SEROVAR PROFILE AND DETECTION OF INVA VIRULENCE GENE AMONG NON-TYPHOIDAL SALMONELLAE SEROVARS ISOLATED FROM ACUTE GASTROENTERITIS CASES IN COASTAL KARNATAKA, SOUTHERN INDIA

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ABSTRACT

Objective: Non-typhoidal salmonellosis is one of the leading zoonosis in the world caused by non-typhoidal *Salmonella* (NTS). Invasive infections with NTS serovars occurs due to the presence of virulence genes like invA along with the immunosuppressive conditions of the patient. The study was conducted to isolate and identify the NTS serovars and their antimicrobial resistance profile from patients with diarrhea and also to detect the virulence marker – *invA* gene among these NTS serovars.

Methods: A prospective cross-sectional study was conducted from January 2015 to December 2016 at the Enteric Diseases Division, Kasturba Medical College, Manipal. 1218 fecal specimens were collected from patients with diarrhea and before antibiotic treatment. NTS serovars were identified, serotyped and then screened for the presence of *invA* virulence gene.

Results: A total of 33 (2.7%) NTS was isolated. *Salmonella typhimurium* (33.34%) was predominant followed by *Salmonella oslo* (30.3%). Out of 33 NTS, *invA* was positive for 28 isolates (84.8%) of which 25 (89.3%) patients were febrile which was statistically significant (p=0.000).

Conclusion: Non-typhoidal salmonellosis is an emerging global infection among immunocompromised patients. Our study showed an association between the *invA* gene and febrile illness among the patients suffering. Thus, this study highlights the importance of *invA* as a significant marker for bloodstream invasion.

Keywords: Non-typhoidal salmonellosis, *invA* gene, Virulence, Non-typhoidal *Salmonella*, Bloodstream infection.

INTRODUCTION

Non-typhoidal salmonellosis is one among the leading causes of zoonosis and gastrointestinal infection in the world [1]. It defines the disorders produced by all serotypes of *Salmonella*, excluding typhi and paratyphi group [2]. The prominent non-typhoidal *Salmonella* (NTS) causing salmonellosis are *Salmonella gallinarum, Salmonella typhimurium, Salmonella choleraesuis, Salmonella enteritidis, Salmonella dublin,* and *Salmonella abortusovis* [3]. Human beings acquire this infection through the consumption of undercooked, uncooked, contaminated food such as eggs, chicken meat, pork, beef, and unpasteurized milk [4]. The clinical manifestations start with an infectious diarrhea that begins within 12–72 h after the intake of infected food [1]. The affected person shows symptoms of diarrhea, vomiting, and abdominal pain [1]. There is a possibility that these non-typhoidal *Salmonella* (NTS) serovars with their diverse invasive virulence genes can cause invasive non-typhoidal salmonellosis resulting in bloodstream infection among adolescents with comorbid conditions such as malaria, malnutrition, and in adults with HIV [5-7]. The clinical manifestation of invasive non-typhoidal salmonellosis is typically a febrile systemic illness which simulates to that of enteric fever, diarrhea is usually not present, and other clinical symptoms vary and are not defined [8]. Risk factors are found to be HIV infection and its worsening illness, malnutrition [8]and malaria [9]. There are several virulence genes such as *invA*, *spv*, *sefA*, *stn*, and *sopB* that are responsible for its colonization, invasiveness, intracellular survival, and damage to host tissues [10].

The bacteria responsible for Non-typhoidal salmonellosis and its invasiveness vary depending on the population and place [5,7,9]. It was observed that no much work has been carried out on this emerging foodborne zoonotic infection from this region of coastal Karnataka. The recent substantial increase in the number of NTS isolates in this part of coastal Karnataka beckons the need for phenotypic and genotypic characterization of the isolates to raise awareness especially regarding their role in foodborne infections. With the growing antimicrobial resistance profile and the severity of invasive cases, we need to have an idea of the likely bacterial serovars and their presumptive antibiotic therapy to improve the outcome of the treatment. The present study was also conducted to detect the *invA* gene as a marker for bloodstream invasion caused by NTS isolated from this region.

METHODS

Fecal specimen of patients suffering from acute gastroenteritis or dysentery admitted to the wards and presenting at the outpatient departments were collected and transported to the Enteric Diseases Division, Kasturba Medical College, Manipal, for a period of 2 years (January 2015 - December 2016).

The study was submitted and approved by the Institutional Ethics Committee. Following the informed consent from patients with diarrhea, a standard clinical questionnaire was completed. The patient's

demographic details were collected and recorded, and it consisted of clinical data (presence of fever, abdominal pain, and vomiting), underlying conditions, physical and systemic examinations, date of the diarrheal episode, frequency of diarrhea, history of travel, type of food consumed and laboratory investigations.

Isolation and identification of NTS from fecal samples

The samples were collected in sterile wide mouthed container following aseptic precautions and immediately transported for further processing according to the protocol given by centers for disease control and prevention (CDC) [11]. Primary inoculation was carried out on MacConkey's agar (HiMedia Laboratories) and Hektoen Enteric agar (Difco, BD), and enrichment was performed on Selenite Feces broth (HiMedia Laboratories) which was subcultured after overnight incubation. Non-lactose fermenting colonies from MacConkey agar plates and green with black centered colonies from Hektoen Enteric agar plates were picked and subjected to further analysis for biochemical tests for the identification of NTS [12].

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* was further serovar identified and its antigenic profiling was done at the national reference center - National Institute of Cholera and Enteric Diseases, Kolkata, India.

Antimicrobial susceptibility test

NTS isolated were subjected to antimicrobial susceptibility testing for various antimicrobials, namely, amikacin (30 μg), ampicillin (10 μg), amoxyclav (30 μg), azithromycin (15 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), and co-trimoxazole (25 μg). *Escherichia coli* 25922 was used as the control strain. The antimicrobial susceptibility testing was performed by modified Kirby-Bauer's disk diffusion method according to Clinical Laboratory Standards Institute guidelines [13].

Detection of virulence-specific marker – *invA* gene of NTS

Salmonella enterica serotype (4,5,12:i:-) was used as the positive control and NTS (n=33) genomic DNA were extracted using the protocol as per De Medici *et al.* [14]. The extracted DNA was quantified using Nanodrop (Eppendorf BioPhotometer D30) with absorbance values at 320 nm. The primer for *invA* gene was designed using the primer 3 output sequence (available at http://bioinfo.ut.ee/primer3-0.4.0/). The forward primer of *invA* gene GTTTACGACCTGAATTACTG and reverse primer GATAAGACGACTGGTACTGA with a base pair of 239 was used in this study. The PCR reaction was carried out following Mir *et al.* [10]. PCR reaction was 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 Milli-Q water was added to make the final volume. The PCR reaction consisted of 1 cycle of an initial denaturation of 94°C for 2 min, followed by 35 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 2 min, and a final extension cycle was performed at 72°C for 10 min. The amplified products were analyzed in a 2.5% (w/v) agarose gel in 1X Tris base, acetic acid and EDTA (TAE) buffer. Ethidium bromide (Sigma-Aldrich, USA) (0.5 μg/mL TAE) stained DNA amplicons were seen using a gel imaging system (Biotron Healthcare).

Statistical analysis

The software version 16.0 IBM SPSS was used to generate descriptive statistics of data. Chi-square test was used to find out the association of *invA* gene and febrile illness with a **p=0.000.

RESULTS

Of the total 1218 diarrheal fecal samples collected, 33 isolates were NTS showing a prevalence of 2.7%. *S. typhimurium* (33.34%, 11/33) was found to be the most common serovar followed by *S. oslo* (30.3%, 10/33). The other NTS isolated are depicted in Fig. 1.

Among the antimicrobial agents screened, resistance was observed in ampicillin and cefuroxime (5/33, 15.1%), amoxicillin (4/33, 12.1%), and ciprofloxacin and cotrimoxazole (3/33, 9.09%). Fig. 2 depicts the antimicrobial resistance pattern of NTS isolates.

Out of the 33 NTS screened for *invA* gene, 28 (84.8%) isolates were positive for *invA* gene this is shown in Fig. 3.

Among the 28 *invA* positives, 25 (89.3%) of the patients were found to be febrile.
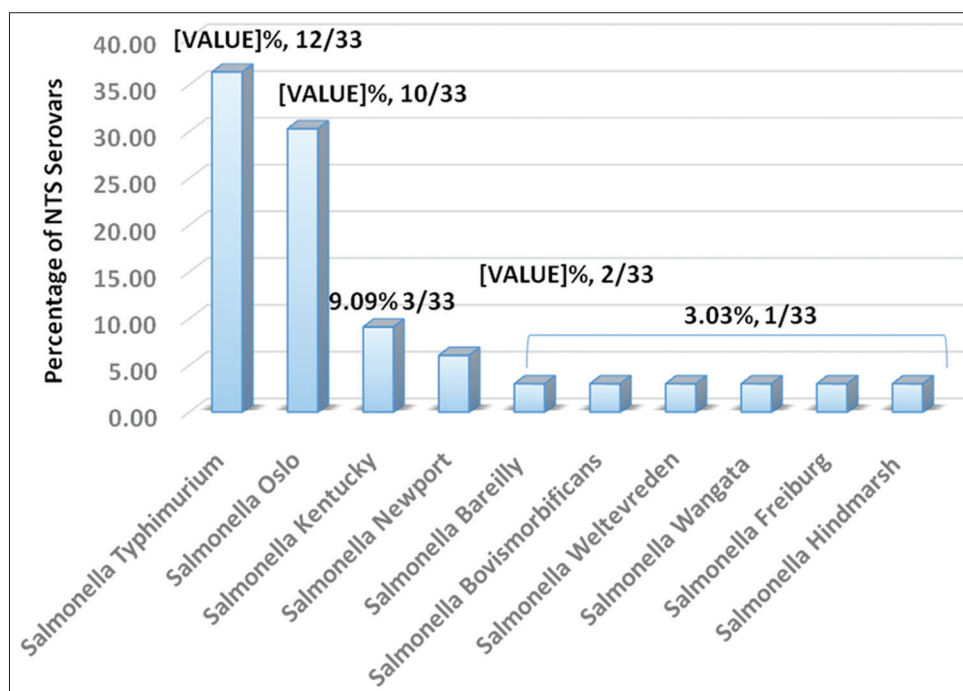

Fig. 1: Non-typhoidal *Salmonella* serovars causing non-typhoidal salmonellosis
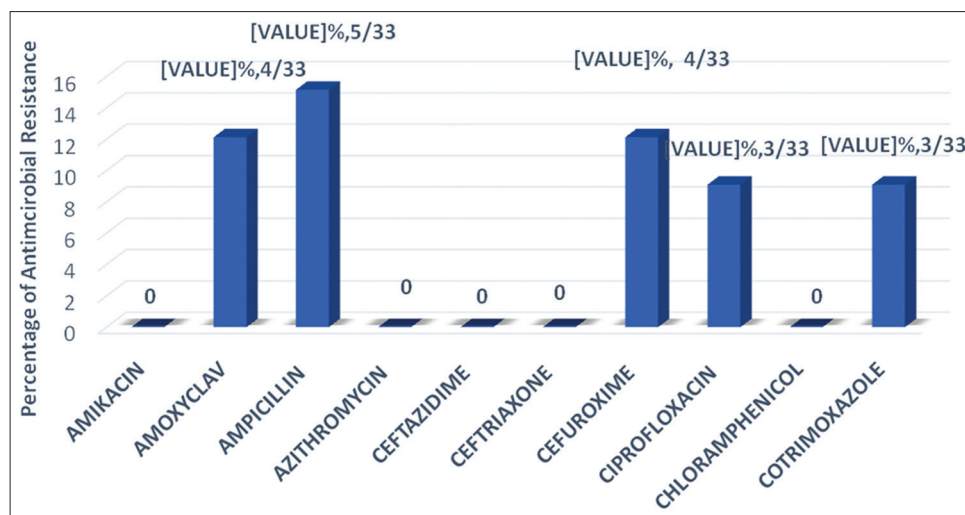
Fig. 2: Antimicrobial resistance pattern of non-typhoidal *Salmonella* isolates

Table 1: Age distribution of patients with Non-typhoidal salmonellosis

Age group (years)	Number
11-20	3
21-30	1
31-40	3
41-50	3
51-60	7
61-70	14
71-80	1
81-90	1
Total	33

The demographic details were analyzed and the age distribution showed a predominance of patients above 60 years to be infected with salmonellosis. This is depicted in Table 1.

The clinical manifestations associated with non-typhoidal salmonellosis were immunosuppressed conditions illustrated in Fig. 4. All the patients had acute gastroenteritis. The symptoms observed in these patients include fever (25/33, 75.8%), vomiting and abdominal pain (7/33, 21.21%), chills (4/33, 12.12%), and headache (2/33, 6.06%).

The most common source of food for non-typhoidal salmonellosis was poultry products (24/33, 72.7%) followed by fruits, vegetables, and water source (3/33, 9.09%), along with dairy products (2/33, 6.06%) and seafood (1/33, 3.03%).

DISCUSSION

Salmonella serovars invariably have the ability to cause bloodstream infections when they have an assemblage of virulence genes in the *Salmonella* pathogenicity islands (SPIs)[15]. SPIs have nearly 60 such genes [15]. These SPIs may be located on a bacterial chromosome or large virulence-associated plasmids [16]. Virulence chromosomal genes of NTS are *invA, spvC, sefA, sopB,* and *stn* [10]. One such virulence gene is *invA,* a local invasion gene essential for the entry of the bacterium from the gut lumen into the epithelial cells [10]. It is the topmost gene of an operon *Salmonella* [10]. *invA* gene is a unit of the SPI 1 that is very much required for the complete cell invasion [15]. Our study showed that of the 33 NTS, 28 (84.8%) isolates were positive for *invA* gene which is in concordance with the study by Ohud *et al.* [17] reporting *invA* gene in 89.9% of the NTS isolates. However, a West African study by Dione *et al.* revealed that *invA* gene was present in 99.5% of the isolates [18]. Many studies have shown that
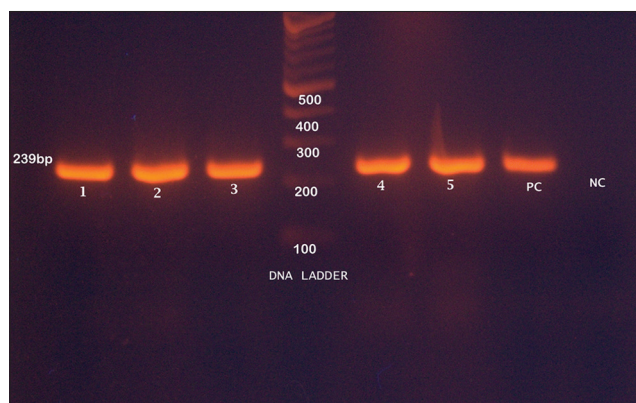

Fig. 3: Gel image showing amplification of *inv*A gene. Bp: Basepair, PC: Positive control, NC: Negative control
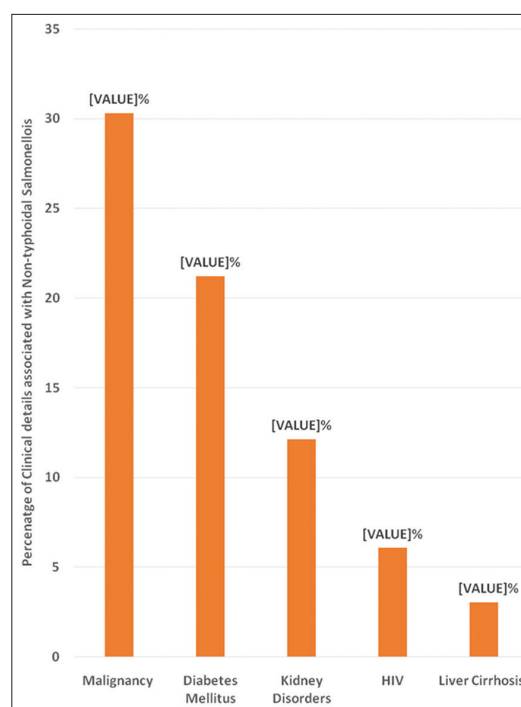

Fig. 4: Clinical details associated with non-typhoidal salmonellosis

invA gene of NTS carries sequences those of which are distinctive for genus *Salmonella*; thus *invA* gene is recognized as an essential target to detect *Salmonella* by PCR [19,20]. The correlation of *invA* gene with the clinical presentation of fever in patients was found to be significant in this study. Out of the 28 patients with *invA* gene in their NTS isolate, 25 (89.3%) patients were found to be febrile which was found to be statistically significant (**p=0.000). This indicates that *invA* gene is possibly responsible for the virulence of the bacteria, facilitating their entry into the bloodstream and resultant consequences including fever. Patients negative for *invA* gene in their NTS serovar did not show any symptom of fever. Thus, there was a good correlation between the *invA* gene and clinical presentation of fever in patients infected with NTS. Non-typhoidal salmonellosis is recognized as a public health issue in previously normal and healthy individuals causing self-limiting gastroenteritis [21]. Although in immunosuppressed and diseased hosts, NTS can cause invasive infections resulting in bacteremia and sepsis [5]. This is termed as invasive non-typhoidal salmonellosis [5]. Immunosuppressed patients with HIV infection, malignancies, steroid use, kidney disorders, liver cirrhosis, diabetes or sickle-cell disease, infants and aged patients are considered as risk groups for invasive non-typhoidal salmonellosis [22]. The observation in our study was that malignancy (30.3%) was the most common immunosuppressed condition followed by acquired immune deficiency syndrome (6.06%). Other comorbid/risk factors include kidney disorders (12.12%) and liver disease (3.03%). A study in Chandigarh reported non-typhoidal salmonellosis associated with malignancy was 9% and renal disorders 7% which is lesser than our study [23]. This finding is similar to a study conducted in Malaysia reporting 23.6% of malignancy cases; AIDS was 20%, diabetes mellitus, renal disorders and liver cirrhosis were 7.3% [24]. It was also observed that patients aged above 60 years were diagnosed of NTS and is in concordant with other studies elsewhere [23,24].

Among the serovars isolated, *S. typhimurium* was the most common serovar followed by *Salmonella oslo*. Similar findings are being reported by studies in Italy by Frasson *et al.* [25]and in Africa by Karuiki *et al.* [26]of *S. typhimurium* being the most frequent serovar causing non-typhoidal salmonellosis. This is in contraindication to studies reported elsewhere in the country. A study conducted by Menezes *et al.* [27], showed *Salmonella agona* as the prevalent serovar followed by *S. typhimurium*. Another study by Taneja *et al.* reported *Salmonella senftenberg* as the prevailing serovar followed by *S. typhimurium* [23].

The predominant food source for non-typhoidal salmonellosis was found to be poultry products such as chicken meat and eggs in 72.7%. CDC has reported that a poultry farm in California in 2013 caused about 300 people to be infected with *Salmonella heidelberg* [28]. NTS generally resides in the gut of poultry, farm animals, and pets. Even the naturally fed poultry can harbor *Salmonella* [29]. Throughout the time usually NTS does not make the birds and farm animals ill, but they can cause severe disorders when transmitted to humans [29]. Antibiotics are used in poultry for their growth fastening process which results in the colonization of antibiotic resistant bacteria [30]. These resistant bacteria can later spread their resistance to human beings and cause infections which would make the treatment difficult [29,30]. To measure the burden and trends of drug resistance pattern in NTS, continuous surveillance and antibiotic susceptibility testing are essential to be performed in different geographical regions throughout the country [31].

CONCLUSION

Non-typhoidal salmonellosis is a major problem among immunocompromised patients. It is also the most common enteric pathogen isolated from Manipal, Karnataka. In the present study, PCR-based techniques with genus-specific primers corresponding to *invA* gene appeared to be rapid, sensitive, and precise. Detection of *invA* gene by PCR throws light on its invasive nature. 89.3% of the patients infected by NTS with *invA* gene had a fever along with diarrhea.

Thus, the *invA* gene proved to be a significant marker for bloodstream invasion and found in almost 90% of the patients suffering from non-typhoidal salmonellosis.

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