PREVALENCE, ANTIBIOTIC RESISTANCE AND GENETIC DIVERSITY OF *SALMONELLA* SEROVARS ISOLATED FROM VARIOUS MEATS OF WET AND HYPERMARKETS IN PULAU PINANG AND PERLIS MALAYSIA

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by

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Hafiz Nidaullah

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LIST OF ABBREVIATIONS

Aml	Amoxicillin
Amp	Ampicillin
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
ºC	The degree Celsius
C	Chloramphenicol
CDC	Center for Disease Control
CLSI	Clinical and Laboratory Standards Institute
Cn	Gentamicin
Caz	Ceftazidime
Cip	Ciprofloxacin
ELFA	Enzyme-linked fluorescent assays
ELISA	Enzyme-linked immunosorbent assays
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FAO	Food and Agricultural Organization
GMP	Good manufacturing practice
g	Gram
HACCP	Hazard analysis critical control points
h	Hour
ISO	International Standard Organization
K	Kanamycin

KBP	kilo base pair
LIA	Lysine Iron Agar
MIC	Minimal Inhibitory Concentration
MKTTN	Muller-Kauffmann Tetrathionate Novobiocin
ml	Milliliter
MHA	Muller Hinton Agar
Na	Nalidixic acid
NCCLS	National Committee for Clinical Laboratory Standards
OIE	Office International des Epizooties
pH	Potential of Hydrogen ion
PCR	Polymerase chain reaction
QMRA	Quantitative Microbial Risk Assessment
RVS	Rappaport-Vassiliadis with Soya
Rl	Sulphamethoxazole
SPIs	*Salmonella* Pathogenicity Islands
SPSS	Statistical Package for Social Science
S	Streptomycin
Te	Tetracycline
TSI	Triple Sugar Iron
TTSS	type III secretion systems
W	Trimethoprim
XLD	Xylose Lysine Deoxycholate
XLT-4	Xylose Lactose Tergitol 4

PREVALENS, KERINTANGAN ANTIBIOTIK DAN KEPELBAGAIAN GENETIK SEROVAR *SALMONELLA* YANG DIPENCILKAN DARIPADA PELBAGAI DAGING DARI PASAR BASAH DAN PASAR RAYA BESAR DI PULAU PINANG DAN PERLIS MALAYSIA

ABSTRAK

Kajian ini telah dijalankan untuk menilai pelbagai nic (*niche*) bagi kewujudan *Salmonella* dalam persekitaran penyembelihan ayam di pelbagai pasar basah dan di kilang pemprosesan ayam berskala kecil di Pulau Pinang dan Perlis, Malaysia. Sebagai tambahan, pencirian risiko kualitatif bagi rintangan antibiotik, penentuan ketahanan genotip, potensi kemudaratan dan kepelbagaian genetik bagi *Salmonella* `serovars` yang dipencilkan daripada pelbagai daging mentah yang dikumpul dari pasar basah dan pasaraya yang berbeza telah dijalankan di Pulau Pinang, Malaysia. Sejumlah 582 sampel termasuk sampel dari persekitaran pemprosesan ayam (182) dan pelbagai sampel daging mentah termasuk daging lembu, ayam, daging kambing, dan ikan yang dikumpul dari pasar basah (240) dan pasaraya (160) telah terkumpul. Keputusan mendedahkan bahawa keseluruhan pemprosesan ayam telah dicemari oleh *Salmonella* (86.1%) beserta daging ayam (100%). Antara daging mentah runcit yang dikumpul di pasar basah dan pasaraya, secara keseluruhannya membuktikan bahawa kewujudan *Salmonella* sebanyak 31.25% (125/400), yang mana 41.67% telah ditemui sebelum ini dan 15.63% telah ditemui setelah itu. Selain itu, daging ayam mentah telah didapati sangat tercemar bagi pasar basah (83.3%) dan pasaraya (35%). Rintangan maksimum telah berlaku pada `tetracycline` (57.6%), diikuti oleh `sulfamethoxazol` (48.8%), `streptomycin`

(25.6%) dan `ampicillin` (14.4%). Model pencirian risko menunjukkan empat kes `very high additional risk` (VHAR) terhadap ketahanan, dua kes bagi isi ikan (`streptomycin` dan `ampicillin`) dan satu kes bagi daging lembu (`streptomycin`) serta daging ayam (`streptomycin`). Penyebaran pelbagai gen SPI (`*Salmonella* pathogenic Island`) di kalangan *Salmonella* serovars menunjukkan 100% penyebaran bagi gen *inv*A (SPI-1). RAPD-PCR mendedahkan bahawa 41, 26. 14, 14 dan 7 strain *S.* Indiana, *S.* Corvallis, *S.* Bareilly, *S.* Newport dan *S.* Senftenberg telah dikumpulankan kepada kluster major 8, 5, 4, 4, 2 dengan kepelbagaian genetik beranggaran dari 31- 100%, 26.5-100%, 28-100%, 25-100% dan 58-100%, bagi setiapnya. Sesetengah strain dari sumber yang berbeza (daging lembu, ayam, ikan, dan kambing) dan lokasi (pasar basah dan pasar raya) berkongsi corak RAPD yang sama dan dikumpulankan dalam kluster persendirian. Dalam bahagian akhir penyelidikan ini, kedua-dua 3M^{TM} MDA dan Sistem 3M^{TM} Petrifilm SALX, menghasilkan persetujuan yang betul dengan kaedah asas-kultur. Sebagai kesimpulan, penyelidikan ini menunjukkan penyebaran yang berskala tinggi, pertahanan antimikrobial serta profil genotip dan kepelbagaian yang luas dan pertimbangan potensi kemudaratan bagi *Salmonella* kepada kesihatan pengguna. Oleh itu, dicadangkan bahawa pengukuran bio-sekuriti yang efisien dan pemantauan yang lebih kerap bagi pelbagai daging mentah di pasar basah dan pasaraya serta rantaian tekanan amat diperlukan untuk membasmi pembawa patogen makanan ini.

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ABSTRACT

Present study has been endeavored to assess various niches of *Salmonella* in the chicken processing environment at various wet markets and small-scale processing plant in Penang and Perlis, Malaysia. In addition, qualitative risk characterization of antibiotic resistance, genotyphic resistance determinants, virulence potential and genetic diversity of *Salmonella* serovars isolated from various raw meats collected from different wet and hypermarkets was carried out in Penang, Malaysia. A total of 582 samples including chicken processing environmental samples (182) and various raw meat samples including beef, chicken, mutton, fish were collected from different wet markets (240) and hypermarkets (160). As a result entire environment encompassing chicken processing line was found contaminated with *Salmonella* (86.1%) along with chicken meat (100%). Amongst retail raw meats collected from traditional wet and hyper markets, overall prevalence of *Salmonella* was 31.25% (125/400), out of which 41.67% was found in former and 15.63% in later case. Further, raw chicken meat was found to be highly contaminated in both wet (83.3%) and hyper (35%) markets. Maximum resistance was found for tetracycline (57.6%), followed by sulfamethoxazol (48.8%), streptomycin (25.6%) and ampicillin (14.4%). Risk characterization model expressed four cases of very

high additional risk (VHAR) of resistance; two cases in fish meat (streptomycin and ampicillin) and one case each in beef (streptomycin) and chicken meat (streptomycin). Distribution of various SPI (*Salmonella* pathogenic Island) genes among *Salmonella* serovars showed 100% prevalence of *inv*A (SPI-1) gene. RAPD-PCR revealed that 41, 26. 14, 14 and 7 strains of *S.* Indiana, *S.* Corvallis, *S.* Bareilly, *S.* Newport and *S.* Senftenberg were grouped into 8, 5, 4, 4, 2 major clusters with a genetic diversity of strains ranged from 31- 100%, 26.5-100%, 28-100%, 25-100% and 58-100%, respectively. Some of the strains from different sources (beef, chicken, fish and mutton) and locations (wet market or hypermarkets) shared the same RAPD patterns and were simultaneously grouped in single clusters. In the last part of this work, both 3M^{TM} MDA and 3M^{TM} Petrifilm SALX System yielded substantial agreement with the culture-based method. In conclusion, this study indicates high prevalence, extensive antimicrobial resistance as well as genotypic profiles, wide diversity and deliberate virulence potential of *Salmonella* to consumer's health. It is thus suggested that efficient bio-security measures and regular surveillances of various raw meats in wet and hypermarkets along with the processing chain would be indispensable to eradicate this foodborne pathogen.

CHAPTER 1

INTRODUCTION

1.1 General background

Emergence of foodborne diseases represents a serious problem with reference to public health and addressing this issue has become a global effort in terms of rapid surveillance studies and designing highly sensitive methods of laboratory detection and identification of infectious bacteria to help in developing efficient strategies for their prevention and control. Each year one out of ten citizens suffers as a result of consuming contaminated food worldwide (Nadon *et al.* 2017). It is well understood that one of the major focus of attention on global level has been to ensure safe food supplies due to the recognition of vast variety of pathogenic bacteria in contaminated food. Most of the foodborne pathogens possesses zoonotic nature, hence causing serious illness categorized into three ways (Ruban *et al.* 2012; Dhama *et al.* 2013), such as;

i) Food infection

ii) Food intoxication

iii) Toxico-infection

Explosive growth in human population and modernization in food consumption styles by following urbanization, income increment, demographic shifts and perceptions of consumer with regard to quality and safety of food has resulted in an increased demand of animal products like meat, milk and eggs as a cheap and high value protein sources (Regmi, 2001; Gehlhar and Coyle, 2001). It is worth mentioning that global meat production would show a rising trend reaching up to 376 million tons per anum with an increased rate of per capita consumption by 2030 as anticipated by Food and Agriculture Organization (FAO, 2013). In spite of modern innovations in the

food chain domain, food safety has been at the forefront for alleviating public health issues throughout the world (Ruban *et al.* 2012). Importantly, infectious bacteria can enter into the food chain that have been exposed to animal feces during processing (Dhama *et al.* 2013) and are ultimately transmitted to the consumers via contaminated food. Amongst other sources, retail raw meat that is contaminated with pathogenic bacteria like *Salmonella*, *Campylobacter* and *Escherichia Coli* etc. has been considered as one of the key sources of foodborne illness (Ruban *et al.* 2011). Moreover, it has been reported by World Health Organization (WHO) that common causes of diarrhea include the consumption of raw or under-cooked meat, eggs, fresh produce and dairy products contaminated with *Norovirus*, *Campylobacter*, *Salmonella* and *E. coli* (WHO, 2015). In addition, it has been estimated that foodborne *Salmonella* is the second largest cause of foodborne illness after *Campylobacter* species (Pathogen, 2011). Most of the people who become infected with *Salmonella* develop diarrhea, fever and abdominal cramps within 12 to 72 hours after the consumption of contaminated foods (Russel, 2012). Approximately 93 million cases were annually recorded worldwide along with associated problems of stomach and 155,000 deaths occur each year due to foodborne *Salmonellosis* (Ta *et al.* 2014). Considering Asia in this respect, the ratio of foodborne illness particularly in Southeast-Asia has become alarming with approximately 22.8 million gastro-enteritis cases and 37600 deaths per anum reported due to *Salmonellosis* (Akbar and Anal, 2014). These bacteria can easily be eliminated from food sources if it is cooked thoroughly, however food contaminations in many cases were caused by food handlers (butchers, workers, vendors) as well due improper hygiene at traditional wet markets, food processing plants and retail shops (Russel, 2012).

The spp. *Salmonella* are capable of causing some serious infections that are quite often foodborne and are clinically manifested as gastroenteritis (Fluit, 2005; Myšková and íšková, 2017). These pathogens survive in three main groups on the basis of host preferences (Pui *et al.* 2011). The first group includes host-restricted serotypes that cause infections only in humans such as *S.* Typhi. In the second group are included different host-adapted serotypes which are associated with one host species but can cause diseases in other hosts as well, for example, serotype such as *S.* Pullorum in avian. The third group encompasses the remaining serotypes that are foodborne and mainly have zoonotic nature. Typically, *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg are the three most frequent serotypes usually recovered each year from humans (Gray and Fedorka-Cray, 2002; Boyen *et al.* 2008).

Generally, human *Salmonellosis* is the outcome of ingesting contaminated food items (Geimba *et al.* 2004; Zaki *et al.* 2009; Park *et al.* 2014). Amongst raw food, poultry meat and eggs are considered to be one of the most important reservoirs from which *Salmonella* is subsequently passed through the food chain and ultimately transmitted to humans (Oliviera *et al.* 2002; Ricke, 2003; Maciorowski *et al.* 2004; CDC, 2009; Finstad *et al.* 2012; Howard *et al.* 2012; Park *et al.* 2014).

With regard to food safety, cross contamination of gut microbiota with meat carcass might occur at any stage during food production and it is an important factor that must be taken into account (Dincer and Baysa 2004: Ruban *et al.* 2011). It is caused by the spreading of *Salmonella* in food processing plants, attachment with various equipments like cutting boards, bleeding drums hence forming biofilms. Typically, raw meat and related food products can get contaminated with the foodborne bacteria that make their way from the intestinal tract or from feacal material attached to the feet or feather of food animals during evisceration and further

processing (Nidaullah *et al.* 2016). Mitigation of microbial contamination has also been a focus of attention in this regard (Arnold and Silvers, 2000).

Modernization and installation of computerized equipments have vastly increased the number of carcasses processed by a single plant every day. The utilization of such modern tools in these plants have a profound impact on increasing the susceptibility of carcass surfaces towards contamination due to the repeated contacts during processing which has ultimately increased the chances for bacterial attachment and hence cross contamination (McEldowney and Fletcher, 1988; Arnold and Silvers, 2000). However, the sources of carcass contamination and the mechanisms by which the organisms spread between meat carcasses during processing are not fully understood as yet. There is a pronounced need to develop knowledge for designing efficient strategies in order to reduce the risks due to foodborne bacteria.

Global consumption of various types of meat such as beef, poultry, mutton and fish has become doubled within the past two decades (Yip *et al.* 2018). An increase in the impulse of per capita meat consumption as well as high rate of population growth at global level aggravated the overall increase of meat production and consumption. It is expected that the world population will surge up to 9 billion by 2050. In this scenario, the share of meat protein for consumers in developing as well as emerging countries is growing as a result of rapid economic growth (Delgado, 2003). According to the latest report meat consumption in the aforementioned countries have been found to increase from an average annual per capita consumption of 10 kg in the 1960s to 26 kg in 2000, reaching 37 kg around the year 2030 (OECD-FAO, 2018). The latest statistics published by "*The Organization for Economic Co-operation and Development* (OECD)" about the meat consumption in different regions of the world has been shown in table 1.1.

Table 1.1: Per capita meat consumption by region (source: OECD/FAO, 2018),

Region	**Beef**	**Sheep**	**Poultry**
North America	24.31054	0.464734	45.64829
South East Asia	5.289314	0.419222	13.11896
European Union	10.83314	1.889887	23.09094
Latin America and Caribbean	17.08654	0.547334	31.25476
Asia and Pacific	2.963895	1.746526	8.641543
Africa	3.738247	2.252353	3.73848

Food and agriculture organization of the United States (FAO, 2017) reported that the global meat production has been raised by a meagre of 0.3 percent to 322 million tones in the year 2017. Whereas, an increased growth rate in the production line is expected in almost all countries, in particular the U. S, Argentina, Brazil and India (FAO, 2017). Moreover, worldwide per capita meat consumption is considered to be stagnate at the rate of 34.6 kg retail weight by 2026. Nevertheless, the increasing trend of population growth in most of the developing countries, it has been estimated that an increase of the total meat consumption is expected up to 1.5% per annum, which will mainly include the consumption of poultry and declination in the pig meat at global level. A positive correlation exist between economic status of a country and the rate of meat consumption. An estimated healthy diet consist of 10 gram of beef, 47 gram of chicken and eggs, and 23 gram of fish, per person, per day on average (Vranken *et al.* 2014). Mostly, the lower rate of income of a country directly related with the decreased level of meat consumption and meat products raise with an

increases in per capita GDP (Allievi *et al.* 2014). An example of such scenario with respect to the global meat consumption observed during early 2000 in the emerging economies, where meat consumption grown from 209 million tons up to 270 million tons (1.3 times). Besides, a constant rate of per capita meat consumption between 70 kg and 90 kg were observed in most of the EU countries including middle-income countries of Eastern Europe. Moreover, in Taiwan, even though it is located in Asia, where food culture differs significantly from that of western countries, per-capita meat consumption has been moving sideways after reaching the same level as that seen in Europe (80 kg). In Asia, meat consumption is stabilized in Japan (50 Kg), which exceptionally considered as low compared with other countries such as Taiwan (80 Kg). Such low level of meat consumption in Japanese society is directly related with the consumption of high level of sea-foods as a source of protein. However, it has been noticed that per capita seafood consumption is mainly recorded higher in South Korea than that of Japan, even though South Korean citizens eat a high quantity of meat. It means that the differences to gain meat protein cannot be estimated by the consumption of seafood only. In low income countries of Asia, meat consumption per capita has been raised from 24 kg to 39 kg in Myanmar and from 38 kg to 57 kg in Vietnam. In most of the countries where the element of a religious taboo exist against eating of meat such as pork and beef, has expressed a lower rate of per-capita meat consumption level such as Indonesia (12 kg) and India (3.4 Kg) respectively. While religious restrictions on the consumption of beef can be considered to be a reason for the low level of meat consumption in India, because the level of consumption of meat other than beef is also low, religious constraints do not appear to be the sole reason. In technologically advanced countries such as USA and Australia, where per-capita consumption has stabilized at a high level of about 120 kg and 100 kg respectively are

still found to increase gradually their meat consumption ratio. Moreover, meat consumption in Latin America is also raising such as Argentina (100 kg) and Brazil (90 kg). It has been noticed that the reason behind high meat consumption in these countries is due to low price and cost of producing beef animals on account of extensive land area for grazing. Also, worldwide, a high percentage of beef meat consumption certainly account for these two countries compared with other countries (Nozaki, 2016).

In Malaysia, food industry is the second largest manufacturing sector after textiles, encompassing fisheries, livestock, fruits vegetables and cocoa (Fernando *et al.* 2014), with meat being the principle source of protein in diet (Kaur, 2010). Malaysia has been self-sufficient in poultry and eggs, however beef and mutton is imported while exports include fisheries and seafood products (MIDA, 2017). The consumption of poultry, beef and mutton is showing a wave of continuous increase in the country with an estimated rate of 2.4% every year (Nor and Rosali, 2015). This high rate of meat consumption is associated with the with the lowest price and high income per capita (Mohamed, 2007; Nor and Rosali, 2015).

Cross-contamination during food processing has become one of the main source of foodborne illness in Malaysia (Adzitey *et al.* 2012; Ngoi and Thong, 2013; Thung *et al.* 2016; Sing *et al.* 2016). In 2007, it has been reported by the Ministry of Health, Malaysia, that more than 50% of food poisoning cases in the country occur due to unhygienic food handling and it has also been confirmed that among foodborne pathogens, *Salmonella* is the leading cause of foodborne illness and regularly monitored by the National Laboratory Surveillance System, Ministry of Health, Malaysia (Soon *et al.* 2011; Salleh *et al.* 2014).

It is cumbersome to figure out the exact rate of food borne illness in Malaysia. Which is due to the lack of foodborne disease investigation and regular surveillance where most outbreaks often go undetected. However, it is interesting to note that in 2006, a total of 6938 cases of food poisoning were reported with an incidence rate of 26.04%, followed by a 100% rise of food poisoning cases in 2007 (incidence rate of 53.19%). The drastic increase in 2007 may not be showing a true increase in food poisoning cases (Soon *et al.* 2011), but the increase may be due to the improvement of the reporting and registration system, through the establishment of the Crisis Preparedness and Response Center (CPRC) in May 2007. Malaysia is one of the country that have high cases of foodborne diseases due to the suitable temperature and condition for the growth of most of the pathogenic bacteria. In a recent outbreak in Terengganu, Malaysia (2014), 49.5% of clinical cases and 18% of food-handlers were confirmed with *Salmonellosis*. Where the major serovar as identified was *S.* typhimurium (Ab Karim *et al.* 2017).

The core risk factors, for instance, unhygienic food handling practices, poor environmental sanitation, raw or lightly cooked food with prolong storage and food handling without safety procedures have been highlighted as key factors in Malaysia (Ezat *et al.* 2013). Numerous scientific studies recently published have confirmed the incidence of *Salmonella* in a variety of food items such as raw, cooked and dried foods, chicken meat, beef and seafood (Modarressi and Thong 2010, Roseliza *et al.* 2011, Thung *et al.* 2016, Budiati *et al.* 2016, Fauzi *et al.* 2017). It has been confirmed that that domestic cooking practice of poultry based meat preparations in home have the risk of *Salmonella* to be survived (Roccato *et al.* 2015). However, the recommended combination of suitable time plus temperature established is considered to 70 °C for 2 minutes, which is merely enough to mitigate most of the heat resistant bacteria in food

matrix. But in some case the reliability of such heat treatment for *Salmonella* is usually affected by the complex composition of meat and meat derived products (fat content, pH, NaCl and water activity) (Silva and Gibbs, 2012). During cross contamination, mostly bacterial pathogens contaminate the surface area of raw meat, but comminuted products such as burgers, nuggets and sausages, if got contaminated, then bacteria goes interiorly and in such cases undercooking of comminuted products is likely to provides greater chances for the survival of interiorly prevailed pathogens (Roccato *et al.* 2015).

Mostly *Salmonella* is present in the intestines or attached with feathers and feet, however, poor cleaning practices as well as sanitation usually lead to the cross contamination. The spreading of *Salmonella* in food processing environment happens because of its attachment with various equipments, for example, cutting boards, bleeding drums, etc. thus forming biofilms and in this way causing cross contamination (Nidaullah *et al.* 2016). Typically, the wet environment encountered in chicken processing plants is ideal for the growth of bacteria and biofilms formation. Studies have shown that mostly *Salmonella* in the contaminated chicken processing environment can be isolated from processing equipment, specifically involved in the slaughtering and evisceration premises (Helke *et al.* 1993; Helke and Wong, 1994; Joseph *et al.* 2001).

In Malaysia, most common serorvars of *Salmonella* prevailed in the food chain are *S.* Enteritidis (28.1%), *S.* Weltevreden (25.7%), *S.* Corvallis (10.3%) and *S.* Typhimurium (6.7%) (Modarressi and Thong, 2010). In one such study, Roseliza *et al.* (2011) reported. *S.* Enteritidis as a second most dominant serovar (12.5%) in various meat samples in Malaysia, which according to them mainly prevailed in poultry meat (23.3%) carrying homogeneity and genotypic persistency in the food chain (Ngoi and Thong, 2013).

Despite the reported prevalence of *Salmonella* in Malaysia, an increasing trend in the resistance potential to even latest generation of antimicrobials such as erythromycin, penicillin, vancomycin, tetracycline (Benacer *et al.* 2010; Tiong *et al.* 2010; Adzitey *et al.* 2011; Ngoi and Thong 2013; Thung *et al.* 2016) have been observed. In a recent investigation reported by Ngoi and Thong (2013), *S.* Entritidis were susceptible to ciprofloxacin but resistant to nalidixic acid (49%), tetracycline (43%), ampicillin (35%), sulfonamide (30%), trimethoprim-sulfamethoxazole (24%), and trimethoprim (23%). Ever increasing risk of antimicrobial resistance provides reduced therapeutic opportunities along with treatment failures and prolonged infections (Parsons *et al.* 2013).

Careless utilization of antibiotics as growth promoters in food animals or abuse in veterinary practices develop resistance in zoonotic bacteria (Van *et al.* 2012) such as *Salmonella spp*., *Campylobacter spp*. and *Escherichia coli* (WHO 2012). Such resistant strains of zoonotic bacteria can be transmitted to humans via food chain (Rahimi, 2012). Poultry meat and related products are considered as main reservoirs of antibiotic resistant *Salmonella*, among other foods (Abd-Elghany *et al.* 2015). This has alarmed the consumers regarding the imprudent uses of various antibiotics in food animal production (Kariuki *et al.* 2015). In recent years, the outbreak of multi-resistant strains has made the treatment even more challenging due to reduced efficacy of antibiotics (Abd-Elghany *et al.* 2015).

In the developing countries, risk of antibiotic resistance in foodborne bacteria is very high (Yang *et al.* 2013). For instance, approximately 92, 68 and 85% of *Salmonella* were resistant to tetracycline, streptomycin and sulfonamides, respectively from animal based foods in Vietnam, Thailand and Malaysia (Van *et al.* 2012). However, a gap exists in regulating the harmonized monitoring of antimicrobial

resistance among clinical, veterinary, agricultural and environmental areas (WHO 2015).

In spite of known multi-drug resistivity of *Salmonella* in Malaysia, the characteristics of drug resistant genes and virulence potential of *Salmonella* is of key importance in modern era of molecular biology. The overuse of antimicrobials in meat-producing animals is considered as the main cause of resistance in *Salmonella*, which ultimately results in difficulties to treat effectively the clinical cases and outbreaks of *Salmonellosis* mainly caused by resistant strains (Cossi *et al.* 2013; Hur *et al.* 2012). One of the primary mechanisms through which these bacteria acquire antimicrobial resistance genes is lateral gene transfer of genetic material (Davies *et al.* 1997). This mechanisms has been linked with the development of resistance of bacteria to multiple antimicrobial agents is the site-specific recombination of integron gene sequence, or integrons (Bailey *et al.* 1988; Collis and Hall, 1995). In fact, integrons are mobile genetic elements that provide the components of a site-specific recombination system allowing the recombination of antimicrobial resistance genes (Ochman *et al.* 2000; McEwen *et al.* 2002).

Besides, the emergence of antibiotic resistance of *Salmonella* serovars, mechanisms involved in the intestinal invasion of non-typhiodal *Salmonella* (NTS) is vital to reveal the pathogenicity and virulence profiles. A range of virulence factors exists among *Salmonella* serovars which are mainly responsible for survival ability and pathogenicity inside the body of hosts (Zhao, 2002). Such virulence factors are mostly specific to each particular serovar of *Salmonella* and includes plasmid mediated virulence genes (*spv*), surface cell structure, flagellin, and pathogenicity islands (SPIs) (Andino and Hanning 2015). Most of virulence factors are assorted at a specific region of chromosome, known as "*Salmonella* Pathogenicity Islands" (SPI) (Santos et al. 2003). These SPIs are usually located on chromosome or plasmid and composed of various

combinations of G/C contents. Usually, they are associated with tRNA, mobile genetic elements, transpons, bacteriophage (Schmidt and Hensel 2004).

In Malaysia, it is rather cumbersome to figure out the exact rate of foodborne illness because the current foodborne disease data is actually collected via physician-based surveillance where it has been predicted that only single out of approximately 38 cases are reported (Soon *et al.* 2011). In principle, thorough-going investigation of foodborne outbreaks demands a highly efficient surveillance system. Effective surveillance of *Salmonella* spp. basically depends upon the accurate isolation, confirmation and precise typing (Adzitey *et al.* 2013). This can be accomplished by using a combination of both conventional cultural methods and numerous molecular techniques to detect and identify different foodborne pathogens (Shea *et al.* 2016). Typically, conventional cultural methods require a preliminary non-selective pre-enrichment step followed by selective enrichment and agar plating, with further confirmation by biochemical and serological based tests (Lee *et al.* 2015). Such cultural based methods are a source of important information like the viability of pathogen and its susceptibility to antibiotics (Poxton, 2005; Mori and Notomi, 2009). However, slow multiplication and difficulty of selective cultivation of many important pathogens often limit the culture based diagnosis (Gilbert, 2002; Mori and Notomi, 2009). On the contrary, rapid assays are used to test the bulk of samples in short time and quarantine the contaminated stuff. Such resources attribute for further confirmation of presumptive positive samples using the established gold standards protocols. Standards are used to validate the sensitivity and specificity of newly rapid diagnostic tests, such as gold standards (Wilkins *et al.* 2010; Lim *et al.* 2013).

Today, a range of rapid diagnostic tests have been developed around the globe for authentic detection of *Salmonella* in food products (Feng *et. al.*, 2001; Boyachuk

et al. 2005). For instance, commercially available 3M™ Petrifilm™ (SALX) and 3M™ Molecular Detection Assay (MDA) are two modern techniques used for a rapid detection of *Salmonella* in many food items, both of which have consequently reduced the overall detection time and investigators workload (Crowley *et al.* 2013[a] Bird *et al.* 2014[a]). Though, both techniques require preliminary enrichments to amplify the targeted pathogen, diluting the effect of inhibitors and other background flora but further confirmation reduced the detection time significantly (Feng, 2007; Ge *et al.* 2009). To introduce these two new assays in Malaysia for the first time, a collaboratory project with 3M™ USA (Malaysia branch) was designed to conduct an exploratory study in this diverse geographical region for *Salmonella* in poultry meat and wet markets environments to estimate the detection efficiency of 3M™ Molecular Detection Assay and 3M™ Petrifilm™ (SALX) versus conventional cultural method (ISO: 6579: 2002). Besides, as per Ministry of Health (MoH) Malaysia statement that approximately 50% food poisoning cases occur in the country due to unsanitary food handling (Soon *et al.* 2011), therefore, the aim of present study was to provide a detailed insight on the prevalence of *Salmonella* serotypes at different stages of poultry processing. Further, to know the contamination aspects of different raw meats with *Salmonella,* its phenotypic and genotypic profiles and the extent of this resistant *Salmonella* from various contaminated meats collected from different wet and hyper markets in Pulau Pinang, Malaysia that could further increase the risk of antimicrobial resistance to the consumers.

1.2 Problem Statement

In Malaysia, Ministry of Health (MoH) reported that 50% food poisoning occurs because of the unsanitary food handling practices (Soon *et al.* 2010). Which,

every year hospitalize around 8,000 patients suffered with stomach upsets and mostly categorized as “Food Poisoning” without proper investigation to confirm the exact etiological agent (A single case reported per 38 cases of *Salmonellosis;* Physician based surveillance) (Soon *et al.* 2010; MOH, 2012). However, a number of studies confirms that raw meat specifically poultry is the main reservoir of food borne MDR (Multi Drug Resistant) *Salmonella* in Malaysia (Learn-Han and Yoke-Kqueen 2008). The emergence and dissemination of multidrug-resistant *Salmonella* is a major public health risk (Ribeiro *et al.* 2011). According to the Food Safety and Inspection Service (FSIS-USDA, 2012) regulations, poultry slaughter facilities must be evaluated for *Salmonella* on an intermittent basis. But, the reported data about foodborne pathogens at national level is the “Tip of Ice Burg (Soon *et al.* 2010). Nevertheless, some of published data of clinical samples (hospitalized patients) confirms that *Salmonella* sp. is the top listed foodborne pathogen in Pulau Pinang (Shunmugam *et al.* 2012). In this context, no information is available that how cross contamination of various raw meats occurs with foodborne *Salmonella* at different retailing in Pulau Pinang, as well as their environmental niches, risk of antibiotic resistance and virulence potential to consumers. Further, worldwide, detection of *Salmonella* sp. in food requires 5-7 days which is a major issue and requires to explore the performance of various modern and swift assays to reduce the detection time of *Salmonella* in contaminated foods.

1.3 Objectives of the study

In the context of aforementioned aspects described in the previous Section, the aims and objectives of the present work include;

- To identify potential niches and distribution of *Salmonella* serovars in chicken processing environments in traditional wet markets

- To determine the prevalence, antibiotic resistance and risk characterization of resistant *Salmonella* isolated from various retail raw meats obtained from wet and hypermarkets.
- To investigate the genotyphic resistance determinants, virulence potential and molecular diversity of *Salmonella* serovars isolated from various raw meats.
- To evaluate modern screening methods for the accurate, sensitive, specific and rapid detection of *Salmonella* in contaminated meats and related processing environment.

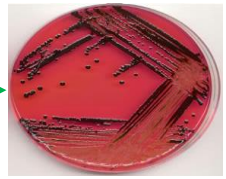
Scheme A

Sample (Poultry and other raw meats; beef, mutton & fish)
(Isolation of *Salmonella* from beef, mutton, fish and chicken of wet and hyper markets)

***Salmonella* Sp. Isolation**

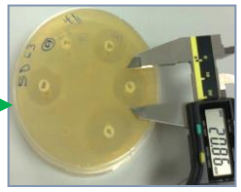
Phenotypic Isolation

Culture Based Method
- ISO: 8579:2002

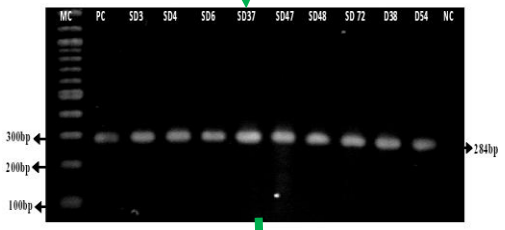


Serotype recognition
(Ipoh Public Health Laboratory)

1. Antimicrobial susceptibility testing (Disk diffusion method)
2. Risk characterization model



Molecular Characterization



PCR Analysis:
- Resistant Genes
- Virulence profile
- RAPD fingerprinting

Figure 1.1 (Scheme A): Flow chart of the present project

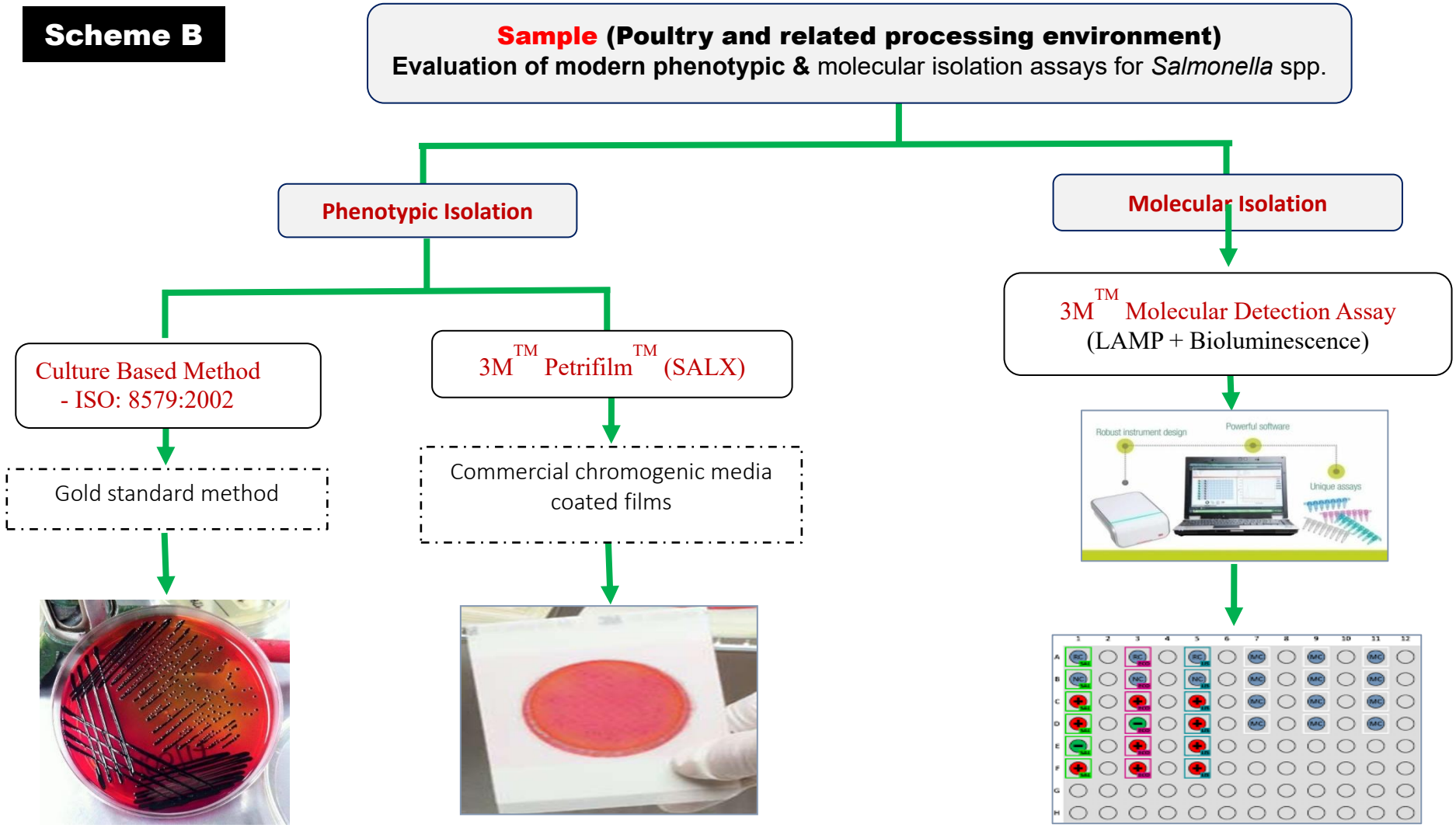


Figure 1.1 (Scheme B): Flow chart of the present project

CHAPTER 2
LITERATURE REVIEW

2.1 The genus *Salmonella*

Salmonella represents a bacterial genus of heterogeneous nature that has been named after Deniel Elmer Salmon (1850–1914), an American veterinarian, who along with his team pioneered in isolating it from porcine intestines in 1885. From evolutionary viewpoint, it has long been considered that due to the close genetic relationship of *Salmonella* and *Escherichia coli* and evolution from a common ancestor such as mammals (Baumler *et al.* 1998), *Salmonella* have been transmuted from the genus *Escherichia coli* more than hundred million years ago (Cotter and Dirita, 2000).

The genus *Salmonella* is enlisted under the domain of bacteria, phylum proteobacteria, class gamma proteobacteria, order enterobacteriales and family enterobacteriaceae (Figure 2.1). They are gram-negative, bacilli, facultative anaerobes, non-spore forming with diameter 0.7-1.5 μm and length 2.0-5.0 μm. They are motile by mean of peritrichous flagella, except *Salmonella* Pullorum and *Salmonella* Gallinarum (non-motile) (Hughes, 2017). Moreover, *Salmonella* spp. thrives at temperature ranging from 5°C to 46°C with an ideal growth at around 37°C, however the growth becomes stunted at freezing temperature.

Numerous intervention techniques have been widely practiced during food processing and storages such as cold or heat treatments are used widely to inactivate or inhibit the growth of foodborne pathogens (Yang *et al.* 2014). As a matter of fact, a decline in the growth rate of *Salmonella* has been observed at 20°C which gets completely inhibited at around 10-11°C.

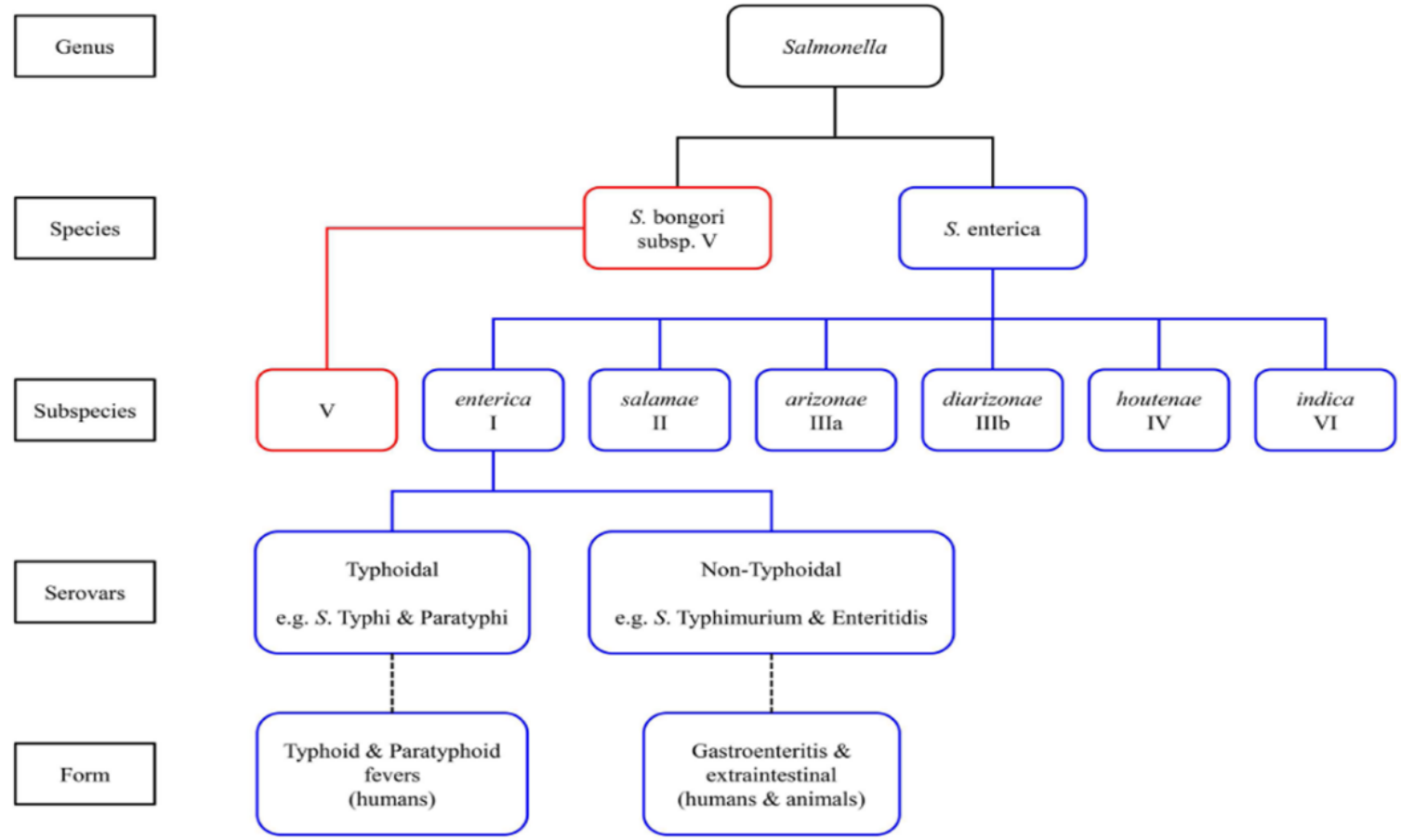


Figure 2.1 Classification of *Salmonella* species and subspecies (Source: Hurley *et al.* 2014)

The unique aptitude of *Salmonella* is to bear a variety of fortuitous conditions, for instance it can propagate in the food products preserved at low (2- 4°C) or elevated (54°C) temperature (Park *et al.* 2014). However, inactivation between 55°C and 70°C has also been reported (Smadi *et al.* 2012).

The genus *Salmonella* has been subdivided into two species; a) *Salmonella* enterica b) *Salmonella bongori*. Further, *S. enterica* is further subdivided in to six sub-species; *S. enterica, S. salamae, S. arizonae*, *S. diarizonae*, *S. houtenae*, *S. indica* and each sub-species consist of various numbers of serovars (Figure 2.1). Till date, around 2557 different serovars have been identified. Moreover, each sub-species of *S. enterica* such as *S. enterica, S. salamae, S. arizonae, S. diarizonae, S. houtenae, S. indica* possess 1531, 505, 99, 336, 73, and 13 serovars, respectively.

The optimum growth of *Salmonella* require water activity around 0.94 to 0.84 and pH about 6.5 to 7.5 (Kemal, 2014). Nutritionally, *Salmonella* have been enlisted in the category of chemo-organotrophic organisms, possessing the capability to metabolize nutrient by fermentative and respiratory pathways (D'Aoust and Maurer, 2007). In addition, majority of the strains are catalase positive, oxidase, indole as well as urease negative and utilize citrate as a sole carbon source, decorboxylate lysine and ornithin. *Salmonella* can ferment various sugars into acid and gas such as glucose and mannitol, except *S.* Typhi which is unable to produce gas. Most of the *Salmonella* produce hydrogen sulphide if grown on triple sugar iron (TSI) agar (Barbara *et al.* 2000). All these traits have been exploited for the presumptive biochemical confirmation of *Salmonella*, following their growth on differential (selective) plating media, such as xylose lysine deoxycholate agar, and Hektoen enteric agar. Moreover, growth on TSI agar medium is indicated by the production of acid and gas. *Salmonella* serotypes exist among various hosts; they can infect warm-blooded animals such as

rodents, birds and cold-blooded animals for instance snakes and other aquacultures (Agbaje *et al.* 2011).

Following the Kauffman and White scheme, sub-species have further been classified into serotypes, on the basis of three main antigens i.e., somatic (O), flagellar (H) and capsular (K). The somatic antigen is heat stable that is chemically made-up of oligosaccharide component of lipopolysaccharide and is located on the outer membrane of *Salmonella*. The flagellar antigen is heat labile that is available in flagella and is concerned with the activation of host immune system (Hu and Kopecko, 2003). Furthermore, capsular antigen is a heat sensitive surface antigen that is composed of polysaccharides and is found on the capsular surface, however they are not very common in *Salmonella* serotypes (Eng *et al.* 2015).

Both "serovar" and "serotype" are synonymous. The Pasteur Institute of the WHO usually follow "serovar," whereas, American Society of Microbiology (ASM) and CDC (Center for Disease Control) frequently uses the term "serotype", although the word "serovar" has been broadly practiced to maintain global uniformity, at present. Further, the name serovar varies on the basis of diseases related to *Salmonella* based infections, geographical importance of isolation and some typical habitats. To date, above 2500 serovars of *Salmonella* have been recognized, where more than 50% belong to *S.* enterica subspecies enterica, which are known for the spreading of most infections in humankind (Guibourdenche et al. 2010).

2.2 Global epidemiology of *Salmonella*

The *Salmonella* enterica, subspecies enterica is further divided into typhoidal and non-typhoidal *Salmonella* (NTS) serovars, both types being exclusively responsible for causing approximately 99% infections in humankind as well as animals. However, it is important to mention that the typhoidal *Salmonella* serovars

are absolutely host restricted (humans) and lack of zoonotic property, for example, serovars such as *S.* Typhi and *S.* Paratyphi A, B. On other hand, the NTS serovars are capable of zoonotic potential and represent important foodborne pathogens that have emerged as the largest cause of foodborne illness after campylobacter (Adzitey et al. 2012).

It has been estimated that the NTS is the first or second most common cause of foodborne diseases, which is mainly transmitted through animal-derived foods such as poultry, eggs, milk, beef and pork (CDC, 2013). Entire serovars of NTS are much diversified and comprise of about 60% of the 2600 identified *S.* enterica serovars. Each year, approximately 93 million cases of stomach upsets and 155,000 deaths occur globally due to foodborne human *Salmonellosis* (Ta *et al.* 2014). Almost 80.3 million of these cases are estimated to be foodborne (Majowicz *et al.* 2010). In United States (US), the annual incidence rate of human *Salmonellosis* as per "FoodNet" surveillance network is 17.6 cases per 100,000 population, whereas *Salmonella* has been marked as one of the main contributor to the death toll (39%) among other prevailed foodborne pathogens (Barton Behravesh *et al.* 2011). Likewise, in many European countries, *Salmonella* is the most common cause of foodborne illness, with 690 clinical cases reported per 100,000 populations (Duggan *et al.* 2012).

In the entire Australian states and territories, *Salmonellosis* is a notifiable disease with an incidence rate of 49.8 cases per 100,000 persons during 2012, while in New Zealand, 24 persons out of 100,000 population were reported to be affected (Ozfood net 2012). Moreover, Thompson (2002) estimated the incidence rate of *Salmonella* infection in Japan, Netherland and Germany as 73, 16, and 120 cases per 100,000 populations every year, respectively. The NTS is endemic in many under-developed nations, especially sub-Saharan Africans where the prevalence is very high

among children below three years of age and also in adults suffering with human immunodeficiency virus (HIV) bearing an estimated mortality rate of 25% (Gordon *et al.* 2008).

In addition, there is also a lack of official data regarding *Salmonella* surveillance in many developing countries. Although, existing data is an outcome of physician based surveillance but it represents only 1 to 10% of the cases that are reported and recorded (Pui *et al.* 2011). Southeast-Asian region has reached to an n alarming situation of food-borne illness at the rate of 22.8 million gastro-enteritis cases and a death toll of 37600 per anum happened due to *Salmonellosis* (Akbar and Anal, 2014). Predominantly, major source of human *Salmonellosis* includes a variety of food items such as meat, eggs, milk, vegetables, and fruits which are contaminated with *Salmonella* (Sudhanthirakodi *et al.* 2016).

Regarding the prevalence of various serovars of *Salmonella*, Enteriditis has been reported as one of most prevailed serovar in Asia, Europe and Latin America and estimated for 38%, 87% and 31% proportion among the prevailed clinical isolates, respectively. Both, *S.* Enteriditis and *S.* Typhimurium were reported the predominated serovars in Africa, with an incidence rate of 26% and 25% among other isolates, respectively. *S.* Typhimurium (29%) followed by *S.* Enteriditis (21%) were the most commonly prevailed serovars in North America (Galanis *et al.* 2006). In Table 2.1, the incidence of *Salmonella* in various animals based foods and distribution of different serovars in different part of the world have been enlisted.

Table 2.1: Global prevalence of *Salmonella* in various foods

Country	**Sample**	**Prevalence (%)**	**Predominant serovar**	**Reference**
USA	Chicken skinned	42.00	Heidelberg, Kentucky, Typhimurium,	Guran *et al.* 2017
	Chicken skinless	17.60	Infantis, Senftenberg, Thompson	
Myanmar	Chicken Carcasses	97.90	Albany, Kentucky, Braenderup, and	Moe *et al.* 2017
			Indiana	
Thailand	Chicken Carcasses	28.70	Typhimurium, Corvallis, Enteritidis,	Trongjit *et al.* 2017
Lebanon	Ready-t-teat food	49.00	Enteritidis, Typhimurium	Fadlallah et a., 2017
Turkey	Fermented sausage	1.04	Not mentioned	Ozbey *et al.* 2017
Taiwan	Raw oysters	66.50	Saintpaul, Newport, Infantis	Lo *et al.* 2017
Morocco	Turkey	52.90	Kentucky, Agona, Reading, Saintpaul,	Amajoud *et al.* 2017
	Chicken	20.90	Corvallis, Typhimurium, Montevideo,	
	Sausage	5.00	Enteritidis, Hadar, Israel, Braenderup	
	Minced meat	12.00		
	Cheese	5.90		
Latvia	Poultry	0.08	Typhimurium, Derby, Enteriditis	Terentjeva *et al.* 2017