CHAPTER 5: DISCUSSION

Bacteria of the genus *Salmonella* are widespread and important causes of foodborne infections in man, and are the most frequent etiologic bacterial agents of foodborne disease outbreaks. Non-typhoidal *Salmonella* serotypes are widely distributed in nature, including in the gastrointestinal tracts of animals. Most clinical infections of humans are transmitted from healthy carrier animals to humans through food. In particular *S*. Typhimurium, became major causes of foodborne illness in the 1980s and 1990s, with an important impact on public health and the economy in industrialized countries (Riemann *et al.,* 2006).

Therefore, the ability to distinguish *Salmonella* Typhimurium strains may be very important and typing of *S*. Typhimurium strains is a tool for the prevention and control of specific infection, and also for risk assessment studies of the sources of zoonotic diseases.

The classical phenotypic methods are hampered with regard to practicability and poor discrimination and therefore have been complemented by the more sensitive DNA-based, molecular techniques. However, there is still a lack of methods with the right combination of rapidity, ease of use, reproducibility, and discriminatory power for typing of *S*. Typhimurium.

PCR-based typing is one of the genotypic methods which has been used popularly in recent years for subtyping of bacterial strains because of its simplicity and ease of operation. These PCR-based methods have been used such as, RAPD, ERIC, Rep-PCR and AP-PCR. For example, in comparison between RAPD, RFLP and Rep-PCR, Vila *et*

al., (1996) found RAPD was more discriminative than RFLP but less discriminative than Rep-PCR. In some studies, RAPD method was successful in differentiate *Salmonella* serotypes (Carraminana *et al.,* 1997 and Betancor *et al.,* 2003).

Arbitrarily Primed PCR (AP-PCR) is DNA-based typing method based on the amplification of genomic DNA with a single primer selected from an arbitrary nucleotide sequence. The multiple products resulting from AP-PCR are then separated according to size by conventional agarose gel electrophoresis, and the DNA banding patterns of different isolates can then be compared.

Restricted AP-PCR (resAP-PCR) is a modification of the AP-PCR method using endonuclease restriction enzyme followed by AP-PCR amplification. In the present study 49 *S*. Typhimurium strains were subtyped by resAP-PCR method to evaluate the usefulness of this method as a subtyping tool.

For the evaluation of each typing method based on the principles of typeability, reproducibility, discriminatory power, ease of performance, 49 selected isolates from sporadic cases of different years (1969 to 2006) were considered.

In term of typeability, all *S*. Typhimurium wee typeable by resAP-PCR (*Hae*III digested DNA) in assigning a defined type to each isolate tested. In terms of reproducibility, stable and reproducible patterns were confirmed for resAP-PCR method. Some findings were also noted that AP-PCR and resAP-PCR has reproducible patterns. For example, AP-PCR fingerprints have been considered reproducible (Berg *et al.,* 1994; Louws *et al.,* 1994 and Fadl *et al.,* 1995). Reproducible patterns for 27 *Salmonella* strains were reported by Bikandi *et al*. (2008).

Also, several findings were reported a number of problems for AP-PCR assays that contribute to a lack of reproducibility and standardization. Since the primers are not directed against any particular genetic locus, many of the priming events are the result of imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns. The use of empirically designed primers, each with its own optimal reaction conditions and reagents, also makes standardization of the technique difficult (Arbeit *et al*., 1994; Meunierand Grimont, 1993, Welsh and McClelland, 1990). Moreover, arbitrary amplification protocols could be affected by other factors, which include DNA extraction methods, ratio of DNA template concentration to primer concentration, batch-to-batch variation in primer synthesis, Mg^{2+} concentration, PCR conditions, model of thermocycler used, and supplier and concentration of *Taq*DNA polymerase. Most of the problems can be overcome by optimizing the reaction conditions for each organism analyzed (Penner *et al*., 1993; Berg *et al*., 1994).

In terms of discriminatory, resAP-PCR assay was able to generate DNA polymorphism in thirty-eight percent of isolates, and therefore clonality and limited diversity were found in most of AP profiles. Since, the discriminatory power of this genotyping assay was low among nonrelated isolates; we conclude that the usefulness of the individual method for molecular typing of *S*. Typhimurium is questionable.

Although, some findings were noted that AP-PCR and resAP-PCR are highly discriminative. For example, Burr and Pepper (1997) believed that high variability was demonstrated in presence-absence scoring of bands in AP-PCR fingerprints, which

often prevented a computer a computer image analysis program from correctly matching known isolates. Also, restricted AP-PCR method has been used by Bikandi *et al*. (2008) and, they found excellent results in genotyping of 27 *Salmonella* strains. They believed that restricted AP-PCR could be used to genotype most of pathogen.

In terms of ease of performance, resAP-PCR method was simple to perform although careful attention must be given to the preparation of template, master mixes and agarose gels to ensure reproducibility and ease of gel to gel comparison. The method was cost-effective.

5.1 Restricted AP-PCR Analysis

The genetic diversities of *Salmonella* Typhimurium isolates from sporadic cases (14 from humans, 35 from animals) were analyzed via the application of restricted AP-PCR method.

Fingerprinting profiles generated by restricted AP-PCR were analyzed with GelCompare II software. The profiles were scored for the presence and absence of DNA bands, and strains differing by one DNA band assigned different restricted AP profiles. Clustering was predicated on the unweighted pair group average method (UPGMA) (Liebana *et al.*, 2001; Woo, 2005). In particular, similarity coefficient and discriminatory index (*D*) were obtained from clustering analysis.

Discriminatory index is a measure of the probability that a method will assign a different type to two unrelated strains sampled randomly and should generate a value of

>0.95 (Blanc *et al.,* 1998). Therefore, unrelated strains should be used to test the discriminatory capacity of a fingerprinting method and, ideally, each strain should give a distinctive fingerprint.

In current study, discriminatory index obtained from clustering analysis was found to be *D*=0.71. This index indicates that if two strains were sampled randomly from the population, then on 71% of occasions they would fall into different types (Hunter and Gaston, 1988).

Restricted AP-PCR fingerprinting discriminated 49 *S*. Typhimurium strains into 19 genotype profiles. Among 14 human strains, there were 9 AP profiles while, among 35 animal strains there were 16 AP profiles. Findings showed there are 8 strains (3 from humans and 5 from animal) had unique AP profiles.

Based on 70% similarity, they were closely related and grouped into two major clusters, cluster A and cluster B. In subcluster A1 which consisted of 24 strains, genetic uniformity was obtained in 14 strains. Fifty-eight percent of strains in subcluster A1 that grouped was together isolated in year 2005. We may suggest, they could be a part of one epidemiological outbreak in 2005.

Furthermore, in subcluster A1, the AP3 profile was presented by 14 strains isolated between years 1970 until 2006. This is a predominant genotype profile which conserved within 36 years. Likewise, this conserved AP profile was shared by human and animal strains and we may suggest human Salmonellosis were the results of the circulation of certain strains between animal and human hosts.

In sucluster A2 which consisted of 8 strains (2 strains from humans and 6 strains from animals) diversity of AP profiles was obtained. AP1, AP14 and AP17 were represented by 8 strains, suggests the infections were from various sources.

In subcluster B1, diversity of AP profiles were obtained. AP8, AP16 and AP2 were presented by 6 strains. Six strains with sporadic sources were grouped together, isolated in different places within different years. Therefore, findings showed this technique could not discriminate among different strains.

In subcluster B2 which consisted of 5 strains, three AP profiles were obtained. Based on findings, animals and humans isolates from various areas over different years, shared same AP profiles. For example, human strain (STM 113254/70) which isolated in year 1970 shared same AP profiles with chicken strain (STM 1204/05) isolated in 2005. We can suggest that certain *S*. Typhimurium may be the prevalent strains and there was a recirculation of certain subtypes of strains between animals and humans.

In summary, the findings showed that this technique was not able to detect any evidence of absolute host specificity for the strains because 41 strains from humans and animals shared same AP profiles and same characteristics. Based on discriminatory index (D = 0.71), restricted AP-PCR was moderate to discriminate of 49 *Salmonella* Typhimurium strains. However, this technique was rapid and simple and highly reproducible.