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Methods for Detection of *Salmonella* spp.

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METHODS FOR DETECTION OF *SALMONELLA* SPP.

by

Parichaya Attaphongse

A report submitted in partial fulfillment of the requirements for the degree of

MASTER OF FOOD MICROBIOLOGY AND SAFETY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

2004
ABSTRACT

Methods for Detection of *Salmonella* spp.

by

Parichaya Attaphongse, Master of Food Microbiology and Safety

Utah State University, 2004

Major Professor: Dr. Jeffery R. Broadbent
Department: Nutrition and Food Sciences

Worldwide concern about food safety and associated health costs is increasing, and *Salmonella* contamination of foods is one of the most important causes of foodborne disease outbreaks. Although conventional detection methods for *Salmonella* offer high sensitivity and low cost, they require many different steps and are very time consuming. Ideally, methods to detect *Salmonella* in foods should be rapid, provide high specificity and sensitivity, be cost-effective, and low labor-intensive. Many rapid tests have been developed to address these goals, including DNA-based tests such as nucleic acid hybridization and PCR, immunoassay-based tests such as ELISA, ELFA and immunomagnetic method, and immuno-latex agglutination based tests. While these tests provide much faster results than conventional tests, additional work is needed to minimize the time required for *Salmonella* in food samples so that results are available within 1 day.  

(57 pages)
ACKNOWLEDGMENTS

I would like to express my gratitude and sincere thanks to my advisor, Dr. Jeffery R. Broadbent, for his patience, encouragement, understanding, and invaluable guidance during the entire period of my report and studies at Utah State University. His comments and suggestions are a valuable source of intellectual stimulation. Without his support, it would not be possible to complete this work.

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Finally, this report is dedicated to my parents, Phaibulya and Suwanna Attaphongse, and my brother, Krisada Attaphongse for their unconditional love, support, understanding, and encouragement.

Parichaya Attaphongse
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INTRODUCTION

The genus *Salmonella* is a typical member of the family *Enterobacteriaceae*. Members of this genus are infectious pathogens which occur in humans and animals throughout the world. It has been recognized as a cause of intestinal disease for more than 100 years. According to the U.S. Centers for Disease Control and Prevention (CDC), in 2001, *Salmonella* is the most common foodborne cause of bacteraemia, with more case numbers than *Campylobacter* or *Shigella* (CDC, 2002a).

The main habitat of *Salmonella* is the intestinal tract of humans and other animals. Some of the 2,200 serovars exist in animals without causing disease (Zhao et al., 2002). Improperly handled or undercooked poultry and eggs are the most common sources of *Salmonella*. Chickens are major reservoirs of *Salmonella*, which accounts for its prominence in poultry products (Rampling, 1993). However, poultry contaminated with *Salmonella* are particularly difficult to identify because infected chickens usually show no signs or symptoms (Apatow, 2004). Since infected chickens have no distinguished characteristics, these chickens go on to lay eggs or to be used as meat which can cause *Salmonella* food poisoning. Other sources of *Salmonella* include unpasteurized milk, dairy foods, ground meat, fish, shrimp, sauces and salad dressing, but other foods can be accidentally contaminated if they come into contact with contaminated material (FDA-CFSAN, 2003). Moreover, children have become ill after playing with turtles or iguanas, and then eating without washing their hands (FDA-HHS, 2003). Because the bacteria are released in the feces for weeks after the infection of *Salmonella*, poor hygiene can allow a carrier to spread the infection to others.
Any of a wide range of mild to serious infections caused by Salmonella is called salmonellosis, including typhoid and paratyphoid fever in humans. Typhoid fever, which is rare in the United States, is caused by a serotype named Salmonella Typhi. But illness due to Salmonella in other strains is called salmonellosis, which is a common type of food poisoning in the United States. Salmonellosis symptoms include diarrhea, vomiting, chills, and painful headaches. According to the CDC’s “Summary of Notifiable Diseases 1999” (Mead et al., 1999), Salmonella is responsible for about 15% of all cases of food poisoning in the United States, and over 40,000 cases of salmonellosis are reported every year. As only about 3% of Salmonella cases are officially reported nationwide, and many milder cases are never diagnosed, the true incidence is certainly much higher (Mead et al., 1999). Salmonellosis is more common in the warmer months of the year. Approximately 500 to 1,000 persons die annually from Salmonella infections in the United States (Mead et al., 1999).

As a foodborne pathogen, Salmonella takes a considerable economic toll, both on victims of salmonellosis for medical care expenses and lost wages, and on the food industry for contamination control costs, quality control costs, and product liability (Ziprin, 1994). The traditional method of Salmonella detection described in the Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) is a culture-based method which generally requires 4 days for negative answers and up to 6 days to confirm the presence of Salmonella in a sample (Andrews and Hammock, 2003). Such lengthy and laborious procedures are a problem in the food industry. Therefore, rapid, sensitive, and simple detection methods for Salmonella in foods that may be contaminated with these organisms are important if incidence of salmonellosis infection from contaminated
foods is to be reduced (Tan and Shelef, 1999). Some of the rapid methods for *Salmonella* detection developed thus far include antibody-based tests like the enzyme-linked immunosorbent assay (ELISA), and DNA-based PCR tests. More improved methods for detection of *Salmonella* in food will save the food industry money and help to reduce the frequency of *Salmonella* infections in humans.

This thesis will review current technology for rapid detection of *Salmonella* in foods, and identify future needs in this area.
CHARACTERISTICS AND PROPERTIES OF *SALMONELLA*

*Salmonella* were discovered by Eberth in 1880 and first cultivated by Gaffky in 1884 (Burrows, 1959). In 1885, D.E. Salmon and T. Smith isolated *Salmonella* Choleraesuis from swine suffering from hog cholera (Le Minor, 1981). Subsequently, in 1900, the genus *Salmonella* was named in honor to D.E. Salmon’s work by Lignieres (Merchant and Packer, 1970). The first laboratory confirmed epidemic of foodborne salmonellosis involved 57 persons who ate meat from sick cows in 1888. *Salmonella* Enteritidis was isolated from organs of the patients who died from this disease and from the meat and blood of infected animals (Merchant and Packer, 1970). Since that time, *Salmonella* have been identified as a major cause gastroenteritis and enteric fever (ICMSF, 1996).

*Salmonella* are Gram-negative, non-sporing, straight, 0.7-1.5 x 2.0-5.0 µm rod shaped, facultatively anaerobic bacteria. They are chemooorganotrophic and display both respiratory and a fermentative metabolism of carbohydrates (Le Minor, 1984; Varnam and Evans, 1991). Most *Salmonella* have peritrichous flagella but some species are non motile. *Salmonella* possess three major antigenic determinants: O or somatic antigens, H or flagella antigens, and Vi or capsule antigens (Giannella, 1996). O antigens are on the external surface of the bacterial outer membrane. This antigen is determined by specific sugar sequences on the cell surface. H antigens are flagella proteins. Most *Salmonella* strains are diphasic, designated as phase 1 (specific) or phase 2 (nonspecific), meaning they express two different flagella antigens. The organisms tend to change from one phase to the other. Vi antigen is a superficial antigen overlying the O antigen.
Salmonella that have Vi antigen such as Salmonella Typhi, Salmonella Paratyphi C and Salmonella Dublin are more virulent than cells without Vi antigens. Antigenic analysis of Salmonella using specific antisera offers clinical and epidemiological advantages. For example, determination of antigenic type permits one to identify the organisms clinically and classify them to particular serogroups (Giannella, 1996).

As with other Gram-negative bacilli, the Salmonella cell envelope contains a complex lipopolysaccharide (LPS) structure that is liberated to some extent upon lysis of the cell during culture (Giannella, 1996). The lipopolysaccharide moiety may function as an endotoxin and be important in determining virulence of these organisms. This macromolecular endotoxin complex consists of three components; an outer O-polysaccharide coat, a middle portion (the R core), and an inner lipid A coat. Lipopolysaccharide structure is important for several reasons. First, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity and may also help determine the virulence of the organism (Giannella, 1996). Salmonella lacking the complete sequence of O-sugar repeat units are called “rough” because of the rough appearance of the colonies on laboratory agar. Rough cells are usually avirulent or less virulent than “smooth” strains which posses a full complement of O-sugar repeat units. Second, antibodies directed against the R core (common enterobacterial antigen) may protect against infection by a wide variety of Gram-negative bacteria sharing a common core structure or may moderate their lethal effects (Giannella, 1996). Finally, the endotoxin component of the cell wall may play an important role in the pathogenesis of many clinical manifestations of Gram-negative infections. Endotoxins evoke fever, activate the serum complement, kinin, and clotting systems,
depress myocardial function, and alter lymphocyte function. Circulating endotoxin may
be responsible in part for many of the manifestations of septic shock that can occur in
systemic infections (Giannella, 1996)

**Nomenclature**

Historically, there was a great deal of confusion over the naming of *Salmonella*
strains as species names were arbitrarily given to serovars for convenient reasons in
medical practice. Some serovar names denoted syndrome (*S. typhi*) or relationship
(*S. paratyphi* A, B, C). Other names were correlated with syndrome and host specificity
which was right in some cases (*S. abortusovis, S. abortusequi*) but wrong in others
(*S. typhimurium, S. choleraesuis*). To avoid possible sources of confusion, names
indicating geographic origin of the first strain of the new serovars (*S. london, S. panama*)
were then used. However, these names are in fact without taxonomic status and wrongly
written as species names for a long time (Popoff and Le Minor, 1997). According to the
report of WHO Collaborating Center for Reference and Research on *Salmonella*,
common serovar names that were formerly italicized are now written in plain font with
the first letter capitalized. For example, *Salmonella typhimurium* has been changed to be
*Salmonella enterica* subspecies *enterica* serovar *Typhimurium*, but is written as
*Salmonella* Typhimurium or *S. Typhimurium* in routine use (Popoff and Le Minor, 1997).
Serovars of the subspecies *enterica* which account for more than 99.5% of isolated
*Salmonella* strains still bear a name while of the other subspecies of *Salmonella enterica*
are designated only by their antigenic formula (Popoff and Le Minor, 1997). Currently,
this nomenclature system has been widely used by most official journals, and *Salmonella*
nomenclature in this report will also follow this format.
Classification

The genus *Salmonella* is a member of the family *Enterobacteriaceae*, which is comprised of closely related genera from which *Salmonella* may be differentiated by biochemical tests. Commonly used differentiating criteria are listed in Table 1 (Varnam and Evans, 1991).

**Table 1** Differentiation of *Salmonella* and other *Enterobacteriaceae*.

<table>
<thead>
<tr>
<th>Produce acid from:</th>
<th><em>Salmonella</em></th>
<th><em>Shigella</em></th>
<th><em>Citrobacter</em></th>
<th><em>Edwardsiella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulcitol</td>
<td>+/-</td>
<td>-</td>
<td>-/+</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-2</td>
<td>4</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Enzyme activity:

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th><em>Salmonella</em></th>
<th><em>Shigella</em></th>
<th><em>Citrobacter</em></th>
<th><em>Edwardsiella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine dihydrolase</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>-2</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>3</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Simmon's citrate</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H₂S production</td>
<td>+</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1. Reactions for *Salmonella* are based on those of ubiquitous serovars of importance in food poisoning. Reactions of *Salmonella* Typhi and other host adapted serovars may differ.
2. *S. enterica* subsp. *arizonae* and some other serovars’ strains are positive.
3. *Shigella sonnei* is regularly positive.
4. Delayed fermentation is a feature of some strains of *Shigella sonnei*.

Source: Adapted from Varnam and Evans (1991)
The genus *Salmonella* currently consists of two species; *Salmonella enterica*, and *Salmonella bongori* formerly called *Salmonella enterica* subsp. *bongori* (Popoff and Le Minor, 1997). *S. enterica* is further divided into six subspecies; *S. enterica*, *S. salamae*, *S. arizonae*, *S. diarizonae*, *S. houtenae*, and *S. indica*. These species and subspecies are distinguished on the basis of the characteristics listed in Table 2, and can be further divided to a level referred to as the Kauffmann-White Scheme (Jay, 1992), which makes use of O, H, and Vi antigens and the fact that each antigen possesses its own genetically determined specificity (Jay, 1992). This serotyping system is useful for identifying and characterizing *Salmonella*, and especially for tracing epidemics or localized outbreaks of foodborne salmonellosis. Through the years, more than 2400 serologically distinct types of *Salmonella* have been described and classified. The number of serovars in each species and subspecies is listed in Table 3.
Table 2 Differential characteristics of *Salmonella* species and subspecies.

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>enterica</th>
<th>salamae</th>
<th>arizonae</th>
<th>diarizonae</th>
<th>houtenae</th>
<th>Indica</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-glucuronidase</td>
<td>d</td>
<td>d</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>Culture with KCN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dulitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>Galacturonate</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(\gamma)-glucuronidase</td>
<td>+(*)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L(+)-tartrate (^{(a)})</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>- (75%)</td>
<td>+ (75%)</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>Lysis by phage O1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Malonate</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mucate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>- (70%)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ONPG (2h)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>Salicine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(d\) = different reactions given by different serovars.

\((*)\) = Typhimurium d, Dublin –

\((a)\) = d-tartrate

+ = 90% or more positive reactions.

- = 90% or more negative reactions.

Source: Adapted from Popoff and Le minor (1997)
In the case of uncommon serovars, no further subdivision may be necessary, but with those commonly encountered such as *Salmonella Typhimurium* and *Salmonella Enteritidis*, only serotyping is not sufficiently discriminatory for epidemiological investigation of *Salmonella* infections. In these cases, different strains of the same serovars may be distinguished by determining differences in cell susceptibility to one or more lytic bacteriophages (Varnam and Evans, 1991). Biotyping has been also used successfully to subdivide serovars of *Salmonella* into biovars which have a different sugar fermentation pattern. Further subdivisions of serovars may be achieved on the basis of the bacteriocin production or sensitivity, resistance to antibiotics, or by genetic typing methods such as plasmid profiling, pulse-field gel electrophoresis (PFGE), polymerase chain reaction (PCR) – based techniques, and ribotyping, which are increasingly used in epidemiological investigations (Varnam and Evans, 1991; Le Minor, 1984).

### Table 3 Numbers of serovars in each species and subspecies of *Salmonella*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Subspecies</th>
<th>Number of serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em></td>
<td>arizonae</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>diarizonae</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>enterica</td>
<td>1435</td>
</tr>
<tr>
<td></td>
<td>houtenaee</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>indica</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>salamaee</td>
<td>485</td>
</tr>
<tr>
<td><em>S. bongori</em></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>2435</strong></td>
</tr>
</tbody>
</table>

Source: Adapted from Ziprin, 1994; Popoff and Le Minor, 1997
For epidemiologic purposes, *Salmonella* are classified into three groups based on the degree of host adaptation (Varnam and Evans, 1991):

1. Serovars adapted to humans. These include serovars such as *S. Typhi*, *S. Paratyphi A* and *S. Sendai* that usually cause serious diseases with septicaemic-typhoidic syndrome (enteric fever). These serovars are not usually pathogenic to animals.

2. Ubiquitous serovars such as *S. Typhimurium*, which affect both humans and a range of animals, cause gastrointestinal infections of varying severity (but usually less severe than enteric fever). In addition to ‘classical’ food poisoning, these serovars are involved in infantile and travelers’ diarrhea.

3. Serovars which are highly adapted to an animal host such as *S. Abortovis* (sheep) and *S. Gallinarum* (poultry). These strains usually produce no or very mild symptoms in humans. However, *S. Choleraesuis*, which has the pig as primary host, also causes a severe systemic illness in human beings (Varnam and Evans, 1991).

Food is the most common source of *Salmonella* for humans, and may have been contaminated because the source, animal or bird, was infected (CDC, 2004). *Salmonella* is particularly common among chickens. Infections in dairy herds may lead to contamination of milk, which if not adequately pasteurized may be consumed directly or used in the preparation of milk products. Other foods can also be the source of *Salmonella*. 
**SALMONELLA IN FOOD**

**Meat and Poultry**

Poultry meats like chicken, turkey, and ducks are considered to be the number one source of *Salmonella* and foodborne outbreaks of salmonellosis (Buxton, 1957). A large number of *Salmonella* serotypes have been isolated from meat and poultry before and during processing, and from the environment of processing plants (Glegan et al., 1966; Jarolmen et al. 1976; Knitvett, 1971; Patterson, 1969; Pivnick, 1970; Surkiewicz et al., 1969; Wilson et al., 1962; Zottola et al., 1970). Moreover, many surveys have found *Salmonella* in dressed and processed chickens and turkey products in retail stores (Bailey et al., 2002; Capita et al., 2003; Cotterill et al., 1977; Kotula and Davis, 1999; Swaminathan et al., 1978). As a result, many methods have been developed to control *Salmonella* contamination of meat and poultry during processing. Common treatments for broiler chickens to decrease the *Salmonella* include spraying with calcium or sodium hypochlorite, lactic acid, or hydrogen peroxide (Nassar et al., 1997; Thomson et al., 1976; Wabeck et al., 1968). In addition, carcass immersion in hot 3% succinic acid or 0.5% glutaraldehyde is also used (Juven et al., 1974; Thomson et al., 1977). Acid dips do not present any known safety concern (Keener et al., 2004). However, these methods can cause changes in the appearance and odor of the finished product. Recently, control of *Salmonella* during processing is also achieved by comparative chilling. USDA regulations state that in the chilling of all carcasses, the internal temperature has to reach 5°C (41°F) or lower within specific time depending on the weight of the carcasses, (FSIS,
1998a). Thus, immersion chilling with agitation and air chilling are broadly used in the poultry industry (Dewaal, 1996).

**Eggs and egg products**

Egg and egg products are one of the major sources of *Salmonella* infection (St. Louis et al., 1988), and the serovar that infects egg is typically *S. Enteritidis* (AEB, 2000). Of the approximately, 46.8 billion shell eggs that are produced each year in the United States, an estimated 2.3 million are infected by *S. Enteritidis* (FSIS, 1998b). From 1993 to 1997, a total of 2,751 outbreaks of foodborne disease were reported in the United States, and *Salmonella* Enteritidis accounted for the largest number of outbreaks, cases, and deaths (CDC, 2000). Most of these outbreaks were attributed to eating eggs (CDC, 2000). In 1993, for example, an outbreak of *Salmonella* Enteritidis associated with homemade ice cream, made with raw eggs, infected 12 people in Florida (CDC, 1994).

Previously, it was thought that *Salmonella* were only found in eggs which had cracked and into which bacteria were subsequently able to penetrate. Eventually, it was shown that there are tiny pores on the shell egg which *Salmonella* can penetrate into the egg after laying (Smith, 2001). It is known also that *Salmonella* can be passed from the infected chicken directly to the egg before the shell has formed around it (CDC, 2003). The sanitary conditions of the farm or place where egg are produced are important in the control of *S. Enteritidis*, and researchers are working to improve the egg quality through implementation of quality assurance programs (Mumma et al., 2004). USDA has developed a National Poultry Improvement Plan to provide a cooperative industry, state, and federal program to improve poultry and poultry products throughout the country.
(APHIS, 2004). Features of this plan can be used to control Salmonella. For example, using hot water to remove soil and organic matter from egg shell can significantly reduce bacterial members (Bierer and Barnett, 1965). Washing shell eggs with sanitizer has also been used with similar results. However, wash methods cannot remove Salmonella after it has penetrated the shell (Bierer and Barnett, 1962). Heat treatment of egg products is the most effective method to control Salmonella. FSIS (2001) recommends that the internal temperature of all cooked egg products reach 71°C (160°F). At this temperature, all Salmonella will be killed.

**Milk and milk products**

In addition to poultry and egg products, Salmonella are also frequent contaminants of unpasteurized milk. S. Typhimurium is a common serotype in milk or milk products collected from infected cattle, and many outbreaks of salmonellosis that have involved milk and milk products. For example, the outbreak of salmonellosis from inadequately pasteurized milk in Kentucky caused 16 cases in 1984 (CDC, 1984). A large outbreak of milk-borne salmonellosis in Illinois in 1985 caused over 1,500 cases (CDC, 1985). More recently, two outbreaks of multidrug-resistant Salmonella serotype Typhimurium DT104 in fresh Mexican-style cheese infected 31 people in northern California in 1997 (Cody et al., 1999). Although Salmonella are killed by pasteurization, one report suggests sub-past heat treatment of skim milk (80-120°C) can actually stimulate the growth of S. Typhimurium (Singh and Mikolajcik, 1971). Contamination of dried milk products can occur if Salmonella contaminate the environment of the spray drying plant (Rowe et al., 1987). In fermented milk foods, Salmonella are usually
damaged by the low pH (pH less than 4.55) of some cheeses and yogurt (Chapman and Sharpe, 1981; Robinson and Tamime, 1981). However, the inhibitory effect of low pH is less in cheese because of the protection of high protein environments provided for bacteria (Rubin, 1985).

Fish

More than 1,300 imported and 768 domestic seafood samples were tested for the presence of Salmonella over a 9 year period (1990 to 1998) and almost 10% of imports and 2.8% of domestic raw seafood were found to be positive for Salmonella contamination (Heinitz et al., 2000). Shellfish such as oysters and clams usually do not harbor the organism, but they can obtain Salmonella by filtering contaminated water through their gastric systems (Huckstep, 2000). Sewage or polluted water is a continuing problem in many parts of the world, which increases the likelihood that shellfish may become contaminated. Salmonella will develop in the tissues of shellfish without causing disease. The primary preventative measure is to ensure that shellfish are grown in water without pollution. For other fish, Salmonella contamination tends to occur during processing and handling.

Salmonella can occur in several different types of foods such as salad dressing, chocolate, and pasta (Varnam and Evans, 1991). Most outbreaks are caused by the use of the contaminated raw products, poor or unsanitized food handling, and poor personal hygiene. These can be prevented by following several critical steps that include adequate cooking or reheating and rapid cooling; preventing cross-contamination; and ensuring good personal hygiene of food handlers.
INFECTION AND DANGER OF SALMONELLA

Salmonellosis in humans usually takes the form of a self-limiting food poisoning but occasionally manifests as a serious systematic infection that requires prompt antibiotic treatment. This infectious disease has three clinical forms: 1) gastroenteritidis, 2) septicemia, and 3) enteric fevers (Volk, 1982).

Gastroenteritidis (food poisoning) is the most common form of Salmonella infection. All species of Salmonella are pathogenic and cause either salmonellosis (gastroenteritidis) or enteric fever (typhoid fever) depending upon the strain of organisms. The incubation period for Salmonella gastroenteritis depends on the ingested dose of bacteria. Symptoms usually begin 10 to 28 hours after ingestion of contaminated food or water and usually take the form of nausea, vomiting, abdominal pain, and diarrhea (Volk, 1982). Myalgia and headache are common; however, the most frequent symptom is diarrhea. Fever (38 °C to 39 °C) and chills are also common (Giannella, 1996). At least two thirds of patients complain of abdominal cramps. The duration of fever and diarrhea varies, but is usually 2 to 7 days, and most cases are not treated with antibiotics (Volk, 1982).

The septicemic type of Salmonella infection is a blood infection which does not involve the gastrointestinal tract. Most cases are caused by S. Cholerasuis, S. Typhi, and S. Paratyphi; however all Salmonella species can cause septicemia (Murray et. al., 1998). Pneumonia, endocarditis, meningitis, or osteomyelitis can result from septicemic infection (Murray et. al., 1998 and Volk, 1982). The severity of the infection may depend on the resistance of the patient and the virulence of Salmonella isolate (Giannella, 1996).
Enteric fevers are severe systemic forms of salmonellosis. Typhoid fever, caused by *S.* Typhi, is the best studied for the enteric fever. Once again, however, any species of *Salmonella* may cause this type of disease (Volk, 1982). Symptoms usually begin after an incubation period of 10 to 14 days (Murray et al., 1998). Enteric fevers may follow gastroenteritis infections, which usually resolve before the onset of systemic disease. The symptoms of enteric fevers include high fever (39 °C to 40 °C), headache, myalgia, anorexia, and malaise then, followed by gastrointestinal symptoms (Murray et al., 1998). Enteric fevers are severe infections and may be fatal if antibiotics are not promptly administered (Giannella, 1996).

The first step in the disease process involves organisms being transmitted to a susceptible host. For *Salmonella*, this is most commonly achieved by the consumption of contaminated food or water. The lowest inoculum needed to initiate infection depends on strains, types of contaminated food, and condition of the host (Giannella, 1996). For typhoidal *Salmonella*, volunteer studies suggest $10^5$ to $10^{10}$ bacteria are required to initiate infection (Wannissorn, 2001). In contrast, depending on the age and health of host and the strain of organisms, the infectious dose of non-typhoidal *Salmonella* can be only 15 to 20 cells (FDA-CFSAN, 2003). To be pathogenic, *Salmonella* must have virulence factors which include the ability to invade cells, a complete lipopolysaccharide coat, the ability to replicate intracellularly, and possibly the elaboration of toxin (Giannella, 1996).
After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and multiply within the epithelium and lymphoid follicles (Giannella, 1996). The mechanism for the epithelial invasion by *Salmonella* involves binding to specific receptors on the epithelial cell surface (Giannella, 1996). When *Salmonella* contact epithelial cells, they develop cell surface invasive appendages that are subsequently used to attach to the host but are shed after colonization (D’Aoust et al., 2001). After colonizing, the organisms will induce ruffling of the enterocyte membrane. *Salmonella* cause the smooth membrane surface to become uneven, which stimulates pinocytosis of the *Salmonella*. After internalization, *Salmonella* can eventually spread further to mesenteric lymph nodes and throughout the body by systemic circulation (Giannella, 1996). Some organisms may infect the liver, spleen, gallbladder, bones, meninges, and other organs. However, the reticuloendothelial system confines and controls spread of the organism, and the extent of infection ultimately depends on the serotype and the efficiency of the host defenses against that serotype (Giannella, 1996). Most serovars are killed rapidly in extraintestinal sites, and gastroenteritis, the most common human *Salmonella* infection, remains confined to the intestine (Giannella, 1996).

After invading the intestinal epithelium and multiplying within the surrounding mucosal cells, most *Salmonella* induce an acute inflammatory response (D’Aoust et al., 2001). For salmonellosis, epithelial invasion induces an inflammatory reaction and diarrhea. The diarrhea is caused by the secretion of fluid and electrolytes by the small and large intestines and the expression of tissue damage (Giannella, 1996). Polymorphonuclear leukocytes rush into the infected mucosa and release prostaglandins, stimulating the production of adenyl cyclase, which increases the level of cyclic
adenosine monophosphate (cAMP) and induces intestinal fluid secretion (D’Aoust et al., 2001). The common symptoms of intestine inflammatory response are fever, chills, abdominal pain, and diarrhea. During the infection, polymorphonuclear leukocytes, blood, and mucus may be formed in the stools of the patients (Giannella, 1996). Pathogenesis of Salmonella enterocolitis and diarrhea is summarized in Figure 1.

Unlike Shigella and Escherichia coli, Salmonella penetrate the intestinal epithelial cells, but do not escape the phagosome. Thus, the area of intercellular spread and ulceration of the epithelium is minimal. In addition, Salmonella strains release toxins such as enterotoxin and cytotoxin which may stimulate intestinal secretion (D’Aoust et al., 2001). Enterotoxin can activate the adenyl cyclase in the epithelial cell and cause the increase of cAMP in the host cells resulting in the diarrhea. Cytotoxin inhibits the protein synthesis and cause the cell lysis which support the spread of Salmonella (D’Aoust et al., 2001).

For systematic illness or enteric fever, Salmonella penetrate the intestinal epithelial mucosa where they get into the bloodstream. Then, they are taken into to the tissue of the liver, the spleen, and the bone marrow from which Salmonella can proliferate during 7 to 28 days. After that, they are released into other areas including the kidney and the gall bladder (D’Aoust et al. 2001).
Ingestion of *Salmonella* 

Colonization of lower intestine 

Mucosal invasion 

*Cytotoxin* 

Acute inflammation 

± Ulceration 
Prostaglandin synthesis 
Enterotoxins 
Cytokines 

Activation of adenyl cyclase 

↑ Cyclic AMP 

Fluid Production 

Diarrhea 

**Figure 1** Summary of the pathogenesis of *Salmonella* enterocolitis and diarrhea 

Source: Adapted from Giannella, 1996.
Epidemiology

Salmonellosis is a zoonotic disease, and a large number of food animal sources have been identified as reservoirs. The most common animal reservoirs are chickens, turkeys, pigs, and cows, but dozens of other domestic and wild animals also harbor these organisms (Giannella, 1996). Salmonella infection; however, is caused primarily by the digestion of undercooked food, and improper handling food or water. Salmonella have been detected in many types of food products. Those most commonly associated with the disease include raw meats, poultry, eggs, milk and milk products, but it has also been recovered from fish, shrimp, frog legs, yeast, coconut, sauces and salad dressing, cake mixes, cream filled desserts and toppings, dried gelatin, peanut butter, cocoa and chocolate (Price, 1999). Alfalfa sprouts were recently implicated in an outbreak of S. Kottbus (CDC, 2002b). Another major source of Salmonella infection is pet turtles. In the early 1970’s, it was estimated that there were about 280,000 cases of turtle-associated salmonellosis in the United States (Mador et. al., 1994). And as a result, it is now illegal to import turtles or turtles eggs, or even to ship domestic turtles with shells less than four inches in diameter across state lines (FDA-HHS, 2003).

The epidemiology of non-typhoidal salmonellosis is rapidly changing. A doubling of salmonellosis incidence in the last two decades has attended modern food industries, centralized production and large scale distribution (Giannella, 1996). Salmonella Enteritidis and S. Typhimurium are currently the most frequently isolated serotypes in countries around the world, and together account for 57-67% of total annual isolates (WHO, 1995). In 1990, 1994, and 1995, S. Enteritidis was the most commonly reported Salmonella serotype in the United States (Hogue et. al., 1997). Salmonella Enteritidis has
become an emerging foodborne pathogen because of its ability to cause infections in egg-laying hens, and subsequent contamination of shell eggs. *Salmonella* Enteritidis can be transmitted vertically from breeding flocks to egg laying hens, which produce contaminated eggs (Giannella, 1996). Once the organism is present in a flock, the infection is difficult to eliminate because transmission is sustained by environmental sources including rodents and manure. Another emerging foodborne *Salmonella* serovars which has become an important public health problem is *S. Typhimurium* Definitive Type 104 which has the ability to resist at least five antimicrobial drugs (Glynn et. al., 1998). Multidrug-resistant isolates of *S. Enteritidis* have also been found in southern Italy (Nastasi et al., 2000), and fluoroquinolone-resistant *S. Choleraesuis* have emerged in Taiwan (Chiu et al., 2002). The unusual characteristics of antimicrobial resistance of these strains emphasize the problem of drug resistance in *Salmonella* serotypes that are commonly cause foodborne disease. The therapeutic use of an antimicrobial agent, in human and animal populations, has created a selective pressure that favors survival of bacterial strains resistant to the agents (Altekruse et al., 1997).

In contrast to gastroenteritis, the epidemiology of typhoid fever and other enteric fevers primarily involves person to person spread because these organisms lack a significant animal reservoir. Contamination of water with human feces which contains *S. Typhi* is the major mode of transmission (Giannella, 1996). Occasionally, contaminated food, usually handled by an individual who harbors *S. Typhi*, may be the vehicle.
In typhoid fever and non-typhoidal salmonellosis, there are two other factors that have epidemiologic significance. First, an asymptomatic human carrier state exists for the agents of either form of the disease. Approximately 0.1% of people infected with non-typhoidal *Salmonella* and 3% of persons infected with *S. Typhi* become chronic carriers (Giannella, 1996). The carrier state may last from weeks to years. Thus, both human and animal reservoirs exist. Second, use of antibiotics in animal feeds and indiscriminant use of antibiotics in humans have increased the prevalence of antibiotic resistance in *Salmonella* (Giannella, 1996).

Because of the serious health hazards posed by foods that are contaminated with *Salmonella*, many microbiological methods have been developed to isolate, detect, and identify these microorganisms. Two of these methods, pulsed-field gel electrophoresis (PFGE) fingerprinting and bacteria phage lysotyping of *Salmonella* isolates, have proved to be very efficient epidemiologic methods for studying outbreaks of salmonellosis and tracing the spread of the organism in the environment (Giannella, 1996).
ISOLATION AND DETECTION OF *SALMONELLA* SPP.

The earliest methods for the isolating and detecting *Salmonella* were developed in 1885 for clinical fecal samples (Le Minor, 1981). Once foods were suspected of being contaminated with this pathogen, these clinical methods were applied to analyze food samples. However, this practice was not suitable for food samples due to several factors. First, *Salmonella* is usually present in much lower numbers in food samples than in clinical specimens. Furthermore, microorganisms in foods have usually been exposed to processing conditions, such as drying or freezing that injure survivors and make recovery more difficult. Thus, a laborious research was initiated to improve methods of isolation and detection *Salmonella* from foods, particularly those types most commonly involved in salmonellosis outbreaks (Andrews, 1992).

Conventional Methods

Conventional isolation and detection techniques for *Salmonella* still remain in general use in many laboratories. These approaches involve pre-enrichment, selective enrichment and selective plating followed by biochemical and/or serological confirmation of identity of suspect colonies (Patel and Williams, 1994). The diagram of a conventional culture method is illustrated in figure 2.
Food

Pre-enrichment broth
(e.g. buffered peptone water)

24-48 hrs

Selective enrichment broth
(e.g. tetrathionate broth and RV broth)

24-48 hrs

Selective and diagnostic agars
(e.g. XLD, HEA, and BSA)

24-48 hrs

Biochemical identification

24 hrs

Serological confirmation

Expression of results

**Figure 2** Overview of conventional methods for detection of *Salmonella* in foods.

Source: Adapted from Patel and Williams, 1994.

Pre-enrichment

In the case of clinical samples, isolation can usually be made by streaking directly onto a suitable selective medium. However, because *Salmonella* is usually present at low numbers and often in impaired condition in foods, other bacteria may interfere the detection. Therefore, more steps are needed to detect *Salmonella* in food samples than in clinical samples. One of these is termed the pre-enrichment step, where in the food
sample is enriched in a nonselective medium to help injured *Salmonella* cells attain a stable physiological state (Bailey et al., 1991). The need for a recovery step is now widely accepted for all types of food, not only for those which have been dried or frozen. A suitable pre-enrichment medium should provide nutrients for *Salmonella* cell multiplication to increase the ratio of *Salmonella* to non-*Salmonella* microorganisms by facilitating cellular repair, rehydration, and dilution of toxic or inhibitory substances (Varnam and Evans, 1991; Poelma et al., 1984). A large number of media have been proposed for the pre-enrichment of *Salmonella*. Examples recommended by FDA-BAM (Andrew et al., 1998) and AOAC (1995) include lactose broth, trypticase soy broth, and reconstituted nonfat dry milk, as well as buffered peptone water recommended by ISO/FDIS 6579: 2002(E) (Andrew et al., 1998; AOAC, 1995; ISO, 2002).

**Selective Enrichment**

After pre-enrichment, food-derived samples are further enriched in a growth-promoting medium that contains selectively inhibitory reagents. This medium allows continued growth of *Salmonella* but limits the proliferation of most other bacteria (Bailey et al., 1991). Many types of agents have been proposed for the selective enrichment of *Salmonella*. The most widely used inhibitors are bile salts, selenite, tetrathionate, and dyes such as brilliant green and malachite green (Varnam and Evans, 1991). These inhibitors have been incorporated, either singly or in combination, into a wide range of media (table 4).
Table 4 Examples of commonly used media for the selective enrichment of *Salmonella*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Inhibitors</th>
<th>Applications and Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrathionate broth</td>
<td>Tetrathionate, brilliant green, ox-bile</td>
<td>Not suitable for host-adapted serovars</td>
</tr>
<tr>
<td>(Muller-Kauffmann)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenite-cysteine broth</td>
<td>Selenite</td>
<td>Cystine enhances <em>Salmonella</em> growth</td>
</tr>
<tr>
<td>Brilliant green - MacConkey</td>
<td>Brilliant green, bile salts</td>
<td>Very effective with <em>S. Cholerasius</em> but not widely used</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rappaport-Vassiliadis</td>
<td>Malachite green, MgCl₂ 'low' pH value</td>
<td>Medium of choice for foods. May fail to recover <em>S. Typhi</em> and <em>S. Dublin</em>. May also be over-selective for other serovars</td>
</tr>
<tr>
<td>(RV broth)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Adapted from Varnam and Evan (1991)

Because a particular selective enrichment broth may inhibit growth of some *Salmonella* spp., use of a second selective enrichment broth is necessary to ensure accurate detection of *Salmonella* spp. (Hammack et al., 1999). Revision A of FDA's BAM 8th edition (Andrew et al., 1998) recommends tetrathionate broth and selenite cystine broth for most foods, while the pair of tetrathionate broth at 43°C and Rappaport-Vassiliadis (RV broth) incubated at 42°C are recommended for the analysis of raw flesh foods, highly contaminated foods and animal feeds. Tetrathionate broth with added novobiocin and Rappaport-Vassiliadis medium with Soya (RVS Broth) incubated at 42°C are a pair of selective enrichment media recommended by ISO/FDIS 6579:2002(E).
Selective plating

Finally, solid selective media agars are used to differentiate *Salmonella* from non-*Salmonella*. Commonly used selective plating media for *Salmonella* are summarized in Table 5. The media are formulated so that *Salmonella* bacteria form distinct colonies while the growth of competing non-*Salmonella* microorganisms is suppressed. This is commonly based on different selective agents used such as bismuth sulphite, bile salt, deoxycholate or brilliant green, and on the inability of most *Salmonella* to ferment lactose and, in some cases, other carbohydrates such as sucrose and salicin (Varnam and Evans, 1991). Bile containing media often use a secondary diagnostic system based on the ability of *Salmonella* to produce hydrogen sulfide (WHO, 1987). This increases their usefulness, particularly when dealing with materials which frequently contain lactose fermenting organisms, although lactose positive, H$_2$S-negative *Salmonella* may also be isolated. Colonies of *Salmonella* might resemble to those of lactose fermenting bacteria such as most strains of *E. coli* but *Salmonella* may be possible to recognize by faster growth rate and larger colony size (Poelma et al., 1984; Varnam and Evans, 1991).
**Table 5** Commonly used media for the selective plating of *Salmonella*

<table>
<thead>
<tr>
<th>Media</th>
<th>Inhibitors</th>
<th>Diagnostic system</th>
<th>Application and limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brilliant green agar</td>
<td>Brilliant green</td>
<td>Fermentation of lactose and sucrose</td>
<td>Widely used in food industry. Not suitable for <em>S. Typhi</em></td>
</tr>
<tr>
<td><em>Salmonella-Shigella</em> agar</td>
<td>Brilliant green, bile salts</td>
<td>Lactose fermentation, ( \text{H}_2\text{S} ) production</td>
<td>Effective with many foods</td>
</tr>
<tr>
<td>Xylose lysine deoxycholate agar</td>
<td>Deoxycholate</td>
<td>Lactose, xylose, and sucrose fermentation, ( \text{H}_2\text{S} ) production</td>
<td>Relatively low selectivity</td>
</tr>
<tr>
<td>Hektoen enteric agar</td>
<td>Bile salts</td>
<td>Lactose, salicin, and sucrose fermentation, ( \text{H}_2\text{S} ) production</td>
<td>Good differentiation, relative low selectivity</td>
</tr>
<tr>
<td>Bismuth sulphite agar</td>
<td>Bismuth sulphite, sodium sulphite, brilliant green</td>
<td>Reduction of sulphite to sulphide in the presence of fermentable carbohydrate</td>
<td>Often recommended for <em>S. Typhi</em>, effective with lactose-positive <em>Salmonella</em>. Performance with foods may be variable</td>
</tr>
</tbody>
</table>

Source: Adapted from Varnam and Evans (1991)
Biochemical confirmation

Biochemical tests are used to obtain a tentative identification of *Salmonella* cultures collected from selective agar (Bailey et al., 1991). A large number of biochemical tests are available for the characterization of cultural isolates obtained from food products. However, it is unnecessary to use complete tests which are designed to identify all members of family *Enterobacteriaceae* in order to differentiate *Salmonella* isolated from foods (Poelma et al., 1984). Biochemical tests used by ISO/FDIS 6579: 2002(E) and revision A of FDA’s BAM 8th edition for identification of *Salmonella* cultures are listed in Tables 6.
**Table 6** Biochemical and serological reactions of *Salmonella*.

<table>
<thead>
<tr>
<th>Test or substrate</th>
<th>Positive</th>
<th>Negative</th>
<th><em>Salmonella</em> species reaction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucose (TSI)</td>
<td>Yellow butt</td>
<td>Red butt</td>
<td>+</td>
</tr>
<tr>
<td>2. Lysine decarboxylase (LIA)</td>
<td>Purple butt</td>
<td>Yellow butt</td>
<td>+</td>
</tr>
<tr>
<td>3. H₂S (TSI and LIA)</td>
<td>Blackening</td>
<td>No blackening</td>
<td>+</td>
</tr>
<tr>
<td>4. Indole test</td>
<td>Violet color at surface</td>
<td>Yellow color at surface</td>
<td>-</td>
</tr>
<tr>
<td>5. Lysine decarboxylase broth</td>
<td>Purple color</td>
<td>Yellow color</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6. Phenol red dulitol broth</td>
<td>Yellow color and/or gas</td>
<td>No gas; no color change</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7. KCN broth</td>
<td>Growth</td>
<td>No growth</td>
<td>-</td>
</tr>
<tr>
<td>8. Malonate broth</td>
<td>Blue color</td>
<td>No color change</td>
<td>-&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>9. Urease</td>
<td>Purple-red color</td>
<td>No color change</td>
<td>-</td>
</tr>
<tr>
<td>10. Polyvalent flagellar test</td>
<td>Agglutination</td>
<td>No agglutination</td>
<td>+</td>
</tr>
<tr>
<td>11. Polyvalent somatic test</td>
<td>Agglutination</td>
<td>No agglutination</td>
<td>+</td>
</tr>
<tr>
<td>12. Phenol red lactose broth</td>
<td>Yellow color and/or gas</td>
<td>No gas; no color change</td>
<td>-&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>13. Phenol red sucrose broth</td>
<td>Yellow color and/or gas</td>
<td>No gas; no color change</td>
<td>-</td>
</tr>
<tr>
<td>14. Voges-Proskauer test</td>
<td>Pink-to-red color</td>
<td>No color change</td>
<td>-</td>
</tr>
<tr>
<td>15. Methyl red test</td>
<td>Diffuse red color</td>
<td>Diffuse yellow color</td>
<td>+</td>
</tr>
<tr>
<td>16. Simmons citrate</td>
<td>Growth; blue color</td>
<td>No growth; no color change</td>
<td>v</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, ≥90% positive in 1 or 2 days; -, ≥90% negative in 1 or 2 days; v, variable.

<sup>b</sup> *S. Paratyphi* A are negative.

<sup>c</sup> *S. enterica* subsp. *arizonae* cultures are negative.

<sup>d</sup> *S. enterica* subsp. *arizonae* cultures are positive.

Source: Adapted from FDA’s BAM 8<sup>th</sup> edition, revision A (Andrew et al., 1998) and ISO (2002).
Serological confirmation

Use of biochemical tests for identification of *Salmonella* cultures can be eliminated if serological tests with the appropriate antisera are performed, since serological testing provides specific identification of *Salmonella* cultures (ISO, 2002). As is outlined on pp 4-5 of this thesis, the genus *Salmonella* is characterized serologically by O, H and Vi antigens (Andrew et al., 1998), and detection of *Salmonella* these antigens is achieved by an agglutination test with the appropriate sera (Table 7). If agglutination is observed, the reaction is considered positive. O antigens are composed of phospholipid polysaccharide complexes which are heat stable and resistant to alcohol and dilute acid. H antigens are protein access in nature, and are heat labile. Vi antigens, the superficial antigens, are present in sufficient amounts to inhibit the agglutination of unheated bacterial suspensions when tested with O antisera (Edwards and Ewing, 1972). Filamentous appendages called fimbriae (protein) may also interfere with O agglutination (Poelma et al., 1984). Strains considered or suspected to be *Salmonella* from biochemical or serological tests should be sent to a recognized *Salmonella* reference laboratory for definitive typing such as DNA-fragment based typing system (ISO, 2002).
**Table 7** Interpretation of confirmatory tests for *Salmonella*.

<table>
<thead>
<tr>
<th>Biochemical reactions</th>
<th>Auto-agglutination</th>
<th>Serological reaction</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical</td>
<td>No</td>
<td>O-, Vi-, H-antigen positive</td>
<td>Strains considered to be <em>Salmonella</em></td>
</tr>
<tr>
<td>Typical</td>
<td>Yes</td>
<td>Not tested (^2)</td>
<td>May be <em>Salmonella</em></td>
</tr>
<tr>
<td>Typical</td>
<td>No</td>
<td>All reactions negative</td>
<td>May be <em>Salmonella</em></td>
</tr>
<tr>
<td>No typical reactions</td>
<td>No / Yes</td>
<td>O-, Vi-, H-antigen positive</td>
<td>Not considered to be <em>Salmonella</em></td>
</tr>
<tr>
<td>No typical reactions</td>
<td>No / Yes</td>
<td>All reactions negative</td>
<td>Not considered to be <em>Salmonella</em></td>
</tr>
</tbody>
</table>

1. The agglutination of bacteria after tested with saline solution only.
2. The strain considered as auto-agglutination shall not be submitted to the following tests.

Source: Adapted from ISO (2002)

In summary, the advantages of traditional culture techniques for the microbiological examination of foods include high sensitivity and relative low cost. However, these methods have many different steps, and so are labor and time intensive. For *Salmonella*, conventional detection methods require 4-6 days to complete, depending on the type of food and the extent of contamination. Increasing public interest in food safety combined with modern technology in food processing and quality assurance have therefore created a need for fast, automated, cost-effective, and more reliable methods for determining microbiological quality and safety.
RAPID DETECTION METHODS

Normally, a large number of food samples from a given lot need to be analyzed to have a reasonable assurance of detecting microbial contamination in that lot (Andrew et al., 1998), and the products are often shipped before such tests are completed. This is due to the fact that conventional methods require 4-6 days before even preliminary results are available (Andrews and Hammock, 2003), and many companies cannot afford to hold food products until confirmatory tests are completed. In most cases, Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Points (HACCP) programs are used as the primary methods to control pathogens contamination (FDA-CFSAN, 2001).

However, if microbial tests conclude or suggest food products are contaminated with pathogenic bacteria, products must be recalled, resulting in significant economic cost and loss of professional reputation (Buzby et al., 2001).

For these reasons, the food industry needs fast, specific, and sensitive detection methods for dangerous microbes. Fortunately, combined advances in immunology, molecular biology, computer technology, biotechnology, and engineering have given rise to a wide range of new techniques for the rapid analysis of foodborne pathogens, including *Salmonella* (Cox and Fleet, 1998). Current rapid detection methods can be divided into three groups; DNA based tests, immunoassay based tests, and immuno-latex agglutination based tests (Dougherty and Kang, 2001).
DNA based tests

These methods provide high sensitivity and specific detection. There are two techniques that are popular and used in commercial settings: nucleic acid hybridization and polymerase chain reaction (PCR). The earliest applications of nucleic acid hybridization methods were based on the development of gene probes to detect and isolate organisms with a specific genotype (Sayler and Layton, 1990). More recently, researchers have concentrated on increasing the sensitivity of DNA detection assays by combining these assays with other detection systems (Sayler and Layton, 1990). The GENE-TRAK colorimetric assay from Neogen Cooperation (Lansing, MI) uses Salmonella-specific DNA probes and a colorimetric system for detecting, for example, Salmonella spp. in enriched food samples. Probes used in the assay are reactive with serovars of all subspecies of S. enterica as well as serovars belonging to the separate species S. bongori. A sample is considered negative for Salmonella spp. if the absorbance at 450 nm (A_{450}) of the test sample is less than or equal to the established cutoff value for the assay. Samples with an absorbance value greater than the cutoff are considered positive for Salmonella spp. The assay is reported to provide relatively low false positive (1.4%) and false negative (2.5%) results (Bailey et al., 1991). This method is effective in the detection of Salmonella spp. in a wide variety of contaminated foods, and can save processors time by reducing the response time in the case of a contamination problem (Chan et al., 1990).
Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) involves the detection of specific gene fragments by enzymatic amplification of the target DNA, followed by detection of the amplified DNA molecule by gel electrophoresis or fluorescent techniques. In PCR, DNA collected from a sample is denatured, then short DNA primers that are specific for a piece of target DNA in the pathogen of interest will anneal to the target DNA sequence (if it is present), and the fragment of target DNA is polymerized. PCR is a highly specific and sensitive method allowing the detection of low numbers less than $10^2$ cells of microorganisms (Riyaz-Ul-Hassan et al., 2004; Whyte et al., 2002; Zhu et al., 1996). However, false-positive reactions can occur if DNA is present from pathogenic organisms that were killed during processing (Norton, 2002). Recently, real time PCR, a new method of PCR quantification, has been invented to reduce the time in gel electrophoresis step. This method uses a DNA-binding fluorescent dye and monitors the fluorescence that is released during the reaction as an indicator of amplicon production during each PCR cycle (Higuchi, 1992; Higuchi, 1993). However, the cost of real time PCR machines is still relatively high, which serves as a disincentive for its use in the food industry (Sambrook and Russell, 2001).

There are currently three commercial *Salmonella* tests based on PCR techniques; Probelia *Salmonella* spp. from Sanofi Diagnostics Pateau (Marnes La Coquette, France), Taqman from Perkin-Elmer Applied Biosystem (Norwalk, CT), and BAX system *Salmonella* from Qualicon (Wilmington, DE). The Probelia *Salmonella* spp. system is based on PCR amplification of the *iagA* gene (involved in the bacterial invasion process of *Salmonella* spp.) followed by probe hybridization (Miras et al., 1995). The sensitivity
was reported to be $10^2$ CFU/ml, and after an 18-h pre-enrichment step, the test could detect viable *Salmonella* in artificially contaminated food samples with 3 CFU/25 g (Fach et al., 1999). In the Taqman *Salmonella* test, the 5' nuclease activity of Taq DNA polymerase is used to digest an internal fluorogenic probe bound to the target DNA. Digestion results in the release of a fluorescent signal, which is used as a positive indicator for the presence of the target gene (Kawasaki et al., 2001; Kimura et al., 1999). Kimura et al. (1999) showed the Taqman assay can detect 3 CFU per 50 µl of PCR reaction mix of *Salmonella* in pure culture (120 CFU/ml of TSB culture) and Kawasaki et al. (2001) reported the TaqMan PCR method is a reliable and rapid method for detecting *Salmonella* in meat products. The BAX system *Salmonella* test has AOAC Performance Test status (Mrozinski et al., 1998). This test combines primers, polymerase, and nucleotides needed for PCR into a single tablet, and then uses a fluorescent detection system to detect PCR products (AOAC, 2002). The BAX system can reportedly provide confirmed test results within 28 hours (Bennet et al., 1998).

**Immunoassay based tests**

Immunoassay tests use antibodies that have been developed to specifically bind target antigens. The technology has been used widely for field analysis because the antibodies can be highly specific, and reactions are relatively quick simple to use (EPA, 2003). The enzyme-linked immunosorbent assay (ELISA) is one of the most popular methods and is based on the principle of antibody-antibody interaction. ELISA is usually performed in a microtiter plate which contains an 8 x 12 matrix of 96 wells. If an antigen from the target bacterium is present in a food sample, it will be captured by antibodies
attached to the wells. The antigen-antibody complex is then detected using monoclonal or polyclonal antibodies conjugated with enzyme such as horseradish peroxidase. These antibodies have a high specificity to the antigen. The results can be obtained by adding enzyme substrate such as H₂O₂ and tetramethylbenzidine (TMB). The colour generated is measured spectrophotometrically. However, false positives can occur due to nonspecific binding of the antibody to non-target antigens from other organisms. If available, the monoclonal antibodies are often used to increase the specificity of the binding and decrease the cross reaction (Robinson, et al., 1983).

Commercial, ELISA-based tests for Salmonella include Assurance Salmonella and Assurance GOLD Salmonella from BioControl (Bellevue, WA), which are both AOAC accepted (AOAC, 2004), MicroELISA from Dynatech Laboratories (Chantilly, VA), BacTrace from KPL, Inc. (Washington, DC), and Salmonella Tek from Organon Teknika (Durham, NC).

Another variation of the ELISA method, termed the sandwich ELISA, requires two antibodies that bind to epitopes that do not overlap on the antigen. This method is valuable especially when the concentration of antigens is low or the samples contain high concentration of contaminating antigens. Wyatt et al. (1993) developed a sandwich ELISA that employed the polyclonal antibodies for the capture stage and monoclonal antibodies for the detection stage. One commercial test that uses a sandwich ELISA configuration is the Salmonella VIA test from TECRA (Sydney, Australia).

Another Ab-based detection technique is the immunomagnetic method. This test uses antibodies bound to magnetic beads that bind target bacteria when the beads are mixed with a food sample. Captured bacteria and beads are readily removed from the
sample by immunomagnetic separation (IMS). One commercial method, the *Salmonella* Enteritidis Screen/Verify from Vicam (Watertown, MA), relies on IMS to selectively remove *Salmonella* from a sample suspension (Cox and Chung, 1999). The Microscreen test from Mercia Diagnostics (Surrey, UK), Reveal for *Salmonella* from Neogen (Lansing, MI), and VIP for *Salmonella* from BioControl (Bellevue, WA) also use immunomagnetic precipitation. After the enrichment step, the sample is mixed with anti-*Salmonella* antibodies which form a complex if the pathogen is present. The antigen-antibody complex is then captured by an additional anti-*Salmonella* antibody, forming a precipitate which provides the positive result (Bird et al., 1999).

Another variation of the Ab-based test, enzyme-linked fluorescent immunoassay (ELFA), employs a fluorescent substrate that binds any antigen-antibody complex present in a sample and the intensity of fluorescence is measured. The principle behind use of the fluorescent dyes is that the fluorescent dye molecules in the sample absorb light of a particular wavelength, which increases the energy of the molecules and causes them to release some of this energy as light of a slightly longer wavelength. One of the most common fluorescent dyes in ELFA test is fluorescent isothiocyanate (FITC), which absorbs light at 460 nm and releases it at the 500 nm. Keith (1997) compared an automated ELFA to a conventional plate method. The detection rate of the ELFA was 96% compared to the conventional method rate. EIAFoss from Foss Electric (Hillerod, Denmark) and VIDAS from BioMerieux Vitek (Durham, NC) are automated commercial methods based on the ELFA test.
**Immuno-latex agglutination based tests**

In this method, latex particles coated with polyvalent *Salmonella* antiserum are used to bind *Salmonella* antigen in food samples and produce agglutination. Latex agglutination methods are usually used for clinical diagnosis and are rapid, easy to perform and cost-effective tests (Benge, 1989). Commercial tests based on immuno-latex agglutination include Spectate from May and Baker Diagnostics (Glasgow, UK), Wellcolex color *Salmonella* (WCS) from Remel (Lenexa, KS), Oxoid *Salmonella* latex test (Ogdensburg, NY), Bactigen from Wampole laboratories (Cranbury, NJ), and Slidex from BioMerieux (Durham, NC).

As an example of these tests, the Wellcolex Color *Salmonella* assay reacts an enriched sample with two grey-brown test reagents that contain antibodies against different *Salmonella* serogroups. In the presence of homologous antigen, one of the colors in the mixture will agglutinate, and the identity of the antigen is indicated by the color of the aggregated particles and a distinct change in the color of the background. Petrova et al. (1992) showed the Wellcolex color *Salmonella* test provided high specificity and gave information on the presence of *Salmonella* species in biological material within 24 hours. The sensitivity of the Wellcolex color *Salmonella* is 98.4% and the specificity is 100% when they were tested on pure cultures received at a reference laboratory (Bouvet and Jeanjean, 1992).
CONCLUSIONS

Salmonellosis is one of the most frequent causes of foodborne disease in North America and Europe. CDC estimates that 1.4 million people in the United States are infected with salmonellosis and 1,000 patients died each year (Frenzen et al., 1999). Thus, *Salmonella* contamination presents an immense and critical challenge to the food industry. Food products infected with *Salmonella*, have been linked to several foodborne disease outbreaks, and have led to the financial ruin of some food manufacturers.

Unfortunately, conventional culture based tests to detect the *Salmonella* usually take about 4-6 days to get preliminary results, which is not fast enough for the food industry. Food companies needed to find a way to move forward in their production processes and quality assurance programs with cost-effective efficiency and a high level of confidence.

As a result, several rapid detection methods have been developed using different molecular-based strategies. Moreover, most commercial methods try to combine as many steps of the test as possible to simplify use, but all of them still require an enrichment step. Thus, future research is needed to minimize the time required for enrichment step so that test results are available within 24 hours.
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