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## Detection of *Salmonella* spp. Presence in Food

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### 1. Introduction

The analysis of food products for presence of pathogenic microorganisms is one of the basic steps to control safety and quality of food. Development of new, fast, and reliable identification methods for biological threats are necessary to meet the safety standards of food products and risk management. *Salmonella* spp., a marker of food products safety, is widely distributed foodborne pathogen.

The standard culture methods to detect the presence of microorganisms in food products are well developed; although these methods require 4 to 5 days to obtain presumptive positive or negative results. These tests are time-consuming and can take up to 7 days depending on the realization of biochemical and serological confirmations. In addition, sensitivity of cultures can be affected by antibiotic treatment, inadequate sampling, and a small number of viable microorganisms in samples.

Standardized classical culture methods are still in use by many labs, especially by regulatory agencies, because they are harmonized methods, looked at as the “gold standards” in food diagnostics and thus overall well accepted. These are important aspects in international trade and compliance testing. A serious drawback is that, although they demand no expensive infrastructure and are rather cheap in consumables, they are laborious to perform, demand large volumes usage of liquid and solid media and reagents, and encompass time-consuming procedures both in operation and data collection.

As an alternative to time-consuming culture methods, several approaches have been developed to accelerate detection of pathogenic microorganisms in food products. In the present work, besides the standard method of *Salmonella* spp. detection in food products (ISO 6579:2003) some alternative detection methods have been presented.

### 2. Taking samples for tests

The first stage of microbiological analysis of food consists in taking and preparing a sample for analyses. Incorrect sampling can lead to obtaining false negative or false positive results. When talking about taking samples, the term “representative sample” is often used. The sample should reflect the image of the product from which it originates as precisely as possible. It is quite easy to take a representative sample from liquid products, e.g. milk, if the milk has been sufficiently mixed before taking the sample. On the other hand, when the subject of examination is a product of high viscosity, with slow flow or of a heterogeneous structure, then it is very difficult to assess the microbiological quality of the entire batch (e.g. a barrel or a

truckload) by examining only one 25-gram sample. The answer to the question concerning the required number of single samples is extremely difficult. In view of the high costs of microbiological tests, the number of samples is generally limited. In a microbiological laboratory, samples are taken with the use of sterile tools, e.g. spoons, scalpels, knives, spatulas and pipettes. Frozen products should be first thawed at below 5°C (for not longer than 12 hours). In the case of deeply frozen samples, sterile drills are used for sampling.

Determination of *Salmonella* sp. in food products always consists in detecting the presence of those bacteria in a specified amount of the product (generally 25g/ml, very rarely 10g/ml), but the number of those microorganisms in food is not determined. Both in the classical method and in its modifications, the first stage of detection is non-selective enrichment. This is crucial, since food production involves its technological treatment, e.g. heating, which can cause the death of most cells or cause sub-lethally injured. Omission of the stage of pre-enrichment of the sample and inoculating the material directly on the solid medium can give false negative results. If the examined material includes a very low number of living cells, or the cells have been sub-lethally damaged during the technological processes, we may not receive macroscopically-visible colonies on the solid medium. In such a case there is a risk of releasing the product to market although it does not satisfy safety criteria. During the storage of such a product, damaged cells can be repaired and bacteria can proliferate to a level that would be hazardous for the consumers.

There are many methods to determine *Salmonella* sp. in food and, for this reason, the present study focuses on the classical culture method – the application of a Vidas device – as the only fully automated one. Additionally, the PCR method (a commonly-applied alternative to the plate method) and the FISH method (which is still not popular, although work on its optimization is ongoing) are also described.

### 3. A classical culture method of detecting *Salmonella*

Detection of the presence of *Salmonella* pursuant to Commission Regulation (EC) No 2073/2005 (microbiological criteria for foodstuff) as amended, is carried out according to the ISO 6579 standard - Microbiology of food and animal feeding stuffs - Horizontal method for detection of *Salmonella* spp. (ISO, 2002). Pursuant to the above regulation, detection of *Salmonella* in food should be carried out for such products as raw meat, meat products intended for consumption in the raw state, gelatine, cheese, butter, cream, unpasteurized milk, powdered milk, eggs and products containing raw eggs, crustaceans, molluscs, fruit and vegetables, unpasteurized juice, powdered infant formulas and dietary food for special medical purposes.

Standard ISO 6579 2003 (Microbiology of food and animal feeding stuffs - Horizontal method for detection of *Salmonella* spp.) includes four stages of the detection process and depending on the need to obtain confirmations, it lasts from 5 to 7 days:

- Pre-enrichment in non-selective liquid medium
- Selective enrichment in liquid media
- Plating on selective media
- Serological and biochemical identification of suspected colonies

During the first stage, in order to proliferate and regenerate damaged cells, the culture is performed on liquid peptone water at 37°C for 18±2 hours. **Buffered peptone water** is applied for non-selective enrichment of *Salmonella* sp. For such products as cocoa or chocolate products, peptone water is applied with an addition of casein or skimmed milk

and brilliant green in order to inhibit the growth of Gram-positive bacteria. In the case of acid and soured food products, peptone water should be used with double concentration of components, while for meat and food of high fat content, pre-enrichment should be performed in lactose broth with the addition of Triton X-100.

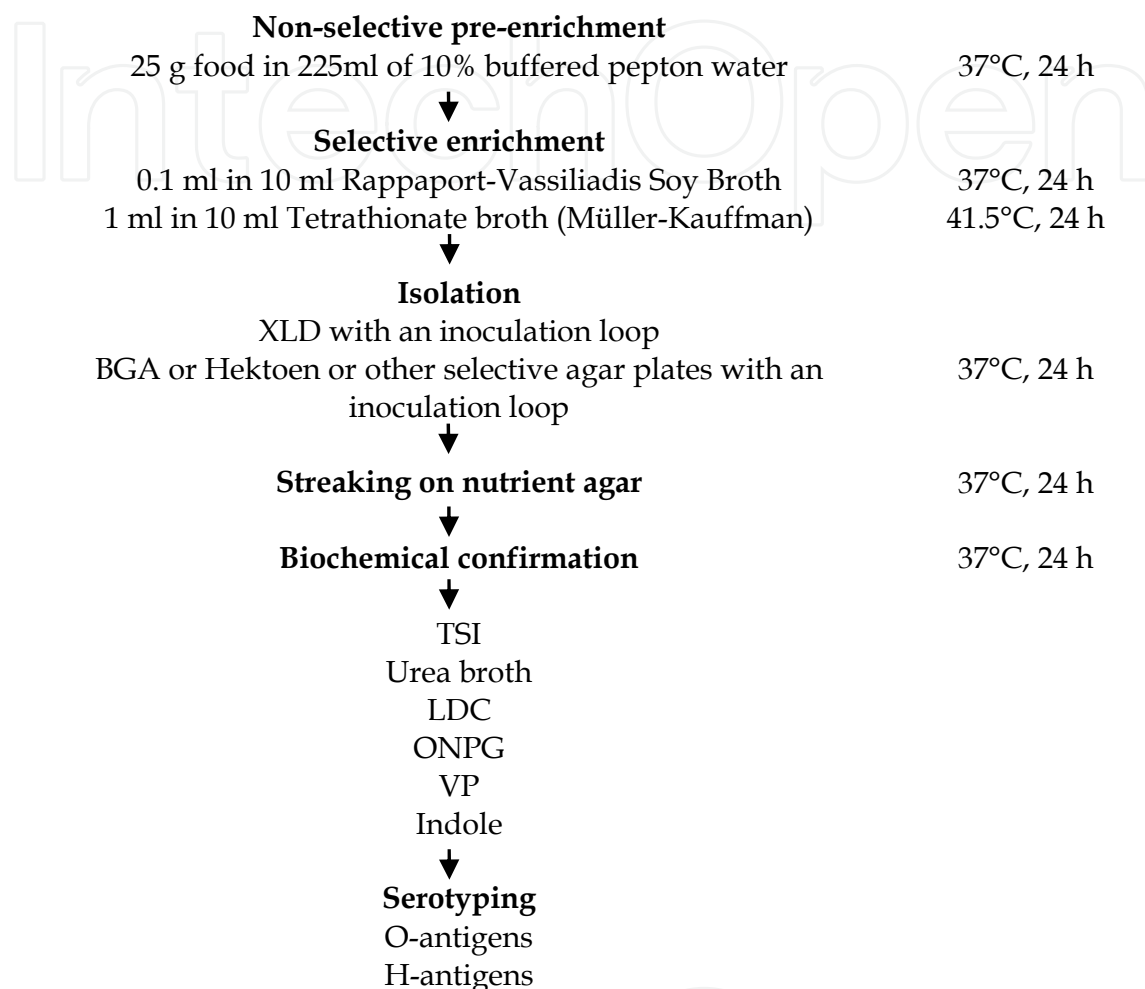


Fig. 1. Flow diagram for detection of *Salmonella*.

After the non-selective pre-enrichment stage, a 0.1cm<sup>3</sup> sample is taken from the culture and inoculated on 10cm<sup>3</sup> of selective medium, Rappaport-Vassiliadis with soya, and on **Muller-Kauffmann** medium in the amount of 1 cm<sup>3</sup>. **Rappaport-Vassiliadis (RVS)** medium is solid, strongly selective and contains malachite green and sodium chloride (inhibiting the growth of accompanying microflora). Soya peptone, pH 5.2, and increased temperature of incubation (41.5°C) favour the growth of *Salmonella* sp. strains. The medium is dark blue and clear. *Salmonella* sp. strains grow on this medium in the form of milky residue, while the colour of the medium itself does not change. The other selective medium, **Muller-Kauffmann broth (MKTn)**, contains sodium thiosulphate and potassium iodide, which react to form a compound known as sodium tetrathionate, inhibiting the growth of the coliforms. *Salmonella* sp. are able to reduce this compound. The broth also contains brilliant green, which, in turn, inhibits the growth of Gram-positive bacteria.

After incubation at 37°C for 48±3 hours, cultures are inoculated on two selective media, so as to receive individual colonies. The first of them is XLD (xylose lysine deoxycholate) agar. The other can be chosen by the laboratory, and it can be BGA (brilliant green agar), Hektoen or Wilson-Blair agar for example.

**XLD agar** contains lactose, saccharose, L-lysine, sodium thiosulphate, sodium deoxycholate, ferric ammonium citrate (III) and phenol red. Differential agents of the agar include: lactose, saccharose, xylose, lysine and sodium thiosulphate, from which hydrogen sulfide is released, forming in reaction with iron salts (III) black residue of iron sulfide in the centre of the colony. The pH indicator is phenol red. The agar makes it possible to determine the sugar fermentation ability. Incubation is carried out at 37°C for 24±3 hours. Typical colonies can be colourless, very light, slightly shiny and transparent (colour of the medium) with a dark tinted centre, surrounded by a light red area and yellow edge, or of pink to red colour, with a black centre or without a black centre. H<sub>2</sub>S (-) colonies are colourless or light pink with darker centres, and lactose (+) colonies are yellow or without the characteristic blackening.

**BGA.** Differential factors of this agar are sugars: saccharose and lactose. Brilliant green is a selective agent. Typical colonies are transparent, colourless or light pink, and the colour around colonies changes from pink to light red.

**Hektoen agar.** Selective agents include bile salts, inhibiting the growth of Gram (+) bacteria. Differential factors are three sugars: lactose, saccharose and salicin. Increased lactose content ensures that bacteria fermenting this sugar with a delay are not omitted. Bacteria colonies producing hydrogen sulfide had a dark centre as a result of the reaction between hydrogen sulfide and iron (III). Typical colonies of *Salmonella* sp. are green, with or without a black centre.

**Wilson-Blair agar.** This is a strongly selective and differential medium for *Salmonella*, including *S. Typhi* isolated from food. *Salmonella* spp., depending on the strain, grow in the form of black colonies surrounded with an area of black medium or dark brown and brown without this area. A characteristic feature of *Salmonella* spp. colonies is a metallic, shining surface as a result of produced hydrogen sulfide, forming a metallic-black residue in reaction with iron ions. The growth of Gram-positive bacteria and other *Enterobacteriaceae*, including *Shigella* spp., is strongly inhibited by brilliant green and bismuth sulfite present in the medium.

**Rambach-agar chromogenic medium** – with sodium deoxycholate, propylene glycol and chromogenic mix. Colonies of *Salmonella* sp. are red as a result of glycol fermentation, lactose positive bacteria from the coli group, due to the activity of galactosidase, destroy a bond between the components of chromogenic mix and released chromophore gives those colonies a blue-violet or blue-green colouring. *Salmonella* Typhi and *Salmonella* Paratyphi form colourless or yellowish colonies on this medium.

New selective media have been developed based on biochemical characteristic of *Salmonella* such as α-galactosidase activity in the absence of β-galactosidase activity, C8-esterase activity, catabolism of glucuronate, glycerol and propylene glycol, hydrolysis of X-5-Gal, and H<sub>2</sub>S production. e.g. SMID agar (BioNerieux, France), Rainbow *Salmonella* agar (Biolog, USA), CHROMagar *Salmonella* (CHROM agar, France), chromogenic *Salmonella* esterase agar (PPR Diagnostics Ltd, UK), Compass *Salmonella* agar (Biokar diagnostics, France), and chromogenic ABC medium (Lab M. Ltd., UK) (Maciorowski et al., 2006; Manafi, 2000; Perry et al., 2007; Schonenbrucher et al., 2008)

MEDIUM	REACTIONS/ENZYMES	RESULTS	
		NEGATIVE	POSITIVE
TSI <sup>a</sup>	Acid production (if the butt is yellow, and the slope is red, acid production is only from glucose)	Butt red	Butt yellow
TSI <sup>a</sup>	Acid production from lactose and/or sucrose	Surface red	Surface yellow
TSI <sup>a</sup>	Gas production	No air bubbles in butt	Air bubbles in butt
TSI <sup>a</sup>	H <sub>2</sub> S production	No black colour	Black colour
UREA BROTH	Urease	Yellow	Rose pink – deep cerise
LCD TEST	Lysine decarboxylase	A yellow/brown colour	A purple colour (and a yellow/brown colour in the LDC control medium if used)
ONPG	β-Galactosidase	Remain colourless	Yellow
VOGES PROSKAUER	Acetoin production	Remain colourless	A pink/red colour
INDOLE	Indole production	Yellow ring	Red / pink ring

Table 1. Interpretation table. <sup>a</sup>Regarding TSI: Read the colour of the butt and of the surface of the medium; ALK: A red colour corresponding to no acid production; NC: No change in the colour of the medium ; A: A yellow colour corresponding to acid production; G: Gas production in the butt; H<sub>2</sub>S production; +: Black colour; -: No black colour

After 48 h incubation at 37°C, a preliminary identification is made on the basis of the appearance of colonies grown on selective media. Five characteristic colonies are selected from each plate and are plating on the nutrient agar medium, followed by biochemical examinations. In order to perform these examinations, biochemical tests are carried out on the following media:

- TSI medium (Triple-sugar iron agar)
- Christensen medium with urea (urease production)
- peptone medium with tryptophan (indole production)
- medium with lysine (lysine decarboxylation)
- Clark medium (V-P reaction)
- ONPG medium (β-galactosidase detection)

Test	Positive or negative reaction	Percentage of <i>Salmonella</i> inoculations showing the reaction <sup>1)</sup>
TSI glucose (acid formation)	+	100
TSI glucose (gas formation)	+	91.9 <sup>2)</sup>
TSI lactose	-	99.2 <sup>3)</sup>
TSI sucrose	-	99.5
TSI hydrogen sulfide	+	91.6
Urea splitting	-	99
Lysine decarboxylation	+	94.6 <sup>4)</sup>
β-Galactosidase reaction	-	98.4 <sup>3)</sup>
Voges-Proskauer reaction	-	100
Indole reaction	-	98.9

<sup>1)</sup> These percentages indicate only that not all strains of *Salmonella* show the reactions marked + or -. These percentages may vary from country to country and from food product to food product.

<sup>2)</sup> *Salmonella* Typhi is anaerogenic.

<sup>3)</sup> The *Salmonella* subspecies III (Arizona) gives positive or negative lactose reactions but is always β-galactosidase positive. The *Salmonella* subspecies II gives a negative lactose reaction, but gives a positive β-galactosidase reaction. For the study of strains, it may be useful to carry out complementary biochemical tests.

<sup>4)</sup> *S. Paratyphi* A is negative.

Table 2. Biochemical results for *Salmonella*.

**Triple-sugar iron agar** is used for differentiation of *Enterobacteriaceae* according to their ability to ferment lactose, sucrose and glucose. The colour of the slope and the butt and gas production are noted. Acid production from fermentation of one or more of the sugars results in a yellow colour because the phenol red indicator turns yellow at low pH. Very little glucose is present in the medium, so if a bacteria, like *Salmonella*, only ferments glucose then only a little acid will be formed. On the slope, the acid will be oxidised by the air and by the breakdown of protein in the medium and the colour will remain red while the butt is yellow. H<sub>2</sub>S production from thiosulphate will be seen as black areas in the medium due to FeS production. Gas production from fermentation of sugars will be seen as gas bubbles in the medium. The medium is only lightly inoculated.

**Christensen medium with urea.** Urea medium tests for high urea activity. It is the most common method to detect urease production by *Enterobacteriaceae* (1):



The phenol red turns red at alkaline pH so a positive reaction is shown as the development of a red-pink colour.

**Tryptone/tryptophane medium for indole reaction.** The media is used for testing the liberation of indole from tryptophane. When Kovacs reagent containing amyl alcohol and p-dimethylaminobenzaldehyde is added, indole can be extracted into the amyl alcohol layer by shaking a little. Indole and p-dimethylaminobenzaldehyde produces a red or pink colour.

**L-Lysine decarboxylation medium for the LDC test.** The LDC broth is used for the test of production of lysine decarboxylase. This enzyme decarboxylates lysine to yield the alkaline

compound cadaverin and  $\text{CO}_2$ . A paraffin oil layer is added after inoculation to keep the pH alkaline. Often glucose is metabolised in the beginning of the incubation period and a yellow colour develops in the media after some hours of incubation, but later the media turns purple if the lysine decarboxylase is present because of formation of the alkaline compound cadaverin. As other compounds in the media could be broken down to alkaline compounds, the LDC control media without lysine is also inoculated, a layer of paraffin oil added and it is incubated at the same time. If both the LDC media and the LDC control media turn purple, it cannot be shown that lysine decarboxylase is present and the test is evaluated as negative.

**Medium VP.** This is a test for acetoin production from glucose. The acetoin produced is oxidised to diacetyl, which produces a red colour with  $\alpha$ -naphthol at alkaline pH. A positive reaction is seen as a very pale red colour.

**ONPG medium.** This medium shows the presence of  $\beta$ -galactosidase producing bacteria.  $\beta$ -galactosidase liberates o-nitrophenol, which is yellow at alkaline pH, from ONPG. The reaction is positive if a yellow colour develops.

**API.** Determination of biochemical features of the examined bacteria can also involve the application of API 20E tests (Biomérieux), aimed at identification of bacteria from the family *Enterobacteriaceae*. The API 20E system facilitates the 24-hour identification of *Enterobacteriaceae* as well as 24 or 48-hour identification of other Gram negative bacteria. The API 20E strip consists of microtubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate (CHO) fermentation. The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. CHO fermentation is detected by colour change in the pH indicator.

**Serological tests.** These tests are carried out for strains of bacteria which have been classified into the *Salmonella* genus on the basis of their biochemical features, in order to detect the presence of somatic O, capsular Vi and flagellar H antigens. The examinations are carried out by slide agglutination on the basis of Kauffmann-White antigenic schema. Polyvalent and monovalent serums should be used to determine somatic antigens, and anti-Vi and anti-H serums to detect the presence of Vi and H antigen. Determination of flagellar antigens makes it possible to determine the serological type of the examined bacteria.

Culture methods are labor intensive and time consuming when handling many samples. In addition, detection can be prevented by the presence of other competing microorganisms during cultural enrichment, and the selective agar media have a very poor specificity creating an abundance of false positives (such as *Citrobacter* or *Proteus*) (Manafi, 2000). Therefore, there is a need for *Salmonella* detection methods that provide results more rapidly with sensitivity similar to or greater than, the conventional methods.

#### 4. Polymerase chain reaction

Due to its high sensitivity, specificity, and rapid results, PCR is an efficient alternative to conventional microbiological culture methods to detect specific types of microorganisms in foods, water, and environmental samples (Mogamedi et al., 2007; Glynn et al., 2006; Píknova et al., 2002). The International Standardization Organization (ISO) recently published standards which address the PCR methodology for the detection of food-borne pathogens (Tomás et al., 2009).



Gene Target	Matrices	Enrichment	Limit of detection	Primers (1-forward; 2-reverse; 3-probe)	References
<i>ttrBCA</i>	chicken minced meat fish	20 h	< 3 CFU/ml	1: CTC ACC AGG AGA TTA CAA CAT GG 2: AGC TCA GAC CAA AAG TGA CCA TC 3: CAC CGA CGG CGA GAC CGA CTT T	Malorny et al., 2004
<i>fimC</i>	ice cream	without	10 <sup>3</sup> CFU/ml	1: ATA AAT CCG GCG GCC TGA TG 2: TGG TAT CGA CGC CTT TAT CTG AGA 3: TTA CAC CGG AGT GGA TTA AAC GGC TGG G	Seo et al., 2006
<i>invA</i>	salmon chicken meat milk	16h	2,5-5 CFU/25g (salmon, chicken) 5 CFU/25 ml (milk)	1: GTG AAA TAA TCG CCA CGT TCG GGC AA 2: TCA TCG CAC CGT CAA AGG AAC CGT AA 3: TTA TTG GCG ATA GCC TGG CGG TGG GTT TTG TTG	Hein et al., 2006
<i>invA</i>	chili powder shrimp	35°C-24h (pre-) 41°C-24h (selective)	0,04 CFU/g	1: AAC GTG TTT CCG TGC GTA AT 2: TCC ATC AAA TTA GCG GAG GC 3: TGG AAG CGC TCG CAT TGT GG	Cheng et al., 2008
<i>oriC</i>	cheddar turkey meat cooked turkey meat	48 h (selective)	6,1 x 10 <sup>1</sup> CFU/ml	1: TCACCTGCGACAGCCATGA 2: TGAGCATCGCCATCGGCAT 3: ATTCCAGCAGTCGGCCATAGCTG (Set I) 1: CATTGATGCCATGGGTGACART 2: CGTGACGATAATCCGTGTAC 3: TACACGAGTCACTAAATCCTTCAGT (Set II)	McCarthy et al., 2009

Table 3. Detection of *Salmonella* using real-time PCR (increase of the released dye concentration). Consequently, we are able to monitor, in real time, whether the product of reaction has been obtained.

#### 4.1 Conventional PCR

PCR – polymerase chain reaction – is an enzymatic reaction replicating a DNA molecule. A critical condition of the PCR course is the presence of an enzyme, thermostable DNA polymerase. Substrates of the reaction are deoxyribonucleotides (building blocks of newly synthesized DNA molecules) and oligonucleotides (starters), which on a complementary basis attach to the replicated DNA strand. Polymerase chain reaction is a thermal process, with cyclically changing temperatures. One cycle leading to synthesis of one copy of DNA from one matrix molecule is composed of the following stages: DNA denaturation (90-95°C), attachment of starters (the so-called annealing, 50-65°C) and strand elongation. Reaction under laboratory conditions is carried out in a device known as thermocycler. Usually, one PCR reaction is made of 35-40 cycles, which result in creating billions of copies of an individual DNA molecule (with a logarithmic increase of the product in each cycle).

#### 4.2 Real-time PCR

Real-time PCR is a polymerase chain reaction observed in real time, is an improvement of the classical PCR method (Al-Soud et al., 2005; Bansal et al., 2006). A real-time PCR thermocycler is equipped with an optical system which collects information about the course of reaction, cycle by cycle. TaqMan® probes labelled with fluorescent dyes make an additional component of PCR real time reactions. A probe is an oligonucleotide designed to bind highly specifically to a replicated fragment of DNA, therefore – in case of the analysed application in food microbiology – to “detect” a sequence which is characteristic for the required pathogen. The probe should bind specifically and strongly, which can be ensured by special protein molecules (a MGB molecule in TaqMan® Applied Biosystems probes). Polymerase, while synthesising a new strand, moves along the matrix, encountering a probe on its way. Because of its exonucleolytic activity, the enzyme starts to “unstick” the probe from the matrix and afterwards to destroy it, releasing a fluorescent dye. An optical system of the thermocycler triggers and then receives dye glow, which becomes more intense with each cycle (a logarithmic increase of the amount of product results in a logarithmic

A forty-cycle real-time PCR reaction lasts about 1.5 hours. After adding the time needed to isolate DNA from the analysed sample (up to 30 minutes), the entire determination of the presence or the lack of pathogen lasts 2 hours (while using the Applied Biosystems TaqMan® Pathogen Detection System). Samples for determination are taken from pre-enrichment cultures on buffered peptone water after 18 h incubation at 37°C, so the total time from the collection of a sample to the final results does not exceed 24 hours.

Chen *et al.* (2000) evaluated the TaqMan system for the detection of *Salmonella* that utilizes primers and probes developed from a novel target sequence (*invA*). The detection limit was below 3 CFU/25 g or 25 ml when raw milk, ground beef and ground pork inoculated with *Salmonella* were pre-enriched overnight. Malorny *et al.* (2004) used specifically designed primers and a probe target within the *ttrRSBCA* locus, and included internal amplification control, which is coamplified with the same primers as the *Salmonella* DNA in the assay. The diagnostic accuracy was shown to be 100% compared to the traditional culture method when 110 various food samples (chicken rinses, minced meat, fish, and raw milk) were investigated for *Salmonella* by real-time PCR including a pre-enrichment step in buffered peptone water.

A very frequent target of species-specific *Salmonella* PCR assays is the invasion protein *invA* gene, and several *invA*-based PCR assays have been already developed and validated (Malorny *et al.* 2003a; b).

Validated PCR methods are available from Bio-Rad, Roche, Qualicon/Oxoid, Genesystems, AES Chemunex, Applied BioSystems, Idaho Technology Inc., Lantmännen, IEH Laboratories and Consulting Group, ADNucleis and BioControl systems. Validation is an important step in the process of standardizing a method because it provides evidence that the new method gives similar results and is in agreement with the currently used reference method (Patel et al., 2006).

One major difficulty with PCR is the presence of compounds that inhibit the PCR reaction. These compounds can contaminate the DNA templates extracted from food samples and may in turn generate false-negative results (Elizaquivel et al., 2008). Therefore, evaluation and elimination of PCR inhibitory compounds are important steps in the development of PCR and real-time PCR assays (Abu Al-Soud et al., 2000). The PCR procedure is sufficiently sensitive such that, in theory, only a few template molecules are required to initiate the synthesis reactions (Uyttendaele et al., 2003). However, an enrichment step is still required to detect small numbers of *Salmonella* in food samples. This step may consist of non-selective enrichment with buffered peptone water (BPW) and selective enrichment with Rappaport-Vassiliadis. These enrichment broths have been directly utilized for *Salmonella* DNA template preparation. However, limited research has been conducted to quantitatively evaluate the effects of the enrichment broths using conventional PCR assays and even less using a real-time PCR protocol. Therefore, identifying and eliminating the PCR inhibitory effects of the enrichment broths is key to enhancing the performance of PCR assays in detecting *Salmonella* in foods.

#### **4.3 Multiplex polymerase chain reaction (PCR)**

Multiplex PCR is a variant of the PCR technique in which two or more loci are simultaneously amplified in the same reaction. Multiplex PCR can be described as a specific and sensitive in vitro amplification of DNA with distinguishable size products from the same or different organisms in a single reaction (Jasson et al., 2010; Fitzgerald et al., 2007). In this methodology several specific primer sets are combined into a single PCR assay. Multiplex PCR is undoubtedly useful to rapidly establish simultaneous detection of multiple virulence factors (Fach et al., 2009) or combined detection of multiple isolates (Kawasaki et al., 2009; Settanni & Corsetti, 2007). A convenient format for Multiplex PCR is the GeneDisc (PALL) (Beutin et al., 2009).

Recently, multiplex real-time PCR assays have been applied to detect more than two gene sequences in a single reaction by using spectrally distinct dye-labeled probes (TaqMan system) (Elizaquivel et al., 2008). This technology could potentially save time and effort in the laboratory and thus may lower testing-related costs incurred by the food industry (Elizaquivel et al., 2008).

### **5. Fish (fluorescent *in situ* hybridization)**

The literature provides a limited number of reports concerning the application of the FISH technique for food examination (Ootsubo et al., 2003), while it is broadly applied in microbiology of environment, histopathology, histoimmunology, cytogenetics. Initially, it was developed in order to identify and to determine the number of bacterial cells in water ecosystem environments (Skowrońska & Zmysłowska, 2006), deposits, rhizosphere and soil.

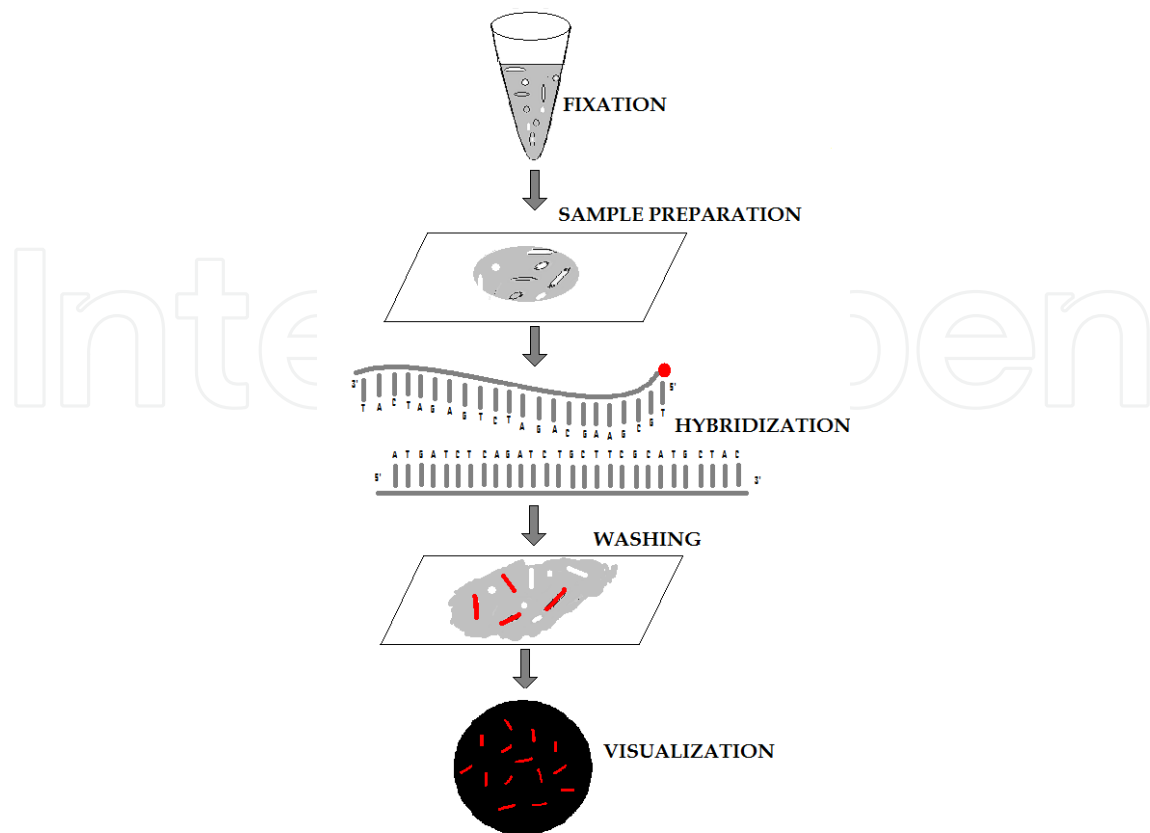


Fig. 1. Flow chart of a typical FISH procedure.

The FISH technique consists in hybridization of the rRNA sequence of immobilized cells by a fluorescently-labelled 16S rRNA oligonucleotide probe (Zwirgmaier, 2005; Baudart et al., 2005). Oligonucleotide probes are short fragments of deoxyribonucleic acid which hybridize or are paired with complementary sequences of DNA or RNA extracted from the analysed microorganisms. They are paired in the same way as double-stranded DNA forms (adenine with thymine and guanine with cytosine). If the sequence of bases on the DNA probe is complementary to the sequence characteristic for the determined microorganism, the probe binds only with the DNA of the identified microorganism. Probes are most often marked on one or on both ends with a fluorescent dye. Molecular probes bind specifically to rRNA in ribosomes of the target cells, identifying them on various taxonomic levels. Such a solution significantly increases the sensitivity of determination – since rRNA is an integral part of bacterial ribosome, it is found in the cell in large number of copies (between 1,000 and 10,000). Another advantage of this solution is the availability of vast information concerning rRNA sequences originating from various microorganisms which are often very closely genetically related, which allows probes to have very high specificity (Sakai et al., 2004; Ercoloni et al., 2005) Due to the range of probe specificity, the following probes can be distinguished: universal, e.g. EUB338 (GCTGCCITCCCGTAGGAGT), specific for *Bacteria* domain, except for the *Planctomycetales* order, antisense, e.g. NON388 (CGACGGAGGGCATCCTCA) designed to detect non-specific probe binding, and specific probes, e.g. for *Salmonella* sp.: Sal3 (5'-AATCACTTCACCTACGTG-3').

The FISH method with the application of fluorescently labelled 16S rRNA oligonucleotide probes is used for determining only the number of physiologically active cells, since rRNA

has a shorter half-life than DNA, which makes rRNA a potentially better indicator of their activity. Ribosomes of quickly-growing cells include a certain number of rRNA copies, usually over 1,000, which is sufficient for inducing a light signal after the labelled probe binds (Bottari et al., 2006). After the death of the cell, rRNA disintegrates, and the rate of this process depends, among others, on the concentration of enzymes – RNAz, as well as on the continuity of the cytoplasmic membrane (Vieira-Pinto et al., 2007). Slowly growing or metabolically inactive cells, containing a small number of ribosome copies, emit light of low intensity. New, more sensitive modifications of the FISH technique lead to amplification of the signal (TSA-FISH also known as CARD-FISH), becoming a useful tool for contemporary microbiology (Fang et al., 2003). 16S rRNA oligonucleotide probes labelled with fluorophores of various molecular weight (e.g. horseradish peroxidase – 44 kDa, fluorescein – 330 kDa) provide a visual assessment of the degree of cell wall permeabilization, on the basis of differences in dye permeation into the cell. An extreme advantage of the FISH technique is the possibility to detect VBNC (viable but not culturable) cells which do not grow on solid media, which makes this method more sensitive in comparison to the plate methods (Pisz et al., 2007, Oliver, 2005)

The most frequent used dyes for the detection of the FISH signals are FITC (fluorescein isothiocyanate) that emits a green fluorescence and dyes with orange or red fluorescence, such as Cy3<sup>TM</sup>, or TexasRed<sup>TM</sup>. Another commonly used fluorochrome in FISH experiments is Cy5<sup>TM</sup> that emits in the far red/close infrared. Since this fluorescence is not visible by eye it would need detection by an infrared sensitive camera mounted on the microscope. (Hoefel et al., 2003; Gatti et al., 2006) There are various other dyes with similar characteristics available from various different vendors. For example, FITC can well be replaced by Rhodamin 110, and Cy3<sup>TM</sup> by TAMRA (carboxytetramethyl- rhodamine) depending on the actual price and for some dyes, depending on the quality of the currently distributed lot (Table 4).

	<b>Dyes</b>	<b>Ex.Max.</b>	<b>Em.Max.</b>
<b>Blue</b>	DAPI	358	461
<b>Turquoise</b>	DEAC	426	480
<b>Green</b>	FITC/R110	494/500	517/525
<b>Yellow</b>	R6G	524	550
<b>Orange</b>	TAMRA/Cy3 <sup>TM</sup>	552/550	575/570
<b>Red</b>	TexRed/Cy3.5 <sup>TM</sup>	590/581	612/596
<b>Near Infrared</b>	Cy5 <sup>TM</sup>	649	670
<b>Infrared</b>	Cy5.5 <sup>TM</sup>	675	694

Table 4. Characteristics of selected fluorescent dyes.

A standard FISH methodology includes preparation and permeabilization of cells, hybridization, washing off the excess unbound probe and detecting the signal with the application of fluorescence microscopy (Jasson et al., 2010; Ootsubo et al., 2003)

Reports concerning the use of fluorescent *in situ* hybridization in food research indicate the possibility of its application of *Salmonella* spp. detection and recommend this method as very sensitive, fast and cheap.

Vieira-Pinto et al., (2005) compared FISH methods with the classical plate method for detection of *Salmonella* spp. Out of 47 samples of pork tonsils, 16 (34%) were positive for *Salmonella* spp detection by the FISH method with the application of 23S rRNA Sal3 probe. Out of 31 negative results obtained by FISH method, one sample was positive for *Salmonella* spp. detection by the plate method. Similar results were obtained by Fang et al., (2003), who detected *Salmonella* sp. species in 56 samples of food products by the FISH method (23S rRNA Sal3), while 28 samples were not positive for *Salmonella* spp. detection by the plate method. Huge number of positive results can derive from the presence of cells slightly damaged or occurrence of factors inhibiting their growth in food products, which can transfer cells to VBNS state. The authors suggest that FISH method seems to be less prone to diverse physical-chemical properties of preserved food products (temperature, concentration of NaCl, pH), which can work as a stress factor for *Salmonella* spp. cells. Presence of microflora can be another reason of high number of positive results obtained by the FISH method as compared to the plate methods.

The conclusions drawn from the research show the need for continuous improvement of the methodology and selecting and/or designing more specific probes. This is related to the varied chemical and microbiological composition of food (the so-called matrix), which can lead to errors in reading. Therefore, a relatively fast assessment of the quality and safety of food requires not only the selection of probes for individual species of microorganisms, but first of all optimal preparation of food samples for examination purposes on the basis of the matrix. Preparation of samples is understood as proper filtration and centrifugation at various parameters in order to eliminate large particles, and also the choice of optimal digestion conditions or permeabilization of the cell wall of microorganisms (e.g. with lysozyme, proteinase K, achromopeptidase, paraformaldehyde, ethanol etc.) occurring in the examined food. Proper preparation of samples and cells prevents non-specific absorption of the probe on cell elements and easier penetration of cell cytoplasm.

## 6. VIDAS

VIDAS™ (BioMérieux) is an automated enzyme-linked fluorescent assay (ELFA) method based on the detection of *Salmonella* by using specific antibodies coated on the inner surface of a tip-like disposable pipette which is introduced into the VIDAS system along with the VIDAS *Salmonella* strip containing the boiled *Salmonella* culture.

VIDAS Immuno-concentration *Salmonella* (ICS) is a fully automated method for the concentration of *Salmonella* from foods. It replaces traditional selective enrichment procedures with an automated immunological capture and specific release process (Yeh et al., 2002). The method is based on multistage reaction. The kit contains so called reagent stripes, that is a set of wells with reagents sealed tightly inside, and pipettes, which inner sides are coated with antibodies against specific antigens. The amount of 500 µl of the sample after selective enrichment stage on RVS is introduced to the first well and a strip is placed in the immunoanalyser chamber. Reaction suspension is cyclically pulled up and down by pipettes. A pipet tip-like device, the solid-phase receptacle (SPR) serves as the solid phase as well as the pipet for the assay. The SPR is coated with anti-*Salmonella* antibodies absorbed on the surface. A final enzymatic step releases the captured *Salmonella* into a well. Detection of *Salmonella* antigens is based on enzyme-linked fluorescent immunoassay performed in the automated VIDAS instrument. ASPR serves as the solid phase as well as the pipet for the assay. The SPR is coated with a cocktail of highly specific monoclonal

antibodies. All of the assay steps are performed automatically by the VIDAS instrument. For the detection of *Salmonella* by VIDAS SLM, the sample is inoculated into lactose broth and incubated for 18 h at 37°C (non-selective pre-enrichment). Subsequently, 0.1 ml of this medium is inoculated into Rappaport-Vassiliadis broth and 1 ml into tetrathionate broth, and then incubated for 8 h at 42°C and 8 h at 37°C, respectively. Then, 1 ml of each broth is inoculated separately into 10 ml of M-broth and incubated at 42°C for 18 h. Finally, 1 ml of each broth is placed in a tube, which is heated for 15 min at 100°C. Following pre-enrichment, immuno-concentration, and postenrichment of test portions, an aliquot of the boiled test suspension is placed into the reagent strip and is cycled in and out of the SPR for a specific length of time. *Salmonella* antigens, if present, bind to the monoclonal antibodies coating the interior of the SPR. All other unbound material is washed away. Antibodies conjugated with alkaline phosphatase are cycled in and out of the SPR, binding to any *Salmonella* antigen bound to the SPR wall. The final wash step removes unbound conjugate. The substrate, 4-methyl umbelliferyl phosphate, is converted by the enzyme on SPR wall to the fluorescent product, 4-methyl umbelliferone.

The intensity of fluorescence is measured by the optical scanner in VIDAS. The fluorescence intensity is measured twice at 450 nm. The first result is related to the background, the second it the value after incubation of the substrate with enzyme. Based on that, the apparatus calculates the result of the test and interprets it as a positive or negative one. RFV (Relative Fluorescence Value) is calculated as the difference between the sample and background fluorescences. The printed report contains the RFV value of the sample, RFV value of the standard, and test value (TV), which is a quotient of the sample value and standard value. A result was interpreted by the apparatus as positive, if  $TV \geq 0.23$ , while as negative if  $TV \leq 0.23$ . Results are interpreted after the test values and control are compared to thresholds stored in the computer. A positive result requires confirmation with classical culture methods, that is streak plating on two plates with selection growth medium. For confirmation, previously prepared and stored under cold conditions broth culture of the investigated sample is used.

Based on the comparative studies with the standard plate method, it can be concluded that the VIDAS system can be use to get fast results; however, because these results can be false positive then they have to be confirmed by culture method (Yeh et al., 2002; Zadernowska et al., 2010; Walker et al., 2001)

Problems with detection of some *Salmonella* spp. serotypes were observed during detection by the immunoenzymatic method. This may be caused by weak binding of antibodies, which is confirmed by results obtained by other authors. Vitek Immunodiagnostic Assay System (VIDAS, BioMérieux) are currently used in the meat and poultry processing industries (Maciorowski et al., 2006). Several validation studies have been reported that the detection rate of VIDAS systems were comparable to that of culture method (Yeh et al., 2002) and real-time PCR (Uyttendaele et al., 2003) for detecting of *Salmonella* in food.

**VIDAS Salmonella Xpress** (VIDAS SLMX) is most rapid method for the detection of *Salmonella* than VIDAS SLM. The results are obtained as little as 17 hours. The method has been simplified with a single enrichment in buffer peptone water and just one pipeting step. A broad incubation time of 16 to 24 hours simplifies the laboratory workflow, enabling all samples to be processed as they arrive during the day. This test is validated for raw beef and veal meats (including frozen), not flavoured and pasteurized egg products.

**VIDAS UP Salmonella** is a new generation of assay based on the latest technology available for pathogen screening: Phage recombinant protein. Bacteriophages are viruses

infecting bacteria. Phages are extremely host-specific. Most bacteria can be infected by particular phages and it is common that a given phage can recognize and infect only one or a few strains or species of bacteria (Hagens & Loessner, 2007). The specificity of these phages is partly mediated by tail-associated proteins that distinctively recognize surface molecules of susceptible bacteria (Kretzer et al., 2007). Bacteriophages or proteins of bacteriophages have been included in various ways in detection methods for pathogens (Favrin et al., 2003)

Although there is a need to perform a collaborative study to further evaluate the methods before it can be concluded that their performances are equal, both the PCR and the ELFA-based assay could provide a rapid and user-friendly screening method for detection of *Salmonella* in food (Uyttendaele et al., 2003; Priego et al., 2009; Kumar et al., 2008; Szabo et al., 2008)

### 7. Conclusion

Numerous and diverse alternative methods for microbial analysis of foods, as described above, exist (Bohaychuk et al., 2005; Wu, 2008) . They are currently brought to the market by various suppliers in a variety of formats as a result of recent developments, particularly in the field of biotechnology, microelectronics and related software development. Many of them have been proven to be equivalent to the “golden standard” reference methods with regard to the performance characteristics of the method.

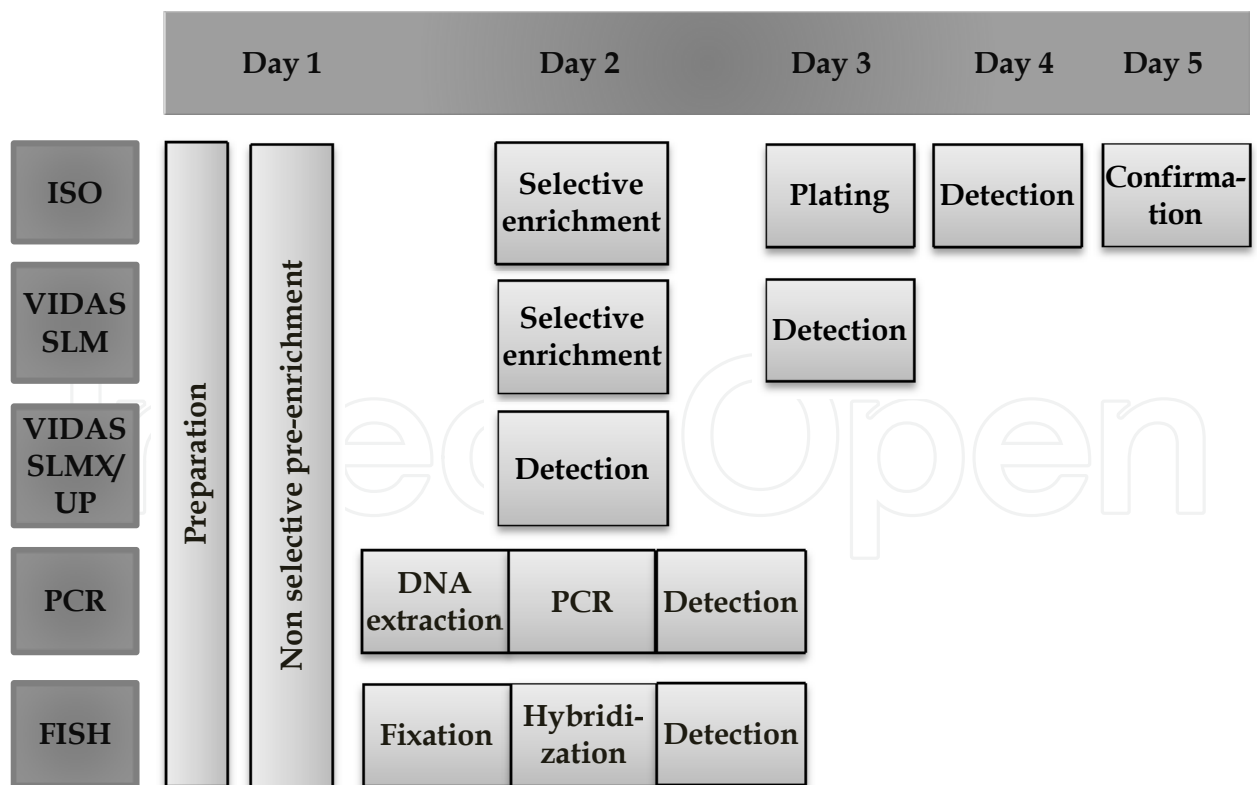


Fig. 2. Detection of *Salmonella* spp. from food.



Due to an overload of alternative methods and/or formats on the market, food business operators or competent authority, for which microbial analysis of food is only a supporting tool in the assurance of food safety, have difficulties in deciding which method is best fit for their purpose in their particular context. (Jasson et al., 2010)

Evolution in alternative rapid methods, mainly immunological and molecular methods, focus on the combination of available techniques e.g. combination of immunocapture and PCR and/or by elaboration of new formats optimizing reading and registration software rather than introducing new principles of detection or enumeration.

## 8. Acknowledgment

This study was supported by the Grant N N312 491340 from the National Science Centre, Poland.

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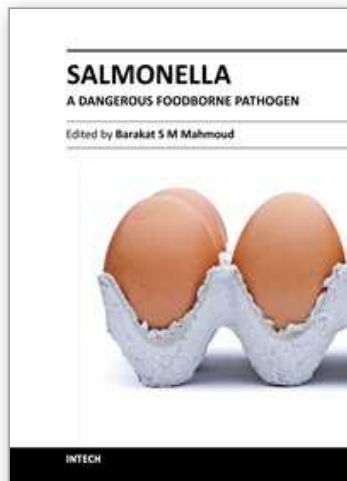
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## **Salmonella - A Dangerous Foodborne Pathogen**

Edited by Dr. Dr. Barakat S M Mahmoud

ISBN 978-953-307-782-6

Hard cover, 450 pages

**Publisher** InTech

**Published online** 20, January, 2012

**Published in print edition** January, 2012

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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