CHAPTER 2
LITERATURE REVIEW

2.1 *Salmonella*

2.1.1 Microbiology

Salmonellae are small, Gram-negative, facultative anaerobic, non-spore-forming bacilli. They belong to the *Enterobacteriaceae* family. Most of them are motile by peritrichous flagellae at a temperature range of 5-45^{0}C, with an optimum temperature between 35^{0}C to 37^{0}C (Bhunia, 2008). A pH between 6.6 and 8.2 is required for the best growth of *Salmonella*. *Salmonella* will form long filament chains when growing at temperatures between 4 and 8^{0}C or at 44^{0}C at pH of 4.4 or 9.4. The presence of 3 to 4% NaCl generally inhibits the growth of *Salmonella* spp. They are unable to tolerate high salt concentration. Brine above 9% is reported to be bactericidal for them. Regarding moisture, the growth of salmonellae is inhibited at a_w values below 0.94 in media with a neutral pH (Jay *et al*., 2005).

Salmonellae catabolize D-glucose and other carbohydrates with the production of acid and gas. They are oxidase-negative and catalase-positive, grow on citrate as a sole carbon source, generally produce hydrogen sulfide, decarboxylase lysine and ornithine, and do not hydrolyze urea. Most members of the *Salmonella* genus do not ferment lactose or sucrose. (D' Aoust *et al.*, 2001)

2.1.2 Serotyping of *Salmonella*

Salmonella are classified based on their somatic (O), flagella (H), and capsular (Vi) antigenic patterns. The somatic (O) antigens are associated with lipopolysaccharides (LPS) on the external surface of the bacteria's outer membrane. These antigens are heat stable, resistant to alcohol and dilute acids. The flagella antigens (H) are associated with the peritrichous flagella; they are heat-labile proteins. The capsular (Vi) antigen, which can be found only in *Salmonella* serovars Typhi,

Paratyphi and Dublin, is a heat-sensitive carbohydrate (Yousef and Carlstrom, 2003). So far, 2,579 *Salmonella* serotypes have been identified which are placed under two species: *Salmonella enterica* and *Salmonella bongori*. Another classification system for *Salmonella* groups them according to their susceptibility to different bacteriophages. This is called phage typing. There are more than 200 definitive phage types (DT), including phase type (PT) 1, 4, 8, 13, 13a, 23, DT104, DT108, DT204, etc. Resistance to antibiotics has also been used as a means of classification. For example, DT204, which is an emerging strain, is resistant to eight to nine antibiotics (Bhunia, 2008).

Table 1. Present number of serovars in each *Salmonella* species and subspecies (Source: Grimont and Weill, 2007)

S. enterica	
subsp. *enterica*	1531
subsp. *salamae*	505
subsp. *arizonae*	99
subsp. *diarizonae*	336
subsp. *houtenae*	73
subsp. *indica*	13
S. bongari	22
Total	**2579**

2.2 Epidemiology

For epidemiological purposes, salmonellae can be placed into three groups: Group 1. highly host adapted and invasive serovars. This group includes species restricted and invasive salmonella such as *S. Pullorum*, *S. Gallinarum* in poultry and *S. Typhi* in humans.

Group 2, non-host adapted and invasive serovars. This group consists of approximately 10-20 serovars that are able to cause an invasive infection in poultry and may be capable of infecting humans. Currently, the most important serovars are *S. Typhimurium*, *S.Hadar*, *S. Arizonae* and *S. Enteritidis*

Group 3, non-host adapted and non-invasive serovars. Most serovars of the genus *Salmonella* belong to this group and are harmless for animals and humans (Hafez, 2008).

The primary habitat of S*almonella* spp. is the intestinal tract of animals such as birds, reptiles, farm animals, humans and occasionally insects. The S*almonella* multiply in the intestinal tract of an animal. As intestinal form, they are excreted in faeces from which they may be transmitted by insects or other living animals and also by water or food that have been contaminated by insects or by other means. When consumed by humans and other animals, *Salmonella* are shed through faecal matter with a continuation of the cycle. This cycle causes a worldwide distribution of *Salmonella* via international shipments of animal products and feeds (Jay *et al*., 2005).

The prevalence of *Salmonella* spp. in broiler flocks in the EU between October 2005 and September 2006 ranged from 0% (Sweden) to 68.2% (Hungary), with a mean of 23.7% of the 7,120 investigated flocks in 23 member states. The five most frequently isolated *Salmonella* serovars were, respectively, in decreasing order *S*. Enteritidis (in 37% of Salmonella-positive flocks), *S*. Infantis (20%), S. Mbandaka (7.5%), *S*. Typhimurium (4.6%) and *S*. Hadar (4.2%). All these serovars, with the exception of *S*. Mbandaka, are frequent causes of *Salmonella* infections in humans in the EU (EFSA, 2007a).

In northern Thailand, the prevalences of *Salmonella* in chickens at the farm level, at slaughterhouses and in chicken meat at markets were estimated as 4%, 9%, and 57%, respectively. The most frequently isolated serovar of *Salmonella* was S. Weltevreden in chickens and humans (Padungtod and Kaneene, 2006).

Since 1990, there has been a substantial increase of human *S.* Enteritidis infections in Thailand. This could suggest that these infections may be associated

with the increased prevalence of *S.* Enteritidis in chickens (Sakai and Chalermchaikit, 1996), but this association has not been proven.

2.3 Salmonellosis

2.3.1 Salmonellosis in humans

Salmonellosis in humans is generally contracted through the consumption of contaminated food of animal origin (meat, poultry, eggs and milk), although many other foods, including green vegetables contaminated from manure, have been implicated in transmissions. The causative organisms pass through the food chain from primary production to households or food-service establishments and institutions. (WHO, 2005)

Human salmonellosis is usually characterized by an acute onset of fever, abdominal pain, nausea, and sometimes vomiting. Symptoms are often mild and most infections are self-limiting, lasting only a few days. However, in some patients the infection may be more serious and the associated dehydration can be life threatening. This situation also occurs when infection with invasive *Salmonella* take place. Salmonellosis has also been associated with long-term and sometimes chronic sequelae e.g. reactive arthritis (EFSA, 2006).

The infective dose of *Salmonella* may vary from 1 to 10^9 cfu g^{-1}. Studies conducted with human adult volunteers showed that a dose range of 10^5-10^{10} is required to cause *Salmonella* disease. The required infectious dose will be lower when agents are consumed with liquid food that transverses the stomach rapidly or with foods such as milk and cheese that neutralize the acid in the stomach (Bhunia, 2008).

In the EU, the reported number of human salmonellosis cases in 2005 was 176,395, relating to an incidence of 38.2 cases per 100,000 population. Incidences ranged from 4.4 to 322 amongst individual member states. The most frequently reported serovars were *S.* Enteritidis, followed by *S.* Typhimurium (Table 2).

Table 2. Reported confirmed salmonellosis cases in humans in the EU by serovars (10 most frequent serovars); BSN and Enter-Net data, 2005 (EFSA, 2006)

Top ten BSN			**Top ten Enter-Net**		
Serovar	N	%	Serovar	N	%
S. Enteritidis	86,536	52.2	*S*. Enteritidis	69,290	69.1
S. Typhimurium	15,058	9.1	*S*. Typhimurium	12,828	12.8
S. Infantis	1,354	0.8	*S*. Hadar	2,064	2.1
S. Bovismorbificans	621	<0.5	*S*. Virchow	1,026	1.0
S. Hadar	577	<0.5	*S*. Infantis	887	0.8
S. Virchow	535	<0.5	*S*. Agona	606	0.6
S. Derby	259	<0.5	*S*. Newport	599	0.6
S. Newport	245	<0.5	*S*. Stanley	535	0.5
S. Anatum	179	<0.5	*S*. Bovismorbificans	533	0.5
S. Goldcoast	173	<0.5	*S*. Derby	481	0.5

S. spp. reported through the BSN, N=56,619 (34.1%); BSN = EU's Basic Surveillance Network (monthly collection of data on approximately 50 notifiable diseases)

S. spp. reported through Enter-Net, N=2,626 (2.6%); Enter Net = EU's surveillance system for human *Salmonella* and *Escherichia* coli O157 infections

In Thailand, the National *Salmonella* and *Shigella* Center of the National Institute of Health of Thailand reported the top ten *Salmonella* serovars from human sources in its annual report for 2006 (Table 3).

Table 3. Top ten *Salmonella* serovars from human sources in Thailand (2006)

Serovars	Isolated	Percentage
S. Enteritidis	541	18.46
S. Stanley	313	10.68
S. Choleraesuis	233	7.95
S. Rissen	224	7.64
S. Weltevreden	202	6.89
S. 14,5,12:i:-	126	4.3
S. Corvallis	122	4.16
S. Anatum	113	3.86
S. Typhimurium	96	3.28
S. Kedougou	86	2.73
other (93 serovars)	881	31.06
Total	2931	100

(Source: www.dmsc.moph.go.th/ifc_nih/applications/files/Annual_report_2006.pdf.)

2.3.2 Salmonellosis in animals

Salmonellosis is of common occurrence in domestic animals. Some of the *Salmonella* serotypes are relatively host specific such as *Salmonella* Pullorum for poultry, *Salmonella* Cholerasuis for pigs and *Salmonella* Dubin for cattle. On the other hand, some serotypes have a wide host range such as *Salmonella* Typhimurium. (Quinn and Markey, 2003).

The most common type of *Salmonella* infection are subclinical infections in which animals may become intermittent or persistent carriers, since organisms may easily spread between animals in a herd or between flocks without detection.

For clinical signs, infected cows may succumb to fever, diarrhoea and abortion. Fever and diarrhoea are less common in pigs than in cattle, and sheep, goats and poultry usually show no signs of infection (EFSA, 2006).

Chickens can be infected with many different serovars of *Salmonella.* Some serovars, such as *S.* Pullorum and *S.* Gallinarum, are host-specific for chickens, whereas other serovars, such as *S*. Typhimurium, *S*. Enteritidis, and *S*. Heidelberg are able to infect a wide range of hosts (Foley *et al*., 2008). There are three main sources of infection:

1. Via parents/breeding stock
2. Via environments such as contact with mice, pests, birds, insects, dust, etc.
3. Via feed contaminated with *Salmonella* (Barbut, 2002)

Infections of *Salmonella* in poultry can be classified into two groups. The first group is infection with non-motile serotypes such as *S.* Pullorum and *S.* Gallinarum, which have been responsible for serious economic losses for poultry producers in the past; however, they were eliminated from commercial flocks with the implementation of control strategies and eradiation programs. The second is infection with motile *Salmonella* serotypes such as *S*. Typhimurium and *S*. Enteritidis. They can infect a wide variety of hosts and also are of concern as a cause of foodborne disease in humans (Gast and Shivaprasa, 2003). Especially *S*. Enteritidis can colonize the ovaries of laying hens. Such transovarian transmission allows these organisms to be present in eggs before the eggshell is formed in the oviduct. The result of this is that eggs stored at room temperature can contain high numbers of *Salmonella* (as high as 10^{11} cells per egg) (Bhunia, 2008).

The virulence of *Salmonella* relates to their ability to invade host cells, replicate in them and resist both digestion by phagocytes and destruction by complement components of the plasma.(Quinn and Markey, 2003)

Salmonella Enteritidis plays a role in poultry as a most common serotype in human cases of Salmonellosis in the EU and are reported to be of second importance in the United States (CDC, 2007, Jong and Ekdahl, 2006). The effective colonization of the reproductive tract in poultry allows *S*. Enteritidis to be transmitted vertically to eggs and subsequently to chicks following hatching. This efficient colonization and transmission likely aided to the rapid spread of *S*. Enteritidis in poultry following the eradication of *S*. Gallinarum (Foley *et al*., 2008).

To control *Salmonella* infection, implementing a closed-herd policy, purchasing animals only from reliable sources and preventing contamination of water and foodstuff are reported as some measures to reduce the risk of exposure of animals to pathogens. Vaccination procedures, where permitted, for enhancing resistance and reducing the likelihood of clinical disease are used in cattle, sheep, pigs and poultry (Quinn and Markey, 2003).

The top-ten *Salmonella* serovars from animal sources in Thailand (Table 4) for 2006 are reported in the annual report of the National *Salmonella* and *Shigella* Center of the National Institute of Health of Thailand.

Table 4. Top ten Salmonella serovars from animals in Thailand (2006)

Serovars	**Number Isolated**	**Percentage**
S. Weltevreden	148	19.12
S. Corvallis	33	13.15
S. Enteritidis	31	12.35
S. Newport	16	6.37
S. Stanley	14	5.58
S. Brunei	11	4.38
S. Typhimurium	9	3.59
S. Virchow	8	3.19
S. Javiana	8	3.19
S. Amsterdam	8	3.19
Other	65	25.9
Total	251	100

(Source: www.dmsc.moph.go.th/ifc_nih/applications/files/Annual_report_2006.pdf.)

2.4 *Salmonella* detection

2.4.1 Conventional methods

Conventional methods for the detection of *Salmonella* generally are based on the following steps:

1. Pre enrichment and selective enrichment

These steps are carried out to resuscitate injured salmonellae and to increase their number to detectable levels. Buffered peptone water is required for pre-enrichment. Modified Semisolid Rappaport-Vassiliadis medium (MSRV) is used for selective enrichment.

2. Isolation of *Salmonella*

Isolation includes streaking enrichments onto selective differential agar media and recognizing presumptive *Salmonella* colonies on the incubated plates. These isolated colonies are then sub-cultured for subsequent identification. Media for *Salmonella* isolation should contain selective components such as bile or deoxycholate salts, brilliant green, and bismuth sulfite. These components inhibit Gram- positive and non-enteric bacteria. According to Annex D of the ISO 6579 standard, Xylose Lysine Deoxycholate (XLD) agar is recommended as the first plating-out medium and Brilliant Green Agar (BGA), Bishmuth Sulfite agar (BS) etc., could be used as second plating-out media.

3. Biochemical identification

According to Annex D: For biochemical testing to confirm *Salmonella*, the ISO 6579 standard recommends using the TSI agar, Urea agar, L-Lysine decarboxylation medium, β-galactosidase, Voges-proskauer (VP) reaction and indole reaction.

4. Serological identification

Isolates are serotyped by agglutination according to the Kauffmann-White scheme using first *Salmonella* polyvalent I (A-E) and *Salmonella* Polyvalent II (F-67) antisera and then *Salmonella* antisera specific to individual groups.

2.4.2 Alternative methods

Alternative methods are becoming popular in the food industry, most of these are based on genetic or immunoassay techniques. The methods do not include the laboratory-intensive and time-consuming biochemical identification steps.

2.4.2.1 Immunoassay-based method

ELISA tests used to detect *Salmonella* include pre-enrichment and selective enrichment steps similar to those applied in the conventional detection protocol. An additional enrichment in mannose broth continues the immunoassay step. By use of the ELISA-based detection method to cull out *Salmonella*-free food samples are fast included, essentially saving detection time (Yousef and Carlstrom, 2003).

2.4.2.2 Genetic-based methods

2.4.2.2.1 DNA Probe Hybridization

In studies by Whyte *et al*. (2002), the PCR assay proved to be a highly specific and sensitive method for detecting *Salmonella,* and the incorporation of a routine PCR test in conjunction with standard culture proved to be effective in providing a more accurate profile of the prevalence of this pathogen in broiler carcasses. In their study, the PCR test was considerably more sensitive (53%) than culture methods (30%) in detecting *Salmonella* within flocks. This suggests that the PCR assay could be used alone as a routine surveillance test where the levels of contamination in flocks are suspected to be low. Where an outbreak occurs, or when the prevalence increases, the combination of both culture and PCR tests could be employed to assess the real levels of infection in flocks and the potential for further transmission. It is probable therefore, that sensitive DNA-based methods used in conjunction with traditional culture methods will in the future enable more accurate assessments to be made of the prevalence of e.g. contaminated carcasses.

2.4.2.2.2 Molecular Typing Methods

PFGE of whole chromosomal DNA: Pulsed-field gel electrophoresis (PFGE) has been recognized as a powerful tool for molecular typing and has been the method of choice applied in numerous epidemiological studies of *Salmonella* (Gorman and Adley, 2006). For PFGE, bacterial isolates, grown either in broth or on solid media, are combined with molten agarose and poured into small molds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are then subjected to in-situ detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of very-large-molecular length DNA fragments ranging from 10 to 800 kb. The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed, and the data can be stored by using one of the commercially available digital systems, such as those manufactured by Alpha-Innotech, Bio-Rad, Hitachi, or Molecular Dynamics. Data analysis can be accomplished by using any of a number of commercially available software packages available from e.g. Applied Math, Bio-Rad, BioSystematics, Media Cybernetics, or Scanalytics (Olive and Bean, 1999).