-target bacterial organisms[a]

| Organism | Strain or Source[b] | Reactivity[c] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | INV | INT | A | C | S | Su | T |
| Salmonellae | | | | | | | | |
| *S. typhimurium* | ATCC 10145 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. typhimurium* | ATCC 14028 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. enteritidis* | ATCC 13076 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. senftenberg* | ATCC 8400 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. alachua* | OLC 57 (Feed) | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. johannesburg* | OLC 58 (Feed) | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S. montevideo* | ATCC 8387 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *S.* Typhimurium DT104 (ACSSuT) | OLC 593 (Bovine) | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| *S.* Typhimurium DT104 (ACSSuT) | OLC 594 (Porcine) | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| *S.* Typhimurium DT104 (ACSSuT) | OLC 595 (Equine) | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| *S.* Typhimurium DT104 (ACSSuT) | OLC 596 (Quail) | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| *S.* Typhimurium DT104 (ACSSuT) | OLC 597 (Chicken) | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| *S.* Typhimurium DT104 (ACSSuT) | ATCC 700407 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| Non-Salmonellae | | | | | | | | |
| *Listeria monocytogenes* | ATCC 15313 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Escherichia coli* | ATCC 11775 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Shigella sonnei* | ATCC 29938 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Enterobacter cloacae* | ATCC 13047 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Proteus vulgaris* | ATCC 13315 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |

**Table 3.2** Continued

| Organism | Strain or Source[b] | Reactivity[c] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | INV | INT | A | C | S | Su | T |
| Non-Salmonellae | | | | | | | | |
| *Citrobacter freundii* | ATCC 8090 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Bacillus cereus* | ATCC 14579 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Bacillus subtilis* | ATCC 6051 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| *Escherichia coli* O157:H7 | ATCC 35150 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |

[a] Bacterial colonies were suspended in 50 µl of PCR buffer and then lysed by addition of an equal volume of 2% Triton X-100 (Sigma), followed by incubation at 100 °C for 10 min. Cell lysates were subjected to the multiplex PCR-CHAS procedure as described in Methods. Gene markers were as follows: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamides; T, tetracycline; INT, integrase; INV, *invA*.

[b] ATCC - American Type Culture Collection; OLC - Ottawa Laboratory Carling Culture Collection, Canadian Food Inspection Agency

[c] No. of replicates giving positive results per total no. tested.

and resistance characteristics of isolates were evaluated according to the disk manufacturer's instructions.

***DNA probe preparation***

DNA probes for immobilization on the arrays were prepared by PCR using primer pairs specific for each of the following antibiotic resistance genes: ampicillin (A-3, A-4), chloramphenicol (C-3, C-4), streptomycin (S-3, S-4), sulfonamide (Su-3, Su-4), and tetracycline (T-3, T-4), as well as the integrase (INT-3, INT-4) and *invA* genes (INV-3, INV-4) (Table 3.1). These probes were designed to be internal to the amplicons from the multiplex PCR system (see below). For the PCR, 10 μl template DNA (10 ng) was added to 90 μl PCR mixture [2.5 units HotStarTaq DNA Polymerase and 1 × PCR buffer with 1.5 mM $MgCl_2$ (Qiagen), 200 μM each dNTP (Promega), 0.5 μM each primer (Table 3.1), and 2 μg BSA/ml (Sigma)]. The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf) using the following program: initial heating at 94 °C for 15.5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 1.5 min, with an additional 2 min at 72 °C following the last cycle. PCR product was quantified using a Hoefer DyNA Quant 200 Fluorometer, ethanol-precipitated and re-suspended in deionized distilled water, and stored at –20 $^0$C until use.

***Multiplex PCR and CHAS procedure***

The general scheme for the multipex PCR and amplicon detection procedure is shown in Figure 3.1. Cell suspensions or purified genomic DNA samples were subjected to a

multiplex PCR incorporating seven primer pairs targeting antibiotic resistance and marker genes (Table 3.1). For the PCR, 10 µl of test sample was added to 90 µl of multiplex PCR mixture [2.5 units HotStarTaq and 1.5 × PCR buffer containing 2.25 mM $MgCl_2$, plus 200 µM each dNTP, 5 µM DIG-11-dUTP (Roche), 0.1 µM of each primer (Table 3.1), and 2 µg BSA/ml]. The PCR was carried out as above, with the exception that an annealing temperature of 52 °C was used.

For the CHAS, polyester cloth (DuPont, Sontara 8100) was cut into 2 × 5 cm strips, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M $MgCl_2$ and 0.15 M NaCl] and lightly blotted immediately prior to use for DNA probe immobilization.

The DNA probes (10 ng/µl in coating buffer) were denatured by heating at 100 °C for 10 min, and placed on ice. Probes were spotted (5 µl) in discrete spots on a cloth strip, followed by incubation at 37 °C for 30 min. DNA probes were cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 $mJ/cm^2$) using a UVP cross-linker (Model CL-1000, VWR Scientific). The strips were pre-hybridized for 1 h at 37 °C with hybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they were washed with a total 5 × 3 ml of 0.01 M phosphate-buffered (pH 7.2)/0.15 M NaCl (PBS) containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips were stored dry at 4 °C until use.

For the assay of DIG-labelled multiplex PCR products, amplicons were denatured by heating at 100 °C for 10 min, and placed on ice. The PCR product (100 µl) was mixed with 900 µl hybridization solution containing 50% (v/v) formamide. The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 30 min at 45 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-DIG-peroxidase conjugate (Roche) diluted 1/2000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 1 ml of tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories), and incubated for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

**Results**

***Detectability of the combined multiplex PCR-CHAS procedure***

The detectability of the PCR-CHAS assay was determined using both purified genomic DNA and whole cells suspended in broth. Various amounts of genomic DNA extracted from an *S.* Typhimurium DT104 strain (chicken isolate) were subjected to the combined multiplex PCR and CHAS method in order to determine the minimum amount of genomic DNA required to produce positive reactions with the immobilized probes on the cloth array. Positive reactions were consistently obtained on the array for all of the gene markers using a minimum of 0.12 pg of genomic DNA input into the PCR, with positive reactions occurring for many of the markers with as little as 0.01-0.03 pg of DNA (Table 3.3), which corresponds to less than one bacterial genome equivalent.

**Table 3.3** Detectability of multiplex PCR-CHAS in the assay of various amounts of genomic DNA from *S.* Typhimurium DT104[a]

| Genomic DNA (pg) | No. positives/total[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | INV | INT | A | C | S | Su | T |
| 1 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.5 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.25 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.12 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 0.06 | 2/2 | 2/2 | 2/2 | 2/2 | 1/2 | 2/2 | 2/2 |
| 0.03 | 2/2 | 2/2 | 2/2 | 1/2 | 2/2 | 0/2 | 2/2 |
| 0.01 | 2/2 | 1/2 | 0/2 | 2/2 | 0/2 | 2/2 | 0/2 |
| 0 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |

[a] Various amounts of genomic DNA from a pentaresistant strain of *S.* Typhimurium DT104 were subjected to the multiplex PCR-CHAS procedure as described in Methods. Gene markers were as follows: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamides; T, tetracycline; INT, integrase; INV, *invA*.

[b] No. of positives per total number of replicates tested.

The detectability of the assay for whole cells was determined by preparing serial dilutions of *S.* Typhimurium DT104 cells in tryptic soy broth, followed by lysis with Triton X-100 (see footnote in Table 3.2) and subjecting the lysates to the combined multiplex PCR-CHAS procedure. A lysate derived from a minimum of *ca* 10 cfu input into the PCR was required to reliably amplify and detect all of the gene markers using the array, with some of the markers (i.e., streptomycin and integrase) becoming undetectable with lower levels of cells (data not shown).

***Specificity of the combined multiplex PCR-CHAS procedure***

The specificity of the combined multiplex PCR-CHAS for the targeted gene markers was ascertained by subjecting colonies of a variety of target and non-target bacteria to the procedure. The antibiotic resistance phenotype of the *S.* Typhimurium DT104 strains was verified by antibiotic susceptibility testing. The six *S.* Typhimurium DT104 strains tested gave positive reactions for all of the gene markers on the array, with the expected reactivities for the different antibiotic resistance markers based on the pentaresistant phenotypes (Table 3.2), thus demonstrating the inclusivity of the procedure. All of the non-DT104 *Salmonella* strains tested produced the expected positive reaction for the *invA* gene, but were negative for the other gene markers. No positive signals were obtained with the non-*Salmonella* bacteria tested, demonstrating the exclusivity of the test. These results confirm the specificity of the multiplex PCR-CHAS procedure for the relevant *Salmonella*-specific and antibiotic resistance markers.

**Discussion**

These limited studies demonstrate the applicability of a multiplex PCR system combined with a simple cloth-based hybridization procedure for the simultaneous assay of multiple amplicons in the characterization of *S.* Typhimurium DT104 isolates. Novel features of this assay system include the simultaneous amplification of a large number of gene markers in a single PCR reaction (up to 7 markers for pentaresistant DT104 isolates), and a rapid hybridization procedure which simplifies the analysis of the multiple amplicons through visualization of the individual PCR products as coloured spots on an array. The detection of multiple amplicons by hybridization with an array of immobilized probes is advantageous in that primers can be designed to generate amplicons of similar size (<300 bp in the present study), which would not be readily distinguishable by conventional agarose gel electrophoresis, increasing the likelihood that all amplicons will be produced with near-equal efficiency during the amplification process. The small differences in amplification efficiency for some of the amplicons are likely a function of the individual primer sequences, and could be addressed by redesigning the primers. However, since this system is intended to serve primarily as a colony screening tool its performance characteristics were deemed satisfactory. A further advantage of the CHAS approach is that the specificity of the amplicons is confirmed by hybridization with immobilized probes, obviating the need for time-consuming restriction endonuclease analysis.

One limitation of this technique is that *S.* Typhimurium DT104 isolates with dysfunctional antibiotic resistance genes may still produce positive results in the PCR-CHAS assay if primer-complementary portions of the genes remain intact. Therefore, the results of this assay should be regarded as presumptive, with confirmation by standard

antibiotic susceptibility testing techniques being required for more definitive characterization. However, given the highly adaptive nature of bacteria in general, it is possible that even a dysfunctional gene could revert to its active state under the right selective conditions, and hence, the presence of such genes may be of public health significance. While in this study only the five key genes conferring the classic pentaresistant pattern were considered, the multiplex PCR and array might be expanded to include the detection of other antibiotic resistance genes which have been observed in some DT104 strains.

The present system exhibited adequate detectability (*ca* 10 cfu) and specificity to enable its use as a reliable tool for the characterization of colonies obtained from foods and clinical specimens using suitable enrichment culture techniques according to the scheme illustrated in Figure 3.1. The combined multiplex PCR-CHAS procedure could be completed within a single day, thus providing information on the nature of isolates at a much earlier juncture than the standard culture-based antibiotic susceptibility techniques, which usually require several days to complete. Therefore, the present approach will provide information within a timeframe enabling a timely response by regulators and the food industry to minimize the spread of contamination of the food supply with *S.* Typhimurium DT104. The principle of the cloth-based hybridization array system demonstrated in this chapter should be broadly applicable to the detection and characterization of other molecular markers of food pathogens, such as virulence and toxin genes.

# CHAPTER 4

## Detection of *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 by Polymerase Chain Reaction and a Simple Cloth-Based Dot Blot Hybridization System

**Abstract**

The development of a method for the specific detection of *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 in food samples after enrichment was attempted. The proposed method involves a three-step process in which test samples are first subjected to a polymerase chain reaction targeting the integrase gene sequence of class 1 integrons found in *S.* Typhimurium DT104, followed by spotting of the resulting PCR products in discrete spots on polyester cloth and subsequent detection of the amplicons by hybridization with a digoxigenin-labelled target-specific DNA probe and immunoenzymatic assay of the bound digoxigenin label. This system provided sensitive and specific detection of the integrase gene in target *S.* Typhimurium DT104 with no cross-reactions when tested against a panel of non-target Gram positive and Gram negative bacteria. The application of the combined PCR and cloth-based dot blot hybridization system (C-DBHS) to the direct detection of *S.* Typhimurium DT104 in various inoculated food samples, including poultry feed, powdered egg, and whole chicken, by subjecting the test samples to enrichment culture techniques (pre-enrichment and selective enrichment) was evaluated. Positive results were obtained for both inoculated and uninoculated samples of poultry feed and whole chicken carcass. The reactivity of the combined PCR and C-DBHS with uninoculated samples suggests that the integrase gene primers detected homologous gene sequences present in indigenous microflora associated with the food samples.

## Introduction

The use of antimicrobial agents in any environment creates selection pressures that favor the survival and proliferation of antibiotic-resistant pathogens (O'Brien, 2002). Antimicrobials are used in food-producing animals for different purposes: (1) treatment of sick animals, (2) prevention of disease among animals susceptible to bacterial infections, and (3) growth promotion (Schwarz and Chaslus-Dancla, 2001). Antibiotics used in disease prevention usually affect a large number of animals because they involve treatment of the whole herd or flock to prevent further expansion of the infection. The use of antibiotics for growth promotion is specific to food-producing animals. Antibiotics used as growth promoters are added to the feed in low doses, and are fed to animals for long periods of time. As growth promoters, the antibiotics in the feed may enhance weight gain in animals by suppressing bacteria interfering with nutrient absorption (McEwen and Fedorka-Cray, 2002). Antimicrobial use in animals leads to the undesired consequence of selecting for resistant foodborne bacterial pathogens that may, in turn, be transmitted to humans as food contaminants (White *et al.* 2002). The emergence of multidrug-resistant foodborne pathogens such as *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 has become a major public health issue for the food industry and regulatory agencies worldwide. Most DT104 strains are resistant to a core group of antimicrobials, including ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), and tetracycline (T), and are referred to as ACSSuT-type. This pathogen causes great concern because the genes encoding the broad antimicrobial resistance are encoded within the chromosome of the bacteria (Briggs and Fratamico, 1999), suggesting that this broad resistance will likely persist in descendants

of the strain even in the absence of selective pressure caused by antibiotic usage. Furthermore, because these antibiotic resistance genes found in DT104 are located on integrons, which are described as very efficient genetic mechanisms by which bacteria can acquire resistance genes (Hall and Stokes, 1993; Carattoli, 2001), DT104 constitutes a potential reservoir for the transfer of antibiotic resistance genes to more serious pathogens. Although human intestinal illnesses are not normally treated with antibiotics, treatment may be needed in immunocompromised individuals, and the development of multidrug-resistant foodborne bacterial pathogens such as *S.* Typhimurium DT104 can potentially compromise human drug treatments (Humphrey, 2001). Given that DT104 ACSSuT-type strains are spreading on a global scale, the development of a rapid and sensitive method specific for this pathogen is an important element in any strategy to control its spread in the food supply, particularly in poultry products, where there is a high incidence of *Salmonella* contamination (Abouzeed *et al.* 2000).

Traditional recovery methods for foodborne salmonellae involve time-consuming, multistep enrichment processes, in which several culture media are used enabling the growth and selection of this organism prior to plating onto differential and selective media. After enrichment culture techniques, the identification of pathogens such as *S.* Typhimurium DT104, involves serological, phage- and antibiotic-susceptibility testing (White *et al.* 2001), a process which is performed in specialized reference laboratories and can take more than 2 weeks to complete.

A cloth-based hybridization array system (CHAS) was featured in Chapter 3 for the characterization of *S.* Typhimurium DT104 isolates on the basis of their antibiotic resistance profiles. In this system, seven marker sequences were chosen, and included

among these are the five common antibiotic resistance genes (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline). In addition to the antibiotic resistance gene markers, the invasion gene, *invA,* is essential for full virulence in *Salmonella* by triggering the internalization required for the invasion of deeper tissues (Galan and Curtiss, 1989) and was therefore chosen to act as a target for *Salmonella* spp. identification. The integrase gene encodes a site-specific recombinase that catalyzes the insertion of gene cassettes, which are in most cases comprised of antibiotic resistance genes, into the integron (Recchia and Hall, 1995). The integrase gene was chosen as a marker to confirm that the *Salmonella* isolate is a DT104 strain. A multiplex PCR was used to simultaneously amplify the seven marker sequences and the amplicons were rapidly detected by hybridization with an array of probes immobilized on polyester cloth followed by immunoenzymatic assay of the bound label. While simple, rapid, and cost-effective, this system was designed to serve primarily as a colony-screening tool for the characterization of DT104 strains based on their antibiotic resistance profiles and is not designed for the direct detection of this pathogen from food samples.

A preliminary screening method is required to rapidly identify presumptive DT104 strains in samples requiring more intensive cultural, biochemical, and molecular analyses to confirm the nature of contaminants, and thus, assure a timely response to contamination incidents involving this serious foodborne pathogen. To enable its application in the routine food testing laboratory, the procedure should be simple, rapid, cost-effective, specific, sensitive, and more importantly, have the ability to process multiple samples at one time. This chapter examines the feasibility of detecting *S.* Typhimiurium DT104 in food samples, such as poultry products, using a cloth-based dot

blot hybridization system. Poultry feed was also included as a commodity to be analyzed in this chapter, since there is evidence that animal feeds (e.g. poultry feeds) are frequently contamined with foodborne bacterial pathogens (e.g. *Salmonella enterica*). Food-producing animals such as chickens can acquire these pathogens by ingestion, and transmit them to humans through the food chain (Crump *et al.* 2002). In this system, test samples (e.g. enrichment broth cultures) are subjected to a PCR targeting the integrase gene, chosen as a marker sequence for the specific detection of *S.* Typhimurium DT104. Resulting amplicons are spotted on a polyester cloth sheet capable of accomodating multiple samples and detected by hybridization with a digoxigenin (DIG)-labelled target-specific DNA probe with subsequent immunoenzymatic assay of the bound label. While the CHAS described in Chapter 3 was suitable for screening single samples (i.e. colonies) for the presence of multiple gene markers, the cloth-based dot blot hybridization system (C-DBHS) is designed for the analysis of multiple samples in a single operation.

## Materials and Methods

### *DNA extraction and PCR primers*

Genomic DNA was extracted from a *S.* Typhimurium DT104 strain exhibiting the penta-resistant phenotype ACSSuT (A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamides and T, tetracycline) selected from the Ottawa Laboratory Carling (OLC) culture collection of the Canadian Food Inspection Agency. Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA was quantified using a Hoefer DyNA

Quant 200 Fluorometer (Amersham Biosciences) and stored at -20 °C until use. This was the source of template DNA for probe production by PCR.

PCR primers targeting the integrase (INT) gene of *S.* Typhimurium DT104 are given in Table 4.1. Primers were synthesized by Sigma Genosys.

***Bacteria***

The *Salmonella* and non-*Salmonella* bacteria examined in this study, including pure cultures from the American Type Culture Collection (ATCC) and the OLC culture collection of the Canadian Food Inspection Agency are listed in Table 4.2. The *S.* Typhimurium DT104 strains were a kind gift from Dr. C. Poppe (Health Canada Laboratory, Guelph, Ont., Canada). All *Vibrio* strains used in this study were routinely grown by plating on Marine Agar (Difco) and incubating for 18 h at 37 °C. *Listeria monocytogenes*, *Enterobacter cloacae*, and *Bacillus cereus* were grown on Tryptone Soya Agar (TSA) (Difco) for 18 h at 30°C. All other strains were cultured on TSA for 18 h at 37 °C. In some instances, bacteria were grown in Tryptone Soya Broth (TSB) for 18 h at their respective optimum growth temperatures. Viable counts were determined by plating serial dilutions of the broth cultures on agar media and incubating as indicated above.

***Foods and Feeds***

Samples were collected from Canadian food and animal feed-manufacturing plants by Canadian Food Inspection Agency inspection staff and submitted for routine microbiological examination at the Ottawa Laboratory (Carling). Commodities examined

**Table 4.1** Oligonucleotide primer sequences used in this study

| Primer | Sequence (5' to 3') | Amplicon size | NCBI accession number[a] |
|---|---|---|---|
| *PCR* | | | |
| INT-1 | CGC ACG ATG ATC GTG CCG TGA TCG A | 235 bp | AF261825 |
| INT-2 | TAC GGC AAG GTG CTG TGC ACG GAT C | | |
| *Probe preparation* | | | |
| INT-3 | GAT CCT TGA CCC GCA GTT GC | 174 bp | AF261825 |
| INT-4 | TGG CTT CAG GAG ATC GGA AG | | |

[a] NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

**Table 4.2** Specificity of the combined PCR and C-DBHS in the assay of target and non-target bacterial organisms[a]

| Strain | Source[b] | No. positives/total[c] |
|---|---|---|
| **Salmonellae** | | |
| *S. typhimurium* | ATCC 10145 | 0/2 |
| *S. typhimurium* | ATCC 14028 | 0/2 |
| *S. enteritidis* | ATCC 13076 | 0/2 |
| *S. senftenberg* | ATCC 8400 | 0/2 |
| *S. alachua* | OLC 57 | 0/2 |
| *S. johannesburg* | OLC 58 | 0/2 |
| *S. montevideo* | ATCC 8387 | 0/2 |
| *S.* Typhimurium DT104 (bovine) | OLC 593 | 2/2 |
| *S.* Typhimurium DT104 (porcine) | OLC 594 | 2/2 |
| *S.* Typhimurium DT104 (equine) | OLC 595 | 2/2 |
| *S.* Typhimurium DT104 (quail) | OLC 596 | 2/2 |
| *S.* Typhimurium DT104 (chicken) | OLC 597 | 2/2 |
| *S.* Typhimurium DT104 | ATCC 7004047 | 2/2 |
| **Non-Salmonellae** | | |
| *Listeria monocytogenes* | ATCC 15313 | 0/2 |
| *Escherichia coli* | ATCC 11775 | 0/2 |
| *Shigella sonnei* | ATCC 29938 | 0/2 |
| *Enterobacter cloacae* | ATCC 13047 | 0/2 |
| *Proteus vulgaris* | ATCC 13315 | 0/2 |
| *Citrobacter freundii* | ATCC 8090 | 0/2 |
| *Bacillus cereus* | ATCC 14579 | 0/2 |
| *Bacillus subtilis* | ATCC 6051 | 0/2 |
| *Escherichia coli* O157:H7 | ATCC 35150 | 0/2 |

[a] Bacterial colonies were suspended in 50 µl of PCR buffer and then lysed by addition of an equal volume of 2% (w/v) Triton X-100 (Sigma), followed by incubation at 100 °C for 10 min. Cell lysates were subjected to the PCR and C-DBHS as described in Methods.
[b] ATCC, American Type Culture Collection; OLC, Ottawa Laboratory Carling culture collection, Canadian Food Inspection Agency.
[c] No. of replicates giving positive results per total no. tested.

include grain-based animal feed (poultry feed), powdered eggs, and whole chicken carcasses.

***Sample inoculation and enrichment culture techniques***

Powdered eggs were analyzed using the standard culture technique (MFHPB-20) recommended by Health Canada (D'Aoust and Purvis, 1998; Anonymous, 2003), whereas the chicken carcass and poultry feed were analyzed by the modified semi-solid Rappaport Vassiliadis (MSRV) method (MFLP-75) (Poppe *et al.* 2004), to ensure that food and feed samples were negative for *Salmonella*. Aerobic colony counts were performed for each of the commodities examined using the standard method (MFHPB-18) recommended by Health Canada (Anonymous, 2001). Once confirmed negative for *Salmonella*, sample preparation and pre-enrichment for the different products varied according to the prescribed method (D'Aoust and Purvis, 1998; Anonymous, 2003). For powdered egg and animal feed, 25 g of sample was added to 225 g of buffered peptone water (BPW). The chicken carcass was rinsed in a sealed stomacher bag by shaking in 1 L of BPW, followed by removal of the carcass from the rinsate.

Samples were inoculated at various levels (*ca* 200 cfu, 20 cfu, 2 cfu, and 0 cfu per 25 g sample for egg and feed, or 100 ml for chicken rinsate) with *S.* Typhimurium DT104 cells by adding 100 µl of each inoculum level to the samples (duplicates) and stomaching (except for the chicken carcass rinsate). All samples were pre-enriched by incubation at 35 °C for 18 to 24 h.

For all samples, 1 ml of pre-enrichment culture was transferred to 9 ml of tetrathionate brilliant green (TBG) broth and 0.1 ml of pre-enrichment culture was

transferred to 9 ml of Rappaport Vassiliadis Soya (RVS) peptone broth. All selective enrichment cultures were incubated at 43 °C for 24 h. All pre-enrichment samples were tested using the MSRV medium (MFLP-75) to confirm the *Salmonella* status of inoculated and uninoculated samples.

***DNA probe preparation***

DNA probe used for detection of amplified DNA was prepared by PCR using primer pairs specific for the integrase gene of *S.* Typhimurium DT104 (INT-3, INT-4) (Table 4.1). The probe was designed to be internal to the amplicon arising from the INT-1 and INT-2 PCR system used in the analysis of foods (see below). For the PCR, 10 µl template DNA (10 ng) was added to 90 µl PCR mixture [2.5 units HotStarTaq DNA Polymerase (Qiagen) and 1 × PCR buffer with 1.5 mM $MgCl_2$ (Qiagen), 200 µM each dNTP (Promega), 5 µM DIG-11-dUTP (Roche), 0.5 µM each primer (INT-3, INT-4) (Table 4.1), and 2 µg BSA/ml (Sigma)]. The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf) using the following program: initial heating at 94 °C for 15.5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 1.5 min, with an additional 2 min at 72 °C following the last cycle. PCR product was purified by ethanol-precipitation and re-suspended in deionized distilled water and stored at -20 °C until use. PCR product was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences).

***PCR and Cloth-based Dot Blot Hybridization System (C-DBHS)***

Samples were subjected to a PCR incorporating a primer pair (INT-1 and INT-2; Table 4.1) targeting the integrase gene of integrons found in *S.* Typhimurium DT104. In instances where samples were comprised of whole cell suspensions (e.g. enrichment broth cultures), the cells were lysed by mixing with an equal volume of 2 % (w/v) Triton X-100 (Sigma) in water, and heating at 100 °C for 10 min. Purified genomic DNA was used directly in the PCR. For the PCR, 10 µl of sample was added to 90 µl of PCR mixture [2.5 units HotStarTaq and 1.5 × PCR buffer containing 1.5 mM $MgCl_2$, plus 200 µM each dNTP, 0.1 µM each primer (INT-1, INT-2) (Table 4.1), and 2 µg BSA/ml]. The PCR was carried out as above, with the exception that an annealing temperature of 52 °C was used. Amplified DNA was analyzed by electrophoresing 10 µl of PCR product in a 1.5% (w/v) agarose gel at 80 V for about 1.5 h, followed by staining in ethidium bromide. DNA on the gels was visualized by fluorescence under UV light and photographed onto Polaroid 667 film. The size of the amplified DNA fragments was determined by including a sample of GeneRuler™ 100 bp DNA ladder (MBI Fermentas) in each gel.

For the C-DBHS, polyester cloth (DuPont, Sontara 8100) was cut into 7 × 7 cm sheets capable of accomodating 36 individual samples spotted with a pipette (see below), and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The sheets were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M $MgCl_2$ and 0.15 M NaCl] and lightly blotted immediately prior to use for PCR product immobilization.

Amplified DNA was denatured by heating at 100 °C for 10 min, and placed on ice. Denatured PCR product was spotted (5 µl) in discrete spots on a cloth sheet,

followed by incubation at 37 °C for 30 min. Spotted product was cross-linked to the cloth by exposing the sheets to UV light for 1 min (254 nm, 100 mJ/cm$^2$) using a UVP cross-linker (Model Stratalinker 1800, Stratagene). The sheets were blocked for 20 min at 37 °C with hybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they were washed five times by saturating the sheets with 0.01 M phosphate-buffered saline [pH 7.2)/0.15 M NaCl (PBS)] containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction.

DNA on the cloth sheets was detected by hybridization for 1 h at 45 °C with DIG-labelled DNA probe diluted in hybridization solution containing 50% formamide at a final concentration of 0.1 μg DNA probe/ml hybridization solution (final volume of 7 ml for 7 × 7 cm cloth sheets), followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Cloth sheets were saturated with 7 ml anti-DIG-peroxidase conjugate (Roche) diluted 1/1000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 7 ml tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories), and incubated for 20 min. Reactions were graded as follows: positive (blue spot), negative (no spot).

**Results**

The proposed method involves a three-step process in which test samples (e.g. enrichment broth cultures) are first subjected to a PCR amplifying the integrase gene

sequence present in the class 1 integrons of *S.* Typhimurium DT104, followed by spotting of the resulting PCR products in discrete spots on polyester cloth and subsequent detection by hybridization with a digoxigenin-labelled target-specific DNA probe and immunoenzymatic assay of the bound label.

***Detectability of the combined PCR and C-DBHS***

The detectability of the combined PCR and C-DBHS was determined using both purified genomic DNA and whole cells suspended in broth. Various amounts of genomic DNA extracted from an *S.* Typhimurium DT104 strain (chicken isolate) (Table 4.2) were subjected to the PCR and C-DBHS procedure in order to determine the minimum amount of genomic DNA required to produce a positive result by hybridization with the DNA probe on polyester cloth. Positive reactions were obtained on the cloth strip using a minimum of 0.01 pg of genomic DNA input into the PCR (Table 4.3), which corresponds to approximately two bacterial genome equivalents.

The detectability of the assay for whole cells was determined by preparing serial dilutions of *S.* Typhimurium DT104 cells in tryptone soya broth, followed by lysis with Triton X-100 (see Methods) and subjecting the lysates to the combined PCR and C-DBHS procedure. A lysate derived from a minimum of *ca* 3 cfu input into the PCR was required to reliably amplify and detect the integrase gene using polyester cloth as a solid phase.

**Table 4.3** Detectability of the combined PCR and C-DBHS in the assay of various amounts of genomic DNA from *S.* Typhimurium DT104[a]

| Genomic DNA (pg) | No. positives/total[b] |
|---|---|
| 20 | 2/2 |
| 10 | 2/2 |
| 5 | 2/2 |
| 2.5 | 2/2 |
| 1.25 | 2/2 |
| 0.6 | 2/2 |
| 0.3 | 2/2 |
| 0.15 | 2/2 |
| 0.07 | 2/2 |
| 0.03 | 2/2 |
| 0.01 | 2/2 |
| 0 | 0/2 |

[a] Various amounts of genomic DNA from a penta-resistant strain of *S.* Typhimurium DT104 (ACSSuT) were subjected to the PCR and C-DBHS as described in Methods.
[b] No. of replicates giving positive results per total no. tested.

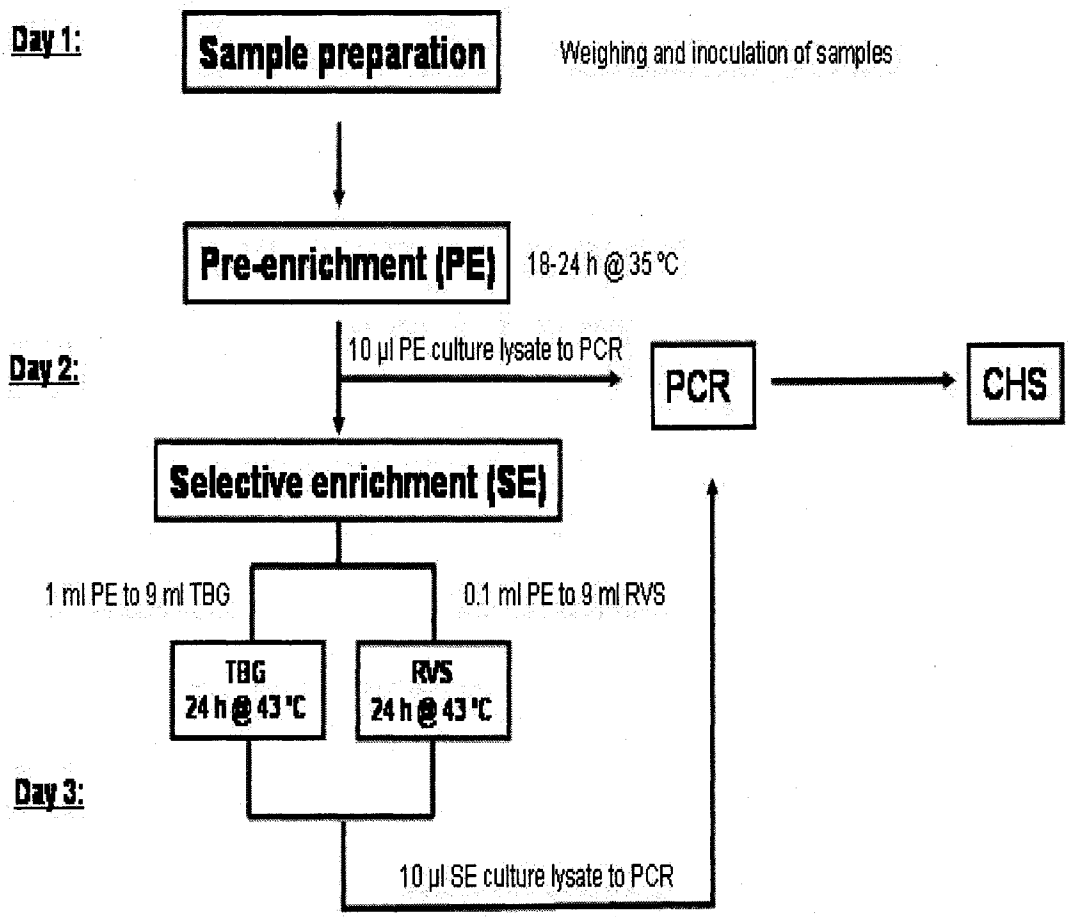
***Specificity of the combined PCR and C-DBHS***

Because foods and related commodities are complex matrices that may contain a variety of different Gram-positive and Gram-negative bacteria, an essential feature of the combined PCR and C-DBHS is that it should be specific for the detection of *S.* Typhimurium DT104 in foods without exhibiting cross-reactions with non-target organisms. Therefore, the specificity of the combined PCR and C-DBHS for *S.* Typhimurium DT104 was ascertained by subjecting colonies of a variety of target and non-target bacteria to the procedure. The six *S.* Typhimurium DT104 strains tested gave positive reactions using the C-DBHS (Table 4.2), thus demonstrating the inclusivity of the procedure. No positive reactions were obtained with the non-DT104 *Salmonella* or any of the non-*Salmonella* bacteria tested, demonstrating the exclusivity of the test (Table 4.2). These results confirm the specificity of the combined PCR and C-DBHS for the detection *S.* Typhimurium DT104 in the form of pure isolates on a plate using the integrase gene as a marker sequence specific for this organism.

***Food inoculation studies***

The feasibility of applying the DT104 PCR and C-DBHS procedure to the detection of *S.* Typhimurium DT104 in foods was determined by examining its performance as a screening tool for pre-enrichment and selective enrichment broth cultures of foods, and animal feeds inoculated with *S.* Typhimurium DT104 cells at different levels according to the scheme presented in Figure 4.1 and as described in Methods. Portions of pre-enrichment and selective enrichment broth cultures were lysed with Triton X-100. Lysates were then subjected to the PCR targeting the integrase gene of *S.* Typhimurium

**Figure 4.1** Scheme for the combined PCR and C-DBHS as applied in the detection of *Salmonella* Typhimurium DT104 from commodities after pre-enrichment and selective enrichment culture techniques.



DT104 and resulting amplicons were analyzed by the C-DBHS and by agarose gel electrophoresis.

Amplification results were consistent for all pre-enrichment cultures of the commodities analyzed, regardless of the method chosen for amplicon detection. Inoculated samples that were pre-enriched and subjected to the PCR gave positive reactions when resulting products were analyzed using the C-DBHS or agarose gel electrophoresis for all commodities analyzed (Table 4.4, Figure 4.2, Table 4.5, and Figure 4.3). In all commodities, increasing the level of inoculation did not have any effect on the intensities of the spots on the cloth sheets (data not shown) or on the intensity of the bands seen on the gel (Figure 4.2 and Figure 4.3). However, positive reactions were observed using both detection methods for uninoculated poultry feed and whole chicken pre-enrichment samples (Table 4.4, Figure 4.2, Table 4.5, and Figure 4.3). Uninoculated powdered egg samples that were pre-enriched and subjected to the PCR gave negative results when analyzed using both methods of detection (Table 4.4 and Figure 4.2).

Amplification results analyzed using the C-DBHS and agarose gel electrophoresis for selective enrichment samples were not consistent. The detection signals obtained on the cloth sheets were generally weak for both poultry feed and powdered egg selective enrichment samples, to non-existant for whole chicken (data not shown). Although weak signals were obtained on the cloth sheets for poultry feed and powdered egg selective enrichment samples, the results were consistent with what was observed on the gel (Table 4.4, Figure 4.4, and Figure 4.5). For poultry feed, positive results were obtained for samples that were initially inoculated with *S.* Typhimurium DT104 after selective enrichment in TBG (Table 4.4 and Figure 4.4). As observed with the pre-

**Table 4.4** Detection of *S.* Typhimurium DT104 from poultry feed and egg powder samples after pre-enrichment and selective enrichment by subjecting samples to the combined PCR and C-DBHS procedure

| Commodity[g] | Inoculation level (cfu)[f] | Reactivity[e] | | |
|---|---|---|---|---|
| | | Pre-enrichment[c] | Selective Enrichment[d] | |
| | | BPW | TBG | RVS |
| Poultry feed #1 | 0 | 3/3 | 0/3 | 0/3 |
| | 2 | 3/3 | 3/3 | 0/3 |
| | 20 | 3/3 | 3/3 | 0/3 |
| | 200 | 3/3 | 3/3 | 0/3 |
| Poultry feed #2 | 0 | 3/3 | 3/3 | 0/3 |
| | 2 | 3/3 | 3/3 | 0/3 |
| | 20 | 3/3 | 3/3 | 0/3 |
| | 200 | 3/3 | 3/3 | 0/3 |
| Egg powder #1 | 0 | 0/3 | 0/3 | 0/3 |
| | 2 | 3/3 | 3/3 | 0/3 |
| | 20 | 3/3 | 3/3 | 0/3 |
| | 200 | 3/3 | 3/3 | 3/3 |
| Egg powder #2 | 0 | 0/3 | 0/3 | 0/3 |
| | 2 | 3/3 | 3/3 | 3/3 |
| | 20 | 3/3 | 3/3 | 3/3 |
| | 200 | 3/3 | 3/3 | 3/3 |
| Positive control[a] | overnight culture | 3/3 | 3/3 | 3/3 |
| Negative control[b] | - | 0/3 | 0/3 | 0/3 |

[a] Positive control (BPW inoculated with *S.* Typhimurium DT104 for pre-enrichment; TBG and RVS inoculated with pre-enrichment positive control for selective enrichment) was set up in parallel with the test samples to ensure the validity of the results.

[b] Negative media control (BPW, TBG or RVS) was set up in parallel with the test samples to ensure the validity of the results.

[c] Commodity samples (inoculated and uninoculated) along with the controls were pre-enriched in buffered peptone water (BPW) and portions of the pre-enrichment samples were subjected to the PCR and C-DBHS procedure as described in Methods.

[d] Portions of pre-enrichment were transferred to each of 9 ml of tetrathionate brilliant green (TBG) broth and rappaport vassiliadis soya (RVS) peptone broth and selectively enriched. Portions of the selective enrichment cultures were subjected to the PCR and C-DBHS procedure as described in methods.

[e] No. of replicates giving positive results on cloth strip per total no. tested.

[f] Samples were inoculated at various levels (*ca* 200 cfu, 20 cfu, 2 cfu, and 0 cfu per 25 g sample for egg and feed) as described in Methods.

[g] Each commodity was analyzed in duplicate.

**Figure 4.2** Detection of *S.* Typhimurium DT104 from poultry feed and powdered egg samples after pre-enrichment by subjecting samples to the PCR and analysis of resulting products by agarose gel electrophoresis. Poultry feed and powdered egg samples were inoculated at various levels (200 cfu, 20 cfu, 2 cfu, and 0 cfu per 25 g sample) with *S.* Typhimurium DT104 and samples were pre-enriched for 18 to 24 h in BPW as described in Methods. Pre-enrichment samples (10 μl) were subjected to the PCR procedure using a primer pair targeting the integrase gene of *S.* Typhimurium DT104 to generate a 235 bp DNA fragment. PCR products were then analyzed by agarose gel electrophoresis. The following pre-enrichment samples (and inoculation levels) were used in the PCR as a source of template DNA: lanes 1 and 2, feed (200 cfu); lanes 3 and 4, feed (20 cfu); lanes 5 and 6, feed (2 cfu); lanes 7 and 8, feed (0 cfu); lanes 9 and 10, egg (200 cfu); lanes 11 and 12, egg (20 cfu); lanes 13 and 14, egg (2 cfu); lanes 15 and 16, egg (0 cfu); lane 17, positive control (BPW inoculated with *S.* Typhimurium DT104); lane 18, negative control (BPW devoid of cells). Lane m contains GeneRuler™ 100 bp DNA ladder.

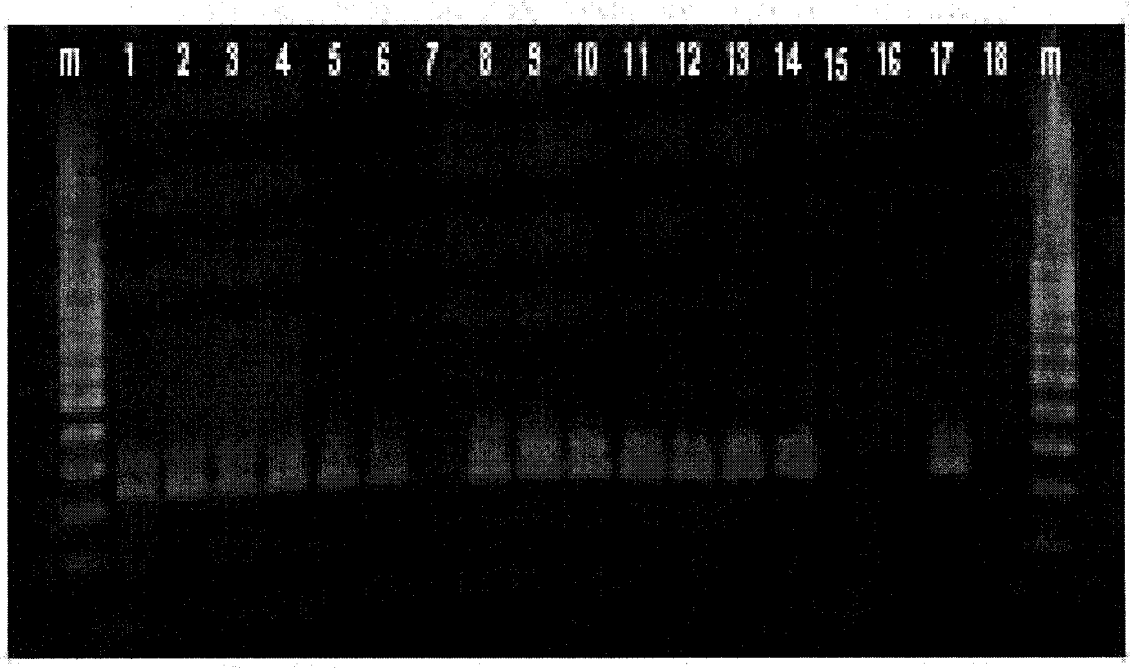


**Table 4.5** Detection of *S.* Typhimurium DT104 from whole chicken rinsate after pre-enrichment and selective enrichment by subjecting samples to the combined PCR and C-DBHS

| Commodity[g] | Inoculation level (cfu)[f] | Reactivity[e] | | |
|---|---|---|---|---|
| | | Pre-enrichment[c] | Selective Enrichment[d] | |
| | | BPW | TBG | RVS |
| Whole chicken #1 | 0 | 3/3 | 0/3 | 0/3 |
| | 2 | 3/3 | 0/3 | 0/3 |
| | 20 | 3/3 | 0/3 | 0/3 |
| | 200 | 3/3 | 0/3 | 0/3 |
| Whole chicken #2 | 0 | 3/3 | 0/3 | 0/3 |
| | 2 | 3/3 | 0/3 | 0/3 |
| | 20 | 3/3 | 0/3 | 0/3 |
| | 200 | 3/3 | 0/3 | 0/3 |
| Positive control[a] | overnight culture | 3/3 | 0/3 | 0/3 |
| Negative control[b] | - | 0/3 | 0/3 | 0/3 |

[a] Positive control (BPW inoculated with *S.* Typhimurium DT104 for pre-enrichment; TBG and RVS inoculated with pre-enrichment positive control for selective enrichment) was set up in parallel with the test samples to ensure the validity of the results.

[b] Negative media control (BPW, TBG or RVS) was set up in parallel with the test samples to ensure the validity of the results.

[c] Commodity samples (inoculated and uninoculated) along with the controls were pre-enriched in buffered peptone water (BPW) and portions of the pre-enrichment samples were subjected to the PCR and C-DBHS procedure as described in Methods.

[d] Portions of pre-enrichment were transferred to each of 9 ml of tetrathionate brilliant green (TBG) broth and rappaport vassiliadis soya (RVS) peptone broth and selectively enriched. Portions of the selective enrichment cultures were subjected to the PCR and C-DBHS procedure as described in methods.

[e] No. of replicates giving positive results on cloth strip per total no. tested.

[f] Samples were inoculated at various levels (*ca* 200 cfu, 20 cfu, 2 cfu, and 0 cfu per 100 ml sample for whole chicken rinsate) as described in Methods.

[g] Each commodity was analyzed in duplicate.

**Figure 4.3** Detection of *S.* Typhimurium DT104 from a whole chicken carcass after pre-enrichment by subjecting samples to the PCR and analysis of resulting products by agarose gel electrophoresis. The chicken carcass rinsate was inoculated at various levels (200 cfu, 20 cfu, 2 CFU, and 0 cfu per 100 ml whole chicken rinsate) and was pre-enriched for 18 to 24 h as described in Methods. Pre-enrichment samples (10 μl) were subjected to the PCR procedure using a primer pair targeting the integrase gene of *S.* Typhimurium DT104 to generate a 235 bp DNA fragment. PCR products were then analyzed by agarose gel electrophoresis. The following pre-enrichment samples (and inoculation levels) were used in the PCR as a source of template DNA: lanes 1 and 2, chicken (0 cfu); lanes 3 and 4, chicken (2 cfu); lanes 5 and 6, chicken (20 cfu); lanes 7 and 8, chicken (200 cfu); lane 9, negative control (BPW devoid of cells); lane 10, positive control (BPW inoculated with *S.* Typhimurium DT104). Lane m contains GeneRuler™ 100 bp DNA ladder.

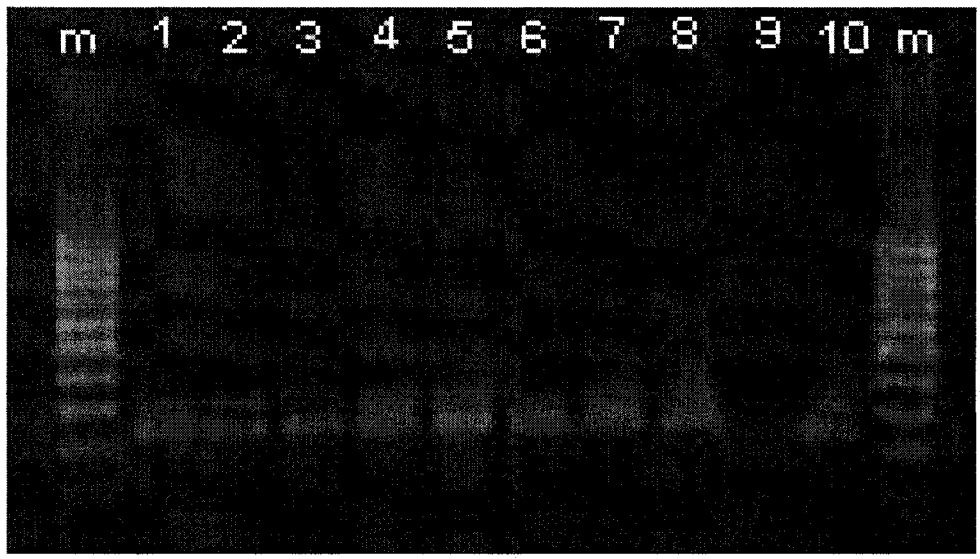


**Figure 4.4** Detection of *S.* Typhimurium DT104 from poultry feed after selective enrichment in TBG broth and RVS broth by subjecting samples to the PCR and analysis of products by agarose gel electrophoresis. Selective enrichment samples (10 µl) were subjected to the PCR procedure using a primer pair targeting the integrase gene of *S.* Typhimurium DT104 to generate a 235 bp DNA fragment. PCR products were then analyzed by agarose gel electrophoresis. The following selective enrichment samples (and initial inoculation levels) were used in the PCR as a source of template DNA: lanes 1 and 2, feed TBG (200 cfu); lanes 3 and 4, feed TBG (20 cfu); lanes 5 and 6, feed TBG (2 cfu); lanes 7 and 8, feed TBG (0 cfu); lane 9 and 10, feed RVS (200 cfu); lane 11 and 12, feed RVS (20 cfu), lane 13 and 14, feed RVS (2 cfu); lane 15 and 16, feed RVS (0 cfu); lane 17, positive control TBG; lane 18, negative control TBG. Lane m contains GeneRuler™ 100 bp DNA ladder.

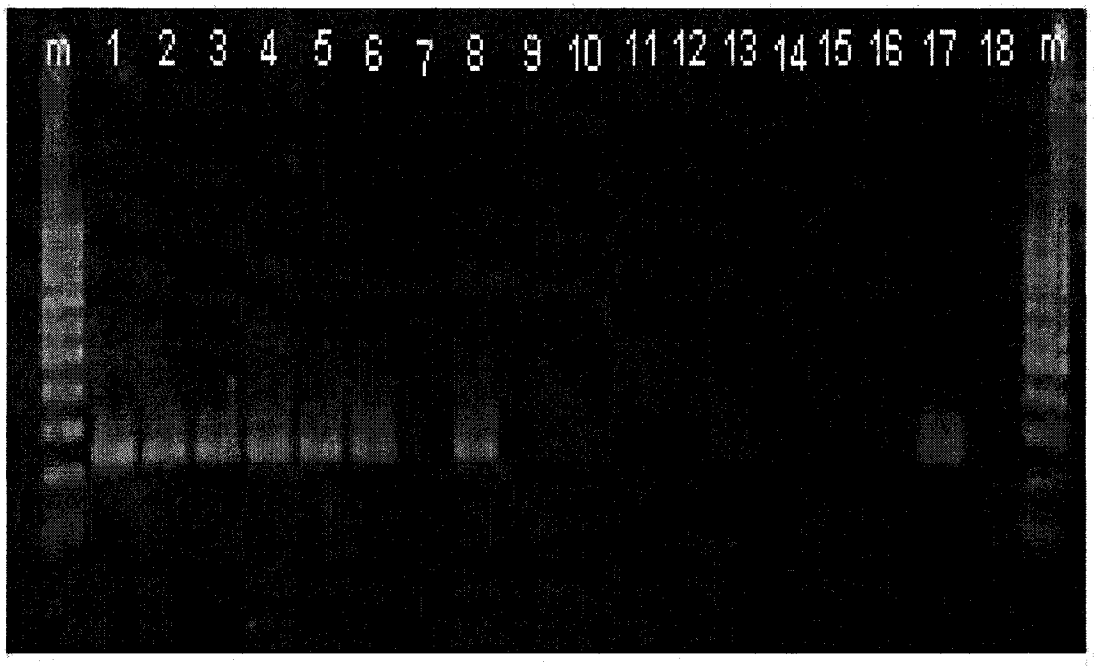
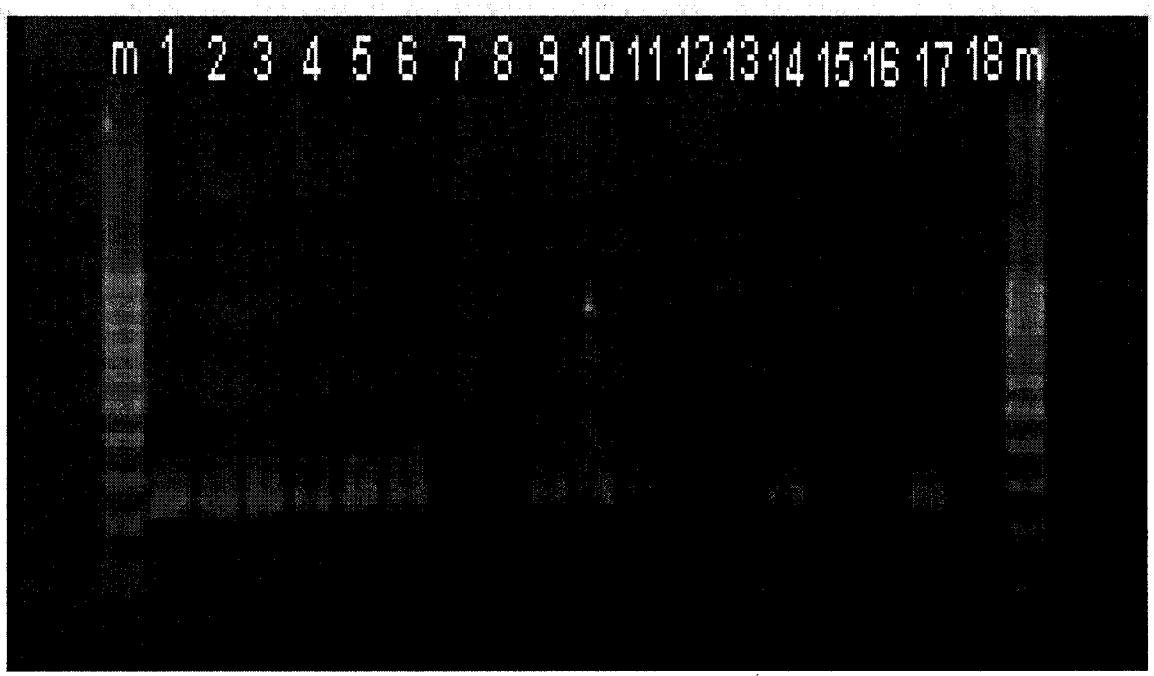
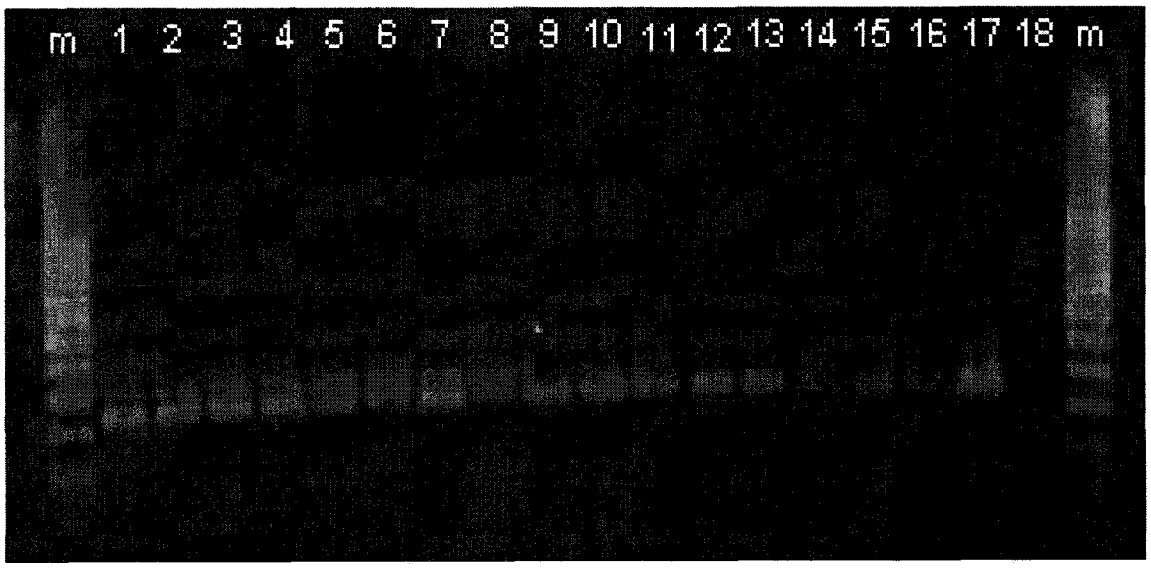


**Figure 4.5** Detection of *S.* Typhimurium DT104 from powdered egg after selective enrichment in TBG broth and RVS broth by subjecting samples to the PCR and analysis of products by agarose gel electrophoresis. Selective enrichment samples (10 μl) were subjected to the PCR procedure using a primer pair targeting the integrase gene of *S.* Typhimurium DT104 to generate a 235 bp DNA fragment. PCR products were then analyzed by agarose gel electrophoresis. The following selective enrichment samples (and initial inoculation levels) were used in the PCR as a source of template DNA: lanes 1 and 2, egg TBG (200 cfu); lanes 3 and 4, egg TBG (20 cfu); lanes 5 and 6, egg TBG (2 cfu); lanes 7 and 8, egg TBG (0 cfu); lane 9 and 10, egg RVS (200 cfu); lane 11 and 12, egg RVS (20 cfu), lane 13 and 14, egg RVS (2 cfu); lane 15 and 16, egg RVS (0 cfu); lane 17, positive control RVS; lane 18, negative control RVS. Lane m contains GeneRuler™ 100 bp DNA ladder.



enrichment samples, the intensities of the spots on the cloth sheets or the bands on the gel did not increase with the initial level of inoculation. One of the duplicate uninoculated poultry feed samples gave positive results on the cloth sheets and on the gel after selective enrichment in TBG (Table 4.4 and Figure 4.4). Negative results were obtained on the sheets and on the gel for all samples selectively enriched in RVS, regardless of the initial inoculation level (Table 4.4 and Figure 4.4). For powdered egg selectively enriched in TBG, positive results were obtained on the cloth sheets and on the gel for samples initally inoculated, whereas uninoculated samples were negative as seen with both detection methods (Table 4.4 and Figure 4.5). Results for inoculated powdered egg samples selectively enriched in RVS were sporadic but consistent for both the cloth and the gel (Table 4.4, Figure 4.5). Both of the duplicate egg powder samples initially inoculated at a level of 200 cfu per 25 g yielded positive results after selective enrichment in RVS, whereas only one of each of the duplicate egg powder samples initially inoculated at 20 cfu and 2 cfu, respectively, gave positive results as seen using both methods of detection. Both of the duplicates of uninoculated egg powder samples selectively enriched in RVS produced negative results using the C-DBHS and agarose gel electrophoresis (Table 4.4, Figure 4.5). Analysis of a whole poultry carcass selectively enriched in both TBG and RVS using the C-DBHS did not yield any visible positive reactions on the cloth sheets for any of the initial inoculation levels (Table 4.5). Analysis of all selectively enriched chicken carcass samples, including initially uninoculated samples, using agarose gel electrophoresis yielded positive reactions (Figure 4.6).

**Figure 4.6** Detection of *S.* Typhimurium DT104 from whole chicken carcass after selective enrichment in TBG broth and RVS broth by subjecting samples to the PCR and analysis of products by agarose gel electrophoresis. Selective enrichment samples (10 µl) were subjected to the PCR procedure using a primer pair targeting the integrase gene of *S.* Typhimurium DT104 to generate a 235 bp DNA fragment. PCR products were then analyzed by agarose gel electrophoresis. The following selective enrichment samples (and initial inoculation levels) were used in the PCR as a source of template DNA: lanes 1 and 2, chicken TBG (200 cfu); lanes 3 and 4, chicken TBG (20 cfu); lanes 5 and 6, chicken TBG (2 cfu); lanes 7 and 8, chicken TBG (0 cfu); lane 9 and 10, chicken RVS (200 cfu); lane 11 and 12, chicken RVS (20 cfu), lane 13 and 14, chicken RVS (2 cfu); lane 15 and 16, chicken RVS (0 cfu); lane 17, positive control RVS; lane 18, negative control RVS. Lane m contains GeneRuler™ 100 bp DNA ladder.



**Discussion**

The combined DT104 PCR C-DBHS was designed for the direct detection of *S.* Typhimurium DT104 in food samples after enrichment (pre-enrichment and/or selective enrichment) to allow for a more rapid response to contamination incidents involving this serious foodborne pathogen. The integrase gene was chosen to serve as a marker sequence for *S.* Typhimurium DT104 in PCR amplification. The integrase gene is an essential part of all integrons, and the presence of these mobile DNA elements has been described as the mechanism of antibiotic resistance found in DT104 (Sandvang *et al.* 1998).

To verify the reliability of this assay system as a tool for screening enrichment broth cultures for the presence of *S.* Typhimurium DT104, initial studies examined the performance characteristics of the combined PCR and C-DBHS procedure using pure cultures of bacteria. Intial results suggested that the system exhibited adequate detectabilities with purified genomic DNA (0.01 pg) (Table 4.3) and whole cells (*ca* 3 cfu) to enable its application in screening enrichment culture samples for the presence of *S.* Typhimurium DT104. The system also demonstrated an apparent high degree of specificity for *S.* Typhimurium DT104 (Table 4.2), thus supporting the suitability of the integrase gene as a potential target for the detection of *S.* Typhimurium DT104 in food samples.

The detection of pathogenic bacteria in foods, however, is a challenging task. Foods and other related samples are often composed of a wide spectrum of ingredients, some of which can affect bacterial cell viability, interfering with pathogen recovery and identification from food samples. Variations in the physical characteristics of foods,

from liquids to solids or semisolids, can make it difficult to isolate bacteria or obtain uniform homogenates for reproducible analysis. Another problem encountered in food safety analysis is that pathogenic bacteria are often found in very low numbers in comparison to the normal competing bacterial flora, and this becomes a critical issue when foods are being examined for pathogens which have low infectious doses. The field of food microbiology has overcome these challenges by relying on multistep enrichment culture of foods in order to enhance the detection of specific pathogens.

This chapter has examined the possibility of applying a preliminary screening method for the rapid identification of presumptive DT104 strains in samples requiring more intensive cultural, biochemical, and molecular analyses to confirm the nature of food contaminants. In Canada, foods are analyzed for *Salmonella* by standard enrichment culture techiques involving pre-enrichment followed by selective enrichment in TBG and RVS broths (D'Aoust and Purvis, 1998; Anonymous, 2003). After selective enrichment, cultures are streaked on selective differential agars for the isolation of *Salmonella*, followed by purification of presumptive isolates, biochemical screening, and serological identification, a process which requires specialized reference laboratories and can take up to 2 weeks to complete. For animal feeds, chicken carcasses, and whole eggs, pre-enrichment cultures can be screened by the MSRV method (Poppe *et al.* 2004), which is based on the ability of *Salmonella* to grow and migrate across a semi-solid agar at 42 °C. Isolates are subsequently confirmed using biochemical and serological tests. The preliminary screening method described in this chapter was designed for detection of *S.* Typhimurium DT104 in enrichment broth cultures (pre-enrichment and selective

enrichment) of foods and animal feeds as illustrated in Figure 4.1, and allowing for a more rapid screening of food samples.

The performance characteristics of the combined PCR and C-DBHS were evaluated in the detection of *S.* Typhimurium DT104 in enrichment broth cultures of foods (e.g chicken carcass and powdered egg) and animal feeds (e.g poultry feed). Pre-enrichment and selective enrichment broth cultures of inoculated food and feed samples were subjected to the PCR targeting the integrase gene and resulting amplicons were analysed by the C-DBHS as well as by standard agarose gel electrophoresis for comparison purposes. The intensity of the spots on the cloth sheets was dependent on the commodity sample analyzed and the enrichment culture step. Signals on the cloth blots were generally weak for all pre-enrichment samples and selective enrichment samples of poultry feed and powdered egg. However, the spots on the cloth sheets were barely visible for selective enrichment samples of whole chicken carcass. These weak assay signals could possibly be caused by compounds found in food matrices (e.g. proteins, fats) which may have been carried over and consequently interfered with DNA binding on the polyester cloth.

The combined PCR and C-DBHS was sensitive enough to allow for the detection of *S.* Typhimurium from all inoculated commodities examined after pre-enrichment (Table 4.4 and Table 4.5). Similar results were obtained when amplicons were analyzed using agarose gel electrophoresis techniques (Figure 4.2 and Figure 4.3). The ability of the combined PCR and C-DBHS to produce positive reactions for inoculated commodity samples after pre-enrichment is promising in that it would allow for a more timely screening of samples for the presence of *S.* Typhimurium DT104 contamination.

However, positive reactions were obtained using both C-DBHS and agarose gel electrophoresis for uninoculated poultry feed and chicken carcass samples after pre-enrichment and selective enrichment (Table 4.4, Table 4.5, Figure 4.2, Figure 4.3, Figure 4.4, and Figure 4.6). These results are not likely due to cross-contamination of the samples since uninoculated samples were always manipulated prior to the inoculated samples. In addition, all pre-enrichment samples were tested using the MSRV medium as described in Methods to confirm the *Salmonella* status of inoculated and uninoculated samples. Uninoculated samples were confirmed negative for motile *Salmonella* using the MSRV method (MFLP-75), suggesting that the positive results observed on the cloth sheets and on the gel are not due to the presence of endogenous *S.* Typhimurium DT104 in the samples. It has been reported recently that class 1 integrons are common in Gram-negative bacteria of the group Enterobacteriaceae, including *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Serratia*, and *Shigella* (Fluit and Schmitz, 2004). The presence of class 1 integrons in other bacteria of the family Enterobacteriaceae could help explain the positive results obtained for uninoculated poultry feed and whole chicken carcass after pre-enrichment. Contamination by members of this group should be expected in these types of commodities, especially in poultry carcasses since the steps involved in poultry processing (e.g. scalding, defeathering, and evisceration) can introduce bacterial contamination in the finished product. Pre-enrichment is intended to favor the repair and growth of stressed or injured *Salmonella* without necessarily repressing or inhibiting competing microorganisms found in the sample. Given this, non-target microorganisms in the sample harboring class 1 integrons would have a chance to multiply during the pre-enrichment step, and hence provide a source of DNA template for

PCR-based detection of the integrase gene. As mentioned above, positive results were also obtained from uninoculated poultry feed and whole chicken after selective enrichment, suggesting that bacterial strains, other than DT104, and harboring class 1 integrons, are providing a source of DNA template for the PCR-amplification of the integrase gene. While the selective enrichment step is intended to favor the proliferation of *Salmonella* through a selective repression or inhibition of competing organisms, these steps are not 100% effective in inhibiting all competing bacteria. For example, TBG broth contains bile which supports the growth of enteric bacteria and inhibits bacteria that are not normal members of the gut flora. The brilliant-green in TBG broth specifically inhibits the gram-positive bacteria. It is therefore likely that gram-negative enteric bacteria harboring class 1 integrons were able to survive in this medium in sufficient numbers to produce positive PCR results. Another possibility, which cannot be ruled out for the positive results obtained for uninoculated samples, is the disadvantage that DNA-based methods such as PCR do not distinguish between living and dead organisms (Herman, 1997; Sheridan *et al.* 1998). DNA molecules can remain intact even though the target organism is dead, and therefore provide a source of DNA for PCR amplification.

Integrons capture and express mobile genes known as cassettes, which are, in most cases, antibiotic resistance genes (Recchia and Hall, 1997). The integrase gene is an essential part of all integrons and it encodes a site-specific recombinase that catalyzes the insertion of gene cassettes into the integron. While I sought to investigate the usefulness of a PCR method targeting the integrase gene for the detection of DT104, the results demonstrated that this maker is not sufficiently unique to permit its use in food testing applications. It has been reported that the gene that confers resistance to

florfenicol and chloramphenicol, *flo*$_{St}$, is unique to *S.* Typhimurium DT104 and multi-drug resistant *S.* Typhimurium (Bolton *et al.* 1999). The combined PCR and C-DBHS procedure could therefore be adapted to target the *flo*$_{St}$ gene for the presumptive identification of *S.* Typhimurium DT104. Also, the issue regarding weak signals on polyester cloth due to food co-extractants would need to be addressed if this technology is to be applied to the detection of this pathogen in food samples. Methods such as DNA purification may need to be applied to the procedure following enrichment (pre-enrichment and/or selective enrichment) in order to increase signal intensity, but this possibility would need to be investigated further.

# CHAPTER 5

## Cloth-Based Hybridization Array System for the Detection of Common Toxin Genes Associated with Major Foodborne Pathogenic Bacteria

**Abstract**

A simple cloth-based hybridization array system (CHAS) was developed for the identification of toxin genes associated with major foodborne pathogenic bacteria, including toxigenic *Escherichia coli*, *Vibrio cholerae*, and *Salmonella*. Bacterial isolates were subjected to a multiplex polymerase chain reaction (PCR) incorporating digoxigenin-dUTP and primers targeting a variety of toxin genes (verotoxin, *Salmonella* enterotoxin, cholera toxin, and heat-labile/stable enterotoxin), followed by hybridization of the amplicons with an array of probes immobilized on polyester cloth and subsequent immunoenzymatic assay of the bound digoxigenin label. This system provided sensitive and specific detection of the different target toxin gene markers in a variety of bacterial isolates, exhibiting the expected patterns of reactivity with a panel of bacteria with defined toxigenicity profiles. The CHAS is a cost-effective tool facilitating the determination of the potential toxigenic profile of bacteria in the food microbiology laboratory, thus contributing valuable information to the risk assessment process in the microbiological analysis of foods.

**Introduction**

Many foodborne illnesses reported worldwide are caused by bacterial pathogens having the ability to synthesize toxins which serve as primary virulence factors, and are transmitted by foods, water, or person-to-person contact. Enteric diseases such as salmonellosis (*Salmonella* spp.), cholera (*Vibrio cholerae*), and other diarrheal infections (enterotoxigenic and enterohemorrhagic *Escherichia* coli) are among the best known

examples with a foodborne origin. Cholera, caused by the toxigenic *Vibrio cholerae*, colonizes the small intestine, produces cholera toxin (CT), an enterotoxin disrupting ion transport by intestinal epithelial cells leading to severe watery diarrhea (Faruque *et al.* 1998). Although *E. coli* is recognized as a member of the normal gut flora, some strains have the potential to cause serious illness. There are four main categories of diarrheagenic *E. coli* categorized on the basis virulence properties: (1) enterotoxigenic *E. coli* (ETEC), (2) enterohemorrhagic *E. coli* (EHEC), (3) enteropathogenic *E. coli* (EPEC), and (4) enteroinvasive *E. coli* (EIEC) (Levine, 1987). Infections by ETEC occur through the ingestion of contaminated food or water and are a common cause of watery diarrhea, traveller's diarrhea, and childhood diarrhea in developing areas (González-Garcia, 2002). The ETEC cause illness by colonizing the small intestine and elaborating heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST) (Levine, 1987). EHEC are associated with a wide spectrum of disease including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Karmali, 1989). EHEC strains producing verotoxins (VT) that are lethal to Vero cells in culture (Konowalchuk *et al.* 1977) are referred to as verotoxin-producing *E. coli* (VTEC). VTEC strains produce either one or both of two immunologically distinct VTs (VT1 and VT2) encoded by bacteriophages (Scotland *et al.* 1983; Smith *et al.* 1983).

The presence of toxigenic bacteria in the food supply is a major public health risk, and their detection to prevent transmission from foods to humans is a major challenge for regulatory agencies and the food industry. While enrichment culture techniques for the isolation of the major pathogenic bacteria from foods are generally well established, there

is a need for methods to facilitate characterization of the toxigenic potential of isolates in order to provide information for judicious risk assessments of the hazards associated with food consumption. Conventional methods for toxin detection involve time-consuming bioassays (Dean *et al.* 1972) or cell culture techniques (Konowalchuk *et al.* 1977) requiring specialized facilities, making them unsuitable for use in the routine food microbiology testing laboratory. Alternative methods for toxin characterization include gene probes (Woodward *et al.* 1990; Thomas *et al.* 1991; Yoh *et al.* 1997) and immunological techniques such as latex agglutination and ELISA (Karmali *et* al. 1999; Kehl, 2002). In addition, a number of sensitive and highly specific polymerase chain reaction (PCR) methods have been developed for the detection of toxin genes in bacteria, such as, for instance, toxigenic *Vibrio cholerae* (Varela *et al.* 1994) and VTEC (Thomas *et al.* 1994).

A further refinement of the PCR concept has been the development of multiplex approaches in which several target DNA sequences are co-amplified in a single reaction, thus increasing the amount of information which can be rapidly generated from isolates in order to assist in investigations of contamination incidents. The analysis of amplicons generated in multiplex PCR systems is commonly achieved on the basis of their differential mobility by agarose gel electrophoresis, as previously described for multiplex PCR systems targeting bacterial toxin genes (Nagano *et al.* 1998; Osek, 2001). Agarose gel electrophoresis analysis can be time-consuming and complex electrophoretic patterns may be difficult to interpret accurately. High-density microarray systems for detection and characterization of gene markers in bacteria have also been developed (Strizhkov *et*

*al.* 2000; Chizhikov *et al.* 2001), but these rely on the use of highly sophisticated instruments to prepare and process the arrays, and are not generally within the scope of analytical capabilities of the basic food microbiology laboratory.

As a practical alternative for the analysis of multiplex PCR products which can be more readily implemented in the routine food microbiology laboratory, I propose a simple low-density array technique based on the use of a macroporous, hydrophobic polyester cloth as a solid phase for the detection of amplicons from a multiplex PCR by their hybridization with an array of immobilized DNA probes. The advantages of polyester cloth as a DNA adsorbent for nucleic acid hybridization assays have been previously demonstrated (Blais and Phillippe, 1995), and were recently applied in the identification of antibiotic resistance and other genes associated with the multidrug-resistant food pathogen *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 (Gauthier and Blais, 2004) (Chapter 3, this thesis). Polyester cloth is a cost-effective support yielding improved reaction kinetics due to a large and readily accessible surface, and is easy to wash between reaction steps to remove unbound reagents. In the proposed cloth-based hybridization array system (CHAS), an array of target-specific DNA probes is immobilized on a polyester cloth strip and hybridized with digoxigenin-labelled amplicons generated in a multiplex PCR targeting multiple toxin gene markers, followed by immunoenzymatic detection of the bound label. In this chapter I demonstrate the applicability of the CHAS in the detection of various toxin genes associated with major foodborne pathogenic bacteria, including verotoxin, *Salmonella* enterotoxin, cholera toxin, and heat-labile/stable enterotoxin genes.

## Materials and Methods

### *DNA extraction and primer design*

Genomic DNA was extracted from bacterial colonies grown on nutrient agar (see below) and purified using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Purified genomic DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20 °C until use.

Oligonucleotide primer sequences targeting a variety of bacterial toxin genes used in the preparation of probes for immobilization on the arrays as well as in the multiplex PCR are given in Table 5.1. All primers were synthesized by Sigma Genosys.

### *Bacteria*

The performance characteristics of the combined multiplex PCR-CHAS procedure were assessed using a variety of reference strains listed in Table 5.2. All *Vibrio* strains used in this study were routinely grown by plating on Marine Agar (Difco) and incubating for 18 h at 37 °C. *Listeria monocytogenes*, *Enterobacter cloacae*, and *Bacillus cereus* were grown on Tryptone Soya Agar (TSA) (Difco) for 18 h at 30°C. All other strains were cultured on TSA for 18 h at 37 °C. In some instances, bacteria were grown in Tryptone Soya Broth (TSB) (or Martine Broth in the case of *V. cholerae*) for 18 h at their respective optimum growth temperatures. Viable counts were determined by plating serial dilutions of the broth cultures on agar media and incubating as indicated above.

**Table 5.1** Oligonucleotide primers used in this study[a]

| Primer | Sequence (5' to 3') | Amplicon size | NCBI[b] Accession number |
|---|---|---|---|
| *Probe Preparation* | | | |
| STN-3 | ATC GCC TCC AGC TGA TCC GG | 168 bp | L16014 |
| STN-4 | ACC CGG ACA GGC TGA CTC AG | | |
| VT1-3 | GTG ACA GCT GAA GCT TTA CG | 124 bp | M19473 |
| VT1-4 | CTT CCC CAG TTC AAT GTA AG | | |
| VT2-3 | CCA TCT TCG TCT GAT TAT TG | 139 bp | X07865 |
| VT2-4 | TGT CCG TTG TCA TGG AAA CC | | |
| LT1-3 | CAT TAC ATT TAA GAG CGG CG | 120 bp | AF242418 |
| LT1-4 | CTC GGT CAG ATA TGT GAT TC | | |
| ST1-3 | CTG AAT CAC TTG ACT CTT CA | 119 bp | M58746 |
| ST1-4 | GTT CAC AGC AGT AAA ATG TG | | |
| CTXA-3 | GAT GTA TTA GGG GCA TAC AGT CC | 179 bp | D30053 |
| CTXA-4 | TGC TGG AGC AAT ATC TAA GTT ACT G | | |
| *Multiplex PCR* | | | |
| STN-1 | CGT CAG CTT TGG TCG TAA AAT AAG G | 227 bp | L16014 |
| STN-2 | ATG AGA CGC TTA AGC GTA TTC AGG C | | |
| VT1-1 | GTG GCA AGA GCG ATG TTA CGG TTT G | 182 bp | M19473 |
| VT1-2 | ATG ATA GTC AGG CAG GAC GCT ACT C | | |
| VT2-1 | ACG AGG GCT TGA TGT CTA TCA GGC G | 200 bp | X07865 |
| VT2-2 | GCG ACA CGT TGC AGA GTG GTA TAA C | | |
| LT1-1* | ATC ATA TAC GGA ATC GAT GGC AGG C | 226 bp | AF242418 |
| LT1-2 | TAC TGA TTG CCG CAA TTG AAT TGG G | | |
| ST1-1 | CTT TCC CCT CTT TTA GTC AGT C | 176 bp | M58746 |
| ST1-2 | AAT AAC ATC CAG CAC AGG CAG G | | |
| CTXA-1 | CCA CTG CAC CCA ACA TGT TTA ACG | 232 bp | D30053 |
| CTXA-2 | ACC TGC CAA TCC ATA ACC ATC TGC | | |

[a] Gene markers are as follows: STN, *Salmonella* enterotoxin; VT1, verotoxin 1 of verotoxin-producing *E. coli* (VTEC); VT2, verotoxin 2 of VTEC; ST1, heat-stable enterotoxin 1 of enterotoxigenic *E. coli* (ETEC); LT1, heat-labile enterotoxin 1 of ETEC; CTXA, cholera toxin of *Vibrio cholerae*.

[b] NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

**Table 5.2** Bacteria examined in this study

| Organism | Source | Toxin Profile[g] | | | | | |
|---|---|---|---|---|---|---|---|
| | | STN | VT1 | VT2 | ST1 | LT1 | CTXA |
| Toxigenic bacteria | | | | | | | |
| (1) *Salmonella typhimurium* | ATCC 10145[a] | + | - | - | - | - | - |
| (2) *S. montevideo* | ATCC 8387[a] | + | - | - | - | - | - |
| (3) *S. enteritidis* | ATCC 13076[a] | + | - | - | - | - | - |
| (4) *S. senftenberg* | ATCC 8400[a] | + | - | - | - | - | - |
| (5) *S. alachua* | OLC 57[b] | + | - | - | - | - | - |
| (6) *S. johannesburg* | OLC 58[b] | + | - | - | - | - | - |
| (7) *S. typhimurium* | ATCC 14028[a] | + | - | - | - | - | - |
| (8) *Escherichia coli* O157:H7 | ATCC 35150[a] | - | + | + | - | - | - |
| (9) *E. coli* O111:NM | 910005[c] | - | + | + | - | - | - |
| (10) *E. coli* O157:H7 | 920082[c] | - | + | + | - | - | - |
| (11) *E. coli* O157:H7 | 920026[c] | - | + | + | - | - | - |
| (12) *E. coli* O157:H7 | 920192[c] | - | + | + | - | - | - |
| (13) *E. coli* O111:H11 | 910093[c] | - | + | - | - | - | - |
| (14) *E. coli* O111:H8 | 910115[c] | - | + | - | - | - | - |
| (15) *E. coli* O111:NM | 910168[c] | - | + | - | - | - | - |
| (16) *E. coli* O26:H11 | 910114[c] | - | + | - | - | - | - |
| (17) *E. coli* O5:NM | 910136[c] | - | + | - | - | - | - |
| (18) *E. coli* O157:H7 | 920027[c] | - | - | + | - | - | - |
| (19) *E. coli* O157:H7 | 920079[c] | - | - | + | - | - | - |
| (20) *E. coli* O157:H7 | 940047[c] | - | - | + | - | - | - |
| (21) *E. coli* O157:H7 | 920409[c] | - | - | + | - | - | - |
| (22) *E. coli* O157:H7 | 920037[c] | - | - | + | - | - | - |
| (23) *E. coli* O159:H4 | 99-4701-1[d] | - | - | - | - | + | - |
| (24) *E. coli* O159:H4 | 99-4711[d] | - | - | - | - | + | - |
| (25) *E. coli* | TD427C2[d] | - | - | - | - | + | - |

**Table 5.2** Continued

| Organism | Source | Toxin Profile[g] | | | | | |
|---|---|---|---|---|---|---|---|
| | | STN | VT1 | VT2 | ST1 | LT1 | CTXA |
| (26) *E. coli* | ATCC43886[a] | - | - | - | - | + | - |
| (27) *E. coli* | H10407[d] | - | - | - | + | + | - |
| (28) *E. coli* | ATCC 35401[a] | - | - | - | + | + | - |
| (29) *Vibrio cholerae* | ATCC 9459[a] | - | - | - | - | - | + |
| (30) *V. cholerae* | E121[e] | - | - | - | - | - | + |
| Non-toxigenic bacteria | | | | | | | |
| (31) *V. cholerae* | C43[e] | - | - | - | - | - | - |
| (32) *V. cholerae* | C41[e] | - | - | - | - | - | - |
| (33) *V. cholerae* | C42[e] | - | - | - | - | - | - |
| (34) *E. coli* | ATCC 11775[a] | - | - | - | - | - | - |
| (35) *V. parahaemolyticus* | ATCC 17802[a] | - | - | - | - | - | - |
| (36) *Listeria monocytogenes* | ATCC 15313[a] | - | - | - | - | - | - |
| (37) *Enterobacter cloacae* | ATCC 13047[a] | - | - | - | - | - | - |
| (38) *Klebsiella pneumoniae* | ATCC 13883[a] | - | - | - | - | - | - |
| (39) *Proteus vulgaris* | ATCC 13315[a] | - | - | - | - | - | - |
| (40) *Staphylococcus aureus* | ATCC 12600[a] | - | - | - | - | - | - |
| (41) *Citrobacter freundii* | ATCC 8090[a] | - | - | - | - | - | - |
| (42) *Bacillus cereus* | ATCC 14579[a] | - | - | - | - | - | - |
| (43) *Morganella morganii* | OLC 641[b] | - | - | - | - | - | - |
| (44) *Shigella sonnei* | ATCC 29938[a] | - | - | - | - | - | - |
| (45) *Shigella flexneri* | HC25[f] | - | - | - | - | - | - |

[a] American Type Culture Collection, Manassas, Virginia, USA; [b] Ottawa Laboratory (Carling) Culture Collection, Canadian Food Inspection Agency, Ottawa, Ontario, Canada; [c] S. Read, Laboratory for Foodborne Zoonoses, Health Canada, Guelph, Ontario, Canada; [d] T. Kruk, National Laboratory for Enteric Pathogens, Health Canada, Winnipeg, Manitoba, Canada; [e] E. Buenaventura, Burnaby Laboratory (Boundary) – Fish Microbiology, Canadian Food Inspection Agency, Burnaby, British Columbia, Canada; [f] C. Kingombe, Bureau of Microbial Hazards, Health Canada, Ottawa, Ontario, Canada.

[g] Gene markers were as follows: STN, *Salmonella* enterotoxin; VT1, verotoxin 1 of verotoxin-producing *E. coli* (VTEC); VT2, verotoxin 2 of VTEC; ST1, heat-stable enterotoxin 1 of enterotoxigenic *E. coli* (ETEC); LT1, heat-labile enterotoxin 1 of ETEC; CTXA, cholera toxin of *Vibrio cholerae*.

***DNA probe preparation***

DNA probes for immobilization on the arrays were individually prepared by PCR using primer pairs (Table 5.1) specific for each of the following toxin gene markers (strain from Table 5.2 used for the preparation of template DNA indicated in parentheses): (1) *Salmonella* enterotoxin (STN-3, STN-4) (strain 7); (2) verotoxin 1 of verotoxin-producing *E. coli* (VTEC) (VT1-3, VT1-4) (strain 11); (3) verotoxin 2 of VTEC (VT2-3, VT2-4) (strain 11); (4) heat-labile enterotoxin 1 of enterotoxigenic *E. coli* (ETEC) (LT1-3, LT1-4) (strain 23); (5) heat-stable enterotoxin 1 of ETEC (ST1-3, ST1-4) (strain 27); (6) cholera toxin of *Vibrio cholerae* (CTXA-3, CTXA-4) (strain 29). PCR probes were designed to be internal to the amplicons from the multiplex PCR system to avoid possible primer dimer-complementary sequences possibly arising in the latter. For the production of PCR probe, 10 μl of appropriate template DNA (10 ng) was added to 90 μl of PCR mixture [2.5 units HotStarTaq DNA Polymerase and 1 × PCR buffer with 1.5 mM $MgCl_2$ (Qiagen), 200 μM each dNTP (Promega), 0.5 μM each primer (Table 5.1), and 2 μg BSA/ml (Sigma Chemical Co.)]. The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf) using the following program: initial heating at 94 °C for 15.5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C (exceptions: 51 °C for ST1, and 60 °C for CTXA) for 30 s, and primer extension at 72 °C for 1.5 min, with an additional 2 min at 72 °C following the last cycle. PCR product was purified by ethanol-precipitation and re-suspended in deionized distilled water and stored at -20 °C until use. PCR product was quantified using Hoefer DyNA Quant 200 fluorometer.

***Multiplex PCR and CHAS procedure***

Samples were subjected to a multiplex PCR incorporating six primer pairs targeting the various toxin gene markers (Table 5.1). In instances where samples were comprised of whole cell suspensions (e.g., enrichment broth cultures), the cells were lysed by mixing with an equal volume of 2 % (w/v) Triton X-100 (Sigma Chemical Co.) in water, and heating at 100 °C for 10 min. Purified genomic DNA was used directly in the PCR. For the PCR, 10 µl of test sample was added to 90 µl of multiplex PCR mixture [2.5 units HotStarTaq and 1 × PCR buffer containing 1.5 mM $MgCl_2$, plus 200 µM each dNTP, 5 µM DIG-11-dUTP (Roche), 0.1 µM each primer (Table 5.1), and 2 µg BSA/ml]. The PCR cycling parameters were as above (with an annealing temperature of 55 °C).

For the CHAS, polyester cloth (DuPont, Sontara 8100) was cut into 2 × 4 cm strips, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M $MgCl_2$, and 0.1 M NaCl] and lightly blotted immediately prior to use for DNA probe immobilization.

The probes were diluted in coating buffer (10 ng/µl) and denatured by heating at 100 °C for 10 min, then placed on ice. Probes were spotted (5 µl) in discrete spots on a cloth strip, followed by incubation at 37 °C for 30 min. DNA probes were cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 $mJ/cm^2$) using a UVP cross-linker (Model Stratalinker 1800, Stratagene). The strips were blocked by incubation for 1 h at 37 °C with hybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1%

(w/v) protein blocking reagent (Bio-Rad)], after which they were washed five times by saturating strips with 0.01 M phosphate-buffered saline [pH 7.2/0.15 M NaCl (PBS)] containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips were air dried and stored at 4 °C until use.

For the assay of DIG-labelled multiplex PCR products, amplicons were denatured by heating at 100 °C for 10 min, and placed on ice. The PCR product (100 µl) was mixed with 900 µl hybridization solution containing 50% (v/v) formamide. The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 30 min at 45 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-DIG-peroxidase conjugate (Roche) diluted 1/2000 in PBST containing 0.05% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 1 ml of tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories), and incubated for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

## Results

### *Detectability of the combined multiplex PCR-CHAS procedure*

The CHAS used in conjunction with a multiplex PCR targeting a panel of toxin genes (Table 5.1) is designed to provide a tool for the characterization of the toxigenic potential of bacterial isolates from foods. While the primary purpose of this procedure is to screen colonies from plating media, maximal detectability for the target genes is highly desirable

to assure the reliability of the method. To verify the performance characteristics of the system in this regard, the detectability of the combined PCR-CHAS procedure was determined for both purified genomic DNA and whole cells suspended in broth. Various amounts of genomic DNA extracted from different toxigenic bacteria were subjected to the multiplex PCR-CHAS to determine the minimum amount of genomic DNA input into the PCR, required to evince positive reactions with the immobilized probes on the cloth array. The minimum quantity of genomic DNA from the different bacterial sources producing positive reactions with their respective relevant probes on the array was variable (Table 5.3). For instance, a minimum of 0.12 pg of *Salmonella typhimurium* genomic DNA was required to obtain a positive reaction with the *Salmonella* enterotoxin (STN) probe on the array, with no reactions occurring with any of the other toxin probes on the array at any DNA level tested; whereas, a minimum of 0.015 pg of *Vibrio cholerae* genomic DNA was required to obtain a positive reaction with the CTXA probe (Table 5.3). All of the other bacteria tested exhibited detectabilities with their respective relevant probes on the array within the 0.015-0.12 pg DNA range, and no heterologous probe reactions were observed for the different bacteria at any DNA level tested. These experiments were repeated and the results were reproducible.

The detectability of the assay for whole cells was determined by preparing serial dilutions of broth cultures of the various toxigenic bacteria (Table 5.3) in Marine broth for *Vibrio cholerae* and in TSB for all other strains, which were then lysed and subjected to the multiplex PCR-CHAS. Lysates from a minimum of *ca* 10 cfu (or less) input into the PCR produced positive reactions with the relevant probes on the array, with no

**Table 5.3** Detectability of multiplex PCR-CHAS in the assay of toxigenic foodborne bacteria[a]

| Organism | Source | Sample | Minimum detected[b] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | STN | VT1 | VT2 | ST1 | LT1 | CTXA |
| *S. typhimurium* | ATCC 14028 | DNA (pg) | 0.12 | - | - | - | - | - |
| | | Whole cells (cfu) | 10 | - | - | - | - | - |
| *E. coli* O157:H7 | 920026 | DNA (pg) | - | 0.06 | 0.06 | - | - | - |
| | | Whole cells (cfu) | - | 5 | 5 | - | - | - |
| *E. coli* | H10407 | DNA (pg) | - | - | - | 0.015 | 0.015 | - |
| | | Whole cells (cfu) | - | - | - | 1 | 1 | - |
| *E. coli* O159:H4 | 99-4701-1 | DNA (pg) | - | - | - | - | 0.06 | - |
| | | Whole cells (cfu) | - | - | - | - | 4 | - |
| *V. cholerae* | ATCC 9459 | DNA (pg) | - | - | - | - | - | 0.015 |
| | | Whole cells (cfu) | - | - | - | - | - | 1 |

[a] Various amounts of genomic DNA (range of 0.01 to 1.0 pg) and whole cells (range of *ca* 0.1 to $10^6$ cfu) from the different bacteria were subjected to the combined multiplex PCR-CHAS procedure as described in Methods. Gene markers are as follows: STN, *Salmonella* enterotoxin; VT1, verotoxin 1 of verotoxin-producing *E. coli* (VTEC); VT2, verotoxin 2 of VTEC; ST1, heat-stable enterotoxin 1 of enterotoxigenic *E. coli* (ETEC); LT1, heat-labile enterotoxin 1 of ETEC; CTXA, cholera toxin of *Vibrio cholerae*.
[b] Minimum amount of genomic DNA (pg) or whole cells (cfu) going into the multiplex PCR, that produced positive reactions with the immobilized probes on the cloth array where results were reproducible (duplicates). –, no reaction.

heterologous probe reactions occurring for the different bacteria at any cell level tested (Table 5.3).

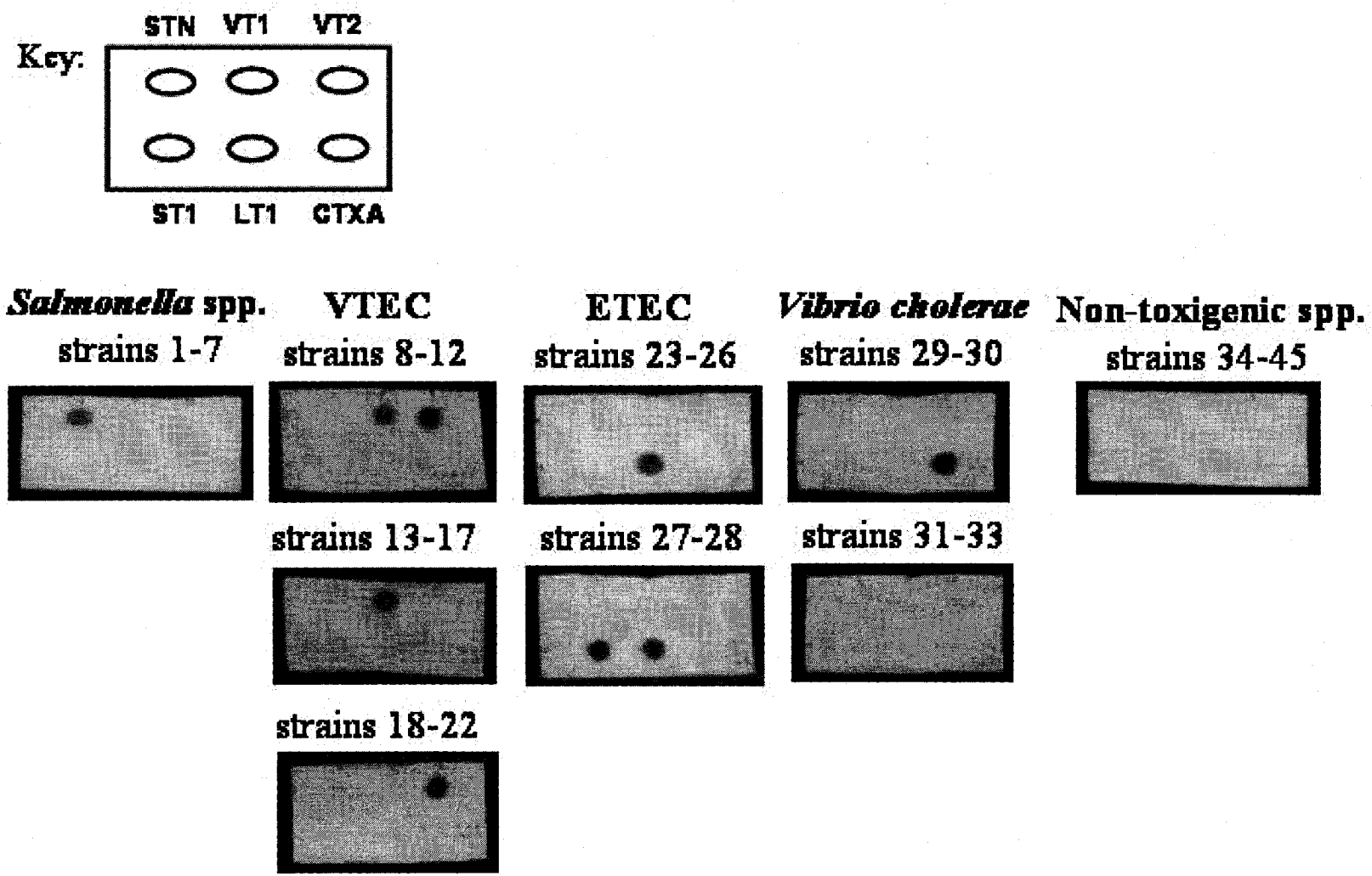
***Specificity of the combined multiplex PCR-CHAS procedure***

The specificity of the combined multiplex PCR-CHAS for the target gene markers was ascertained using colonies of various toxigenic and non-toxigenic bacteria. The toxin profiles of the various organisms used in this study is shown in Table 5.2. All toxigenic bacteria displayed the expected reactivity patterns on the array, and no heterologous hybridization reactions were observed (Figure 5.1). Thus, the inclusivity of the multiplex PCR-CHAS is demonstrated by the homologous reactions of the different toxigenic bacteria with their respective probes on the array, and its exclusivity is suggested by the lack of heterologous reactions between amplicons from toxigenic bacteria and the probes on the array. None of the various Gram-positive and Gram-negative non-toxigenic bacteria tested produced positive reactions with any of the toxin probes on the array, further demonstrating the exclusivity of this procedure. These experiments were repeated and the results were reproducible. These results confirm the specificity of the multiplex PCR-CHAS procedure in the characterization of toxigenic bacteria.

**Discussion**

These studies demonstrate the applicability of a multiplex PCR system combined with a simple cloth-based hybridization procedure for the detection of various common toxin genes associated with major foodborne pathogenic bacteria. The system described here

**Figure 5.1** Specificity of the combined multiplex PCR-CHAS. A variety of toxigenic and non-toxigenic bacteria isolated on plating media were subjected to the combined multiplex PCR-CHAS procedure targeting various toxin genes. For each isolate tested, a single colony was suspended in 50 µl of 1 × PCR buffer and then subjected to the lysis procedure described in Methods. Typical array reactivity patterns are shown pictorially for all of the strains tested. Gene markers are as follows: STN, *Salmonella* enterotoxin; VT1, verotoxin 1 of verotoxin-producing *E. coli* (VTEC); VT2, verotoxin 2 of VTEC; ST1, heat-stable enterotoxin 1 of enterotoxigenic *E. coli* (ETEC); LT1, heat-labile enterotoxin 1 of ETEC; CTXA, cholera toxin of *Vibrio cholerae*.



exhibited adequate detectability characteristics for all the toxin gene markers tested to enable its reliable application in screening colonies of food isolates (Table 5.3). Moreover, this system was highly specific in that the expected reactivity patterns (Table 5.2) with the array of probes were obtained using the multiplex PCR products from various bacteria, with the combined multiplex PCR-CHAS distinguishing toxigenic from non-toxigenic bacteria and correctly identifying the specific toxin genes harboured by the different bacteria (Figure 5.1). The combined multiplex PCR and CHAS procedure could be completed within a single day, thus providing information on the nature of the isolates at a much earlier juncture than standard bioassay or cell culture techniques, enabling its use as a diagnostic tool for the rapid and specific characterization of colonies isolated from food samples or clinical specimens using suitable enrichment culture techniques. The CHAS facilitates the detection of different amplicons generated in the multiplex PCR and provides confirmation of their identity by hybridization with immobilized probes, obviating the need for time-consuming restriction endonuclease analysis as used with conventional agarose gel electrophoresis techniques. Thus, the specificity of the present system is doubly assured through the use of target-specific PCR primers and probes. While in this study I focused on the detection of six toxin genes associated with major foodborne pathogens, the multiplex PCR and CHAS procedures can be expanded to include the identification of other toxin or virulence genes of interest.

# CHAPTER 6

## Cloth-Based Hybridization Array System for the Detection of *Clostridium botulinum* Type A, B, E, and F Neurotoxin Genes

**Abstract**

A simple cloth-based hybridization array system (CHAS) was developed for the characterization of *Clostridium botulinum* isolates based on the botulinum neurotoxin (BoNT) serotype. Bacterial isolates were subjected to a multiplex PCR incorporating digoxigenin-dUTP and primers targeting the four BoNT gene serotypes (A, B, E, and F) predominantly involved in human illness, followed by hybridization of the amplicons with an array of toxin gene-specific oligonucleotide probes immobilized on polyester cloth, and subsequent immunoenzymatic assay of the bound digoxigenin label. This system provided sensitive and specific detection of the different BoNT gene markers in a variety of *C. botulinum* strains, exhibiting the expected patterns of reactivity with a panel of target and non-target organisms. The CHAS is a cost-effective tool facilitating the characterization of *C. botulinum* serotypes in the food microbiology laboratory, thus contributing valuable information to the risk assessment process in the microbiological analysis of foods.

**Introduction**

*Clostridium botulinum* is an anaerobic spore-forming bacterium with the ability to produce highly potent botulinum neurotoxin (BoNT) responsible for the paralytic disease known as botulism. BoNTs are classified into seven different types (A through G), based on the serological specificity of the toxin. Strains of *C. botulinum* are divided into four groups (designated Groups I to IV), based on physiological differences. Group I includes the proteolytic strains of toxin types A, B, and F. Group II contains toxin type E

strains and the nonproteolytic strains of toxin types B and F. Group III contains strains of toxin types C and D and are responsible for animal botulism. Group IV contains strains producing type G toxin. *C. botulinum* strains involved in human illness fall into Groups I and II (Hatheway, 1990; Rhodehamel *et al.* 1992).

A foodborne source of the illness is confirmed for patients exhibiting the clinical syndrome of botulism when neurotoxin and/or *C. botulinum* cells are detected in suspect food samples (Austin and Blanchfield, 1997). The standard method for toxin detection and the characterization of bacterial isolates is the mouse bioassay (Kautter and Solomon, 1977; Austin and Blanchfield, 1997), which requires specialized facilities, is costly, labour-intensive, and time-consuming. There is a need for more rapid and practical methods for the characterization of bacterial isolates with respect to botulinum toxin production.

Molecular-based detection methods such as the polymerase chain reaction (PCR) technique have revolutionized the field of diagnostic microbiology. PCR offers high sensitivity and specificity in the detection of gene markers associated with a variety of pathogenic microorganisms. Several PCR-based methods have been reported for the detection and characterization of *C. botulinum* based on the amplification of BoNT gene sequences (Aranda *et al.* 1997; Braconnier *et al.* 2001; Craven *et al.* 2002; Franciosa *et al.* 1994; Hielm *et al.* 1996; Takeshi *et al.* 1996). The use of multiplex PCR approaches in which several target DNA sequences are co-amplified in a single reaction might increase the amount of information which can be rapidly generated from isolates in order to assist in trace back investigations of incidents of foodborne botulism. The analysis of

amplicons generated in multiplex PCR systems is commonly achieved on the basis of their differential electrophoretic mobility by agarose gel electrophoresis, as previously described for a multiplex PCR system targeting botulinum toxin genes (Lindström *et al.*, 2001). Agarose gel electrophoresis analysis is time-consuming and the complex electrophoretic patterns obtained with multiplex PCR can be difficult to interpret. Furthermore, the identity of individual amplicons can be difficult to confirm, especially if restriction endonuclease analysis is required. High-density microarray systems have been developed for the analysis of DNA fragments from bacteria (Strizhkov *et al.* 2000; Chizhikov *et al.* 2001), but these rely on the use of highly sophisticated instruments and procedures to process the arrays, and are not generally within the means of the basic food microbiology laboratory.

As a practical alternative for the analysis of multiplex PCR products, I propose a simple low-density array technique based on the use of macroporous, hydrophobic polyester cloth as a solid phase for the detection of amplicons from a multiplex PCR targeting BoNT gene serotypes A, B, E, and F by their hybridization with immobilized oligonucleotide probes. The advantages of polyester cloth as a DNA adsorbent for nucleic acid hybridization assays have been previously demonstrated (Blais and Phillippe, 1995) and were recently applied in the identification of antibiotic resistance and other genes associated with the multidrug-resistant food pathogen *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104 (Gauthier and Blais, 2004) (Chapter 3, this thesis) and toxin genes in a variety of food pathogens (Chapter 5, this thesis). Polyester cloth is a cost-effective solid support yielding improved reaction

kinetics due to a large and readily accessible surface, and is easy to wash between reaction steps to remove unbound reagents. In the proposed cloth-based hybridization array system (CHAS), an array of target-specific oligonucleotide probes is immobilized on a polyester cloth strip and hybridized with digoxigenin-labelled amplicons generated in a multiplex PCR targeting multiple BoNT gene markers, followed by immunoenzymatic detection of the bound label. In this chapter I demonstrate the applicability of the CHAS in the characterization of *C. botulinum* isolates based on their BoNT gene serotypes. In previous chapters describing the use of CHAS, double-stranded DNA fragments generated by PCR were used as DNA probes on the arrays. In this chapter, the use of oligonucleotide probes for immobilization on cloth strips was investigated. Oligonucleotide probes offer a number of advantages over PCR probes. Oligonucleotide probes are short, single-stranded, defined nucleotide sequences which can be synthesized, to produce large amounts of uniform sequences. The ability to chemically synthesize oligonucleotide probes is more convenient, and saves considerable amounts of time compared to PCR probe production, which involves time-consuming cycling conditions, ethanol precipitation techniques to purify the probe, and quantification. Oligonucleotide probes are also more cost-efficient than PCR probes as they do not require the use of expensive reagents such as DNA polymerase for their production.

## Materials and Methods

### *DNA extraction and primer design*

Genomic DNA was extracted and purified from broth cultures of *Clostridium botulinum* strains (strains 1, 4, 10, and 14, Table 6.1) by Brigitte Cadieux (Botulism Reference Service, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada) as previously described (Hyttiä *et al.* 1999). Purified genomic DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20 °C until use.

The oligonucleotide primer sequences targeting the four BoNT gene serotypes (A, B, E, and F) used in the multiplex PCR are presented in Table 6.2. All primers were synthesized by Sigma Genosys.

### *Bacteria*

The performance characteristics of the combined multiplex PCR-CHAS procedure were assessed using a variety of reference strains listed in Table 6.1. Three strains of *C. botulinum* type A, three strains of group I *C. botulinum* type B, three strains of group II *C. botulinum* type B, three strains of *C. botulinum* type E, three strains of group II *C. botulinum* type F, and 12 other *Clostridium* species were used in this study (Table 6.1). *Clostridium* strains were routinely grown anaerobically (10% $H_2$, 10% $CO_2$, 80% $N_2$) at their respective optimum temperatures (35 °C for *C. botulinum* type A, group I *C. botulinum* type B, and other *Clostridium* species; 25 °C for group II *C. botulinum* type B, *C. botulinum* type E, and *C. botulinum* type F) for 17 hours in 8 ml special peptone-peptone-glucose-yeast extract broth (SPGY) [5% (w/w) special peptone (Oxoid), 0.5%

**Table 6.1** Bacteria examined in this study

| Organism | Group | Strain | Serological Profile[i] | | | |
|---|---|---|---|---|---|---|
| | | | BoNT A | BoNT B | BoNT E | BoNT F |
| ***Clostridia*** | | | | | | |
| 1) *C. botulinum* | I | 62A[a] | + | - | - | - |
| 2) *C. botulinum* | I | 17A[a] | + | - | - | - |
| 3) *C. botulinum* | I | A6[a] | + | - | - | - |
| 4) *C. botulinum* | I | 13983IIB[a] | - | + | - | - |
| 5) *C. botulinum* | I | 368B[a] | - | + | - | - |
| 6) *C. botulinum* | I | IB1-B[a] | - | + | - | - |
| 7) *C. botulinum* | II | 17B[a] | - | + | - | - |
| 8) *C. botulinum* | II | 2B[a] | - | + | - | - |
| 9) *C. botulinum* | II | DB-2[a] | - | + | - | - |
| 10) *C. botulinum* | II | Russ[a] | - | - | + | - |
| 11) *C. botulinum* | II | Bennett[a] | - | - | + | - |
| 12) *C. botulinum* | II | Gordon[a] | - | - | + | - |
| 13) *C. botulinum* | II | 70F[a] | - | - | - | + |
| 14) *C. botulinum* | II | 610F[a] | - | - | - | + |
| 15) *C. botulinum* | II | 190F[a] | - | - | - | + |
| 16) *C. aurantibutyricum* | n/a | ATCC 17777[b] | - | - | - | - |
| 17) *C. baratii* | | 4624[c] | - | - | - | - |
| 18) *C. beijenrinckii* | | A401[d] | - | - | - | - |
| 19) *C. bifermentans* | | ATCC 638[b] | - | - | - | - |
| 20) *C. butyricum* | | ATCC 19398[b] | - | - | - | - |
| 21) *C. difficile* | | ATCC 9689[b] | - | - | - | - |
| 22) *C. hastiforme* | | ATCC 33268[b] | - | - | - | - |
| 23) *C. novyi* B | | ATCC 27606[b] | - | - | - | - |
| 24) *C. perfringens* | | ATCC 13124[b] | - | - | - | - |

**Table 6.1** Continued

| Organism | Group | Strain | Serological Profile[i] BoNT A | BoNT B | BoNT E | BoNT F |
|---|---|---|---|---|---|---|
| 25) *C. sordellii* | | ATCC 9714[b] | - | - | - | - |
| 26) *C. sporogenes* | | ATCC 3584[b] | - | - | - | - |
| 27) *C. tetani* | | A064[d] | - | - | - | - |
| ***Salmonella*** | | | | | | |
| 28) *S. typhimurium* | | ATCC 14028[b] | - | - | - | - |
| 29) *S. montevideo* | | ATCC 8387[b] | - | - | - | - |
| 30) *S. enteritidis* | | ATCC 13076[b] | - | - | - | - |
| 31) *S. senftenberg* | | ATCC 8400[b] | - | - | - | - |
| 32) *S. alachua* | | OLC57[f] | - | - | - | - |
| 33) *S. johannesburg* | | OLC58[f] | - | - | - | - |
| ***Escherichia coli*** | | | | | | |
| 34) *E. coli* | | ATCC 11775[b] | - | - | - | - |
| 35) *E. coli* O157:H7 | | ATCC 35150[b] | - | - | - | - |
| 36) *E. coli* O111:H11 | | 910093[g] | - | - | - | - |
| 37) *E. coli* O111:H8 | | 910115[g] | - | - | - | - |
| 38) *E. coli* O26:H11 | | 910114[g] | - | - | - | - |
| 39) *E. coli* O157:H7 | | 920026[g] | - | - | - | - |
| 40) *E. coli* O157:H7 | | 930413[g] | - | - | - | - |
| 41) *E. coli* O159:H4 | | 99-4701-1[b] | - | - | - | - |
| 42) *E. coli* | | TD427C2[h] | - | - | - | - |
| 43) *E. coli* | | TD231C2[h] | - | - | - | - |
| 44) *E. coli* | | ATCC 35401[b] | - | - | - | - |
| 45) *E. coli* | | ATCC 43886[b] | - | - | - | - |

**Table 6.1** Continued

| Organism | Group | Strain | Serological Profile[i] BoNT A | BoNT B | BoNT E | BoNT F |
|---|---|---|---|---|---|---|
| ***Other spp.*** | | | | | | |
| 46) *Listeria monocytogenes* | | ATCC 15313[b] | - | - | - | - |
| 47) *Shigella sonnei* | | ATCC 29938[b] | - | - | - | - |
| 48) *Enterobacter cloacae* | | ATCC 13047[b] | - | - | - | - |
| 49) *Klebsiella pneumoniae* | | ATCC 13883[b] | - | - | - | - |
| 50) *Proteus vulgaris* | | ATCC 13315[b] | - | - | - | - |
| 51) *Vibrio parahaemolyticus* | | ATCC17802[b] | - | - | - | - |
| 52) *Staphylococcus aureus* | | ATCC 12600[b] | - | - | - | - |
| 53) *Citrobacter freundii* | | ATCC 8090[b] | - | - | - | - |
| 54) *Bacillus cereus* | | ATCC 14579[b] | - | - | - | - |
| 55) *Vibrio cholerae* | | ATCC 9459[b] | - | - | - | - |
| 56) *Shigella flexneri* | | HC25[e] | - | - | - | - |
| 57) *Shigella dysenteriae* | | HC8[e] | - | - | - | - |

[a] Botulism Reference Service, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada; [b] American Type Culture Collection, Manassas, Virginia, USA; [c] Dr. L.V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA; [d] Laboratory Centre for Disease Control, Population and Public Health Branch, Health Canada, Ottawa, Ontario, Canada; [e] C. Bin-Kingombe, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada; [f] Ottawa Laboratory (Carling) Culture Collection, Canadian Food Inspection Agency, Ottawa, Ontario, Canada; [g] S. Read, Laboratory for Foodborne Zoonoses, Health Canada, Guelph, Ontario, Canada; [h] T. Kruk, National Laboratory for Enteric Pathogens, Health Canada, Winnipeg, Manitoba, Canada; [i] Gene markers are as follows: BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; and BoNT F, botulinum neurotoxin type F.

**Table 6.2** Oligonucleotide primers and probes used in the combined multiplex PCR-CHAS procedure[a]

| Primer or Probe | Sequence (5' to 3') | Amplicon Size | NCBI[b] Accession number |
|---|---|---|---|
| BoNT A-1 | CAT GGT AAA TCT AGG ATT GC | 125 bp | AF461540 |
| BoNT A-2 | CAT AGC TGC CTC CGT AGC TT | | |
| Probe A | CGT GTT TAT ACA TTT TTT TCT TCA GAC - TAT GTA AA | 35 bp | AF461540 |
| BoNT B-1 | CAA GAT CCC AGC ATC ATA AGT | 122 bp | AF295926 |
| BoNT B-2 | AAT GTT AGG ATC TGA TAT GC | | |
| Probe B | TAG AGG GAT AGT TGA TAG ACT TAA CAA - GGT TTT AG | 35 bp | AF295926 |
| BoNT E-1 | GAC AGG TTC TTA ACT GAA AG | 144 bp | X62089 |
| BoNT E-2 | TCT CCC AAG ATT GAT CCA TG | | |
| Probe E | GAG AAT ATG ATG AGA ATG TCA AAA CGT - ATT TAT TG | 35 bp | X62089 |
| BoNT F-1 | CAT GCA GAT ACC ATA TGA AG | 113 bp | X81714 |
| BoNT F-2 | AAA TCA CTA GGA TCC GTT CC | | |
| Probe F | GAG ATT ATG CGT AAT GTT TGG ATA ATT - CCT GAG AG | 35 bp | X81714 |

[a] BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; and BoNT F, botulinum neurotoxin type F.
[b] NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

(w/w) peptone (Becton Dickinson and Co.), 0.4% (w/w) glucose (Difco), 2% (w/w) yeast extract (Difco), 0.1% (w/w) sodium thioglycollate (Sigma), pH 7.2]. In instances where tests were carried out on isolated colonies, *Clostridium botulinum* strains were plated on McClung Toabe agar plates (7.5% [w/w] McClung Toabe agar base [Difco], 0.5% [w/w] yeast extract [Difco], 5.0% [w/w] egg yolk in 0.85 % [w/v] NaCl, pH 7.6 [Difco]) and incubated anaerobically for 48 h at their respective optimum growth temperatures as indicated above. The growth and manipulation of Clostridia strains mentioned above was done by Brigitte Cadieux (Botulism Reference Service, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada).

A variety of non-*Clostridium* bacteria were also used in this study (Table 6.1). All *Vibrio* strains were routinely grown on Marine Agar (Difco) for 18 h at 37 °C. *Listeria monocytogenes*, *Enterobacter cloacae*, and *Bacillus cereus* were grown on Tryptone Soya Agar (TSA) (Difco) for 18 h at 30°C. All other strains were cultured on TSA for 18 h at 37 °C. Bacteria cell counts were determined by performing serial dilutions of an overnight culture of the target organism and performing a direct microscopic count using a Petroff-Hausser counting chamber.

***Oligonucleotide probes***

Oligonucleotide probe sequences for immobilization on the arrays were selected on the basis of nucleotide sequence information deposited in the National Center for Biotechnology Information (NCBI) database as indicated in Table 6.2. The probes were designed to target sequences located within the individual serotype-specific BoNT

amplicons generated in the multiplex PCR (Table 6.2). Oligonucleotide probes were synthesized by Sigma Genosys, and rehydrated at a concentration of 100 μM in Tris-EDTA buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) (Sigma), then stored at -20 °C until use.

***Multiplex PCR and CHAS procedure***

Samples were subjected to a multiplex PCR incorporating four primer pairs targeting BoNT gene serotypes A, B, E and F (Table 6.2). In instances where samples were comprised of whole cell suspensions (e.g. enrichment broth cultures), the cells were lysed by mixing with an equal volume of 2% (w/v) Triton X-100 (Sigma Chemical Co.) in water, and heating at 100 °C for 10 min. Purified genomic DNA was used directly in the PCR. For the PCR, 10 μl of test sample was added to 90 μl of multiplex PCR mixture [2.5 units HotStar Taq and 1 × PCR buffer containing 1.5 mM $MgCl_2$, plus 200 μM each dNTP, 5 μM DIG-11-dUTP (Roche), 0.5 μM each of primers BoNT A-1, BoNT A-2, BoNT B-1, and BoNT B-2, and 0.2 μM each of primers BoNT E-1, BoNT E-2, BoNT F-1, and BoNT F-2 (Table 6.2), and 2 μg BSA/ml]. The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf) using the following program: initial heating at 94 °C for 15.5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s, and primer extension at 72 °C for 1.5 min, with an additional 2 min at 72 °C following the last cycle.

For the CHAS, polyester cloth (DuPont, Sontara 8100) was cut into 1.5 × 5 cm strips, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled

water on a filter with vacuum suction. The strips were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M $MgCl_2$ and 0.15 M NaCl] and lightly blotted immediately prior to use for oligonucleotide probe immobilization. The oligonucleotide probes were diluted in coating buffer (10 μM) and heated at 100 °C for 10 min (to disrupt any possible secondary structures), then placed on ice. Probes (5 μl) were pipetted in discrete spots on a cloth strip, followed by incubation at 37 °C for 30 min. Probes were cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 $mJ/cm^2$) using a UVP cross-linker (Model Stratalinker 1800, Stratagene). The strips were blocked by incubating for 1 h at 37 °C with hybridization solution [5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they were washed five times by saturating strips with 0.01 M phosphate-buffered saline [pH 7.2/0.15 M NaCl (PBS)] containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips were air-dried and stored at 4 °C until use.

For the assay of DIG-labelled multiplex PCR products, amplicons were denatured by heating at 100 °C for 10 min, and placed on ice. The PCR product (100 μl) was mixed with 900 μl ice-cold hybridization solution containing 50% (v/v) formamide. The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 10 min at 37 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-DIG-peroxidase conjugate (Roche) diluted 1:2000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST,

the strips were saturated with 1 ml tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories), and incubated for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

**Results**

The proposed method involves a two phase process in which test samples (e.g., a colony from a plate) are first subjected to a multiplex PCR amplifying DNA sequences unique to the type A, B, E and F BoNT genes, followed by hybridization of the PCR products with an array of oligonucleotide probes specific for each toxin gene immobilized on a polyester cloth strip. To assure its reliability as a tool for the characterization of *C. botulinum* toxin types in colonial isolates, the combined multiplex PCR-CHAS procedure must exhibit suitable performance characteristics with respect to its detectability and spectrum of reactivity with various clostridia and non-clostridial strains.

***Detectability of the combined multiplex PCR-CHAS procedure***

The minimum quantity of genomic DNA input into the PCR required to obtain positive reactions with the immobilized probes on the cloth array was determined using various amounts of genomic DNA extracted from a representative strain of each of the four *C. botulinum* serotypes (Table 6.3). The detectability of the combined multiplex PCR-CHAS procedure for genomic DNA varied with the serotype (Table 6.3). For instance, at least 0.5 pg of *C. botulinum* serotype F genomic DNA was required to obtain a positive

**Table 6.3** Detectability of the combined multiplex PCR-CHAS for different *Clostridium botulinum* serotypes[a]

| Organism (strain)[c] | Sample | Minimum detected[b] | | | |
|---|---|---|---|---|---|
| | | BoNT A | BoNT B | BoNT E | BoNT F |
| *C. botulinum* type A (strain 1) | DNA (pg) | 0.06 | - | - | - |
| | Whole cells (cfu) | 4 | - | - | - |
| *C. botulinum* type B (strain 4) | DNA (pg) | - | 0.03 | - | - |
| | Whole cells (cfu) | - | 4 | - | - |
| *C. botulinum* type E (strain 10) | DNA (pg) | - | - | 0.015 | - |
| | Whole cells (cfu) | - | - | 4 | - |
| *C. botulinum* type F (strain 14) | DNA (pg) | - | - | - | 0.5 |
| | Whole cells (cfu) | - | - | - | 420 |

[a] Serial doubling dilutions of genomic DNA (range of 0.01 to 1.0 pg) and ten-fold dilutions of whole cells (range of *ca* 0.4 to 4 X $10^6$ cfu) from different serotypes of *C. botulinum* were subjected to the combined multiplex PCR-CHAS procedure as described in Methods. BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; BoNT F, botulinum neurotoxin type F.
[b] Minimum amount of genomic DNA (pg) or whole cells (cfu) into the PCR producing positive reactions with the immobilized probes on the cloth array. –, no reaction.
[c] Strain number designated in Table 6.1.

reaction with the BoNT F gene probe on the array, with no reactions occurring with any of the other BoNT gene probes at any DNA level tested; whereas, a minimum of 0.015 pg of *C. botulinum* serotype E genomic DNA was required to obtain a positive reaction with the BoNT E gene probe (Table 6.3). The two other *C. botulinum* serotypes tested (A and B) exhibited detectabilities with their respective relevant probes on the array within the 0.015-0.5 pg DNA range, and no heterologous probe reactions were observed for the different bacteria at any DNA level tested.

The detectability of the assay for whole cells was determined by preparing serial dilutions of the cells of the various strain serotypes (Table 6.3) in SPGY broth (as described above), which were then lysed and subjected to the multiplex PCR-CHAS. For the panel of bacteria tested, lysates derived from at least 0.4 to 4 cfu (or 42 to 420 cfu for serotype F) input into the PCR produced positive reactions with the relevant probes on the array, with no heterologous probe reactions occurring for the different serotypes at any cell level tested (Table 6.3). These experiments were repeated and the results were reproducible.

***Specificity of the combined multiplex PCR-CHAS procedure***

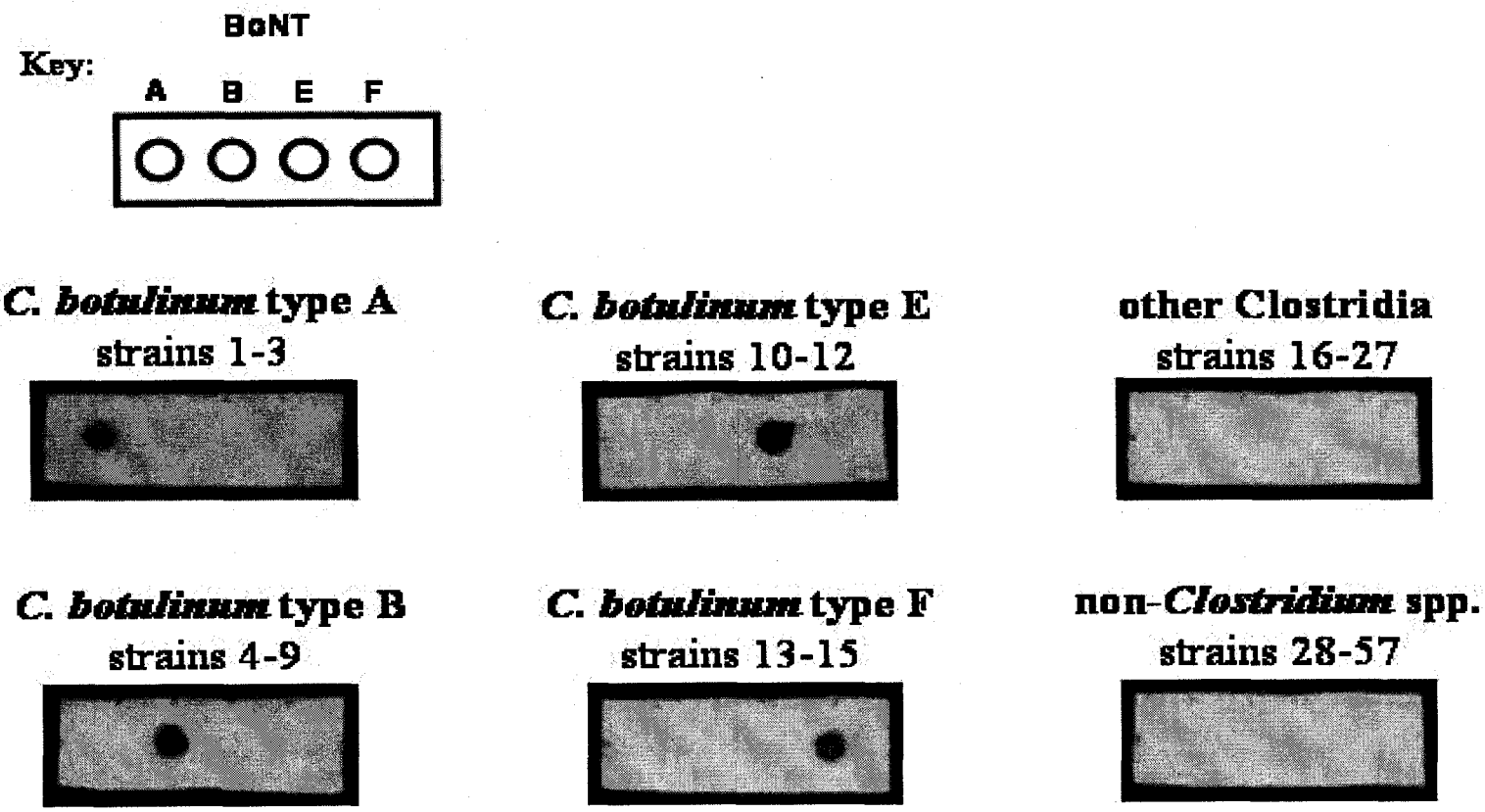
The specificity of the combined multiplex PCR-CHAS for the target gene markers was ascertained by subjecting lysates from individual colonies of various target and non-target bacteria to the procedure. The bacterial strains used in this study are detailed in Table 6.1. All *C. botulinum* strains displayed the expected reactivity patterns on the array, with the different *C. botulinum* BoNT serotypes producing positive reactions with

their corresponding probes on the array (Figure 6.1). No heterologous hybridization reactions were observed. Thus, the inclusivity of the multiplex PCR-CHAS is demonstrated by the homologous reactions of the different *C. botulinum* strains with their respective probes on the array, and its exclusivity is suggested by the lack of heterologous reactions between amplicons from target bacteria and the probes on the array. None of the PCR products obtained with lysates from the various other non-target bacteria tested (other clostridia and non-*Clostridium* strains) produced positive reactions with any of the toxin gene probes on the array, further demonstrating the exclusivity of this procedure. These experiments were repeated and the results were reproducible.

**Discussion**

The CHAS used in conjunction with a multiplex PCR targeting multiple BoNT gene serotypes is designed to provide a tool for the characterization of *C. botulinum* isolates based on their toxin gene profiles. The system described here exhibited adequate detectability characteristics for all the BoNT gene markers tested to enable its reliable application in the characterization of colonies isolated on plating media (Table 6.3). The comparatively poorer detectability for the BoNT serotype F gene does not preclude the use of this method for the characterization of colonial isolates since the level of cells present in a colony would not be a limiting factor. However, the applicability of this method to the detection of BoNT serotype F strains in enrichment broth cultures from foods will need to be investigated. This method was highly specific for the BoNT toxin gene serotypes A, B, E and F, with the combined multiplex PCR-CHAS correctly

**Figure 6.1** Specificity of the combined multiplex PCR-CHAS. A variety of target (*Clostridium botulinum* type A, B, E, and F) and non-target (other Clostridia and non-*Clostridium* spp.) bacteria were subjected to the combined multiplex PCR-CHAS procedure. For each isolate tested, a single colony was suspended in 50 µl of 1 × PCR buffer and then subjected to the lysis procedure described in Methods. BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; BoNT F, botulinum neurotoxin type F.



identifying the different *C. botulinum* BoNT toxin genes in the various serotypes tested (Figure 6.1). The combined multiplex PCR and CHAS procedure could be completed within a single day, thus providing information regarding the serotype of the isolates at an earlier juncture than the conventional mouse bioassay, enabling its use for the rapid and specific characterization of *C. botulinum* strains isolated from food samples or clinical specimens using suitable enrichment culture techniques. The CHAS facilitates the detection of different amplicons generated in the multiplex PCR and provides confirmation of their identity by hybridization with immobilized probes, obviating the need for time-consuming restriction endonuclease analysis as used with conventional agarose gel electrophoresis techniques. Thus, the specificity of the present system is doubly assured through the use of BoNT gene-specific PCR primers and oligonucleotide probes. While in this study I focused on the identification of the four serotypes known to be involved in outbreaks of human illness, the multiplex PCR and CHAS procedures can be expanded to include the detection of all seven BoNT gene serotypes.

# CHAPTER 7

## General Conclusion

The molecular characterization systems described in this thesis were designed to enhance the ability of the food microbiology laboratory to provide information on the nature of foodborne isolates within a timeframe enabling a timely response by regulators and the food industry to minimize the spread of contamination of the food supply with bacterial pathogens. This thesis has examined the development of cloth-based hybridization systems as simple and cost-effective tools for the rapid detection and characterization of common foodborne pathogenic bacteria.

Different methods for labelling PCR products with non-radioactive labels such as biotin and digoxigenin (DIG), for their detection by hybridization with immobilized DNA probes were compared in Chapter 2. DIG was found to be the label of choice for non-radioactive labelling of PCR products as it provided greater sensitivity than biotin. The incorporation of DIG into amplicons in the form of 5'-DIG-labelled oligonucleotide primers resulted in better assay signals and was more economical than the use of DIG-labelled dUTP. However, for the purpose of my experiments, which rely on multiplex PCR systems, I chose to label PCR products using digoxigenin in the form of DIG-dUTP since the labelling of each individual primer set (e.g. up to 7 in Chapter 3) would incur a greater collective cost compared to labelling using DIG-dUTP.

A simple cloth-based hybridization array system (CHAS) was developed in Chapter 3, for the detection of antibiotic resistance and other marker genes associated with the emerging multidrug-resistant food pathogen *Salmonella enterica* serotype Typhimurium DT104. This system provided specific and sensitive detection of the different genetic markers in the *S.* Typhimurium DT104 genome, making it a simple and

cost-effective tool for the identification of *S.* Typhimurium DT104 in foods and their production environment.

Chapter 4 described an attempt to develop a method for the direct detection of *S.* Typhimurium DT104 from food samples after enrichment. The proposed method involved a multi-step process in which food enrichment samples were subjected to a polymerase chain reaction targeting the integrase gene of class 1 integrons found in *S.* Typhimurium DT104, followed by spotting the resulting PCR products on sheets of polyester cloth and subsequent detection of the amplicons by hybridization with a target-specific probe and immunoenzymatic assay of the bound label. The system provided sensitive and specific detection of the integrase gene in target *S.* Typhimurium DT104 with no cross-reactivities when tested with pure cultures of a panel of non-target Gram-positive and Gram-negative bacteria. However when investigating the application of the system in the direct detection of *S.* Typhimurium DT104 in food samples after enrichment, it was found that this assay produced positive reactions for inoculated as well as uninoculated food samples, suggesting that the target chosen is not suitable for the specific detection of *S.* Typhimurium DT104 from foods.

Another problem facing food microbiologists is the identification of the toxigenic potential of foodborne bacteria. A simple cloth-based hybridization system was developed in Chapter 5 for the identification of toxin genes associated with major foodborne pathogenic bacteria. The system provided sensitive and specific detection of the different target toxin gene markers in a variety of bacterial isolates, exhibiting the expected patterns of reactivity with a panel of bacteria with defined toxigenicity profiles.

This system is a cost-effective tool facilitating the determination of the potential toxigenic profile of bacteria in the food microbiology laboratory, thus contributing valuable information to the risk assessment process in the microbiological analysis of foods.

A special case of toxigenic bacteria occurring in foods are *Clostridium botulinum* strains, which produce a highly potent neurotoxin. Chapter 6 described the development of a cloth-based hybridization system for the characterization of *Clostridium botulinum* isolates based on the botulinum neurotoxin serotype. This system provided sensitive and specific detection of the different BoNT gene markers in a variety of *C. botulinum* strains, exhibiting the expected patterns of reactivity with a panel of target and non-target organisms. These results demonstrate the use of this system as a cost-effective tool facilitating the characterization of *C. botulinum* serotypes in the foods.

Pathogenic bacteria present in foods are commonly in an injured state and require a period of recovery (pre-enrichment) before they regain their full growth potential. In addition, pathogenic bacteria are usually found in very low numbers in food samples, and require selective enrichment to increase their numbers to a detectable level. Because a minimum of approximately $10^4$ bacteria/ml in suspension is required for reliable detection by a typical 30-cycle PCR (Rijpens and Herman, 2002), the direct detection of pathogenic bacteria in food products is difficult to achieve without prior enrichment culture techniques. The cloth-hybridizations systems described in this thesis can be applied in two ways: (1) as identification or characterization tools for culture confirmation (Chapters 3, 5, and 6), and (2) shorten conventional methods for the

detection of bacterial pathogens in foods through their application to pre-enrichment or selective enrichment media (Chapter 4). In both scenarios, the food testing laboratorian can obtain presumptive information on the nature of the samples (isolated colonies or enrichment samples) at a much earlier juncture than with the conventional scheme for the isolation and identification of foodborne pathogens, which involve a battery of biochemical and serological tests (Figure 1.6). These systems could be implemented in the routine food testing laboratory as they do not require sophisticated equipment for their operation, they are simple to perform, and incur minimal costs to generate results in the analysis of food products. Positive results generated using these systems can be confirmed by standard methods if desired.

Various applications of cloth-based hybridization systems were described in this thesis. The cloth-based hybridization systems described herein were found to be better suited for the identification and characterization of foodborne isolates (Chapters 3, 5, and 6) than for the detection of specific foodborne pathogens directly from enrichment cultures of foods (Chapter 4). The cloth-based hybridization array system (CHAS) has a number of advantages, making it an attractive array technology for the detection and characterization of foodborne pathogens. The CHAS is a rapid procedure. In combination with a multiplex PCR, the procedure can be completed within a single day, thus providing important information to the food testing laboratorian on the nature of foodborne isolates at a much earlier juncture than conventional isolation and identification procedures. The CHAS is a very simple procedure. It can be implemented in any routine food testing laboratory since it does not require highly sophisticated

equipment for its operation. Its rapid hybridization procedure simplifies the analysis of multiple amplicons through the visualization of individual PCR products as coloured spots on an array. The CHAS is sensitive and specific. The hybridization procedure increases the sensitivity that would not normally be detected by standard agarose gel electrophoresis. The specificity of the CHAS is doubly assured through the use of target-specific primers and the confirmation of amplicons by hybridization with immobilized target-specific probes. The CHAS is a great alternative to using sophisticated microarray technology for the characterization of foodborne isolates, especially in instances where a small number of marker genes are targeted. The CHAS principle should be broadly applicable to the detection and characterization of other molecular markers of food pathogens, such as virulence genes. This system is not limited to the field of food microbiology, it could also be applied to other diagnostic fields.

# REFERENCES

Abouzeed, Y.M., Hariharan, H., Poppe, C., and Kibenge, F.S.B. (2000) Characterization of *Salmonella* isolates from beef cattle, broiler chickens and human sources on Prince Edward Island. *Comparative Immunology, Microbiology and Infectous Diseases* **23**: 253-266.

Altwegg, M. (1995) General problems associated with diagnostic applications of amplification methods. *Journal of Microbiological Methods* **23**: 21-30.

Anderson, E.R., Koplan, J., Henney, J.E., and Thomas, T.J. (2001) Diagnosis and management of foodborne illnesses: a primer for physicians. *Morbidity and Mortality Weekly Report* **50**: 1-69.

Angulo, F.J., and Griffin, P.M. (2000) Changes in antimicrobial resistance in *Salmonella enterica* serovar Typhimurium. *Emerging Infectious Diseases* **6**: 436-437.

Anonymous (1996) VTEC O157 infection in West Yorkshire associated with the consumption of raw milk. *Communicable Disease Report Weekly* **6**: 181.

Anonymous (2000) Waterborne outbreak of gastroenteritis associated with a contaminated municipal water supply, Walkerton, Ontario, May-June 2000. *Canada Communicable Disease Report* **26**: 170-173.

Anonymous (2001) Determination of the aerobic colony count in foods. MFHPB-18. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_2/pdf/e_mfhpb18.pdf

Anonymous (2003) Supplement to all methods that detect *Salmonella*. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/appendix/pdf/e_appendix_j.pdf

Aranda, E., Rodríguez, M.M., Asensio, M.A., and Córdoba, J.J. (1997) Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe. *Letters in Applied Microbiology* **25**: 186-190.

Arcangioli, M.A., Leroy-Sétrin, S., Martel, J.L., and Chaslus-Dancla, E. (1999) A new chloramphenicol and florfenicol resistance gene flanked by two integron structures in *Salmonella typhimurium* DT104. *FEMS Microbiology Letters* **174**: 327-332.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.C., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L., and Tonat, K.

(2001) Botulinum toxin as a biological weapon. *The Journal of the American Medical Association* **285**: 1059-1070.

Austin, J.W. and Blanchfield, B. (1997) Detection of *Clostridium botulinum* and its toxins in suspect foods and clinical specimens. MFHPB-16. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_2/e_index.html).

Bell, B.P., Goldoll, M., Griffin, P.M., Davis, M.A., Gordon, D.C., Tarr, P.I., Bartleson, C.A., Lewis, J.H., Barrett, T.J., Wells, J.G., Baron, R., and Kobayashi, J. (1994) A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. *The Journal of the American Medical Association* **272**: 1349-1353.

Blais, B.W., Gaudreault, M., and Phillippe, L.M. (2003) Multiplex enzyme immunoassay system for the simultaneous detection of multiple allergens in foods. *Food Control* **14**: 43-47.

Blais, B.W., and Phillippe, L.M. (1995) Macroporous hydrophobic cloth (Polymacron) as a solid phase for nucleic acid probe hybridizations. *Biotechnology Techniques* **9**: 377-382.

Blais, B.W., and Phillippe, L.M. (2000) A cloth-based enzyme immunoassay for detection of peanut proteins in foods. *Food and Agricultural Immunology* **12**: 243-248.

Blais, B.W., Phillippe, L.M., and Vary, N. (2002) Cloth-based hybridization array system for detection of transgenic soy and corn by multiplex polymerase chain reaction. *Biotechnology Letters* **24**: 1407-1411.

Blais, B.W., and Yamazaki, H. (1997) Use of macroporous hydrophobic cloth as a solid phase for enzyme immunoassays in microbiological food safety testing. *Recent Research and Development in Microbiology* **1**: 155-170.

Blais, B.W., Yamazaki, H., and Rigby, C.E. (1989) Use of hydrophobic cloths as antibody adsorbents for enzyme immunoassay: detection of brucella antigens. *Veterinary Microbiology* **20**: 155-163.

Bolton, L.F., Kelley, L.C., Lee, M.D., Fedorka-Cray, P.J., and Maurer, J.J. (1999) Detection of multidrug-resistant *Salmonella enterica* serotype *typhimurium* DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. *Journal of Clinical Microbiology* **37**: 1348-1351.

Boyce, T.G., Swerdlow, D.L., and Griffin, P.M. (1995) *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *The New England Journal of Medicine* **333**: 364-368.

Braconnier, A., Broussolle, V., Perelle, S., Fach, P., Nguyen-The, C., and Carlin, F. (2001) Screening for *Clostridium botulinum* type A, B, and E in cooked chilled foods containing vegetables and raw material using polymerase chain reaction and molecular probes. *Journal of Food Protection* **64**: 201-207.

Briggs, C.E., and Fratamico, P.M. (1999) Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrobial Agents and Chemotherapy* **43**: 846-849.

Carattoli, A. (2001) Importance of integrons in the diffusion of resistance. *Veterinary Research* **32**: 243-259.

Carlson, S.A., Bolton, L.F., Briggs, C.E., Hurd, H.S., Sharma, V.K., Fedorka-Cray, P.J., and Jones, B.D. (1999) Detection of multiresistant *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. *Molecular and Cellular Probes* **13**: 213-222.

Cloeckaert, A., and Schwarz, S. (2001) Molecular characterization, spread and evolution of multidrug resistance in *Salmonella enterica* Typhimurium DT104. *Veterinary Research* **32**: 301-310.

Chizhikov, V., Rasooly, A., Chumakov, K., and Levy, D.D. (2001) Microarray analysis of microbial virulence factors. *Applied and Environmental Microbiology* **67**: 3258-3263.

Collignon, P. (2000) Antimicrobial resistance. *Emerging Infectious Diseases* **6**: 1-2.

Craven, K.E., Ferreira, J.L., Harrison, M.A., and Edmonds, P. (2002) Specific detection of *Clostridium botulinum* types A, B, E, and F using the polymerase chain reaction. *Journal of AOAC International* **85**: 1025-1028.

Crump, J.A., Griffin, P.M., and Angulo, F.J. (2002) Bacterial contamination of animal feed and its relationship to human foodborne illness. *Clinical Infectious Diseases* **35**: 859-865.

D'Aoust, J.-Y., and Purvis, U. (1998) Isolation and identification of *Salmonella* from foods. MFHPB-20. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_2/e_mfhpb2001.html

Davis, M.A., Hancock, D.D., Besser, T.E., Rice, D.H., and Gay, J.M. (2000) Reply to Drs. Angulo and Collignon. *Emerging Infectious Diseases* **6**: 437-438.

Dean, A.G., Ching, Y.-C., Williams, R.G., and Harden, L.B. (1972) Test for *Escherichia coli* enterotoxin using infant mice. Application in a study of diarrhea in children in Honolulu. *The Journal of Infectious Diseases* **15**: 407-411.

De Boer, E., and Beumer, R.R. (1999) Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology* **50**: 119-130.

Faruque, S.M., Albert, M.J., and Mekalanos, J.J. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiology and Molecular Biology Reviews* **62**: 1301-1314.

Feng, P. (1995) *Escherichia coli* serotype O157:H7: novel vehicles of infection and emergence of phenotypic variants. *Emerging Infectious Diseases* **1**: 47-52.

Feng, P. (1997) Impact of molecular biology on the detection of foodborne pathogens. *Molecular Biotechnology* **7**: 267-278.

Fluit, A.C., and Schmitz, F.-J. (2004) Resistance integrons and super-integrons. *Clinical Microbiology and Infection* **10**: 272-288.

Franciosa, G., Ferreira, J.L., and Hatheway, C.L. (1994) Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. *Journal of Clinical Microbiology* **32**: 1911-1917.

Gabig, M., and Wegrzyn, G. (2001) An introduction to DNA chips: principles, technology, applications and analysis. *Acta Biochimica Polonica* **48**: 615-622.

Galán, J.E., and Curtiss III, R. (1989) Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proceedings of the National Academy of Science USA* **86**: 6383-6387.

Gauthier, M., and Blais, B.W. (2004) Cloth-based hybridization array system for the detection of multiple antibiotic resistance genes in *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104. *Letters in Applied Microbiology* **38**: 265-270.

Gauthier, M., and Blais, B.W. (2003) Comparison of different approaches for the incorporation of non-radioactive labels into polymerase chain reaction products. *Biotechnology Letters* **25**: 1369-1374.

Glynn, M.K., Bopp, C., Dewitt, W., Dabney, P., Mokhtar, M., and Angulo, F.J. (1998) Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *The New England Journal of Medicine* **338**: 1333-1338.

González Garcia, E.A. (2002) Animal health and foodborne pathogens: enterohaemorrhagic O157:H7 strains and other pathogenic *Escherichia coli* virotypes (EPEC, ETEC, EIEC, EHEC). *Polish Journal of Veterinary Sciences* **5**: 103-115.

Griffin, P.M., and Tauxe, R.V. (1991) The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews* **13**: 60-98.

Hall, R.M., Brookes, D.E., and Stokes, H.W. (1991) Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Molecular Microbiology* **5**: 1941-1959.

Hall, R.M., and Stokes, H.W. (1993) Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetica* **90**: 115-132.

Hatheway, C.L. (1990) Toxigenic Clostridia. *Clinical Microbiology Reviews* **3**: 66-98.

Heer A.H., Keyszer G.M., Gay R.E., and Gay S. (1994) Inhibition of RNA polymerases by digoxigenin-labeled UTP. *BioTechniques* **16**: 54-55.

Heller, M.J. (2002) DNA microarray technology: devices, systems, and applications. *Annual Review of Biomedical Engineering* **4**: 129-153.

Herman, L. (1997) Detection of viable and dead *Listeria monocytogenes* by PCR. *Food Microbiology* **14**: 103-110.

Hielm, S., Hyytiä, E., Ridell, J., and Korkeala, H. (1996) Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. *International Journal of Food Microbiology* **31**: 357-365.

Hill, W.E. (1996) The polymerase chain reaction: applicatons for the detection of foodborne pathogens. *Critical Reviews in Food Science and Nutrition* **36**: 123-173.

Humphrey, T. (2001) *Salmonella* Typhimurium definitive type 104: a multi-resistant *Salmonella*. *International Journal of Food Microbiology* **67**: 173-186.

Hyytiä, E., Björkroth, J., Hielm, S., and Korkeala, H. (1999) Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. *International Journal of Food Microbiology* **48**: 179-189.

Institute of Food Technologists. (2004) Bacteria associated with foodborne diseases. Scientific status summary of the Institute of Food Technologists. Chicago. Ill.

Jorgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D.R., Bolton, F.J., Frost, J.A., Ward, L., and Humphrey, T.J. (2002) Prevalence and numbers of Salmonella and Campylobacter spp. on raw, whole chickens in relation to sampling methods. *International Journal of Food Microbiology* **76**: 151-164.

Kaper, J.B., Morris Jr., J.G., and Levine, M.M. (1995) Cholera. *Clinical Microbiology Reviews* **8**: 48-86.

Karch, H., Bielaszewska, M., Bitzan, M., and Schmidt, H. (1999) Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Diagnostic Microbiology and Infectious Disease* **34**: 229-243.

Karmali, M.A. (1989) Infection by verocytotoxin-producing *Escherichia coli*. *Clinical Microbiology Reviews* **2**: 15-38.

Karmali, M.A., Petric, M., and Bielaszewska, M. (1999) Evaluation of a microplate latex agglutination method (Verotox-F assay) for detecting and characterizing verotoxins (shiga toxins) in *Escherichia coli*. *Journal of Clinical Microbiology* **37**: 396-399.

Kautter, D.A., and Solomon, H.M. (1977) Collaborative study of a method for the detection of *Clostridium botulinum* and its toxins in foods. *Journal of AOAC International* **60**: 541-545.

Keene, W.E., McAnulty, J.M., Hoesly, F.C., Williams Jr., L.P., Hedberg, P.H.K., Oxman, G.L., Barrett, T.J., Pfaller, M.A., and Fleming, D.W. (1994) A swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonnei*. *The New England Journal of Medicine* **331**: 579-584.

Kehl, S.C. (2002) Role of the laboratory in the diagnosis of enterohemorrhagic *Escherichia coli* infections. *Journal of Clinical Microbiology* **40**: 2711-2715.

Khan, A.A., Nawaz, M.S., Khan, S.A. and Cerniglia, C.E. (2000) Detection of multidrug-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. *FEMS Microbiology Letters* **182**: 355-360.

Kim, E.H., and Aoki, T. (1993) Drug resistance and broad geographical distribution of identical R plasmids of *Pasteurella piscicida* isolated from cultured yellowtail in Japan. *Microbiology and Immunology* **37**: 103-109.

Konowalchuk, J., Spiers, J.I., and Stavric, S. (1977) VERO cell response to a cytotoxin of *Escherichia coli*. *Infection and Immunity* **18**: 755-779.

Lantz, P.G., Abu Al-Soud, W., Knutsson, R., Hahn-Hägerdal, and Rådström, P. (2000) Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. *Biotechnology Annual Review* **5**: 87-130.

Lee, L.A., Puhr, N.D., Maloney, E.K., Bean, N.H., and Tauxe, R.V. (1994) Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. *Journal of Infectious Diseases* **170**: 128-134.

LeBlanc, J.J. (2003) Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. *Critical Reviews in Microbiology* **29**: 277-296.

Levine, M.M. (1987) *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *The Journal of Infectious Diseases* **155**: 377-389.

Lindström, M., Keto, R., Markkula, A, Nevas, M., Hielm, S., and Korkeala, H. (2001) Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Applied and Environmental Microbiology* **67**: 5694-5699.

Martin R., James D., and Levesque C.A. (2000) Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology* **38**: 207-239.

McEwen, S.A., and Fedorka-Cray, P.J. (2002) Antimicrobial use and resistance in animals. *Clinical Infectious Diseases* **34**: S93-S106.

McQuaid, S., McMahon, J., and Allan, G.M. (1995) A comparison of digoxigenin and biotin labelled DNA and RNA probes for in situ hybridization. *Biotechnic and Histochemistry* **70**: 147-154.

Mead, P.S., and Griffin, P.M. (1998) *Escherichia coli* O157:H7. *Lancet* **352**: 1207-1212.

Mead, P.S., Slutsker, L., Dietz, V., McCraig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerging Infectious Diseases* **5**: 607-625.

Montecucco, C., Schiavo, G., Tugnoli, V., and de Grandis, D. (1996) Botulinum neurotoxins: mechanism of action and therapeutic applications. *Molecular Medicine Today* **2**: 418-424.

Monteiro L., Cabrita J., and Megraud F. (1997) Evaluation of performances of three DNA enzyme immunoassays for detection of *Helicobacter pylori* PCR products from biopsy specimens. *Journal of Clinical Microbiology* **35**: 2931-2936.

Morris, R.G., Arends, M.J., Bishop, P.E., Sizer, K., Duvall, E., and Bird, C.C. (1990) Sensitivity of digoxigenin and biotin labelled probes for detection of human papillomavirus by in situ hybridization. *Journal of Clinical Pathology* **43:** 800-805.

Nagano, I., Kunishima, M., Itoh, Y., Wu, Z., and Takahashi, Y. (1998) Detection of verotoxin-producing *Escherichia coli* O157:H7 by multiplex polymerase chain reaction. *Microbiology and Immunology* **42**: 371-376.

Ng, L.-K., Mulvey, M.R., Martin, I., Peters, G.A., and Johnson, W. (1999) Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. *Antimicrobial Agents and Chemotherapy* **43**: 3018-3021.

O'Brien, T.F. (2002) Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clinical Infectious Diseases* **34**: S78-S84.

Olsen, J.E., Aabo, S., Hill, W., Notermans, S., Wernars, K., Granum, P.E., Popovic, T., Rasmussen, H.N., and Olsvik, Ø. (1995) Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *International Journal of Food Microbiology* **28**: 1-78.

Osek, J. (2001) Multiplex polymerase chain reaction assay for identification of enterotoxigenic *Escherichia coli* strains. *Journal of Veterinary Diagnostic Investigation* **13**: 308-311.

Poppe, C., Smart, N., Khakhria, R., Johnson, W., Spika, J., and Prescott, J. (1998) *Salmonella typhimurium* DT104: a virulent and drug-resistant pathogen. *The Canadian Veterinary Journal* **39**: 559-565.

Poppe, C., Mann, E.D., Shaw, S., Warburton, D., and Sewell, A. (2004) Procedure for the isolation of *Salmonella* species by the modified semi-soli rappaport vassiliadis (MSRV) method. MFLP-75. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_3/pdf/e_mflp75.pdf

Rabbani, G.H., and Greenough III, W.B. (1999) Food as a vehicle of transmission of cholera. *Journal of Diarrhoeal Diseases Research* **17**: 1-9.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtiss III, R., and Gyles, C.L. (1992) Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6**: 271-279.

Recchia, G.D., and Hall, R.M. (1995) Gene cassettes: a new class of mobile element. *Microbiology* **141**: 3015-3027.

Recchia, G.D., and Hall, R.M. (1997) Origins of the mobile gene cassettes found in integrons. *Trends in Microbiology* **5**: 389-394.

Reidl, J., and Klose, K.E. (2002) *Vibrio cholerae* and cholera: out of the water and into the host. *FEMS Microbiology Reviews* **26**: 125-139.

Rhodehamel, E.J., Reddy, N.R., and Pierson, M.D. (1992) Botulism: the causative agent and its control in foods. *Food Control* **3**: 125-143.

Ridley, A., and Threlfall, J.E. (1998) Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT104. *Microbial Drug Resistance* **4**: 113-118.

Rijpens, N.P., and Herman, L.M.F. (2002) Molecular methods for identification and detection of bacterial pathogens. *Journal of AOAC International* **85**: 984-995.

Riley, L.W. (1987) The epidemiologic, clinical, and microbiologic features of hemorrhagic colitis. *Annual Review of Microbiology* **41**: 383-407.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *The New England Journal of Medicine* **308**: 681-685.

Sack, D.A., Sack, R.B., Nair, G.B., and Siddique, A.K. (2004) Cholera. *Lancet* **363**: 223-233.

Salyers, A.A., and Whitt, D.D. (2003) Bacterial pathogenesis: a molecular approach 2nd edition. ASM Press. Washington. D.C.

Sambrook, J., and Russell, D.W. (2001) Molecular cloning: a laboratory manual. (eds) Cold Spring Harbor: Cold Spring Harbor Laboratory. New York.

Sandvang, D., Aarestrup, F.M., and Jensen, L.B. (1998) Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiology Letters* **160**: 37-41.

Schmitt, C.K., Meysick, K.C., and O'Brien, A.D. (1999) Bacterial toxins: friends or foes? *Emerging Infectious Diseases* **5**: 224-234.

Schwarz, S., and Chaslus-Dancla, E. (2001) Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Veterinary Research* **32**: 201-225.

Scotland, S.M., Smith, H.R., Willshaw, G.A., and Rowe, B. (1983) Vero cytotoxin production in strain of *Escherichia coli* is determined by genes carried on bacteriophage. *Lancet* **2**: 216.

Sheridan, G.E.C., Masters, C.I., Shallcross, J.A., and Mackey, B.M. (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology* **64**: 1313-1318.

Smith, W.H., Green, P., and Parsell, Z. (1983) Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chicken and pigs. *Journal of General Microbiology* **129**: 3121-3137.

Strizhkov, B.N., Drobyshev, A.L., Mikhailovich, V.M., and Mirzabekov, A.D. (2000) PCR amplification on a microarray of gel immobilized oligonucleotides: detection of bacterial toxin- and drug-resistant genes and their mutations. *BioTechniques* **29**: 844-857.

Swaminathan, B., and Feng, P. (1994) Rapid detection of food-borne pathogenic bacteria. *Annual Review of Microbiology* **48**: 401-426.

Takeshi, K., Fujinaga Y, Inoue, K, Nakajima, H., Oguma, K., Ueno, T, Sunagawa, H., and Ohyama, T. (1996) Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction. *Microbiology and Immunology* **40**: 5-11.

Tauxe, R.V. (1991) *Salmonella*: a postmodern pathogen. *Journal of Food Protection* **54**: 563-568.

Thomas, A., Jiggle, B., Smith, H.R., and Rowe, B. (1994) The detection of Vero cytotoxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 in faecal specimens using polymerase chain reaction gene amplification. *Letters in Applied Microbiology* **19**: 406-409.

Thomas, A., Smith, H.R., Willshaw, G.A., and Rowe, B. (1991) Non-radioactively labelled polynucleotide and oligonucleotide DNA probes, for selectively detecting *Escherichia coli* strains producing Vero cytotoxins VT1, VT2, and VT2 variant. *Molecular and Cellular Probes* **5**: 129-135.

Threlfall, E.J. (2000) Epidemic *Salmonella typhimurium* DT104 – a truly international multiresistant clone. *Journal of Antimicrobial Chemotherapy* **46**: 7-10.

Threlfall, E.J., and Rowe, B. (1998) Multiresistant *Salmonella typhimurium* DT104 and salmonella bacteraemia. *Lancet* **352**: 287-288.

Threlfall, E.J., Rowe, B., and Ward, L.R. (1993) A comparison of multiple drug resistance in salmonellas from humans and food animals in England and Wales, 1981 and 1990. *Epidemiology and Infection* **111**: 189-197.

Tietjen, M., and Fung, D.Y.C. (1995) Salmonellae and food safety. *Critical Reviews in Microbiology* **21**: 53-83.

Tilden, J., Young, W., McNamara, A.M., Custer, C., Boesel, B., Lambert-Fair, A., Majkowski, J., Vugia, D., Werner, S.B., Hollongsworth, J., and Morris, J.G. (1996) A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *American Journal of Public Health* **86**: 1142-1145.

Turton, K., Chaddock, J.A., and Acharya, K.R. (2002) Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *TRENDS in Biochemical Sciences* **27**: 552-558.

U.S. Food & Drug Administration (1992) *Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxin Handbook.* http://www.cfsan.fda.gov/~mow/chap5.html

Varela, P., Pollevick, G.D., Rivas, M., Chinen, I., Binsztein, N., Frasch, A.C.C., and Ugalde, R.A. (1994) Direct detection of *Vibrio cholerae* in stool samples. *Journal of Clinical Microbiology* **32**: 1246-1248.

Wall, P.G., Morgan, D., Lamden, K., Ryan, M., Griffin, M., Threlfall, E.J., Ward, L.R., and Rowe, B. (1994) A case control study of infection with an epidemic strain of multi-resistant *Salmonella typhimurium* DT104 in England and Wales. *Communicable Disease Report Review* **4**: R130-R135.

Wang, H., Blais, B.W., and Yamazaki, H. (1995) Rapid and economical detection of *Salmonella enteritidis* in eggs by the polymyxin-cloth enzyme immunoassay. *International Journal of Food Microbiology* **24**: 397-406.

White, D.G., Zhao, S., Simjee, S., Wagner, D.D., and McDermott, P.F. (2002) Antimicrobial resistance of foodborne pathogens. *Microbes and Infection* **4**: 405-412.

Wolcott, M.J. (1992) Advances in nucleic acid-based detection methods. *Clinical Microbiology Reviews* **5**: 370-386.

Woodward, M.J., Kearsley, R., Wray, C., and Roeder, P.L. (1990) DNA probes for the detection of toxin genes in *Escherichia coli* isolated from diarrhoeal disease in cattle and pigs. *Veterinary Microbiology* **22**: 277-290.

White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D., and Meng, J. (2001) The isolation of antibiotic-resistant *Salmonella* from retail ground meats. *The New England Journal of Medicine* **345**: 1147-1154.

Ye, R.W., Wang, T., Bedzyk, L., and Croker, K.M. (2001) Applications of DNA microarrays in microbial systems. *Journal of Microbiological Methods* **47**: 257-272.

Yoh, M., Takagi, K., Eda, J., Ohtomo, M., Takarada, Y., Shibata, S., and Honda, T. (1997) Evaluation of enzyme-labeled oligonucleotide probes to identify enterohaemorrhagic *Escherichia coli*. *Microbiology and Immunology* **41**: 879-882.

Zhao, J., and Aoki, T. (1992) Nucleotide sequence analysis of the class G tetracycline resistance determinant from *Vibrio anguillarum*. *Microbiology and Immunology* **36**: 1051-1060.