Chapter 2: LITERATURE RIVIEW

2.1 *Salmonella* background

2.1.1 Biology

The genus *Salmonella* is a member of the Enterobacteriaceae family, and is a Gram-negative, nonspore-forming bacillus. Salmonellae are motile (except *S.* Pullorum and *S.* Gallinarum) and express peritrichous flagella. They are facultative anaerobes that can grow in a temperature range of 5–45 °C with optimum temperature of 35–37 °C. They are able to grow at low pH and are generally sensitive to increased concentrations of salt. *Salmonella* forms long filamentous chains when grown at temperature extremes of 4–8 °C or at 44 °C and also when grown at pH 4.4 or 9.4. All salmonellae are facultative intracellular pathogen and considered pathogenic and can invade macrophages, dendritic and epithelial cells. The virulence genes responsible for invasion, survival, and extraintestinal spread are distributed in *Salmonella* pathogenicity islands (SPI) (Ohl and Miller, 2001).

Members of the genus are usually motile by peritrichous flagella, reduce nitrates to nitrites, usually produce gas from glucose, produce hydrogen sulfide on triple-sugar iron agar, and grow on citrate as the sole carbon source. They are indole- and urease-negative and lysine- and ornithinedecarboxylase- positive and sucrose-, salicin-, inositol-, and amygdalin-negative. Phenylalanine and tryptophan are not oxidatively deaminated, and lipase and deoxyribonuclease are not produced (Le Minor, 1984).

2.1.2 History and classification

Historically, *Salmonella* nomenclature had been based on the places of origin such as *S.* Miami, *S.* London, *S.* Richmond, *S.* Dublin, *S.* Indiana, *S.* Kentucky, *S.* Tennessee and according to the disease they caused (e.g. *S.* Enteritidis, *S.* Typhi, *S.* Paratyphi, *S.* Abortus equi, and *S.* Bovismorbificans), or the animals from which they were isolated (Kelterborn, 1967). For example, *S.* Gallinarum and *S.* Pullorum were important pathogens in poultry, *S.* Choleraesuis an important swine pathogen (Löffler, 1892). A limited number of serotypes were named after the person who isolated it (e.g. *S.* Virchow).

The systems explained above for classification is now discontinued and Salmonellae have been grouped based on their somatic (O) and flagellar (H) based on Kauffmann–White scheme. The Kauffmann–White scheme, which at present includes more than 2500 serotypes, is the most successful bacterial typing scheme in history. *Salmonella* serotypes are often correlated with clinical severity, reservoir, and occurrence of resistance. Information about the distribution of different serotypes, as well as subtypes, in different animal species, food and man may be used to quantify the relative importance of different sources of *Salmonella* infections (Altekruse *et al*., 1993; Hald *et al*., 2004).

Salmonella serotypes, are now placed under two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* now has six subspecies, which are designated by roman numerals: I (enterica), II (salamae), IIIa (arizonae), IIIb (diarizonae), IV (houtenae), and VI (indica). For example, a *Salmonella* isolate is designated as

Salmonella enterica subspecies I serovar Enteritidis. Under the modern nomenclature system, often, the subspecies information is omitted and the culture is called *S. enterica* serovar Enteritidis and in subsequent appearance, it is written as *S.* Enteritidis. Scientists are thus encouraged to follow this system of *Salmonella* classification and nomenclature to bring uniformity in the reporting and to avoid further confusions (Tindall *et al.,* 2005).

2.1.3 *Salmonella* infections

Salmonella is one of the major foodborne pathogens of significant public health concern in both developed and developing countries (Bhunia, 2008).

2.1.3.1 Transmission of *Salmonella*

Salmonella are present in the intestinal tract of birds, reptiles, turtles, insects, farm animals, and humans. Poultry are a major source for human foodborne salmonellosis, in part due to high-density farming operations which allow colonized birds to quickly spread salmonellae to other birds within a flock. Intestinal colonization by salmonellae increases the risk for contamination during slaughter. Eggs are also reservoirs for *Salmonella*, particularly serovar Enteritidis, as this organism can colonize the ovary of the laying hen. Such transovarian transmission allows bacteria to be present in the egg before the eggshell is formed in the oviduct. As a result, eggs stored at room temperature can contain high concentrations of *Salmonella* (Bhunia, 2008).

Human salmonellosis is generally foodborne and is contracted through consumption of contaminated food of animal origin such as meat, milk, poultry, and eggs. Dairy products including cheese and ice cream were also implicated in the outbreak. However, fruits and vegetables such as lettuce, tomatoes, cilantro, alfalfa-sprouts, and almonds have also been implicated in recent outbreaks (Andrews, 2005).

2.1.3.2 *Salmonella enterica* serotypes infections

Salmonella causes three forms of disease: typhoid fever, gastroenteritis, and bacteremia. *Salmonella enterica* serovar Typhi is the most invasive type and it causes typhoid fever, a systemic disease in humans. *S. enterica* serovar Paratyphi causes typhoid-like infection in humans. *Salmonella enterica* serovar Typhimurium and serovar Enteritidis cause self-limiting gastroenteritis or enterocolitis, which is mostly localized to the gastrointestinal tract and these two are the most common serovars responsible for human infections. *S. enterica* serovar Typhimurium causes typhoid-like infection in mice. *S. enterica* serovar Choleraesuis, a swine-adapted pathogen, causes septicemia (paratyphoid) in pigs. A bovine-adapted *S. enterica* serovar Dublin causes bacteremia, inflammation in the digestive tract and abortion in cows, and serotype Arizonae infects reptiles. Importantly, these serovars (Choleraesuis, Dublin, and Arizonae) occasionally cause infections in humans. *S. enterica* serovar Pullorum and *S. enterica* serovar Gallinarum cause infection in poultry (Bhunia, 2008).

2.1.3.2.1 Typhoid Fever

Salmonella enterica serovar Typhi causes systemic febrile illness called typhoid fever, characterized by the ingestion of food/water contaminated with human feces. Symptoms of typhoid fever included high fever, malaise, headache, nausea, myalgia, anorexia, constipation, chills, convulsions, and delirium (Zhang *et al.*, 2003).

2.1.3.2.2 Gastroenteritis

The non-typhoid *Salmonella* serotypes such as *S*. Enteritidis and *S*. Typhimurium are widely distributed in nature, including in the gastrointestinal tracts of mammals, reptiles, birds, and insects. Most clinical infections of humans are transmitted from healthy carrier animals to humans through food. The main clinical manifestation of human infection with non-typhoid *Salmonella* is an acute gastrointestinal illness and, less frequently, septicemia (Mølbak *et al.*, 2002). *Salmonella* Typhimurium causes serious illness in children, and immune compromised individuals, often resulting in systemic infection. In healthy individuals, symptoms include fever, diarrhea, abdominal pain, and sometimes vomiting (Bhunia, 2008)

2.2 Typing Approaches

The ability to discriminate or subtype foodborne pathogens below the level of species has been applied successfully to aid the epidemiological investigation of outbreaks. Reliable, sensitive and informative subtyping methods are required to

recognize outbreaks of infection, match case isolates with those from potential vehicles of infection and discriminate these from unrelated strains. Sensitive and discriminatory subtyping methods also are required for surveillance programs to identify new or emergent strains or clones that may present a new risk to public health. These methods also are fundamental to epidemiological research projects to identify potential reservoirs of strains that cause disease in humans, identify routes of transmission and improve our understanding of the epidemiology of foodborne disease (Swaminathan et *al.*, 2001).

2.2.1 Phenotypic Methods

Traditionally, bacterial pathogens have been identified by the examination of phenotypic characteristics of organisms. The methods of characterization used include biochemical profiles based on metabolic activity combined with methods such as serotyping and phage typing. These and other phenotypic-typing techniques utilize the expression products of particular genes that may be present in the different strains of bacteria to separate organisms. Phenotypic subtyping methods include biotyping, which discriminates between strains on the basis of specific biochemical reactions, and their ability to grow in the presence of certain chemicals, pH gradients, temperatures or gaseous atmospheres. Biotyping is often used to identify isolates to species level, but the method has poor discriminatory power and a limited ability to differentiate between isolates below the level of species (Foley and Grant, 2006).

Serotyping uses differences in the somatic (O) and flagellar (H) surface antigens to separate strains into distinct serotypes (Voogt *et al.*, 2002). For serotyping, the

suspension of bacteria is mixed and incubated with a panel of antisera specific for a variety of O and H epitopes. Specific agglutination profiles are used to determine the serotype of the isolate being tested. Kauffmann–White scheme is an important tool for classification of *Salmonella* and more than 2500 serotypes are recognized by *Salmonella* Serotyping (Popoff, 2000).

Phage typing utilizes the selective ability of bacteriophages to infect certain strains of bacteria (Schmieger, 1999). This differential ability of bacteriophages to infect bacteria is related to the phage receptors present on the surface of the bacteria (Snyder and Champness, 1997). There are a number of phage typing schemes available for discriminating among foodborne pathogens including *Salmonella, E. coli,* and *Campylobacter* (Frost *et al.,* 1999; Barrett *et al.*, 1994 and Humphrey, 2001).

Phage typing has been shown to be useful in the description of pandemic clones such *S. enterica* serovar Typhimurium definitive type 104 (DT104), a relatively common cause severe gastrointestinal illness in humans and is typically resistant to multiple antibiotics and the pathogenic *S. enteric* serovar Enteriditis phage types 4 and 8 (Humphrey, 2001).Thus far, many phenotypic methods are not universally applicable and are useful for all species. Variability in gene expression and the acquisition or loss of DNA-carrying genes, which encode phenotypic traits, can lead to changes in the phenotype displayed by the strain. This can lead to closely-related strains exhibiting different phenotypic traits and unrelated strains exhibiting indistinguishable subtypes (Riemann, 2006).

2.2.2 Genotypic Methods

The limitations of phenotypic subtyping methods and the rapid growth of molecular biological techniques have led to the development of a range of molecular subtyping methods. Molecular subtyping methods target genotypic variation within the DNA sequence of the organism (Thomas, 2003).

The majority of the molecular methods developed for characterization of bacteria have been applied to *Salmonella* both for epidemiological investigation and for research. The choice of method depends on the serotype and specific circumstances, and often more than one method is used to improve the quality of the typing (Riemann, 2006).

Plasmid profiling was originally the most popular method for molecular typing of *Salmonella*. It offers excellent discriminatory power for many serotypes, where plasmids of varying sizes occur frequently (Olsen, 2000). It can be particularly useful in the investigation of foodborne disease outbreaks, but is less useful for large-scale, population-based studies covering extended periods of time because the plasmid genotype can be relatively labile due to loss or uptake of plasmids.

Chromosome-based typing methods primarily consist of restriction endonuclease methods, where the chromosome is cut into fragments of various sizes. The results are subsequently analyzed by interpretation of the patterns produced by gel electrophoresis (DNA fingerprints), or in combination with hybridization to DNA-blots to highlight variation in selected loci by use of hybridization probes (RFLP-pattern). RFLP-based

methods, such as ribotyping (Grimont and Grimont, 1986) and IS*200* typing (Stanley *et al*., 1991) were previously used extensively with *Salmonella,* but are now uncommon.

Pulsed field gel electrophoresis (PFGE) is an applicable method for typing and good standardized protocols have been developed for use with *Salmonella* (Swaminathan *et al*., 2001).

PFGE profiling effectively subdivides most serotypes, where other methods fail to do so (Olsen, 2000). However, discrimination within clones, such as phage type DT104, is less efficient (Liebana *et al*., 2002). Lack of stability of DNA during the analysis is an obstacle with some serotypes (Stanley *et al*., 1995; Baggesen *et al*., 1996), but substitution with HEPES buffer for the normal Tris-borate buffer during preparation may help overcome this problem (Koort *et al*., 2002).

The PCR is a rapid and reliable method for detection and identification of foodborne pathogens such as *Salmonella* (Hill, 1996). Recent methods for DNA fingerprinting have been developed using polymerase chain reaction (PCR) amplification of random genomic DNA fragments with single or multiple arbitrary primers (Welsh and McClelland, 1990; Williams *et al*., 1990). One advantage of arbitrarily primed PCR (AP-PCR) is that it can be used without previous knowledge of the nucleotide sequence of the target DNA (Caetano- Anolle´s *et al*., 1991). AP-PCR typing method is based on low-stringency PCR amplification by using a single, 10 to 20-mer primer of arbitrary sequence. In the early cycles of the PCR reaction, the primer anneals to multiple sequences with partial homology, and fragments of DNA lying within less than a few kb between annealing sites on opposite DNA strands are amplified. After additional cycles, a strain-specific array of amplified DNA segments of

various sizes is obtained. This simple and rapid technique has been successfully applied to genotypic strain delineation and genetic population analysis of a broad range of microbial pathogens such as, bacteria. All isolates are typeable and no prior knowledge of target genome sequences is necessary. Discrimination is good and correlates well with other genotyping techniques. The discriminatory power is variable according to number and sequence of arbitrary primers an amplification conditions. In spite of its attractive efficiency, AP-PCR typing suffers from problems in lack of consensus rules for interpretation of pattern differences **(**Maslow & Mulligan 1996, Struelens *et al.*, 1996**).**

Restricted AP-PCR (ResAP-PCR) is described as a modification of the AP-PCR technique employing endonuclease restriction enzymes. To perform this technique, the genomic DNA of the selected strain has to be digested with HaeIII restriction enzymes prior to amplification with three 9 mer oligonucleotid primers. The primers have been selected based on genome sequence of each strain, and their usage to genotype member of the same species is likely to yield comparable bands patterns by agarose gels electrophoresis. This technique is only available for organisms with appropriate C+G content (around 50% or higher) at their genome. Few data have been published on the applicability of the resAP-PCR for the analysis of bacterial strains. Bikandi *et al*. (2008) applied this technique to differentiate 27 *Salmonella* isolates belonging to 13 serotypes of *S*. enteric where they have shown that resAP-PCR technique is discriminatory and fingerprints of the test stains were highly reproducible.

In the current study, resAP-PCR was used as a subtyping tool for 49 *S.* Typhimurium strains.