Chapter 2

Collection, isolation, screening and identification of *Salmonella* strains from clinical and animal originated food sources

2.1. INTRODUCTION

Salmonella enterica remains one of the consistently associated with food borne illness throughout the world, even though many other pathogens have recently received considerable media attention. In recent years, the incidence of salmonellosis has been reported in many developing countries including India, Egypt, Brazil and Zimbabwe (El-Aziz, 2013). Based on the disease causing ability, *Salmonella enterica* from human infection can be subdivided into two groups Typhoidal (Enteric group) and Non-Typhoidal group. Generally, *Salmonella* infections can be manifested in three forms: Gastroenteritis, Enteric fever and Septicaemia (Todar, 2005). There are several transmission routes for salmonellosis, but the majority of human infections are derived from the consumption of contaminated foods especially those of animal origin (Hernandez *et al*., 2005). A variety of food products, particularly meat products (beef, pork, poultry and egg) including minced meat are the frequent vectors responsible for the transmission of this bacteria to human sources (Jay, 2000; Ejeta *et al*., 2004; Orji *et al*., 2005; Chittick *et al*., 2006; Dechet *et al*., 2006; Aissa *et al*., 2007; Kumar *et al*., 2008; Andrews *et al*., 2009; Yan *et al*., 2010 and Guo *et al*., 2011).

Worldwide NTS represents one of the important public health problems. Especially in India and Kenya, the disease is endemic and carries a significant morbidity and mortality in both paediatric and adult populations (Doris *et al*., 2010). Now a days, in developing countries NTS accounts for a steadily increasing proportion of human infections and are the major agents of causing food poisoning and acute gastroenteritis (Graham *et al*., 2000a; Grisaru *et al*., 2004; Gordon, 2008b and Imran *et al*., 2010). In addition, they can also cause severe diseases such as septicaemia and local infections at any site of the body. The infection outbreaks have been associated with a wide variety of food and its dominant serotypes from clinical cases vary with

geographical region. For example, until the 1980s, *S*. Typhimurium was considered the clinically important and most commonly isolated serovar from human worldwide. But in the late 1980s, *S*. Enteritidis has emerged as the most common cause of salmonellosis in Europe and during the 1990s, it became the most prevalent serovar round the globe (Mishu *et al*., 1994; Poppe, 1999; Herikstad *et al*., 2002; Cogan and Humphrey, 2003 and Schroeder *et al*., 2005). The reasons for this worldwide serovar shift are still unknown. NTS can become invasive, leading to bacteraemia, sepsis and focal infections in immunocompromised, debilitated hosts and infants less than three months of age (Adak *et al*., 2002; Kennedy *et al*., 2004 and Vugia *et al*., 2004).

Enteric or typhoid fever is one of the most common causes of systemic infection in India which causes significant traveller associated illness each year in developed countries such as South Asia, South America and parts of Africa (Ekdahl *et al*., 2005; Health Protection Agency, 2007 and Kothari *et al*., 2008). *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi B and *S*. Paratyphi C are causative agents of the enteric fever. These are strictly human-adapted pathogen associated with an invasive infection in immunocompromised persons. There are an estimated 16 million to 21.6 million cases of typhoid fever each year, with the highest incidence being reported from Southeast Asia. The CDC, USA estimated that 21 million cases of typhoid fever and 2,00,000 deaths occur worldwide (CDC, 2013) and the global human health impact of NTS is high, with an estimated 93.8 million illnesses, of which an estimated 80.3 million are foodborne, and 1,55,000 deaths each year (Majowicz *et al*., 2010). The acquisition of the organism occurs by ingestion of food or water contaminated with human excreta and associated with poor sanitation and hygiene (Mezrioui *et al*., 1995 and Mollie *et al*., 2003).

Epidemiological reports suggested that animal originated food or meat (poultry, pork, cattle and sea food) is still the primary cause of food poisoning (Mulder, 1999). In animal originated food, Salmonellosis is one of the important food borne diseases. It causes a serious health problem and one of the most addressed problems by food industry. It leads to major economic losses in food industry through mortality and reduced production. Particularly, in India “The Hindu-CNN IBN State of the nation survey” conducted by the Centre of the Study Developing Society in 2006 found that

60% of Indians are non-vegetarians and fish is consumed as a country's favourite meat, chicken comes a close second followed by lamb meat and beef a distant third and fourth. In India, a number of investigators have reported contamination by *Salmonella* in seafood and poultry (Hatha and Lakshmanaperumalsamy, 1997; Shabarinath *et al*., 2007; Ruban *et al*., 2010; Selvaraj *et al*., 2010 and Kaushik *et al*., 2014).

Earlier, most of the studies on the prevalence of *Salmonella* in food samples were conducted by using conventional culture methods. During recent years, molecular techniques have been increasingly used for detection of pathogens in food because of its efficiency and high recovery rate when compared to conventional method (Shabarinath *et al*., 2007). PCR has become a powerful and increasingly popular tool in microbial identification. It may also be used to identify direct detection of bacteria in clinical/food samples (Hein *et al*., 2006 and Wolffs *et al*., 2006). Several PCR assays have been developed by targeting various *Salmonella* genes commonly encoding target protein *HilA*, *InvA*, *InvE* and *Hns* (Rahn *et al*., 1992; Jones *et al*., 1993; Stone *et al*., 1994 and Pathmanathan *et al*., 2003). In addition, many reports suggested that *InvA* is one of the most often used gene to detect *Salmonella* spp. (Rahn *et al*., 1992; Bulte and Jakob, 1995; Wang *et al*., 1995; Malorny *et al*., 2003 and Bisi-Johnson *et al*., 2011). The *InvA* gene codes for protein in the inner membrane of bacteria, necessary for invasion to epithelial cells and entry into the intestinal mucosa (Darwin and Miller, 1999). Hence, an attempt has been made to investigate the presence of *Salmonella* from animal originated food and human origin. Monitoring the occurrence and distribution of *Salmonella* serovars from human and animal sources is important to know the possible outbreaks and to identify the possible sources of infection.

2.2. MATERIALS AND METHODS

2.2.1. Collection, transport and processing of samples

The standard method described by World Health Organization (WHO) manual 2010 and Food and Drug Administration (FDA, 2011) were followed for collection, isolation and identification of *Salmonella* from clinical samples. During the study period of 2011 to 2013, for the isolation of *Salmonella*, stool and blood samples were collected from the patients admitted to government K.A.P.Viswanatham Medical College and Hospital, Tiruchirappalli, Tamil Nadu from time to time with diarrhoea

and enteric fever symptoms. Patients who have had antibiotic therapy prior to sample collection were excluded from the study.

2.2.2. Stool sample

Freshly passed stool samples were collected and placed immediately in Cary-Blair transport medium (Oxoid Ltd-UK) and transported to the laboratory within 2 h of collection. All the samples were directly inoculated into the Xylose Lysin Deoxycholate Agar (XLD-Difco Ltd-USA) and Hektoen Enteric Agar (HEA- Difco Ltd-USA) and incubated at 37ºC for 24 h. Simultaneously, all stool sample swabs were inoculated into 10 ml of Selenite F broth (SFB-Difco Ltd-USA) for enrichment and incubated at 37ºC for 24 h and then sub cultured on XLD and HEA agar at 37ºC for 18-24 h.

2.2.3. Blood sample

About 5 ml of blood from adult and 2 ml from children were drawn aseptically and transferred into culture bottles containing 50 and 20 ml of Brain Heart Infusion (BHI) broth (Difco Ltd-USA) with 0.05% Sodium Polyanethole Sufonate (SPS) respectively. A minimum blood transferred into broth was 1 in 10 ratio (Collee *et al*., 1989). Then the broth was incubated at 37ºC for seven days and checked for sign of bacterial growth daily up to 7 days and bottles which showed turbidity were sub cultured onto XLD and HEA agar.

2.2.4. Collection and processing of animal originated food samples

Poultry (chicken neck, chicken breast and egg), different meat (pork and beef) and seafood (finfishes and crustaceans) were used to isolate the *Salmonella* strains collected from retail shop, slaughter house and fish market in Tiruchirappalli city, India. All the samples were collected in new plastic bags and transported to the laboratory immediately in an ice chest (4ºC) and processed within 2 h of collection and bacteriological examination were made.

The samples were processed following conventional method prescribed in WHO protocol (2010) and The United States Department of Agriculture (USDA, 2011) manual. Briefly, 25 g of tissues from all samples were homogenised and transferred to a 500 ml conical flask containing 225 ml of sterile Buffered Peptone Water (BPW) and

incubated at 37ºC for 18-24 h. After incubation, 0.1 ml, 0.2 ml and 1 ml enriched culture from all samples were transferred to 10 ml of Rappaport-Vassiliadis Soy Broth (RVS), SFB and Tetrathinate Broth (TTB) respectively. The tubes were incubated at various temperature such as 41.5±0.5ºC for RVS, whereas 36±1ºC for SFB and TTB for 18-24 h. At the end of selective enrichment, a loop full of selective enrichment culture medium was streaked onto XLD and HEA selective agar plates. The plates were incubated at 36±1ºC for 24 h. After completion of the incubation, 3-5 typical *Salmonella* like colonies were randomly picked up from all the plates and streaked onto Trypticase Soy Agar (TSA-Difco Ltd-USA) plates. All the purified colonies from TSA were subjected to various biochemical characterization tests.

2.2.5. Biochemical identification of *Salmonella*

All isolated colonies were identified through series of biochemical reaction as per WHO protocol (2010), which included Triple Sugar Iron agar reaction (TSI-Difco Ltd-USA), Urease production assay (Difco Ltd-USA), Lysine Iron Agar reaction (LIA-Difco Ltd-USA), Motility Indole Ornithine test (MIO- Difco Ltd-USA) and Citrate Utilization test (Difco Ltd-USA).

2.2.6. Molecular identification of *Salmonella*

2.2.6.1. DNA extraction

Template DNA was prepared from biochemically identified *Salmonella* strains by growing a single bacterial colony in 3 ml of Trypticase Soy Broth (TSB- Difco Ltd-USA) at 37ºC for 24 h and the sample was transferred to 1.5 ml capacity micro centrifuge tube. Then, the cell suspension was centrifuged at 14,000 rpm at 4ºC for 10 min. The supernatant was discarded carefully. Then, the pellet was washed in 200 µl of sterile milli-Q water by means of vortexing. Again the tubes were centrifuged at 14,000 rpm for 5 min and the supernatant was discarded carefully. Again the pellet was washed in 200 µl of milli-Q water by vortexing. Finally, the suspension in microcentrifuge tube was placed in boiling water at 100ºC for 10 min followed by immediate chilling on ice. After that the cell lysate was centrifuged at 14,000 rpm for 3 min and the supernatant containing template DNA was carefully transferred to a sterile new micro centrifuge tube and stored at -20ºC for PCR amplification test (De Medici *et al*., 2003).

2.2.6.2. PCR amplification test

The presence of *Salmonella* specific invasive (*InvA*) gene was evaluated by a simplex PCR using *InvA* specific primers

InvA F-5'-GTGAAATTATCGCCACGTTCGGGCAA-3' and

InvA R-5'-TCATCGCACCGTCAAAGGAACC-3' (Rahn *et al*., 1992).

PCR was performed with 25 µl of reaction mixture. The reaction mixture contained the following reagents 2.5 µl of 10 X PCR buffer, 2.0 µl of 1.24mM dNTPs, 1 µl of 10pmol each primer, 0.2 µl of 5U Taq Polymerase and 3 µl of template DNA. These mixtures were made up to 25 µl with nuclease free water.

The amplification was carried out in 0.2 ml of thin walled, nuclease free PCR tubes (Axygen, USA) using Eppendorf thermocycler. The PCR condition maintained was 30 cycles of initial denaturation at 94ºC for 60s, denaturation at 94ºC for 30s, annealing at 64ºC for 30s, extension at 72ºC for 30s and final extension for 7 min at 72ºC. All runs were included with positive (*S*. Typhi-Reference strain) and negative (Nuclease free water) control. At the end of the reaction all tubes were stored at -20ºC.

2.2.6.3. Electrophoresis

PCR products were checked for DNA amplification by standard electrophoresis procedures with 100bp ladder. Products were analysed by gel electrophoresis in 2% (w/v) ultra-pure agarose in 0.5 X Tris/Borate/EDTA buffer stained with ethidium bromide (0.5 µg ml^{-1}) at 100 V using horizontal gel electrophoresis apparatus. The amplified products were visualized in gel documentation system (UVITECH, Cambridge) and the image was saved as TIFF format.

2.2.7. Serogrouping of *Salmonella*

All *Salmonella* isolates identified through biochemical and molecular techniques were subjected to serological tests by using slide agglutination tests with poly 'O' and single O-groups A, B, C, D and E antisera (Difco Ltd- USA). These strains were further tested against poly 'H' antisera. Those strains identified biochemically as *S*. Typhi were also tested against Vi antisera.

2.2.7.1. Serotyping

Serotyping of *Salmonella* species isolates was performed after serogrouping on the basis of phase 1 and phase 2 flagellar antigens by tube agglutination tests with known antisera according to the Kaufmann–White scheme (Patrick and Francois, 2007).

2.2.7.2. 'O' antigen detection

All *Salmonella* isolates were tested for the presence of O antigen using polyvalent (A-G) and individual (A, B, C1, C2, D and E) O-group antisera by slide agglutination method. Part of the colonies from the plate were suspended in 10 µl normal saline on a glass slide and mixed with equal volume of 'O' antisera. The slide was rocked gently for one minute and then examined for the presence of agglutination reaction. The bacterial suspension was mixed with normal saline as a negative control.

2.2.7.3. 'H' antigen detection

Salmonella isolates that showed positive with any single O serum were further tested for polyvalent H phase 1 and 2 and polyvalent phase 2 antigens by tube agglutination method. Phase 1 H antigen was detected using Rapid *Salmonella* Diagnostic 'H' antisera (RSD) plus H-I. Phase 1 and phase 2 H antigens were detected using tube agglutination methods. All the test organism was incubated overnight in Luria-Bertani Broth (LB) (Oxoid, Ltd-UK) at 37ºC. One ml of this culture was diluted in 9 ml LB and incubated with shaking at 37ºC for 3-4 h. The culture was then diluted with 10 ml of 1% saline (until it matches to a MacFarland 0.5 standard). For each agglutination reaction, 1 ml of diluted culture was mixed with 5 µl of H antisera in Dreyer's tube (VWR International, Lutterworth, UK) and incubated at 50ºC in an water bath for 2 h. Tubes with presence of agglutination were taken as positive reaction for the respective H antigens.

2.2.7.4. Phase change

Phase conversion was made for all *Salmonella* isolates which had only one phase. Ditch plate method was used to convert phase 1 to phase 2 and vice versa. Ditch with the size of 50 x 20 mm was made on a well dried Nutrient Agar (NA). A sterile filter paper strip was placed across the ditch at right angles. A drop of specific antisera

which gave a positive result in the tube agglutination test was placed in the centre of the bridge. The organism was inoculated at the very tip of the filter paper. The set up was incubated at 37ºC for two days. Organisms that crossed the bridge appeared on the other half of the plate and were then tested by tube agglutination test (H1; H2), to confirm change in expression of flagellar antigens.

2.3. RESULTS

2.3.1. Screening of *Salmonella* from clinical and different food samples

Distribution of *Salmonella* in clinical, poultry, pork, beef and seafood samples are presented in Table 1. A total of 305 samples were examined for the presence of *Salmonella* consisting of clinical (blood N = 39 and stool N = 45), poultry (N = 40), pork (N = 33), beef (N = 33) and sea food samples (N = 115) for the present investigation. Out of 305 samples examined for the presence of *Salmonella*, only 107 (35%) samples showed positive for *Salmonella.* In clinical samples, 51 (61%) samples exhibited positive for *Salmonella*. However, in food samples, pork was found to be associated with maximum number of *Salmonella* (91%), followed by poultry (40%) and seafood (13%) samples and there was no *Salmnella* detected in egg, beef and crustaceans.

Table 1. Prevalence and distribution of *Salmonella* from clinical and different food samples

S.No	Sources (N)	Type of sample examined	Total number of sample examined	Number of *Salmonella* positive sample obtained (%)
1	**Clinical (84)**	Blood	39	51 (61)
		Stool	45	
2	**Poultry[a] (40)**	Chicken neck	13	16 (40)
		Chicken Breast	13	
		Egg	14	
3	**Meat[b] (66)**	Pork	33	30 (91)
		Beef	33	ND
4	**Seafood[c] (115)**	Finfishes	75	10 (13)
		Crustaceans	40	ND
Total			305	107 (35)

[a] Poultry sample obtained from retail shop ND-Not detected
[b] Meat samples obtained from slaughter house
[c] Finfish and crustaceans obtained from fish market

2.3.2. Isolation of *Salmonella* from clinical specimens (blood and stool samples)

A total of 39 blood samples and 45 stool samples were collected from typhoid and diarrhoeal patients admitted to KAPV Government Medical College Hospital, Tiruchirappalli, India from 2011 to 2013. Out of 39 blood and 45 stool samples tested through biochemical confirmation, all 39 (100%) blood samples possessed *Salmonella*, whereas only 12 (27%) from stool samples harboured the enteric pathogen *Salmonella*. All biochemically confirmed isolates were found to possess *InvA* gene through PCR technique using *InvA* primer (Table 2).

Table 2. Detection of *Salmonella* in clinical sample by biochemical and molecular technique

S.No	Sample (N)	Total number of isolates	No. of positive *Salmonella* by biochemical test (%)	No. of positive *Salmonella* by PCR test (%)
1	**Blood (39)**	39	39 (100)	39 (100)
2	**Stool (45)**	45	12 (27)	12 (100)
	Total	**84**	**51 (61)**	**51 (100)**

2.3.3. Recovery of *Salmonella* in different food samples by biochemical and molecular techniques in various enrichment broth

The role and efficacy of three different selective enrichment broths such as SFB, TTB and RVS were evaluated for the recovery of *Salmonella* in different food samples and the number of *Salmonella* positive isolates recovered from different food samples are summarized in Table 3. A total of 804 suspected *Salmonella* isolates were picked up from all the food samples from all the enrichment broths. Out of three enrichment broths it was found that SFB was suitable enrichment media and enhanced the maximum growth (360 isolates) of suspected *Salmonella* followed by TTB (236 isolates) and RVS (208 isolates). However, after biochemical confirmation maximum typical *Salmonella* was obtained from TTB with 85 isolates followed by SFB (58 isolates) and RVS (38 isolates). A total of 181 *Salmonella* isolates were obtained from animal originated food samples through biochemical characters. All the 181 confirmed isolates were subjected to PCR molecular diagnosis technique. The results revealed that only 173 isolates were confirmed as

Salmonella and this was shared by 84 isolates from TTB, 57 isolates from SFB and 32 isolates from RVS. From the results it was evident that the enrichment media TTB was the suitable media to recover maximum *Salmonella* present in food samples.

Table 3. Detection of *Salmonella* in different food samples by biochemical and molecular technique in various enrichment broth

S.No	Sample	Enrichment broth			Total number of suspected isolates	No. of positive *Salmonella* by biochemical test (%)			Total number of isolates (%)	No. of positive *Salmonella* by PCR test (%)			Total number of isolates (%)
		SFB[1]	TTB[2]	RVS[3]		SFB[1]	TTB[2]	RVS[3]		SFB[1]	TTB[2]	RVS[3]	
1	**Poultry**	**86**	**141**	**96**	**323**	**36 (42)**	**56 (40)**	**23 (24)**	**115 (36)**	**35 (97)**	**55 (98)**	**20 (87)**	**110 (96)**
	Chicken neck (13)	28	40	32	101	17	24	8	49 (49)	17	23	9	49 (100)
	Chicken breast (13)	43	77	48	171	19	30	15	64 (37)	18	32	11	61 (95)
	Egg (14)	15	24	16	51	ND	2	ND	2 (4)	ND	ND	ND	ND
2	**Pork (33)**	**10**	**17**	**13**	**40**	**10 (100)**	**17 (100)**	**13 (100)**	**40 (100)**	**10 (100)**	**17 (100)**	**11 (85)**	**38 (95)**
3	**Beef (33)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
4	**Finfishes (75)**	**264**	**78**	**99**	**441**	**12 (5)**	**12 (15)**	**2 (2)**	**26 (6)**	**12 (100)**	**12 (100)**	**1 (50)**	**25 (96)**
5	**Crustaceans (40)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
	Total	**360**	**236**	**208**	**804**	**58**	**85**	**38**	**181 (23)**	**57 (98)**	**84 (99)**	**32 (84)**	**173 (96)**

SFB[1] - Selenite F-broth
TTB[2] - Tetrathionate broth
RVS[3] - Rappaport Vassiliadis Soy broth
ND - Not Detected

2.3.4. Distribution of *Salmonella* in poultry sample

A total of 40 poultry samples were screened for *Salmonella*, only 16 (40%) samples showed growth in *Salmonella* selective media (Table 1). Using different selective enrichment broth, totally 323 *Salmonella* suspected isolates were obtained after 24 h of incubation (Table 3). The result revealed that a total of 115 isolates (36%) out of 323 were biochemically identified as *Salmonella* and this was shared by TTB (56) followed by SFB (36) and RVS (23). From these 110 isolates were confirmed as *Salmonella* by PCR using *InvA* gene (Fig 1). Among the different region of poultry meat, the prevalence of *Salmonella* was high in chicken neck (100%) and chicken

breast (95%) as confirmed through PCR technique. However, only two isolates were confirmed in biochemical identification from egg and not through PCR.

2.3.5. Detection of *Salmonella* in pork and beef meat sample

In this study, totally 66 meat (33 pork and 33 beef) samples were screened for *Salmonella* contamination. In pork samples, out of 33 samples screened, 30 (91%) samples were positive for *Salmonella* (Table 1). But there was no *Salmonella* like colonies from any of beef samples tested. In different selective enrichment broth, a total of 40 strains of suspected *Salmonella* were obtained in selective media. This was shared by 10 isolates from SFB, 17 from TTB and 13 from RVS. All suspected isolates exhibited positive reaction for *Salmonella* in biochemical identification test. In molecular identification test, out of 40, 38 (95%) isolates were found to possess *InvA* gene. Comparing the various enrichment broths, SFB and TTB supported 100% Salmonella growth, but RVS yielded only 85% Salmonella positive in molecular identification technoque.

2.3.6. Detection of *Salmonella* in seafood sample

Totally, 115 seafood (75 finfishes and 40 crustaceans) samples were tested to screen the *Salmonella* isolates. Out of which, only 10 (13%) samples were positive for *Salmonella* in finfishes and none of the isolates were positive for *Salmonella* in crustaceans (Table 1). In 75 different finfish samples, totally 441 *Salmonella* suspected colonies were recovered (Table 3). However, after biochemical and molecular identification it was found that low incidence in different enrichment broth. In biochemical identification test, the recovery was 15% from TTB, 5% from SFB and 2% from RVS; but in molecular identification test, all (100%) biochemically identified strains from TTB and SFB possess *InvA* gene and only 50% strain from RVS possess *InvA* gene (Table 3).

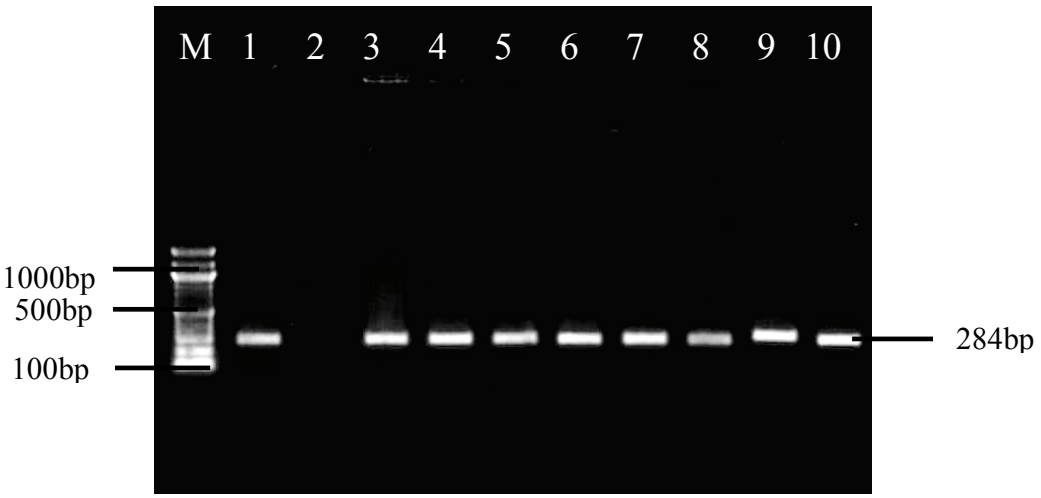

Figure 1. Simplex PCR analysis for identification of *Salmonella enterica* isolated from different food and clinical samples. Lane M: DNA marker (100bp), Lane 1: *S.* Typhi - Reference strain (Positive control), Lane 2: Nuclease free water (Negative control), Lane 3 and 4: *Salmonella enterica* isolates from poultry samples, Lane 5 and 6: *Salmonella enterica* isolates from pork samples, Lane 7 and 8: *Salmonella enterica* isolates from seafood samples and Lane 9 and 10: *Salmonella enterica* isolates from clinical samples.

2.3.7. Distribution of *Salmonella* serovars in different samples

The total number of *Salmonella* serovars and distribution of different serovars in different food samples are presented in Fig 2.

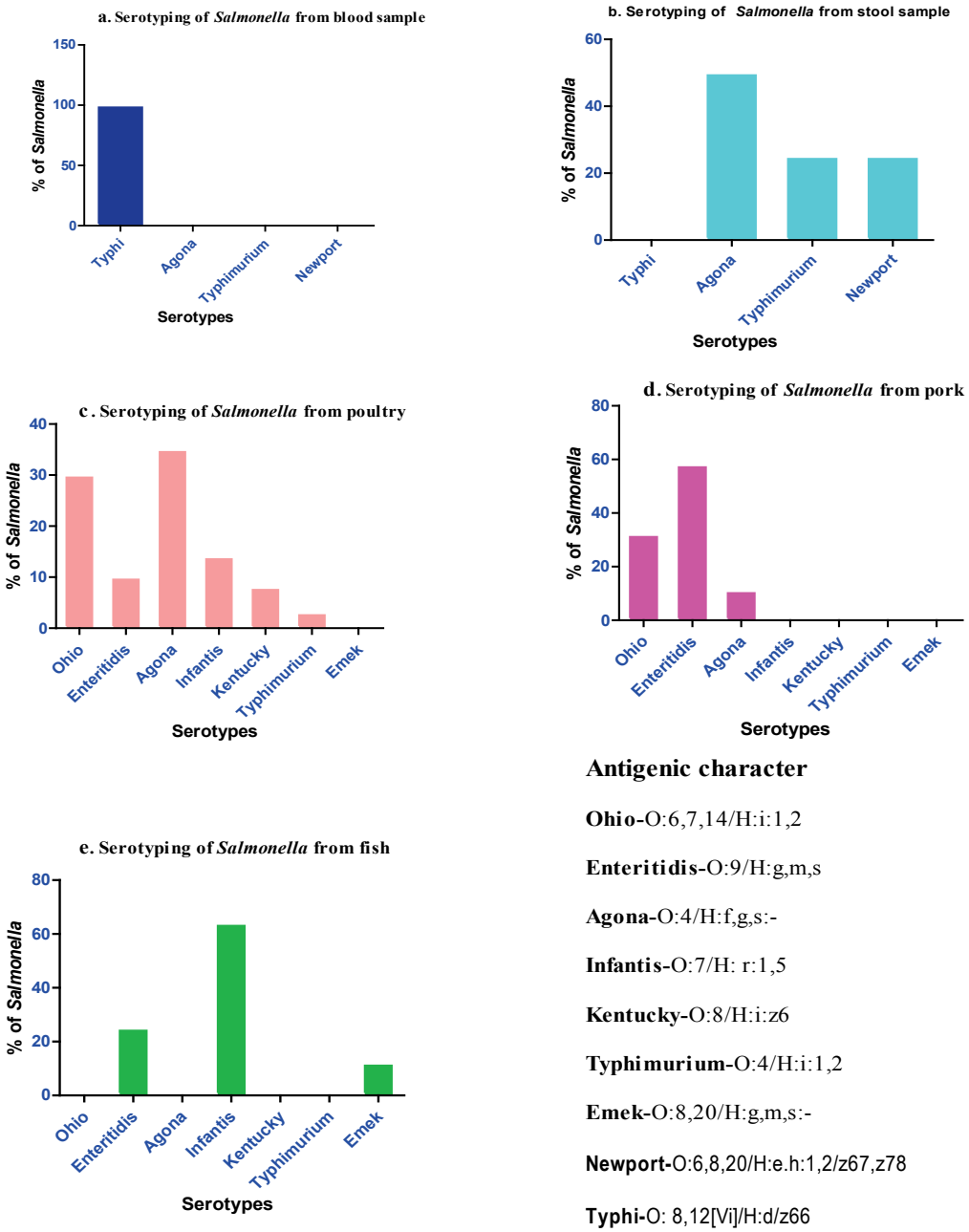

Figure 2. Distribution of different *Salmonella* serovars from different clinical and food samples.

2.3.7.1. Distribution of different *Salmonella* serovar in clinical samples

All 51 *Salmonella* from clinical samples were subjected to different agglutination test by using commercially available antisera. In all clinical strains, only four different serotypes were identified such as *S*. Typhi, *S*. Agona, *S*. Typhimurium and *S*. Newport. Among these, *S*. Typhi (100%) was the predominant serotype and it was found only in the blood sample (Fig 2a). NTS serotypes were also recovered from stool samples and from these *S*. Agona (50%) was frequently isolated followed by *S*. Typhimurium (25%) and *S*. Newport (25%) (Fig 2b).

2.3.7.2. Distribution of different *Salmonella* serovar in food samples

A total of 173 *Salmonella* isolates consisting of 7 different serovars were isolated and identified in different food samples. *S*. Ohio was the predominant serotype in all food samples followed by *S*. Agona, *S*. Enteritidis, *S*. Infantis, *S*. Kentucky, *S*. Typhimurium and *S*. Emek (Fig 2c, d and e). Among the food samples, poultry samples harboured the highest number of *Salmonella* serotypes in the present investigation. *S*. Agona (35%) was found predominant followed by *S*. Ohio (30%), *S*. Infantis (14%), *S*. Enteritidis (10%), *S*. Kentucky (8%) and *S*. Typhimurium (3%). Among these *S*. Agona and *S*. Ohio were often isolated from the poultry sample. In pork sample, only 3 serotypes were identified and this was shared by *S*. Enteritidis (58%), *S*. Ohio (31%) and *S*. Agona (11%). Similarly, in fish (seafood sample) also only 3 different serotypes were isolated such as *S*. Infantis (64%), *S*. Enteritidis (24%) and *S*. Emek (12%).

2.4. DISCUSSION

Salmonellosis has been recognized as a world-wide problem particularly in many developing countries such as the Indian subcontinent, South and Central America and Africa, where there is inadequate sanitation system with rapidly increased population growth, increased urbanization, limited safe drinking water, infrastructure and health problem (Panhotra *et al*., 1980; Ivanoff *et al*., 1994; Crump *et al*., 2004 and Kothari *et al*., 2008). Worldwide, true incidence of salmonellosis in both human and animal is difficult to evaluate because of lack of epidemiological surveillance system, particularly in developing countries, due to ubiquitous occurrence and high incidence of salmonellosis (Rodriguez *et al*., 2006; CDC, 2008; Foley *et al*., 2008 and Pezzoli *et al*., 2008), distribution of a vast variety of *Salmonella* serovars, latent infections and

faecal shedding by the infected as well as silent carriers (Popoff and LeMinor, 1997). In *Salmonella* spp. except a few host-adapted serovars, most of the *Salmonella* causes more or less similar type of disease in different species of animals and in human beings. A periodic surveillance of the level of *Salmonella* contamination in different food animals, food products and environment is necessary to know the status of the disease, furnish disease burden, control the spread of the pathogen and infection to human (Dawson *et al*., 1992 and Arumugaswamy *et al*., 1995).

In India, the epidemiological surveillance of *Salmonella* infection is challenging because of the very limited scope of the studies and lack of coordinated surveillance system. A few studies conducted in India revealed the presence of salmonellosis in humans (Tankhiwale *et al*., 2003; Vaishnavi *et al*., 2005 and Anand and Ramakrishnan, 2011). In the present study, a total of 305 samples (84 clinical, 40 poultry, 66 meat and 115 seafood) were screened for Typhoidal and NTS contamination. The overall prevalence of *Salmonella* contamination in tested food samples was 35%. This result indicates that the widespread occurrence and distribution of *Salmonella* in various samples obtained from hospital, retail shop, slaughter house and fish market in Tiruchirappalli city, Tamil Nadu, India. The result of the present study showed that in clinical sample *Salmonella* was frequently isolated from blood sample than from stool sample; in serotyping analysis of the isolates, blood samples provide 100% positive for *S*. Typhi and there is no NTS strains were recovered. But, NTS such as *S*. Agona, *S*. Newport and *S*. Typhimurium was recovered only from stool samples. Although the percentage of recovery rate of *Salmonella* was low in stool samples, the present findings may support previous study result, which suggested that majority of the *Salmonella* isolates were recovered from blood followed by stool and pus (Abhijit and Sunita, 2011). The serotyping analysis of *Salmonella* from clinical sample in the present study is in concordance with a few previously conducted studies in India at different time, which also reported that *S*. Typhi was the commonest isolates in clinical sample (Abhijit and Sunita, 2011) and NTS recovery rate in clinical sample was a low compared with Typhoidal *Salmonella* such as 5.8% (Vaishnavi *et al*., 2005) and 9.1% in stool sample (Sharath *et al*., 2012). Because *S*. Typhi was a host restricted serovar, it mostly causes the disease through food and water, cross contaminated with faecal excreta of infected persons. But NTS are not host restricted serovars, they cause disease

to both human as well as animal. Tracing the route of transmission of this disease is difficult to identify. The results of the present study showed that NTS retrieval in clinical sample was higher (27%) than the previous study reports.

The major sources for human salmonellosis are farm animals and poultry, which may be intestinal carriers of the organism. *Salmonella* can be shed in the faecal material and transmitted to humans via direct contact with the contaminated faecal material or indirectly via faecal contamination of food or water. The present study determines the prevalence of *Salmonella* in different meat samples (retail poultry, pork, beef and seafood) by using different enrichment broth (SFB, TTB and RVS) and to identify the predominant serovars in each source. This is intended to elucidate the sources of *Salmonella* infections in Tiruchirappalli city, Tamil Nadu, India. The highest incidence of *Salmonella* recovery was from pork than poultry and seafood samples. This result has been correlated with previous study reports, in which among the various retail meat samples tested, percentage of *Salmonella* prevalence was high in pork (Zaidi *et al*., 2006) and In 2012, Lertworapreecha *et al*. also reported that the prevalence of *Salmonella* in pork was 82% in Phatthalung Province, Thailand, these results were to some extent lower than our present study result. In this finding relies, the association of such *Salmonella* in pork sample from slaughter house, clearly implies that pork was found to be the carrier of *Salmonella*. Whereas, in seafood samples, the lowest percentage (23%) of *Salmonella* prevalence was reported as previously stated by Hatha and Lakshmanaperumalsamy (1997) and Heinitz *et al*. (2000) that up to 20% of *Salmonella* incidence was observed in fish samples. Kumar *et al*. (2008) reported the prevalence of *Salmonella* was 23.2% in seafood samples. This is in line with the present findings. Some researchers have, however, stated that the products of aquaculture are rarely involved in outbreaks of salmonellosis (Feldhusen, 2000 and Lunestad *et al*., 2007). This might not be true in all countries, for example *S*. Weltevreden which is a common isolate from shrimp culture environments and shrimp products and is a common serotype involved in human infection in Thailand (Bangtrakulnonth *et al*., 2004), Vietnam (Phan *et al*., 2005) and Malaysia (Yasin *et al*., 1995). Even this low level of prevalence may pose human health risk if aquaculture products are consumed raw. The presence of *Salmonella* in aquatic organisms is

associated with poor sanitation and inadequate hygiene practices (Dalsgaard, 1995; Panisello *et al*., 2000 and Popovic *et al*., 2010).

Poultry sample had the second (40%) leading *Salmonella* contamination among the tested samples. Comparison could also be made with the other studies, in which high level of *Salmonella* contamination was detected in chicken meat (Arumugaswamy *et al*., 1995; Carraminana *et al*., 1997; Dominguez *et al*., 2002 and Hassanein *et al*., 2011). In different region of the chicken samples tested, the prevalence of *Salmonella* was slightly higher in chicken neck than the breast region due to contamination from the digestive tract or from the environment of the slaughter line. Similar results were also reported in broilers in the Czech Republic (Svobodova, 2012). Among the other poultry samples, egg and egg products were most often identified as a route of transmission in the *Salmonella* outbreaks (Braden, 2006). But in the present study, only 4% of egg samples were positive for *Salmonella* in biochemical test. This is due to some physical barriers which may negatively influence penetration of *Salmonella* in egg, principally, temperature plays an important role in the permeability of pathogenic bacteria (Messens *et al*., 2006).

But beef and crustaceans did not possess any *Salmonella* isolates in the present study, which was contradictory with Hatha and Lakshmanaperumalsamy (1997) results, in which 17.39% of crustaceans were found to be contaminated with *Salmonella*. Hyeon *et al*. (2011) has established the prevalence of *Salmonella* in retail beef meat samples. Busani *et al*., 2005 reported that among the different animal originated food sample tested, crustaceans did not possess any *Salmonella*. Fluctuation of *Salmonella* prevalence in different samples were observed in different region of the world (Martinez-Urtaza *et al*., 2003 and Danielle *et al*., 2005). The reason for *Salmonella* prevalence spread through the meat, when meat is cut into pieces, more microorganisms are added to the surfaces of exposed tissue. Raw meats, particularly minced meats have very high total counts of microorganisms and salmonellae are likely to be present in large numbers (Tegegne and Ashenafi, 1998).

The overall recovery rate of *Salmonella* using different selective enrichment broth indicated that TTB has grater efficacy to recover the *Salmonella* strain compared

with SFB and RVS. The result is also in agreement with the previous study of Schrank *et al*. (2001) and Yue *et al*. (2014). This might be due to the alkaline condition in TTB media than RVS media where the pH is acidic in nature.

There are more than 2500 *Salmonella* serotypes have been identified (Popoff, 2001), but only a few of them have clinical importance and are distributed worldwide. NTS infections have emerged as a public health problem since the 1950s (Tauxe, 1991; Olsen *et al*., 2001 and Bangtrakulnonth *et al*., 2004). In India, more than 720 *Salmonella* serovars are prevalent and it has been isolated from wild, pet, domestic animals and also from human beings (Saxena *et al*., 1989; Verma and Gupta, 1995; Verma *et al*., 2001 and Singh, 2007). The present study showed that seven different *Salmonella* serotypes were recovered from various animal originated food samples. The most frequently isolated serotypes are *S*. Ohio, *S*. Agona, *S*. Enteritidis and *S*. Infantis. A previous study conducted by Hendriksen *et al*. (2011) and CDC (2013) revealed that *S*. Enteritidis and *S*. Typhimurium were recovered in the highest frequency. *S*. Agona was also isolated most frequently but the overall proportion was less. These findings are however somewhat inconsistent with the present study results. Seven different *Salmonella* serovars were identified in tested meat samples; most of the *Salmonella* serovars were isolated from poultry sample.

A wide variety of *Salmonella* serotypes was found in poultry than from other examined sources, in which *S*. Agona and *S*. Ohio was predominant serovar. These serovars have occasionally been the etiological agent of human gastroenteritis (Macfarlane, 1986; Rabsch *et al*., 2005; Bertrand *et al*., 2010 and Hendriksen *et al*., 2011). The present study was supported by Orji *et al*. (2005) who isolated *Salmonella* from poultry droppings and other environmental sources with different serovars. This was also endorsed by Sivaramalingam *et al*. (2013) in poultry sample. In contrast, from pork and seafood samples, a few serovars such as *S*. Enteritidis and *S*. Infantis were only isolated. In India, Shabarinath *et al*. (2007) and Rakesh Kumar *et al*. (2008) detected *S*. Weltevreden as the dominant *Salmonella* serotype in fish and shrimp samples. Many of the *Salmonella* serotypes isolated in this study are known to be pathogenic to man because it was positive for *InvA* gene by PCR identification method. *InvA* is a component of SPI which encodes a protein in the inner membrane of bacteria

that is responsible for invasion to the epithelial cells of the host (Moulder, 1985 and Darwin and Miller, 1999).

Thus, the exact source of microbial contamination was difficult to be determined. However, it is possible to check the contaminated feed, water, cross-contamination in slaughter line, secondary contamination through food handlers and environmental condion. The CDC and Foodborne Diseases Active Surveillance Network (FoodNet) indicate that outbreaks and clusters of food-borne infections may be peak during the warmest months of the year (CDC, 2001). The reasons for this seasonal pattern are not clearly known, but they may be due to: (i) increased prevalence of the pathogens in cattle or other livestock or vehicles of transmission during the summer; (ii) greater human exposure to contaminated foods during the cook-out months; and (iii) improper handling (e.g., temperature abuse) or incomplete cooking of products.

The results from this study indicated that pork and poultry samples are the major reservoirs for *Salmonella*. The serotypes frequently reported in the study may not be the true representation of all regions in India. Hence, thorough studies are needed to corroborate these findings.