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# Laboratory Typing Methods for Diagnostic of *Salmonella* Strains, the “Old” Organism That Continued Challenges

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## 1. Introduction

*Salmonella* are enteric gram negative organisms that are widely dispersed in nature. These organisms can reside as common commensals in the gastrointestinal tracts of animals and man or cause disease states that range from self-limited diarrhea to bacteremia with enteric fever or invasion of vascular structures, bone or other localized sites (Hook, 1990).

Organisms can be highly host adapted, where they infect only a limited number of species, or can be much more ubiquitous. The most significant human host-adapted organism is *S. typhi*, the cause of typhoid fever. Man remains the only known reservoir for these isolates. Similarly, *S. pullorum* and *S. gallinarum* are poultry associated organisms that are so host-adapted that even upon transmission to man they usually remain non-pathogenic (Ziprin & Hume, 2001). More frequently, animal host-adapted organisms can be transmitted to man causing symptomatic disease. *S. choleraesuis* is normally a porcine organism though it can cause gastroenteritis and enteric fever, when transmitted to man (especially in children). Other organisms, such as *S. typhimurium*, have a broad host range and these serotypes are responsible for the majority of human infections.

Thus, *Salmonella* strains, the well and “old” pathogens, continued threat to public health. In fact, despite that, the incidence of salmonellosis has decreased substantially especially in developed country, recent events and several articles illustrate continued challenges in *Salmonella* control. The first challenge in *Salmonella* control is the widespread distribution of food; in fact contaminated food produced in one country may cause illness far away demonstrating the importance of robust control programmes. Likewise, this organism cause substantial economic loss resulting from mortality, morbidity, poor growth of infected animals, poultry and human beings; hazardous of transmitting food poisoning with gastroenteritis to human and so represents a serious problem for the food industry (Khan et al., 2007).

The second challenge is traceability, in fact, the complexity of the food supply chains and/or the lack of identifying markers on foods can make it extremely difficult to trace back to their origin.

The third is antimicrobial resistance; in fact, over the last decade, strains of *Salmonella enterica* with multiples drug resistance have been distributed widely in many countries.

The fourth is capacity building to enhance outbreak detection through routinely subtyping certain *Salmonella* using molecular methods.

To contain this organism, it is essential to maintain continued vigilance, including rapid identification of similar strains and the immediate sharing of information within the public health community. Many nations have established extensive surveillance systems to track *Salmonella* infections and disrupt epidemic spread. Most of these surveillance projects rely on traditional serotype and phage type analyses to identify trends and potential outbreaks. Many clinical outbreaks cluster among a few serotypes so further discrimination is often needed.

Molecular epidemiological techniques have been used to enhance surveillance and discriminate outbreak strains within these common serotypes. The institution of these techniques has led to enhanced detection of outbreaks worldwide. In this chapter, we review the theoretical and practical basis of laboratory typing method for diagnostic of *salmonella* strains with emphasis on molecular methods which would contribute to the monitoring of human and animal *Salmonella* infections. Overall, traditional serotype surveillance in association with one or several molecular typing techniques, appears to provide the most reproducible and comparable discrimination of epidemiologically-linked isolates.

## 2. General properties of the genus *Salmonella*

*Salmonella* are Gram negative, short plump shaped rods, nonsporeforming, noncapsulated, aerobic and facultatively anaerobic organisms and classified under the family Enterobacteriaceae (Freeman, 1985).

*Salmonella* nomenclature has changed many times and still is not stable. The genus *Salmonella* was previously differentiated into two species: *Salmonella enterica* and *Salmonella bongori*. However, a new species, *Salmonella subterranea* was identified and validated (Shelobolina et al., 2004; Validation List No: 102, 2005). Among them, the species *Salmonella enterica* (*S. enterica*) is further divided into the six subspecies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). Formerly, *S. bongori* was the subspecies V, but later considered as a separate species (Fluit, 2005).

Fermentation of selected substances, such as dulcitol, malonate, sorbitol, d-tartrate, galacturonate, mucate, salicine, ONPG, and lactose, as well as production of enzymes such as gelatinase,  $\beta$ -glutamyl-transferase or  $\beta$ -glucuronidase, but also lysis by phage O1 allow a differentiation between the different species and subspecies (Le Minor 1984).

Furthermore, the genus composed of over 2500 serotypes differentiated according to three different types of surface antigens discussed bellow in more detail. 99% of these serotypes belong to *S. enterica* and nearly 60% of them are in *S. enterica* subsp. *enterica*. The average DNA sequence similarity between *Salmonella* serotypes is 96-99% (Edwards et al., 2002).

## 3. Bacterial isolations

A standard technique was used to isolate *Salmonella* strains in many laboratories. The technique is explained bellow.

3.1 Food samples

Samples were analysed according to French Norm for *Salmonella spp.* NFV 08-052/97. From each sample, 25 g was pre-enriched in 275 ml buffered peptone water (Oxoid, Dardilly Cedex, France) at 37°C for 24h. Afterwards, 0.1 ml of the pre-enrichment sample was incubated in 9.9 ml of buffered Rappaport-Vassiliadis medium (Oxoid, Dardilly Cedex, France) and 2 ml in 20 ml of buffered selenite cystine medium for another 24 h at 42 °C and 37 °C, respectively. The enrichment samples were then applied onto Hecktoen and Kampelmacher agar. Both selective media were incubated during 24 h at 37 °C. Suspicious colonies were identified by Gram staining performed according to the conventional method and also with biochemical test (oxydase reaction). Both Gram-negative and oxidase-negative isolates were further tested. Biochemical tests other than oxidase test were done by using API 20E test kit (bioMérieux, Inc., France). The plastic strips holding twenty mini-test tubes were inoculated with the saline suspensions of the cultures according to manufacturer's directions. This process also rehydrated the desiccated medium in each tube. A few tubes were completely filled (CIT, VP and GEL), and some tubes were overlaid with mineral oil such that anaerobic reactions could be carried out (ADH, LDC, ODC, H2S, URE) (Figure 1).



Fig. 1. Typical *Salmonella* reaction of API 20E test kit.

After incubation in a humidity chamber for 18-24 hours at 37°C, the colour reactions were read (some with the aid of added reagents as supplied by the kit). The data were analysed by the manufacturer’s software and positive results with ≥89% probabilities were confirmed as *Salmonella*. The list of the biochemical tests performed by API 20E test kit and typical reactions exhibited by *Salmonella spp.* are given in Table 1.

3.2 Stool sample

Each stool sample was streaked onto Hecktoen agar and pre-enriched in selenite broth at 37 °C for 24 h. The pre-enrichment sample was streaked onto Hecktoen agar, and after incubation at 37 °C for another 24 h, the suspicious colonies were identified with biochemical test (as mentioned above).

3.3 Environmental water samples

From each sample, 100 ml was pre-enriched in 100 ml double concentrated buffered peptone water (Oxoid, Dardilly Cedex, France) at 37 °C for 24 h. Afterwards, 0.1 ml of the pre-enrichment sample was incubated in 9.9 ml of buffered Rappaport-Vassiliadis medium (Oxoid, Dardilly Cedex, France) and 1 ml in 9 ml of buffered selenite cystine medium for another 24 h at 37 °C; The enrichment samples were then applied onto Hecktoen and

Kampelmacher agar. Both selective media were incubated during 24 h at 37 °C. The suspicious colonies were identified with biochemical test (as mentioned above).

Tests	Substrate	Reaction	(-) Results	(+) Results	<i>Salmonella</i> <i>spp.</i>
ONPG	ONPG	betagalactosidase	colorless	yellow	-
ADH	arginine	Arginine dihydrolase	yellow	red/orange	-
LDC	lysine	Lysine decarboxylase	yellow	red/orange	+
ODC	ornithine	Ornithine decarboxylase	yellow	red/orange	+
CIT	citrate	Citrate Utilization	pale to green/yellow	blue-green/blue	-
H2S	Na thiosulfate	H2S production	colorless/gray	black deposit	+
URE	urea	Urea hydrolysis	yellow	red/orange	-
TDA	tryptophan	deaminase	yellow	brown-red	-
IND	tryptophan	Indole production	yellow	red (in 2 min)	-
VP	Na-pyruvate	Acetoin production	colorless	pink/red (in 10 min)	-
GEL	charcoal gelatin	Gelatinase	no diffusion of black	black diffusion	-
GLU	glucose	fermentation/oxidation	blue/blue-green	yellow	+
MAN	mannitol	fermentation/oxidation	blue/blue-green	yellow	+
INO	inositol	fermentation/oxidation	blue/blue-green	yellow	-
SOR	sorbitol	fermentation/oxidation	blue/blue-green	yellow	+
RHA	rhamnose	fermentation/oxidation	blue/blue-green	yellow	+
SAC	sucrose	fermentation/oxidation	blue/blue-green	yellow	-
MEL	melibiose	fermentation/oxidation	blue/blue-green	yellow	+
AMY	amygdalin	fermentation/oxidation	blue/blue-green	yellow	-
ARA	arabinose	fermentation/oxidation	blue/blue-green	yellow	+

Table 1. Biochemical reactions involved in API 20E (bioMérieux, Inc., France) test kits and typical *Salmonella* reactions.



4. Laboratory typing methods

The determination of the relatedness of strains within a *Salmonella* serotype is a prerequisite for the identification of the sources of infection and for tracing the routes of *Salmonella* dissemination in outbreaks. Since biochemical analysis did not further differentiate between the bacteria assigned to the same *S. enterica* subspecies, other phenotypic and molecular methods have been used (Riley, 2004).

4.1 Phenotypic methods

4.1.1 Serotyping

Serotyping is the initial step for routine diagnostics of *Salmonella* strains and performed with commercially available omni-, poly- and monovalent antisera. Up to date, over 2500 serotypes of *Salmonella* has been identified and classified in the Kaufmann-White scheme. This scheme differentiates between O (=somatic) antigens of the cell surface, H1 and H2 (=flagellar) antigens of the phase 1 or phase 2, respectively (Selander et al., 1996) and the Vi (=capsular) antigens which, however, may only be present in very few serotype, such as *Typhi*, *Paratyphi C* or *Dublin*.

Each *Salmonella* serogroup has a group specific O-antigen. Within each O-group, different serovars are distinguished by the combination of O- and H-antigens that are present. Each serotype has a specific antigenic formula where the O-antigens are indicated by Arabic numbers, the H1-antigens by lower case letters and the H2- antigens again by Arabic numbers. In these formulas, underlined antigens may only be expressed once the culture is lysogenised by the corresponding converting phage whereas letters or numbers in brackets indicate antigens which may be present or absent without relation to phage conversion (Le Minor, 1984).

For most of the isolates assigned to *S. enterica* and the subspecies I, antigenic formula corresponds to a serotype name. In contrast, serotypes identified after 1996 in the subspecies *salamae*, *houtenae* and *indica* and in the subspecies *bongori* are designated only by antigenic formula (Brenner et al., 2000).

Serotype	O-antigen(s)	H1-antigen(s)	H2-antigen(s)
<i>S. Enteritidis</i>	<u>1</u> , 9, 12	[f], g, m, [p]	[1, 7]
<i>S. Dublin</i>	<u>1</u> , 9, 12 [Vi]	g, p	-
<i>S. Gallinarum</i>	<u>1</u> , 9, 12	-	-
<i>S. Typhimurium</i>	<u>1</u> , 4, 5, 12	i	1, 2
<i>S. Virchow</i>	6, 7	r	1, 2
<i>S. Infantis</i>	6, 7, <u>14</u>	r	1, 5

Table 2. Examples for the antigenic formulas of *Salmonella enterica* subsp. *enterica* serotypes according to Kaufmann-White scheme (Poppoff and Le Minor, 2001).

The detection of the presence of *Salmonella* O- and H- antigens were tested by slide agglutination with the commercially available antisera. One loop of appropriate antisera was dropped onto a cleaned glass slide. One loop of overnight culture grown on agar was dispersed in the drop to obtain a homogeneous and turbid suspension. The slide was rocked gently for 30 s and clumping was monitored by a magnifying glass. The scheme to obtain the serotype was given in **Figure 2**.

Serotyping is easy to perform and standardized antisera are commercially available. However, it only allows the assignment of *Salmonella* strains to a specific serotype, and no further differentiation between strains of the same serotype is achieved.

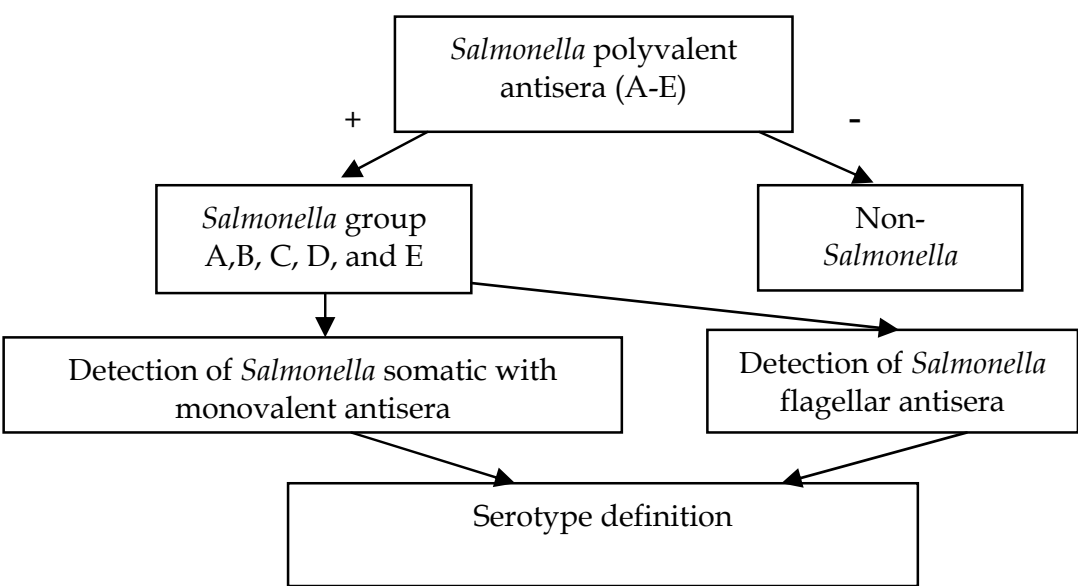


Fig. 2. Serotyping analysis scheme for *Salmonella*

During the 1980's, a tremendous increase in *S. enteritidis* was identified, particularly in the Northeastern U.S. (Rodrigue et al.,1990). Studies linked *S. enteritidis* to contaminated shell eggs or foods that contained eggs (Mishu et al., 1994). During 1987-1997, five serotypes accounted for 66% of all clinical infections in which a *Salmonella* isolate was identified to the serotype level. *S. typhimurium* accounted for 24% of these isolates, *S. enteritidis* (22%), *S. heidelberg* (9%), *S. newport* (5%) and *S. hadar* (4%) followed (Olsen et al., 2001). When clinical outbreaks were distinguished from sporadic infections, *S. enteritidis* was implicated in 55% of *Salmonella* cases associated with a clinical outbreak (Olsen et al., 2001).

In Tunisia, from 1994 to 2004, 16.214 *Salmonella* isolates were reported to the national Centre of Enteropathogenic bacteria at Pasteur Institute, Tunis, Tunisia. (Ridha et al., 2007). The largest proportion of *Salmonella* isolates was from human origin (n=6815) followed by isolates from food (n=5539). During the surveillance period, the top five reported *Salmonella* serotypes were: *Enteritidis*, *Anatum*, *Corvallis*, *Braenderup*, and *Livingstone*. These five serotypes accounted for 3479 strains of all *Salmonella* isolates from food. (Ridha et al., 2007). Finally, *Salmonella* isolates reported from environmental origin came in last position (n=1611) after isolates from animal origin (n=2249) (Ridha et al., 2007).

Serological analysis usually remains the first step in an epidemiological investigation of *Salmonella* and may be sufficient for epidemiological investigations associated with uncommon serotypes (Threlfall & Frost, 1990). However, smaller labs often do not have access to the pools of serum required for this analysis and may need to rely on other techniques to analyze isolates. The multiplex PCR, an easier molecular method, has been developed to differentiate between the most common serotypes of *Salmonella enterica* subsp. *enterica* (Imen et al. 2010).

#### 4.1.2 Phage typing

Individual isolates of many *Salmonella* serotypes vary in their susceptibility to lysis by different bacteriophages and this has led to a typing scheme based on reactivity to a panel of bacteriophage. Therefore, a *Salmonella* strain is subjected to a specified set of typing phages and the lytic pattern obtained commonly allows the assignment to a specific phage type. The strains exhibiting a lytic pattern that does not correspond to a known phage type are classified as RDNC (= Reacting with the typing phage, but lytic pattern Did Not Correspond to any recognized phage types).

Phage typing is mostly performed for serotypes such as *S. Typhimurium*, *S. Enteritidis*, *S. Typhi* or *S. Paratyphi*, although phage typing systems are also available for a number of additional serotypes, including *S. Virchow*. Phage typing has led to the discrimination of over 200 *S. typhimurium* phage types (Threlfall & Frost, 1990) and, together with antimicrobial susceptibility analyses, led to detection of several large-scale, international epidemics including the dissemination of a multi-drug resistant clone of *S. typhimurium* DT104, (definitive phage type, DT, 104) (Threlfall, 2000). In Denmark, phage typing as described by the World Health Organization (WHO) Collaborative Centre for phage typing of Salmonella (Health Protection Agency (HPA), Colindale, United Kingdom) has been applied for surveillance of *S. Enteritidis* and *S. Typhimurium* in humans, food and food production animals. Phage typing has proven to be an important tool for strain characterisation and the results obtained have been used since the mid-90s in surveillance, source attribution and outbreak investigations (Baggesen & Wegener, 1994; Hald et al., 2007)

In general, phage typing is only performed by the National Reference Centers, since only these institutions have access to the defined sets of typing phages. The interpretation of the results requires considerable experience (Riley, 2004). Although, phage typing in *Salmonella* epidemiology has been used since the 1950s, the stability of phage types can be limited by phage type conversion (Rabsch et al., 2002), even during an outbreak (Mmolawa et al., 2002). This is due to the acquisition of a temperate phage or a plasmid. Besides, host-controlled phage defence mechanisms such as restriction/modification systems and phage adsorption inhibition are also responsible for the phage typing difficulties of a *Salmonella* strain.

By means of a sterile inoculation loop, the test culture was inoculated into a test tube containing 4 mL double strength nutrient broth with a special care for heavy inoculum to give visible turbidity for *S. Enteritidis* and a very light inoculum for *S. Typhimurium* to give a barely visible turbidity.

The culture was incubated by shaking at 200 rpm at 37°C for 1-1.5 h for *S. Enteritidis* and for *S. Typhimurium* 1.5 h without agitation to obtain a very light growth in early log phase. After incubation, it was flooded over the surface of double strength nutrient agar using a flooding pipette and the excess of culture was removed. As soon as the surface of agar dried, the appropriate typing phages at routine test dilutions were applied to the dried surface by a multipoint inoculation loop. When the phage spots dried, the agar plate was incubated at 37°C for 18 h. At the end of the incubation, the agar plate was read using a magnifying glass through the bottom of the plate (Ward et al., 1987).

Phage susceptibilities were evaluated by means of the plaque number, size and transparency. The pattern was compared with known phage type patterns in the database and defined. If the culture did not react with any of the typing phages, it was defined as non-typable (NT); and if the culture reacted with the typing phages, but gave a different



pattern other than those in the database, it was considered as reacting with the typing phages, but lytic pattern did not correspond to any recognized phage types, so called RDNC (= Reacting with the typing phage, but lytic pattern Did Not Correspond to any recognized phage types). But, we must note that phage typing analyses needs typing phage sets to be performed.

In brief, phage typing can play an important role in surveillance and control of the common *Salmonella* serotypes. However, this requires strengthened efforts to make the system available to more laboratories internationally, possibly a simplification of the system to enhance its robustness even though this may slightly compromise its discriminatory power, and finally improved external and internal quality assurance systems.

## 4.2 Molecular methods

Phenotypic typing methods requiring enough time, personnel and reagent have led to the development of typing methods based on genotypic information. Currently used molecular typing methods are based on restriction endonuclease digestion, nucleic acid amplification, or nucleotide sequencing techniques.

### 4.2.1 Plasmid profiling

Plasmid profile analysis was one of the earliest DNA-based subtyping schemes. It is particularly important, since most of the plasmids harbour virulence and antimicrobial resistance properties in *Salmonella*. Plasmid content of the host within the same serotype reveals the differentiation according to the profile (the number and molecular sizes of plasmids) obtained. The different plasmid profiles within a serotype points the lateral transfer by gaining or losing the plasmid(s). The plasmids found in *Salmonella* differ in size 2 – 200 kb with different functionalities (Rychlik et al., 2006).

The detection method is based on the isolation of plasmids followed by agarose gel electrophoresis. Different protocols can be used (Helmuth et al., 1985). To view the plasmid pattern, agarose gel must be stained with ethidium bromide solution and then visualised under UV light.

Plasmid analysis has several limitations. Plasmids can rapidly be acquired or lost. Also, single predominant plasmids have become endemic within various serotypes. In sporadic isolates of *S. enteritidis* from Maryland, 88% of isolates contained a single 36-Mda plasmid (Morris et al., 1992). Similarly, only 1 of 56 *S. typhimurium* isolates failed to encode a 90 kb plasmid, which is thought to be a serotype specific virulence plasmid. Despite the ubiquitous nature of the 90 kb plasmid, profiling of the entire complement of plasmids in each strain was able to discriminate *S. typhimurium* strains isolated from a single poultry flock or closely related flocks (Millemann et al., 1995).

Plasmid analysis was also able to identify a multi-state outbreak of chloramphenicol resistant *S. newport* in humans that could be traced back to contaminated beef and to dairy farms (Riley et al., 1983). In a testament to the power of combining a strong traditional epidemiological analysis with serological and genotypic tests, a peak of *S. muenchen* was noted in Ohio, Michigan, Georgia and Alabama. Epidemiological studies failed to identify a common food source responsible for this outbreak, but a strong correlation with marijuana use was identified. Marijuana obtained from affected households was contaminated with *S. muenchen* and the isolates from the different states showed a similar plasmid fingerprint suggesting interstate transfer of the contaminated drug (Taylor et al., 1982).

Plasmid profiling is most useful in an outbreak setting that is limited temporally and geographically (Mendoza & Landeras, 1999). Furthermore, this technique will only be successful if the serotype of interest carries multiple plasmids of differing sizes.

#### 4.2.2 PFGE (pulsed field gel electrophoresis)

PFGE has been considered as the “gold standard” among other molecular typing methods. By cutting the bacterial DNA with rare-cutting restriction endonucleases and running with special electrophoresis separation technique which use pulsed currents that change polarity at defined intervals, it separates the large fragments of DNA up to 12000 kb and yields strain specific patterns.

The choice of restriction endonuclease is somewhat empiric, but the most commonly used enzymes in *Salmonella* have been XbaI, SpeI and NotI. Comparisons of patterns from multiple enzymes can elucidate new subtypes and increase the discriminatory power of this technique (Liebisch & Schwarz, 1996).

PFGE of 60 *S. enteritidis* isolates revealed 28 different XbaI restriction profiles and 26 with SpeI, yet when the patterns generated from both enzymes were combined, 32 different pulsed-field types could be identified (Ridley et al., 1998). PFGE was used to determine whether molecular subtyping was able to detect unsuspected clusters or outbreaks of *S. typhimurium* (Bender et al., 2001). In fact, during a four-year period, 16% of isolates were linked to common source outbreaks. Of these, the authors felt that 62% of outbreak strains would have been missed without the use of PFGE molecular subtyping (Bender et al., 2001). PFGE has also been used to track outbreak strains occurring across national boundaries (Lyytikainen et al., 2000).

PFGE is characterized by a high degree of reproducibility both within and between laboratories (Swaminathan et al., 2001). The recent introduction of computerized gel-based data collection and analysis systems allows better standardization between laboratories thus creating the ability to rapidly compare restriction fragment patterns from isolates analyzed from remote locations (Swaminathan et al., 2001). Large databanks that house PFGE patterns from isolates around the world will greatly enhance *Salmonella* outbreak detection. PulseNet, a molecular subtyping network for foodborne bacterial disease surveillance, has been active in developing standardized PFGE protocols and establishing a national database. An outbreak of *S. agona* linked to contaminated cereal was identified in 1998. PFGE, in association with PulseNet, was used to identify cases in adjoining states that were not initially thought to be at risk (Swaminathan et al., 2001). In fact, combining typing methods such as PFGE and information from food chains, it was possible to identify related strains and common source of contamination. This type of approach may be useful in order to improve *Salmonella* spp. surveillance systems.

PFGE, however, is not always successful. Some serotypes, especially those with certain distinct phage types, can be so genetically homogeneous that multiple genotypic techniques fail to discriminate outbreak from non-outbreak strains. Ahmed et al. (Ahmed et al., 2000) evaluated PFGE to differentiate *S. enteritidis* DT8 strains that developed during a Canada-wide outbreak of gastroenteritis that was eventually traced to contaminated cheese. Successful discrimination was only achieved with a combination of intensive epidemiological, genotypic and phenotypic methods (Ahmed et al., 2000). Additionally, certain serotypes may be more susceptible to genetic rearrangements that can alter the PFGE pattern, even within an outbreak (Echeita & Usera, 1998).

Despite that PFGE is usually considered as the method of choice to determine the molecular relatedness among *Salmonella* strains; this method is relatively slow, often taking three days to complete, and requires the presence of expensive specialized equipment, high quality chemicals, and a considerable experience in the preparation of the DNA-containing agarose slices. Moreover, single genetic events, such as point mutations, integration, deletion or recombination events, can result in differences in the fragment patterns (Herschleb et al., 2007).

#### 4.2.3 Ribotyping

The Fingerprinting of rRNA coding sequences, termed ribotyping, describes the hybridization of restriction-digested DNA fragments with probes specific for rDNA.

Multiple copies of the rRNA operon are present within the *Salmonella* chromosome (Mendoza & Landeras, 1999). The rRNA genes themselves are quite homologous among these copies and between isolates, but the intervening sequences vary in length and nucleotide composition.

Ribotyping begins with separating endonuclease-digested chromosomal DNA on agarose gels, DNA then is transferred to a membrane and fragments are hybridized to a probe that recognizes 16S and 23S rRNA. Analysis of multiple restriction endonucleases can improve the discriminatory powers of ribotyping (Millemann et al., 1995).

Ribotype analysis is clearly able to subtype some of the isolates that fall within some common serotypes and phage types (Landeras et al., 1996). Lin et al. (Lin et al., 1996) detected 7 different ribotypes among 17 *S. enteritidis* PT 8 isolates when chromosomal DNA was digested with SphI. Using rRNA gene restriction patterns to investigate the relatedness of *S. Enteritidis* strains isolated in São Paulo, from 1975 to 1995; Fernandes et al. showed that ribotyping is a genomic profiling method that is reproducible and suitable for tracing the spread of *S. Enteritidis*. They found that the restriction endonuclease SphI discriminated best between subtypes of this serotype. Dambaugh et al. presented evidence suggesting that the ribotyping of *Salmonella* using the restriction enzyme PvuII increased the incidence of discreet ribotype patterns for the most common *Salmonella* serovars. This study evaluates the potential of PvuII to generate serotype-specific DNA fingerprints. However, studies have identified isolates that belong to different phage types yet demonstrate identical ribotypes (Fontana et al., 2002). Therefore, ribotyping is considered not suitable for local epidemiological studies or surveillance studies in a restricted region (Riley, 2004).

Comparisons of ribotyping with PFGE have been somewhat unpredictable and often depend on the enzymes used for digestion as well as the nature of the population being tested. Several studies have found PFGE to be more discriminating than ribotype analysis (Fontana et al., 2002) while others have found the two procedures equivalent (Navarro et al., 1996) or ribotype analysis superior (Liebana et al., 2001). Ribotype analysis using two restriction enzymes, Pst I -SphI or HindIII - EcoRV, can improve discrimination (Liebana et al., 2001). Particular care must be taken when analyzing chromosomal patterns of *S. typhi*. The rapid genomic reassortment that occurs in *S. typhi* can affect ribotype analysis (Ng et al., 1999).

Though most laboratories continue to perform ribotyping manually, machinery has been developed to perform this entire procedure in an automated fashion. Data is stored

electronically and the banding pattern from a particular organism can be compared to the entire databank stored in the computer. In contrast to PFGE, the time required to perform automated ribotyping is minimal; hybridization results can be obtained within 4 hours. A recent study tracking the rise of a multi-drug resistant, cephalosporin-resistant *S. newport* proposes to use automated ribotyping as a way to rapidly identify the *newport* serotype and PFGE to further evaluate strain associations (Fontana et al., 2002). The major drawbacks of automated ribotyping are the high reagent costs per isolate and the cost of the automated riboprinter itself.

Laconha et al. and Ridley et al. investigated the genotypic differences between strains of *Salmonella* by plasmid analysis, ribotyping and pulsed-field gel electrophoresis (PFGE). The results obtained by those researchers indicated that PFGE may offer a better level of discrimination of *S. Enteritidis* types than other genotypic methods. Conversely, other epidemiological studies of *S. Enteritidis* have demonstrated that PFGE methodology has a lower discriminatory capacity than ribotyping (Olsen et al. 1994; Thong et al. 1998).

#### 4.2.4 Insertion sequence (IS) typing

IS200 is a mobile element found in a variety of eubacterial genera, such as *Salmonella*, *Escherichia*, *Shigella*, *Vibrio*, *Enterococcus*, *Clostridium*, *Helicobacter*, and *Actinobacillus*. IS200 elements are very small (707-711 bp) and contain a single gene. Unlike typical mobile elements, IS200 transposes rarely. A consequence of IS200 self-restraint is that the number and distribution of IS200 elements remain fairly constant in natural populations of bacteria. This stability makes IS200 a suitable molecular marker for epidemiological and ecological studies, especially when the number of IS200 copies is high. IS200 typing, has been used to evaluate the molecular relationships between *Salmonella* isolates. In *Salmonella enterica*, IS200 fingerprinting is extensively used for strain discrimination. It is a 708 bp insertion sequence that is present in multiple copies within the *Salmonella* chromosome (Lam & Roth, 1983). Hybridization of digested chromosomal DNA with an IS200 probe has been useful in describing the clonal heritage of *Salmonella* from various serotypes, but has not been as discriminating as phage typing itself for *S. enteritidis*, *S. typhi* and others (Threlfall et al., 1994). For certain phage types of *S. typhimurium*, such as the multidrug resistant DT204c and 193 types common in the U.K., IS200 typing can result in strain discrimination and in some studies has been superior to PFGE and ribotyping (Jeoffreys et al., 2001). More frequently, PFGE has performed better than IS200 typing (Amavisit et al., 2001).

#### 4.2.5 RAPD (randomly amplified polymorphic DNA)

The standard RAPD technology (Williams et al., 1990) utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Decamer primers are commercially available from various sources (e.g., Operon Technologies Inc., Alameda, California). PCR amplification with primers shorter than 10 nucleotides [DNA amplification fingerprinting (DAF)] has also been used producing more complex DNA fingerprinting profiles (Caetano-Annoles et al., 1991).

Although these approaches are different with respect to the length of the random primers, amplification conditions and visualisation methods, they all differ from the standard PCR



condition (Erlich, 1989) in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required.

At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other.

The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Recently, sequence characterised amplified regions (SCARs) analysis of RAPD polymorphisms (Bardakci & Skibinski, 1999) showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile.

Although the RAPD method is relatively fast, cheap and easy to perform in comparison with other methods that have been used as DNA markers, the issue of reproducibility has been of much concern since the publication of the technique. In fact, ordinary PCR is also sensitive to changes in reaction conditions, but the RAPD reaction is far more sensitive than conventional PCR because of the length of a single and arbitrary primer used to amplify anonymous regions of a given genome. This reproducibility problem is usually the case for bands with lower intensity. The most important factor for reproducibility of the RAPD profile has been found to be the result of inadequately prepared template DNA (Welsh & McClelland, 1994). Differences between the template DNA concentration of 2 individuals' DNA samples result in the loss or gain of some bands (Bardakci, 1996).

Since RAPD amplification is directed with a single, arbitrary and short oligonucleotide primer, DNA from virtually from all sources is amenable to amplification. Therefore, DNA from the genome in question may include contaminant DNA from infections and parasites in the material from which the DNA has been isolated. Special care is needed for keeping out the DNA to be amplified from other sources of DNA.

Finally, due to the amplification conditions, RAPD method is sensitive to slight changes within amplification parameters, thus it is hard to achieve reproducibility. However, ribotyping is a supplementary tool in conjunction with other typing methods (Yan et al., 2003).

#### **4.2.6 AFLP (amplified fragment length polymorphism)**

Also termed infrequent restriction site PCR (IRS PCR). It, has been developed by Vos et al. (1995). L'AFLP analysis belongs to the category of selective restriction fragment amplification techniques, which are based on the ligation of adapters (i.e., linkers and indexers) to genomic restriction fragments followed by a PCR-based amplification with adapterspecific primers.

The optimal number of scorable bands (50–100) can easily be set by selection of the appropriate AFLP primers and restriction enzymes. These characteristics make AFLP a powerful fingerprinting technique which can be used in identification, epidemiology and taxonomy (Folkerstma et al. 1996; Huys et al. 1996; Janssen et al. 1996). In addition, the technique can be used to generate large numbers of molecular markers for linkage studies (Ballvora et al. 1995; Becker et al. 1995; van Eck et al. 1995).



For AFLP analysis, only a small amount of purified genomic DNA is needed; this is digested with two restriction enzymes, one with an average cutting frequency (like EcoRI) and a second one with a higher cutting frequency (like MseI or TaqI).

Double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation, which allows simultaneous restriction and ligation, while religated fragments are cleaved again.

An aliquot is then subjected to two subsequent PCR amplifications under highly stringent conditions with adapter-specific primers that have at their 3' ends an extension of one to three nucleotides running into the unknown chromosomal restriction fragment.

An extension of one selective nucleotide amplifies 1 of 4 of the ligated fragments, whereas three selective nucleotides in both primers amplify 1 of 4,096 of the fragments. The PCR primer which spans the average-frequency restriction site is labeled.

After polyacrylamide gel electrophoresis a highly informative pattern of 40 to 200 bands is obtained. The patterns obtained from different strains are polymorphic due to (i) mutations in the restriction sites, (ii) mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and (iii) insertions or deletions within the amplified fragments.

Optimization of restriction enzymes and adapter-specific primers is ongoing for the *Salmonella* (Garaizar et al., 2000), but the technique appears more reproducible than ribotyping techniques (Savelkoul et al., 1999). Some of the studies have shown specificity to the serotype level with occasional subserotype discrimination (Garaizar et al., 2000).

Alternative AFLP typing procedures are based on one enzyme with a single adapter and analysis by agarose gel electrophoresis (Gibson et al., 1998). A major improvement has been obtained using a fluorescent amplified fragment length polymorphisms (FAFLP) technique that followed the same principles of AFLP yet the adapter-specific primers were tagged with a fluorescent moiety (Tamada et al., 2001). Fluorescent tagged fragments are then accurately sized on an automated sequencer.

FAFLP analysis of *S. typhimurium* generated 45-50 fragments ranging in size from 80-430 bp, though only a subset of these fragments were polymorphic among the strains. FAFLP grouped the isolates into four distinct clusters while PFGE generated three clusters.

Sizing was enhanced by incorporation of a fluorescent internal marker (Tamada et al., 2001). This accurate sizing, combined with the ability to acquire and analyze the data as a gel image, electrophorogram or in a tabular data format will allow comparison of patterns among different laboratories or within databanks (Savelkoul et al., 1999).

FAFLP appears quite promising. Disadvantages include the need for a greater technical expertise. In fact, despite that AFLP has been considered as a highly discriminative method, it remains a labour- and cost-intensive technique (Riley, 2004). Set up costs may be prohibitive until automated sequencers become more affordable.

#### 4.2.7 MLST (multilocus sequence typing)

A recently developed methodology (Maiden et al., 1998) called multilocus sequence typing (MLST) may provide an ideal balance of high discriminatory power and a powerful data analysis capability requiring minimal human input. Multilocus sequence typing (MLST) is a molecular typing strategy that compares DNA sequences from portions of housekeeping or virulence genes and/or rRNA sequences which varies due to mutation or recombination events (Maiden et al., 1998). Nucleotide differences in the individual genes

are combined and used to determine the differentiation of strains (Yan et al., 2003). MLST provides data similar to those obtained by multilocus enzyme electrophoresis, but in substantively greater detail, because it has the ability to assess individual nucleotide changes rather than to screen for changes in the overall charge and expression of the enzyme under study (Maiden et al., 1998).

This method is extremely useful for long-term epidemiological studies or phylogenetic analyses. Over 230 *Salmonella* isolates were recently characterized by MLST based on sequences from the 16S RNA, pduF, glnA and manB genes (Kotetishvili et al., 2002). These results were compared to PFGE and serotype analysis. MLST was able to differentiate strains better than PFGE, though not all genes performed equally. Among the four loci, only manB demonstrated clusters among the clinical and environmental strains. As expected, the 16S rRNA locus showed significant homogeneity among the isolates and grouped most isolates together.

MLST shows great promise for accurate strain discrimination with data that can be accurately shared between laboratories. However, like FAFLP, the universal appeal of this technique will be improved when automated sequence machinery becomes more affordable and labs can develop familiarity with complicated DNA sequence analysis and statistical software

#### 4.2.8 Multiplex PCR

##### Theoretical basis of multiplex PCR method: Critical Parameters

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 (Chamberlain et al., 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions (Henegariu et al., 1994), mutations (Shuber et al., 1993) and polymorphisms (Mutirangura et al., 1993), or quantitative assays (Mansfield et al., 1993) and reverse transcription PCR (Crisan, 1994).

The role of various parameters that may influence the performance of standard (uniplex) PCR has been discussed (Robertson & J., 1998). However, fewer publications discuss multiplex PCR (Henegariu et al., 1997).

The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (Polz & C. M., 1998). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (Brownie et al., 1997). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or reduce such non-specific interactions.

Compatibility among the primers within the reaction mixture such that there is no interference, is of great technical importance. Primer selection followed simple rules (i) primer length of 18–24 bp or higher and (ii) a GC content of 35%–60%, thus having an annealing temperature of 55 °C–58 °C or higher. Longer primers (28–30 bp) allowed the reaction to be performed at a higher annealing temperature and yielded less unspecific products.

Combining the primers in various mixtures and amplifying many loci simultaneously required alteration/optimization of some of the parameters of the reaction. When the

multiplex reaction is performed for the first time, it is useful to add the primers in equimolar amounts. The results will suggest how the individual primer concentration and other parameters need to be changed. Special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered (Robertson & J., 1998). Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures and should not display significant homology either internally or to one another (Henegariu et al., 1997). Also, the extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs), and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance have been directed towards the factors affecting annealing and/or extension rates. Therefore, in multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme concentrations, PCR buffer constituents and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products (Chamberlain et al., 1989).

Variation in concentrations of reaction components above those used in uniplex PCR probably reflects the competitive nature of the PCR process. The desired target DNA can be outcompeted by the more efficient amplification of other targets (including nonspecific products), leading to decreases in the efficiency of the amplification of the desired targets and hence sensitivity of the reaction (Raeymaekers, 1995).

Various authors recommend dimethyl sulfoxide (DMSO) and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific products) of PCR, when used in concentrations varying between 5%–10% (vol/vol) (Innis & D.H., 1990). Also bovine serum albumin, or betaine, has been reported to be of benefit in multiplex PCRs (Jackson et al., 1996). The components may act to prevent the stalling of DNA polymerization, which can occur through the formation of secondary structures within regions of template DNA during the extension process (Hengen, 1997). Also it can act as destabilizing agents, reducing the melting temperature of GC-rich sequences, or as osmoprotectants, increasing the resistance of the polymerase to denaturation (Hengen, 1997).

A straightforward solution to difficulties encountered in the development of multiplex PCR has been the use of hot start PCR (Chou et al., 1992) and/or nested PCR (Zheng et al., 1995). The former often eliminates nonspecific reactions (particularly production of primer dimers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermocycling (Chou et al., 1992). The procedure has recently been made more practicable through the use of a nonmechanical hot start methodology which involves the use of a form of Taq polymerase, for example, Ampli Taq Gold (Roche Diagnostics), which is activated only if the reaction mixture is heated in first denaturation step at approximately 94°C for 10 min (Kebelmann-Betzing et al., 1998).

Nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. Although this adaptation is undoubtedly effective in most cases, it also considerably complicates the practical application of PCR. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation.

### Practical test of multiplex PCR method: Application and results in *Salmonella* serotyping

During the last decade, a number of studies have demonstrated the practicality of identifying *Salmonella* serovars using multiplex PCR (mPCR) (Kim et al., 2006). In addition, the technique has been shown to be a powerful and cost-effective tool for *Salmonella* serotyping. For these reasons, we optimize a mPCR protocol to type the most common *Salmonella enterica* subsp. *enterica* serovars. This method is based on detection of genes present in specific serotypes. These genes were selected from analysis of previous work including whole-genome sequencing (Porwollik et al., 2004, 2005).

The first step is to extract bacterial DNA. In this study, it was prepared by boiling (Agarwal et al. 2002). Then, we prepared the final PCR volume (34 µl) that included: dNTPs mixture (0.2 mM); MgCl<sub>2</sub> (2 mM); TaqDNA polymerase (5.0 units); primer(s) (50 ng each); genomic DNA template (5 µl) and deionised water to make up the volume (Imen et al. 2010).

All assays used the same cycling parameters under the following conditions: enzyme activation at 94°C for 5 min and then an additional 40 cycles with heat denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s, and DNA extension at 72°C for 1 min. After the last cycle, samples were maintained at 72°C for 5 min to complete the synthesis of all strands.

The PCR products (10 µl) were separated by electrophoresis on 2% Tris-acetate EDTA agarose gel stained with ethidium bromide, visualized with UV induced fluorescence, and photographed (Imen et al. 2010).

The first multiplex PCR for *Salmonella* serotyping was applied using five primer sets in the same reaction mixture. Using these five STM primers with the 19 *Salmonella* serovars, we can identify four distinct groups (Imen et al. 2010). In a second approach, we validated the mPCR for *Salmonella* serovars detection by using STY primers. Thus, the 19 different tested *Salmonella* serovars could be classified into three groups on the basis of scoring the presence or absence of appropriately size amplicons (Imen et al. 2010). To further evaluate the discriminatory method for *Salmonella* serotyping and to increase identified serovars, we combined molecular results of both the STM and STY primers (Imen et al. 2010).

In this study, using suitable primers for the two five-plex PCRs methods for molecular *Salmonella* serotyping, we could easily discriminate all the tested *Salmonella* serotypes that represented 100% of all *Salmonella* isolates in our laboratory. Also, a high rate of correlation was found between traditional and molecular serotyping. However, one exception was found with *Salmonella Anatum* serotype (Imen et al. 2010).

These results have been found elsewhere (Perch et al. 2003). Whereas, we have noted a resemblance in molecular amplicon code in some *salmonella* serovars that can be explained by the presence of a very similar region in these serovars. It can also be explained by deletion problems that can concern a specific region and so the absence of appropriately sized amplicons with specific primers (Garaizar et al. 2002). A secondary discrimination problem that was interesting to note was that for *Anatum* serovar more than one amplicon code can be detected which may reflect intraserovar variation.

To further discriminate each serovar, we can associate to this multiplex PCR serotyping the PFGE analysis, or the 16 S\23 S r RNA ribotyping. These methods provided a high degree of intraserovar discrimination.

In this way, we describe the mPCR as a rapid, specific, and cost-effective molecular method that has demonstrated its efficient discrimination in serotyping of the most common clinical



and food isolates of *S. enterica* subsp. *enterica* in our region. This technique can be used as an alternative method of standard serotyping in many clinical laboratories.

## 5. Conclusions and perspectives

Overall the *Salmonella* demonstrate significant phenotypic diversity. Several phenotypic typing techniques have been developed and have been used successfully for decades. Over the years, serotype and phage type analyses have been particularly useful as evidenced by the success of the National *Salmonella* Surveillance System, and many other national surveillance projects throughout the world. However, these techniques have often been relegated to reference laboratories making rapid analysis by an individual laboratory difficult. An ideal typing method should fulfil the following six criteria: typeability, reproducibility, discriminatory power, and ease of interpretation, easy to use, and low cost. It is clear, that any method used currently for typing of *Salmonella* strains is an ideal method alone in terms of these criteria, but all methods exhibit benefits and also limitations. It is obvious that it is difficult to find a single method, which is most suitable for typing of *Salmonella* strains. As a consequence, the best discrimination has resulted from combinations of techniques, often a combination of phenotypic and genotypic techniques. At this time, major reference institutions rely on serotype analysis followed by PFGE as the gold standard for strain discrimination. PCR-based techniques, though, are more rapid and within a particular laboratory can be used as a primary screening tool for strain discrimination. Better standardization between laboratories will be required before any of the PCR techniques can become the method of choice. Additionally, validation in outbreak situations involving varied serotypes will be required to prove these techniques effective in the field.

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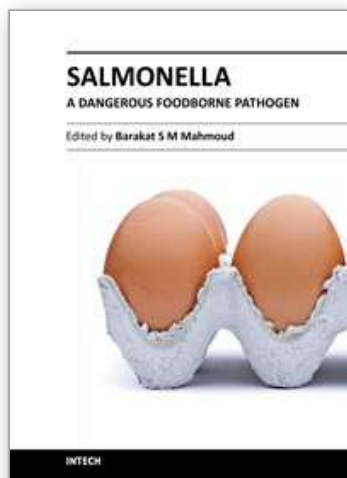
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## **Salmonella - A Dangerous Foodborne Pathogen**

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More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at \$2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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