2. LITERATURE REVIEW

2.1. *Salmonella*

2.1.1. Microbiology

Salmonellae are gram-negative bacteria belonging to the genus *Salmonella* of the family *Enterobacteriaceae*. They are straight rods of 0.7-1.5x2-5 μm that have the capacity to grow under either aerobic or anaerobic conditions (Krieg and Holt, 1984). They are non-encapsulated and non-sporular bacteria. The bacteria grow optimally at 37 ^{o}C on ordinary culture media, where they develop small colonies of 2 to 4 mm in diameter which are smooth, shiny and homogenous in color (Krieg and Holt, 1984). Metabolic characteristics of *Salmonella* usually include the utilization of citrate as a sole carbon source and the production of gas from glucose. Lactose is generally not fermented by salmonellae, except for some strains of *S. diarizonae* (Table 1, Holt *et al.*, 2000, Hanes, 2003). Like most bacteria, their optimum pH for growth is neutral (pH 6.5-7.5), although growth may still occur in a wide pH range (4.5 to 9.5) depending on the surrounding conditions. The lowest temperature at which *Salmonella* has been found to grow is 2 ^{o}C and the highest is 54 ^{o}C (for *S.* Typhimurium). *Salmonella* require water activity (a_w) above 0.94 (Hanes, 2003) and growth inhibition has been reported at a_w below 0.93 (D' Aoust *et al*., 2001). A salt content of 3-4% generally inhibits the growth of *Salmonella*, but increasing the temperature increases salt tolerance in the range of 10 to 30 ^{o}C (D' Aoust *et al*., 2001). However, a salt content above 8% is bactericidal for salmonellae (Jay, 1996).

Table 1: Biochemical profile of *Salmonella*

Test or substrate	*Salmonella* result[a]	Indicating agent	Media colour
Glucose	+	Phenol red	Yellow butt
Lysine decarboxylase	+	Bromocresol purple	Purple butt
H2S	+	-	Blackening
Urease	-	Phenol red	No color change
Lysine decarboxylase broth	+	Bromocresol purple	Purple color
Phenol red dulcitol broth	+[b]	Phenol red	Yellow color and/or gas
KCN broth	-	-	No growth
Malonate broth	-[c]	Bromothymol blue	No color change
Indole test	-	Kovac's reagent	Yellow color at surface
Phenol red lactose broth	-[c]	Phenol red	No gas, no color change
Phenol red sucrose broth	-	Phenol red	No gas, no color change
Voges-Proskauer test	-	Alphanaphthol, Ethylalcohol, KOH	No color change
Methyl red test	+	Methyl red	Diffuse red color
Simmons citrate	v	Bromothylmol brue	Growth, blue color Or no growth, no color change

[a] +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable

[b] Majority of *S. arizonae* cultures are negative

[c] Majority of *S. arizonae* cultures are positive

Source: Hanes (2003), Quinn *et al*. (1999)

The vast majority of salmonellae is motile and propelled by peritrichous flagella with the exception of rare non-motile *Salmonella* serotypes such as *S.* Gallinarum and *S.* Pullorum (Krieg and Holt, 1984, D' Aoust *et al*., 2001). The movement is linear most of the time, but may be interrupted by a brief moment of 'tumbling' (Krieg and Holt, 1984). Like other flagellated cells, the motile salmonellae may lose their ability to develop flagella under the effect of sub-lethal 'stress', caused by external physicochemical influence such as refrigeration or high temperatures (Krieg and Holt, 1984, D' Aoust *et al*., 2001).

2.1.2. Taxonomy

In recent years, there has been a change in the taxonomy of *Salmonella*. In the early development of taxonomic schemes, each *Salmonella* serotype was treated as a species. However, according to the new taxonomic scheme based on DNA-hybridization and enzyme electrophoretic characterizations, all salmonellae have been placed into two species, *S. enterica* and *S. bongori*. *S. enterica* is divided further into six subspecies or groups (Table 2), the main one being *Salmonella enterica* subspecies *enterica*, which represents nearly 99% of the salmonellae isolated in medical practice. It should be noted that the old way of naming serotypes is no longer valid. For example, *Salmonella typhimurium* should be *S. enterica* serotype Typhimurium, or simply *Salmonella* Typhimurium (note that 'typhimurium' is capitalized and not italicized).

Table 2: *Salmonella* species and subspecies

Salmonella species and subspecies	No. of serotypes
Salmonella enterica	2,443
S. enterica subspecies *enterica*	1,454
S. enterica subspecies *salamae*	489
S. enterica subspecies *arizonae*	94
S. enterica subspecies *diarizonae*	324
S. enterica subspecies *houtenae*	70
S. enterica subspecies *indica*	12
Salmonella bongori	20
TOTAL	2463

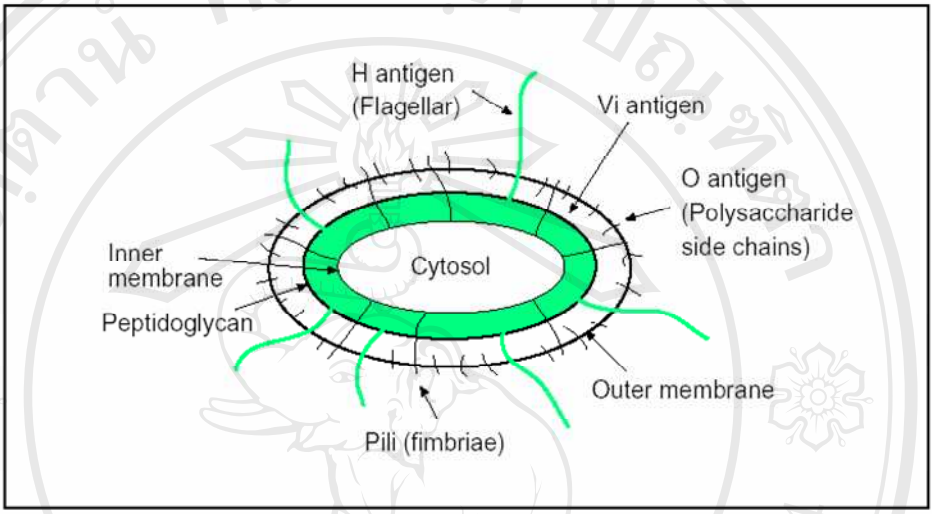
Source: D' Aoust *et al.* (2001)

2.1.3. Serotypes

According to the Kaufman-White classification scheme, there are 2,463 serotypes (serovars) of *Salmonella*, defined by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in Paris, France in the year 2000 (Table 2) (D' Aoust *et al.*, 2001). All serotypes in subspecies *enterica* are named whereas serotypes in other subspecies (except for some in subspecies *salamae* and *houtenae*) and *S. bongori* are not named but designated by antigenic formulae.

The serologic typing of salmonellae has led to the identification of a large number of strains. According to the Kaufman-White scheme, organisms are represented by the numbers and letters given to the different somatic (O) lipopolysaccharides (LPS) on the external surface of the bacterial outer membrane, to flagella (H) antigens associated with the peritrichous flagella, and to capsular (Vi) antigen appearing in *Salmonella* serotypes Typhi, Paratyphi C and Dublin. The Vi antigen is located in an external polysaccharide microcapsule and is associated with virulence for particular hosts (Figure 1) (Krieg and Holt, 1984, D' Aoust *et al.*, 2001).

Figure 1: Schematic representation of the antigen structure of *Salmonella* Typhi showing the relative locations of O, H and Vi antigens



Source: Axelsson and Sorin (1997)

These antigens are heterogeneous structures, and antigenic specificity is determined by the composition and linkage of the O group lipopolysaccharides. Mutations that affect the lipopolysaccharides may lead to new O antigens. In many serotypes the flagellar H antigens can switch between two types, called phase 1 and phase 2. This switching results in two alternative sets of H antigens. Because H antigens are less heterogenous than the carbohydrate side chains, considerable fewer H antigenic serotypes exist. Presently, *Salmonella* serotypes are placed into 67 serogroups (A to 67) designated with letter or numbers according to similarities in content of one or more O antigens (e.g. *S.* Typhi, *S.* Enteritidis, *S.* Gallinarum are serogroup D because all have the same somatic O antigen 9 and 12) (Krieg and Holt, 1984). The antigenic formulae for some salmonellae are shown in Table 3.

Table 3: Examples of antigenic structure formulae for some common salmonellae

Group	Species/Serotypes	O antigen	H Antigens	
			Phase 1	Phase 2
A	*S.* Paratyphi A	1, 2, 12	a	[1,5]
B	*S.* Typhimurium	1, 4, [5], 12	i	1, 2
C1	*S.* Choleraesuis	6, 7	[c]	1, 5
	S. Paratyphi C	6, 7, [Vi]	c	1, 5
D	*S.* Typhi	9, 12, [Vi]	d	-
	S. Enteritidis	1, 9, 12	g, m	[1, 7]
	S. Gallinarum	1, 9, 12	-	-
E1	*S.* Anatum	3, 10	e, h	1, 6

Symbols: [ ], may be absent; ( ) not well developed (weakly agglutination). The underlined antigens are associated with phage conversion

Source: Krieg and Holt (1984)

2.2. Distribution of *Salmonella* in pigs

The primary habitat of *Salmonella* is the intestinal tract of animals such as birds, reptiles, farm animals, humans, and occasionally insects (Jay, 1992, Hanes, 2003). Although their primary habitat is the intestinal tract, they may be found in other parts of the body (Jay, 1992, Hanes, 2003). As intestinal forms, the organisms are excreted in faeces from which they may be transmitted by insects and other living creatures to many places such as to water, soils and building surfaces. In pig production, the two important factors of introducing *Salmonella* into the herds are the feeds and new animals (Lo Fo Wong and Hald, 2000).

The contribution of management to the prevalence of *Salmonella* in farms has been illustrated in various studies. For example, increasing herd sizes would increase the within-herd seroprevalence of *S. enterica* (Mousing *et al.*, 1997). However, this depends on the type of management, feeding system, cleaning and disinfection and bio-security systems (Christensen and Rudemo, 1998). Van der Wolf *et al.* (2001) have indicated that small to moderate herd sizes (<800 finishers) were associated with a higher *Salmonella* seroprevalence than herds that were larger because the larger farms are more hygiene-conscious than the smaller farms. Beloeil *et al.* (2004) and van der Wolf *et al.* (2001) found that the risk for *Salmonella* shedding at the end of the fattening period was increased when dry feed (versus wet feed) was provided. The trough feeding was also associated with a higher *Salmonella* infection level compared to the other type of feeding systems (van der Wolf *et al.*, 1999). In cases where the herds were infected by other diseases such as *Lawsonia intracellularis* and/or PRRS (Porcine Reproductive and Respiratory Syndrome), the prevalence of *Salmonella* in those herds was higher because *Lawsonia intracellularis* disturbs the ecology of the intestine and gut flora, while PRRS induces immunosuppression (Beloeil *et al.*, 2004).

Table 4 shows the prevalence of *Salmonella* in pork, beef and chicken meat in different countries. However, the sensitivity of the test used, sample size and the distribution of the proportions of infected animals within herds have influence on the results (Steinbach *et al*., 2002). Thus, the real number of *Salmonella* carriers might be much higher than shown by bacteriological and serological examination (Steinbach *et al*., 2002).

The distribution of *Salmonella* serotypes shows in Table 5. In Denmark, Canada, the United States and Japan, the most frequently serotypes found in pigs were *S.* Typhimurium and *S.* Derby. In Thailand, there was no report of serotypes isolated from pigs. The serotypes isolated from human cases in Thailand show in Table 5, that *S.* Weltevreden was the serotype most frequency isolated, followed by *S.* Enteritidis and *S.* Anatum.

Table 4: Prevalence of *Salmonella* in raw meats or products

Product	Country	Number of Samples	
		Tested	Percent Positive
Beef	Denmark, 1995[a]	2,559	1.3
	Germany, 1991[b]	18,242	5.1
	United States, 1993[b]	2,112	2.7
Pork	Canada, 1985[b]	448	10.0
	Mexico, 1994[a]	50	76.0
	Portugal, 1987[b]	405	5.4
	Thailand, 1986[a]	130	21.5
Chicken	Cuba, 1990[b]	200	62.5
	Denmark, 1995[b]	4,099	45.7
	France, 1994[a]	616	19.8
	Germany, 1994[b]	630	28.6
	United States, 1995	1,297	20.0
	Mexico, 1993[a]	70	68.6

[a] Retail samples

[b] Post slaughter carcasses

Source: D' Aoust (2001)

Table 5: *Salmonella* serotypes isolated in the different countries

Country	Origin	Serotype	Percentage	Reference
Denmark	Pigs	*S.* Typhimurium *S.* Derby *S.* Altona	75 6 4	Sorensen *et al.* (2004)
Japan	Diarrhea pigs	*S.* Typhimurium O 4, 12: d:- *S.* Derby	91.9 13.1 7.1	Asai *et al.* (2002b)
United States (North Carolina)	Pigs	*S.* Derby *S.* Typhimurium *S.* Schwarzengrund *S.* Heidelberg	6.3 5.7 3.7 3.2	Davies *et al.* (1997)
United States (North Carolina)	Pigs	*S.* Typhimurium *S.* Derby	47.7 7.8	Funk *et al.* (2005)
Canada (Alberta)	Pigs	*S.* Typhimurium *S.* Derby *S.* Infantis *S.* Califonia *S.* Enteritidis	24.1 22.0 14.6 7.5 5.0	Rajic *et al.* (2005)
Thailand	human cases	*S.* Weltevreden *S.* Enteritidis *S.* Anatum *S.* Derby *S.* Typhimurium *S.* Rissen *S.* Stanley	12.5 11.4 7.4 6.6 5.3 5.3 3.8	Bangtrakulnonth *et al.* (2004)

2.3. Foodborne Salmonellosis

Eggs, poultry and raw meat products are the most important food vehicles of *Salmonella* infection in humans, with *S.* Typhimurium and *S.* Enteritidis being the most commonly isolated food-borne serotypes (Krieg and Holt, 1984, Jay, 1996). In Thailand, the most common serotypes isolated from humans were *S.* Weltevreden and *S.* Enteritidis: these serotypes are increasingly isolated from humans and other reservoirs, e.g. chicken, seafood and ducks (Bangtrakulnonth *et al.*, 2004). Symptoms of *Salmonella* usually develop 12 to 14 hours after exposure, although shorter or

longer incubation times have been reported. Symptoms consist of nausea, vomiting, abdominal pain (not as severe as staphylococcal food poisoning), headache, chills and diarrhea. These symptoms are usually accompanied by prostration, muscular weakness, faintness, moderate fever, restlessness and drowsiness. Symptoms usually persist for 2 to 3 days. *Salmonella* generally disappear rapidly from the intestinal tract after recovery from the disease. However, up to 5% of patients may become carriers upon recovery from the disease (Jay, 1996). The pathogenesis of salmonellosis may involve two toxins – an enterotoxin and a cytotoxin. Numbers of cells in the order of 10^7-10^9/g are generally necessary for salmonellosis (Krieg and Holt, 1984). But from one salmonellae outbreak, numbers of cells as few as 100 cells/100 grams of food (*S.* Eastbourne in chocolate) have been reported to make people sick (Jay, 1996).

Determinant factors of salmonellosis are not limited to the immunological heterogeneity within human populations and to the virulence of infecting strains; they may include the chemical composition of incriminated food vehicles. A common determinant of the foods associated with low infectious doses is the high fat content in chocolate (cocoa butter), cheese (milk fat), and meat (animal fat). Suggestively, entrapment of salmonellae within hydrophobic lipid micelles would provide protection against the bactericidal action of lipid moieties in the duodenum, the viable salmonellae would resume their infectious course in search of suitable points of attachment in the lower portion of the small intestine (colonization) (D' Aoust *et al.*, 2001). And commensal *Salmonella* may be found in healthy carriers who are in a state of convalescence, but there are also permanent carriers who contribute to the spread of the illness. However, the true incidence of *Salmonella* infection is difficult to determine. Reported cases represent only a small proportion of the actual number. Normally only large outbreaks are investigated and documented; sporadic cases are underreported, mainly because only patients with protracted diarrhea report to a health care provider for microbiological evaluation (Hanes, 2003).

A study by Hanes (2003) showed a close relationship between the *Salmonella* serotypes most often responsible for human infection and those isolated from animals

in any one geography. These similarities document the importance of nonhuman reservoirs of *Salmonella* in epidemiology of infection in human.

2.4. *Salmonella* Detection

The 2 most used diagnostic methods for detection of *Salmonella* infections in pigs are the microbiological examination of faeces, faecal contents, swab samples of lymph nodes and the serological examination of blood samples or meat juices (Lo Fo Wong and Hald, 2000, Sorensen *et al.*, 2000). Examination of faeces is a useful tool for determining the current infection level in a pig herd. A positive isolation of *Salmonella* will leave little doubt of the presence of the bacteria in the animal or in the samples. Therefore, this method is often defined as the 'gold standard' when comparing results with those obtained from alternative tests (Lo Fo Wong and Hald, 2000). However, present culturing methods are time consuming and laborious, requiring pre-enrichment, selective enrichment, indicative plating and bio/serotyping. Therefore, there is a need for *Salmonella* tests that provide results more rapidly with a similar sensitivity to, or greater than, the conventional methods. These tests should be simple and reproducible and have a specificity that minimizes false-positive results (Axelsson and Sorin, 1997).

Thus, immuno-serological tests have been developed for the detection of *Salmonella*. These can be broadly divided into those based on enzyme-labeled antibodies (ELISA), fluorescent antibody staining, radio immunoassay and other methods. The most popular test for routine use is ELISA (Enzyme-Linked Immunosorbent Assay) technology. This technique takes only about 2 hours to perform. ELISA has the disadvantage that we can not be sure that the infection is still present at the farm at the moment of positive testing. Furthermore, it will not detect infections that occurred shortly (1-2 weeks) before sampling (van der Wolf *et al.*, 2001).

Some studies show the correlation between conventional culture methods and serology in individual pigs. In general most *Salmonella* infections are silent in pigs, they nevertheless undergo an infectious process resulting in an immune response. Thus, serological and bacteriological results generally have a poor correlation (Davies *et al.*, 2003). While Sorensen *et al.* (2004) found that there was a strong association between herd serology and the prevalence of *Salmonella* bacteria measured at tree sampling sites: faecal-content, pharynx and carcass surface. For these sites, the odds for being culture-positive for *Salmonella* varied from 1.3 to 1.5 for each increase of 10% in herd serology. In a study of Asai *et al.* (2002a), *Salmonella* was isolated from 26 (28.9%) of 90 antibody-positive pigs and 21 (11.9%) of 117 antibody-negative pigs at 4 months of age. The authors found that sero-conversion generally occurred during the last third of the fattening phase from 140 days of age to slaughter (Asai *et al.*, 2002a, Beloeil *et al.*, 2003), while shedding was considerable in the first half of the fattening period (Beloeil *et al.*, 2003), particularly in pigs between 4 to 5 months of age (Asai *et al.*, 2002a). According to the above studies, if the intention is to monitor *Salmonella* pre-harvest, measures of herd serology or faecal content are appropriate (Sorensen *et al.* 2004). For more precise results, the prevalence in fattening pigs should be investigated in the late stage of the fattening period or before slaughtering. If the transmissions within the herd are to be studied, it should be done during the first half of fattening period.

Sensitivity regarding bacteriological detection will be relatively high where the animals examined suffer from an acute infection and harbor a high number of microorganisms, and it will be low if only a small number of microorganisms remain in the animal body. Regarding serological diagnosis, there may be differences in sensitivity depending on the intensity of the infection process among the herd and the time lag between infection and examination. The specificity of serological detection of *Salmonella* may become reduced by microorganisms not belonging to *Salmonella*, but inducing antibodies which react with the *Salmonella* antigen (Steinbach *et al.*, 2002). Malorny, *et al.* (2003), found that the inter-laboratory diagnostic accuracy, (i.e. diagnostic specificity and sensitivity) was shown to be 97.5% when detecting

Salmonella by the PCR based method. This was conducted in 5 laboratories, one in Spain, one in France and three in Germany.

2.5. Control of *Salmonella* in pigs

For safety reasons, European Regulations concerning food products stipulate a *Salmonella* contamination rate of less than 1 bacterium per 25 grams. This means that in practice a total absence of the organism is intended. It is important to note that all types of *Salmonella*, whatever their serotype, are considered undesirable and they are tested for. To fulfill this purpose and to respond to the consumers' and society's expectations about food safety, most countries with developed pork production, especially in countries that export pork, have in slightly different ways developed standards for swine production that are run by producer associations (e.g. the Canadian Pork Quality Assurance system, and the PQA system of the U.S. National Pork Producer Council), or by industry associations (e.g. the Quality Assurance System of the UK meat and Livestock Council, or the Dutch Produktschapt voor Vee and Vlees with the renowed IKB-program = Integrate Keten Beheersing), or with laws or ordinances issued by governments that set the basic standards (as in the European Union with the "Zoonosis Directive" or in Germany with the "Schweinehaltungshygiene-Verordnung" or in Denmark with the "National *Salmonella* Control Program in the Danish Pork Industry").

Several studies have shown that the implementation of preventive measures could reduce the prevalence of contamination. Berends *et al.* (1998) reported the implementation of GMP codes from farm to cutting/retail could reduce the current levels of *Salmonella*-positive pigs and pork by 50-60%. If pigs were bred according to the rather costly 'specific pathogen free' (SPF) concept, the prevalence of contaminated carcasses and pork could in total be reduced by 95%. Berends *et al.* (1998) believe that the current EU Regulation, in relying on hazard analysis of critical control points (HACCP)-inspired production in cutting plants, will not be effective in reducing the prevalence of *Salmonella* in pork. This is because there is currently an

almost steady stream of *Salmonella* positive carcasses that enter slaughter and the cutting process and when contaminated carcasses are being processed, further cross contamination during working hours is unavoidable. No steps in the carcass-cutting process are intentionally designed to effectively reduce the risks of the consequences of cross contamination of cuts and retail-ready products (Berends *et al.*, 1998). However, from the study by D' Aoust (2001) in the United States, the preliminary results indicate that after implementation of HACCP in pig and poultry plants, *Salmonella* prevalence in broiler carcasses dropped from 20% to 10.4% and for swine carcasses, the prevalence dropped from 8.7 to 5.5%. Although these are preliminary data, they suggest that HACCP programs can reduce salmonellae in the food supply to a certain animal.

However, controlling *Salmonella* in pork needs a lot of investment. From a study by van der Gaag *et al*. (2004), seven stages can be distinguished in a pork supply chain: breeding and multiplying, finishing, transportation, lairage, slaughtering, processing and retailing, and household. Van der Gaag *et al*. (2004) concluded that the most cost-effective strategy for the pork supply chain is to implement interventions firstly in the slaughterhouse; especially at the lairage stage, secondly in the finishing farms. An additional result from this study is that the reduction of *Salmonella* in the pork chain to a level where the average prevalence, plus standard deviation, is below 2%, can be achieved when at least 4.5 Euro per pig is invested. This is relatively expensive, but it has to be stated that almost all interventions in order to reduce *Salmonella* in the pork chain are also effective in reducing other pathogens. In other words, the direct benefits are outside the pork supply chain, i.e. for society. An indirect benefit is the increased trust of the consumers, the improved image of pork and the strengthened position on the global market for pork (van der Gaag *et al*., 2004).

Up to now the pre-harvest stages of the pork supply chain cannot ensure a zero prevalence of contaminated carcasses. Therefore, the next stages (processing, storage at retail and storage and preparing the pork by the consumer) are also important. For instance, the consumer can reduce the risk of food-born salmonellosis by cool storage

and through heating the pork and by avoiding cross-contamination in the kitchen (van der Gaag *et al.*, 2004). Continuous surveillance and careful reporting of *Salmonella* isolates also contributes to the control of the disease. This surveillance improves awareness of new serotypes, common sources, antibiotic resistance, and carrier state (D' Aoust, 2001).