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ORIGINAL ARTICLE

Non-typhoidal salmonellosis: Detection of genes responsible for virulence - A hospital based study from Manipal, India

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Abstract

Background: Non-typhoidal Salmonella (NTS) is one of the four critical worldwide reasons for diarrhoeal infections and causes a noted zoonotic infection termed non-typhoidal salmonellosis. Non-typhoidal salmonellosis generally causes self-limited gastroenteritis, whereas in immunocompromised conditions can result in invasive infections. *Aim and Objectives:* To detect the likely NTS serovars causing non-typhoidal Salmonellosis and their virulence genes. *Material and Methods*: This is a prospective cross-sectional research work carried out at the Enteric Diseases Division, Central Research Lab, Kasturba Medical College, Manipal from January 2016 - June 2018. Stool samples were collected from patients with diarrhoea admitted to the tertiary care centre in Udupi district, India. Stool samples were processed according to the World Health Organisation laboratory protocol. Non-typhoidal Salmonella isolated were subjected to PCR for the detection of virulence genes. *Results*: Of the 1599 diarrheal samples processed, 55 NTS were isolated with a prevalence of 3.43 %. *invA* gene was existent in 83.6% of the isolates, *spvC* gene in 61.8%, *stn* in 100% *sopB* in 96.4% and *sefA* in 5.45%. *Conclusion:* The presence of virulence genes among NTS increases the complications of non-typhoidal salmonellosis. Routine antimicrobial susceptibility tests for salmonellae should be carried out and then reported to prevent the associated morbidity and mortality.

Keywords: Non-typhoidal Salmonella, Salmonellosis, invasiveness, virulence genes

Introduction

Non-typhoidal Salmonellosis is an infection brought about by one of the Gram-negative bacteria termed Non-typhoidal Salmonella (NTS). It is one of the leading food-borne infections and zoonosis in the world. The symptoms associated with the infection include diarrhoea, vomiting and abdominal cramps [1]. An estimated 155,000 deaths occur annually among immunocompetent individuals throughout the world. Among immunosuppressed individuals including those undergoing cancer chemotherapy and those with inborn or acquired immunodeficiencies, non-typhoidal salmonellosis associated with septicaemia result in 681,000 deaths globally per year [2]. There is a possibility that NTS serovars in the presence of immunosuppressed conditions of patients and their virulent genes might play a vital role in causing bacteraemia and septicaemia. Non-

typhoidal salmonellosis is notified in healthy individuals as self-limited gastroenteritis, whereas among immunocompromised and diseased conditions of the hosts, it might be invasive resulting in bacteraemia, sepsis and focal infections like meningitis. Patients who are immunosuppressed because of HIV infection, malignancy, steroid use, diabetes, ongoing renal or liver illness, sickle-cell sickness, old aged and newly-conceived children are considered as vulnerable groups. Non-typhoidal Salmonella can cause invasive infections because of the presence of an assemblage of virulence genes present in plasmids and genetic material [1]. The *invA* gene is a local invasion gene located in the Salmonella pathogenicity island that confers the assemblage of proteins from the type III secretion system which is responsible for the entry of bacterium into the epithelial cells. Salmonella plasmid virulence (*spv*) operon comprises five genes - *spvRABCD* which is essential for intracellular persistence and multiplication resulting in septicaemia. Intestinal adhesion is mediated by *Salmonella enteritidis* fimbriae -*sefA* gene. *sopB* gene codes for Salmonella outer protein B, an important component of Type III secretion system causing Salmonella-induced gastroenteritis and invasion. *stn* gene codes for Salmonella enterotoxin which is a putative virulence factor and the causative agent of diarrhoea [1, 3]. The present study was undertaken to determine the prevalence and virulence gene profile of NTS isolated from gastroenteritis cases in a tertiary care hospital in Manipal, India. The study was also focused to demonstrate the association of virulence genes with febrile illness among patients suffering from non-typhoidal salmonellosis. With the developing antimicrobial resistance profile and the severity of salmonellosis cases around the globe, it is essential to recognise the non-typhoidal salmonella serovars with their virulence profile to improve the outcome of the treatment.

Material and Methods

This research work was submitted and endorsed by the Institutional Ethics Board. Subsequently, on acquiring the informed consent from patients with diarrhoea, the patient's demographic details were collected and recorded. Stool samples of patients suffering from acute gastroenteritis and dysentery admitted to the wards and also attending the outpatient departments were collected and transported to the Enteric Diseases Division, Department of Microbiology, Kasturba Medical College, Manipal. The samples were transported with ice pack specimen carrier which maintains a temperature of 2-8^{0}C. The study was conducted for a period of two and a half years (January 2016-June 2018).

Isolation and identification of NTS from faecal samples:

The samples were collected in a sterile wide-mouthed container following aseptic precautionary measures and promptly transported for additional processing agreeing to the guidelines of the World Health Organisation (WHO) Laboratory Protocol [4]. Primary inoculation was carried out on MacConkey's agar (HiMedia Laboratories) and Hektoen Enteric agar (Difco, BD). The enrichment media used was Selenite Faeces broth (HiMedia Laboratories) which was sub-cultured after overnight incubation. Non-lactose fermenting colonies from MacConkey agar plates and green with black centred colonies from Hektoen Enteric agar plates were selected and subjected to biochemical tests for the identification of NTS.

Antigenic profiling

Isolates of Salmonella were serotyped with specific polyvalent O and H antisera (Remel Diagnostics, Fisher Scientific). The strains confirmed as *Salmonella enterica* subspecies Enterica were transported to the National reference centre - NICED (National Institute of Cholera and Enteric Diseases), Kolkata, India for serotyping.

Detection of virulence genes in NTS

Genomic DNA of NTS (n=55) was extracted using the protocol as per Medici *et al.* [5]. The extracted DNA was evaluated employing Nanodrop (Eppendorf BioPhotometer D30) with absorbance values at 320 nm. The primers for this study - *invA, spvC, stn , sopB* and *sefA* genes were designed through primer 3 output sequence (available at http://bioinfo.ut.ee/primer3-0.4.0/) (Table 1). The primers were sequenced at Bioserve Ltd (India) & Integrated DNA Technologies (USA) and procured from the same.

Salmonella enterica serotype (4,5,12:i:-) was used as the positive control for *invA, spvC, sopB Salmonella enterica* strain Typhimurium DT96 *for stn and Salmonella* Enteritidis149539for *sefA gene*. *Escherichia coli* ATCC 25922 was used as the negative control. The PCR reaction was completed following the methodology of Mir *et al.*, –[3]. PCR reactions were carried out in a total volume of 12.75 µl, consisting of primers (0.5 µl each), 5 ng of DNA (1 µl), taq polymerase master mix (6.25 µl) (Go Green, Promega Corporation, USA) and sterile MilliQ water was added to make the final volume. The PCR protocol consisted of an initial denaturation step at 95^0C for 2 minutes followed by 30 cycles of DNA denaturation at 95^0C for 30 seconds with 30 cycles of primer annealing for *invA* gene at 50^0C, for *spvC* gene at 54^0C, for *sopB* and *sefA* gene at 55^0C, for *stn* gene at 59^0C for 30 seconds and primer extension at 72^0C for 45 seconds. After the last cycle, a final extension step at 72^0C for 7 min was added. The amplified products were analysed in a 2.5% (w/v) agarose gel in TAE buffer. Ethidium bromide (Sigma-Aldrich, USA) (0.5 µg/mL TAE) stained DNA amplicons were seen using a gel imaging system (Biotron Healthcare).

Table 1: Details of primers of virulence genes used in this study

Gene	Sequence	Size (bp)
invA	FP –GTTTACGACCTGAATTACTG RP – GATAAGACGACTGGTACTGA	239
spvC	FP-GGTAGATAAGTGGAAAGTGA RP-GGATTCCAGACACTCTATAA	163
sopB	FP-CTTCTATCACTCAGCTTCAC RP-CTGATGCTGTAGATAATTCC	184
sefA	FP-ATCTGCAGTAGCAGTTCTT RP-AGTAATGCTGAGAGTACCAA	196
stn	FP-CTATCGGTAACAGTGATGAT RP-ATTACTCACTCCCTGAATCT	230

Statistical analysis

The software version 20.0 IBM SPSS, (The IBM SPSS® software, USA,) was utilized to create descriptive statistics of data. Chi-square test and odds ratio were utilized to figure out the association between virulence genes and febrile illness.

Results

Of the 1599 diarrheal samples processed from January 2016 to June 2018, 55 isolates were found to be NTS showing a prevalence of 3.43 %. The other bacterial pathogens isolated during the period were *Aeromonas spp.* (6.95%), *Vibrio cholerae* (4.17%), *Plesiomonas shigelloides* (2.78%), *Vibrio spp.* (2.78%), *Shigella flexneri* (2.78%), *Shigella sonnei* (1.39%) and diarhheagenic *E. coli* (1.39%). NTS serovars causing acute gastroenteritis are *Salmonella oslo* (17/55, 30.91%), the prevalent serovar followed by *Salmonella typhimurium* (14/55, 27.27%) which was the second commonest serovar causing salmonellosis in our study. Additional to this, other serovars were *Salmonella kentucky*, *Salmonella newport* were 7.27% (4/55), *Salmonella hindmarsh*, *Salmonella wangata* were 5.45% (3/55), *Salmonella bareilly*, *Salmonella agona, Salmonella weltevreden* were 3.64% (2/55) and *Salmonella freiburg*, *Salmonella saintpaul*, *Salmonella enek* were 1.82% (1/55). *invA* gene was present in 83.6% (46/55) of the NTS isolates, *spvC* in 61.8% (34/55), *stn* in 100% (55/55), *sopB* in 96.4% (53/55) and *sefA* in 5.45% (3/55). Figures 1-5 displays the bands which are positive for the virulence genes (*invA, spvC, stn, sopB* and *sefA* gene*).* The distribution of virulence genes among the different NTS serovars is depicted in Table 2.

Table 2: Human NTS serovars with virulence genes

NTS Serovars	*invA*	*spvC*	*sefA*	*sopB*	*stn*
S. oslo (17)	14 (30.44%)	9 (26.47%)	0	17 (32.07%)	17 (30.9%)
S. typhimurium (15)	12 (26.08%)	9 (26.47%)	0	15 (28.3%)	15 (27.27%)
S. newport (4)	4 (8.69%)	3 (8.82%)	0	4 (7.54%)	4 (7.27%)
S. kentucky (4)	3 (6.52%)	4 (11.76%)	0	4 (7.54%)	4 (7.27%)
S. hindmarsh (3)	3 (6.52%)	2 (5.88%)	0	3(5.66%)	3 (5.45%)
S. wangata (3)	3(6.52%)	2 (5.88%)	3 (5.45 %)	3 (5.66%)	3 (5.45%)
S. bareilly (2)	1 (2.17%)	2 (5.88%)	0	2 (3.77%)	2 (3.63%)
S. agona (2)	2 (4.35%)	0	0	2 (3.77%)	2 (3.63%)
S. weltevreden (2)	1 (2.17%)	2 (5.88%)	0	2 (3.77%)	2 (3.63%)
S. freiburg (1)	1 (2.17%)	0	0	0	1 (1.81%)
S. saintpaul (1)	1 (2.17%)	0	0	0	1 (1.81%)
S. enek (1)	1 (2.17%)	1 (2.94%)	0	1(1.88%)	1 (1.81%)
Total	**46 (83.4%)**	**34 (61.8%)**	**3 (5.45%)**	**53 (96.4%)**	**55 (100%)**

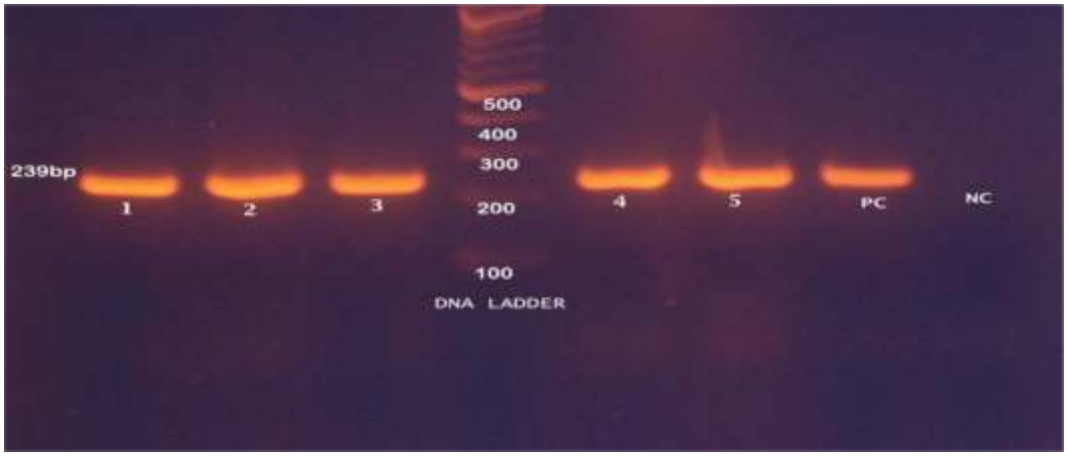

Figure 1: Gel image showing amplification of *invA* gene Bp: Basepair, PC: Positive control, NC: Negative control

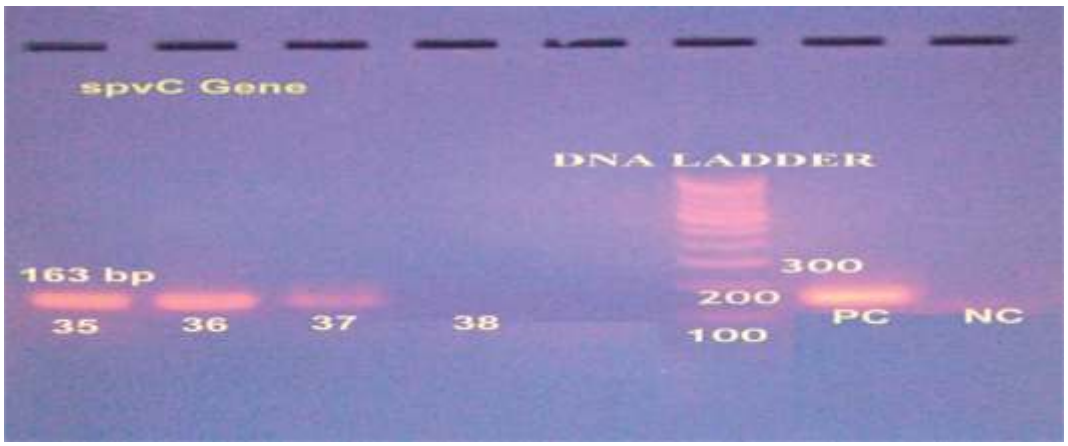

Figure 2: Gel image showing amplification of *spvC* gene Bp: Basepair, PC: Positive control, NC: Negative control

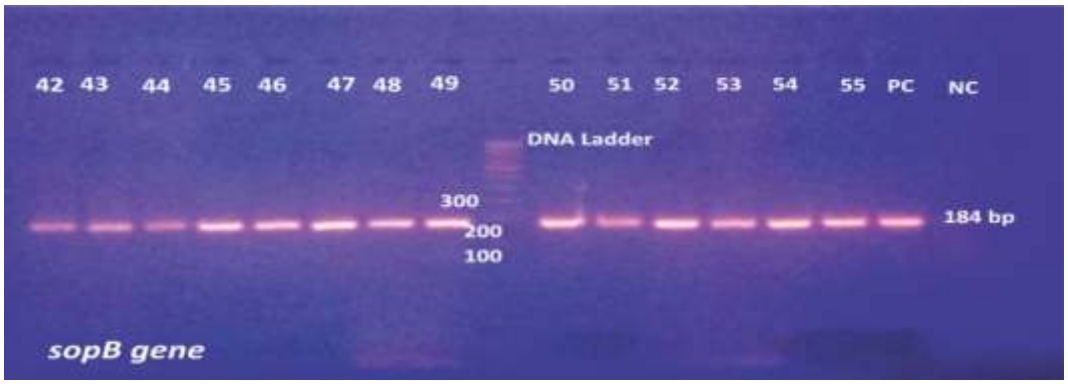

Figure 3: Gel image showing amplification of *sopB* gene Bp: Basepair, PC: Positive control, NC: Negative control

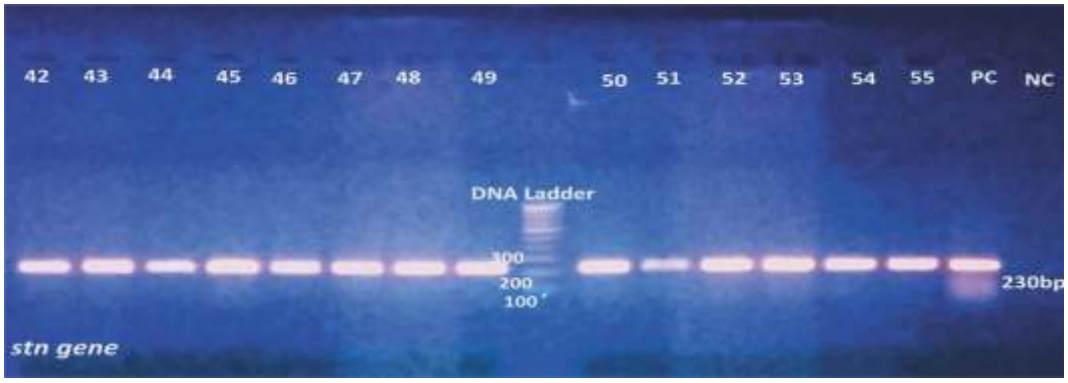

Figure 4: Gel image showing amplification of *stn* gene Bp: Basepair, PC: Positive control, NC: Negative control

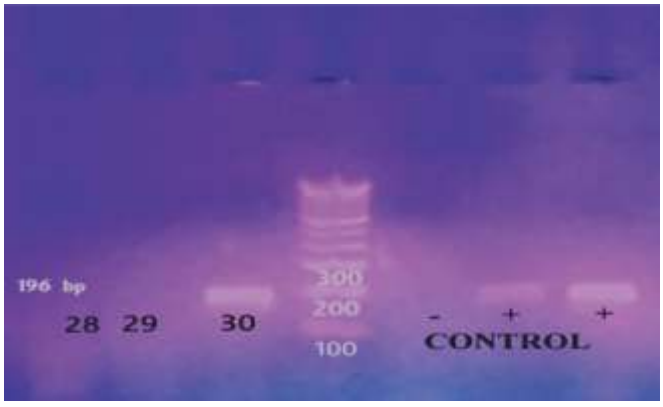

Figure 5: Gel image showing amplification of *sefA* gene Bp: Basepair, PC: Positive control, NC: Negative control

Discussion

Non-typhoidal salmonellosis is a significant public health problem worldwide. It is now being recognized as a leading cause of bacterial diarrhoea universally among human beings and livestock but reports from our country are few.The prevalence of NTS from diarrheal cases in our study was found to be 3.43 %.The presumptive phenotypic characteristics of NTS identified in our study were non-lactose fermenting colonies on MacConkey's agar, green with black centred colonies on Hektoen Enteric agar, gram negative bacilli, short rods and motile. The key biochemical reactions were oxidase-negative, oxidative-fermentative, typical red slope with yellow butt reaction in triple sugar iron (alkaline slope and acid butt) with the production of high amounts of hydrogen sulphide and gas, negative indole test, citrate utilised, lysine decarboxylated and negative urease reaction.The prevalence rate is higher than that of the study carried out in north India at Chandigarh where it was only 0.81% [6]. Asian countries like China had a prevalence rate of 4.5% higher than our prevalence rate [7]. Bangladesh and Malaysia had a prevalence rate of 1.3% and 2.22% respectively which are slightly lower compared to our study [8-9]. The maximum prevalence rate of 43.5% was

seen in an African country, Nigeria [10]. The United States of America also showed a higher prevalence rate of 11% [11]. .

In our study, *S.oslo* was the most prevalent serovar followed by *S. typhimurium*. Our earlier study in 2016 in a similar region revealed *S. typhimurium* as the primary common serovar [1]. This determines that over the three years *S. oslo* supplanted *S. typhimurium* as the primary common serovar in our district. Studies done in the northern district of India revealed *S. senftenberg* as the overwhelming serovar shadowed by *S. typhimurium* [6]. A study conducted by Menezes *et al*. which dealt with the isolation of NTS from three cities of south India showed *S. agona* as the widespread serovar trailed by *S. typhimurium* [12]. The variation in the serovar may be due to the changing epidemiological trend, the regional variation and the specific patient population. Research done in the African continent, Italy and Saudi Arabia has put forward *S. typhimurium* as the utmost, consecutively occurring serovar causing non-typhoidal salmonellosis [13-15]. Similar findings were also observed in a hospital-based cross-sectional study conducted in Iraq showing the most commonly detected serovar in their country was *S. typhimurium* constituting about 54% [16]. Bangladesh one of the Asian countries has also reported *S. typhimurium* as the most common serovar among patients suffering from acute gastroenteritis [8]. A Chinese study also showed *S. typhimurium* as the commonest serovar followed by serovar - 4,5,12:i:-. However, the study also described that among children aged 3–5 and >5 years, *S. enteritidis* was the common NTS followed by *S. typhimurium* [7]. Non-typhoidal Salmonella infections in the USA reported *S. enteritidis* as the most prevailing serovar [17]. *S. corvallis* was the most important strain causing acute gastroenteritis in humans in a Malaysian study [9].

NTS can cause invasive infections because of the presence of an assemblage of virulence genes located in plasmids and chromosomes. *invA* gene is conserved among a majority of the NTS and carries sequences distinctive to the NTS, helping in the identification of salmonella from clinical, food and environmental sources [18]. *invA* gene was found in 83.6% (46/55) of the NTS isolates in our study. However, a West African study through Dione *et al*. concluded that *invA* gene was present in 99.5% of the isolates [19]. The proportion of the *invA* gene in our study is lesser in contrast with different investigations completed which concluded the presence of *invA* gene in all of the NTS isolates [3, 14]. PCR analysis of the NTS isolates in a study done by Smith *et al*. in Nigeria for the detection of *invA* gene indicated its proportion as 96.1% [18]. The presence of *invA* gene at 55% from the poultry NTS isolates was reported in dual studies one from northeast India and the other from Egypt [20-21]. The authors have previously reported the proportion of *invA* gene as 74.13% among the NTS isolated from poultry sources in the same district [22].

Symptoms associated with patients suffering from NTS observed in our study were diarrhoea (100%), fever (61.82%), vomiting (45.45%), abdominal pain (36.37%) and chills (12.73%). The association of *invA* gene with the clinical presentation of fever in patients was found to be significant in this study (OR=20.3). Out of the 46 patients with *invA* gene in their NTS isolate, 34 (73.9%) patients were found to be febrile which was found to be statistically significant (**p=0.001). This finding specifies that the *invA* gene coding for invasion and existence in

macrophages can lead to the invasion of cells thereby causing invasive infections and clinical symptoms among the patients infected [23].

The genes *spvC, sopB, stn* and *sefA* were observed in 61.8%, 96.4%, 100% and 5.45% of the NTS serovars correspondingly. The heterogeneous dissemination of these virulence factors in Salmonella isolated from different sources has been observed. Salmonella plasmid virulence (*spv*) operon comprises five genes -*spvRABCD* which is essential for the intracellular persistence and replication causing septicaemia. The *spvC* gene plays a vital role in causing systemic infection and studies have shown their presence in NTS isolated from food samples causing food poisoning [23]. The proportion of *spvC* in our study was found to be 65.46% which is less contrasted with the studies done by Mir *et al*. and Rowlands *et al*. which showed the extent of gene as 100% and 80.9% respectively [3, 23]. Out of the 36 patients with *spvC* gene in their NTS isolate, 19 (52.78%) patients were observed as febrile. Thus, there was 52.78% *spvC* gene positivity along with the occurrence of fever presentation. This statistically showed less association (OR<1) and was not found to be significant. It is well-defined that *spvC* gene was predominantly present in certain specific serovars such as Typhimurium, Enteritidis, Dublin, Choleraesuis, Gallinarum, Pullorum and Abortusovis [24]. This is upheld by our finding which showed that all the 14 *S. typhimurium* were found to be positive for *spvC* gene showing a proportion of 100%, and among *S. oslo* the proportion of *spvC* gene was 58.82%. Likewise, it is also reported that the important NTS serovars, Typhimurium and Enteritidis had the *spvC* gene in 86% of their strains [23].

The *sopB* gene which codes for Salmonella outer protein B, is an important component of the Type III secretion system causing Salmonella-induced gastroenteritis and invasion [3, 23]. The proportion of *sopB* gene among the NTS isolates in our study was 96.4%. Studies done in north India have shown the proportion of *sopB* gene among the NTS isolates was 100% in poultry samples and 90.9% among the clinical cases [3]. In the present study, the *sopB* gene was present in all the NTS serovars like Typhimurium, Oslo, Newport, Hindmarsh, Kentucky, Wangata, Bareily, Agona, Infantis and Weltevreden except for Freiburg and Saintpaul. Other studies have also shown that *sopB* gene was found to be present in serovars like Typhimurium, Enteritidis, Gallinarum, Virchow, Agona, Choleraesuis, Paratyphi B, Bareilly and Newport [3,19]. The percentage of febrile illness among salmonellosis patients with s*opB* gene was 64.15% (34/53), this showed a strong association (OR>1) but statistically was not found to be significant.

The *stn* gene codes for Salmonella enterotoxin, a putative virulence factor and contributory segment for initiating diarrhoea [25]. The proportion of *stn* gene in our study was found to be 100% present in all NTS serovars isolated in our study. This is in concordance with many studies carried out in the isolation of NTS from human as well as environmental sources [3, 25]. Thus, there was 100% *stn* gene positivity with the occurrence of diarrhoea presentation among patients.

Intestinal adhesion is mediated by *Salmonella enteritidis* fimbriae - *sefA* gene [23]. However, in our study the *sefA* gene was found in only 3 NTS isolates of *S. wangata*. This implies that this gene could have been specific only to *S.wangata*. Earlier studies carried out have also shown the

presence of *sefA* gene only in *S. enteritidis* and being negative for many serovars such as *S. typhimurium*, *S. agona*, *S.infantis*, *S.brandenburg* and *S.saintpaul* which is a likely outcome because the genes of the *sef operon* (*sefABCD*) are constrained only to the serovars of serogroup O:9 which comprises *S. typhi*, *S. gallinarum*, *S. enteritidis*, *S. dublin*, and *S. berta*. *sefA* gene has been used as a molecular tool for differentiating *S. enteritidis*. In addition to the *sefA* gene, there are bacterial adhesion molecules called adhesins which play a vital role in the pathogenesis of infection by adhering to the infection site. Some of the adhesins are found to be fimbriae or pili, pilus-associated adhesins which are said to be monomeric or oligomeric proteins and curli. These adhesins can identify host cell surface receptors such as collagen, fibronectin, laminin and heparan sulfate [26].

A multicentric study involving common objectives in explaining the association between the virulence genes and theclinical presentations would be necessary to achive a dependable outcome. According to the literature search, there are no studies done on the association of virulence genes among human NTS serovars and their correlation with the clinical features of the patients. However, there are studies done where in the presence of virulence genes such as *VT1*, *VT2* and *eaeA* in diarrheagenic *E.coli* are correlated with the clinical presentation of diarrhea initiated by it [27]. The major predominant factors which favour diarrheal infections including salmonellosis are poor hygiene and sanitation [28]. Due to the expanding world population, there is increment in the production of large scale farms in order to fulfil the rising food needs. Subsequently, the NTS serovars which have nonspecific hosts circulate effectively among the farm animals which in turn marks the elimination process of NTS from farms and their yields challenging [29].

Conclusion

The present study vividly summarizes the prominence of this neglected microbe in this portion of South India. NTS generally produces self-limited gastroenteritis in healthy entities whereas, among the elderly and non-immune patients, the result could be an extremely fatal illness. This study endorses that the NTS serovars possess virulence genes which can cause human disease with varying invasive virulence in humans. Some of the virulence genes were confined to certain specific NTS serovars. Statistically, there was strong an association between *invA*, *sopB* gene with the fever presentation in patients, implying that these genes are probably accountable for the virulence of the microbe. Early diagnosis is not only necessary for the detection of NTS but also to prevent fatal complications like septicaemia. Consequently, it is essential to contemplate food hygiene programs to prevent salmonellosis Further analysis on accurate distribution of virulence genes among NTS is also needed to develop accurate preventive measures.

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