https://doi.org/10.3329/cbmj.v12i1.64287

Phenotypic and Genotypic Characterization of *Salmonella Typhi* isolated from Human Blood with Distribution of Virulence Genes and Sequencing

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Abstract

A cross-sectional study was conducted in the Department of Microbiology at tertiary care hospital in Bangladesh from July 2019 to June 2020, to see the phenotypic and genotypic characterization of *Salmonella Typhi* isolated from human blood with distribution of virulence genes and sequencing. Clinically suspected enteric fever patients irrespective of age and gender who attended either the Department of Microbiology or Medicine out-patient department (MOPD) in a tertiary level hospital in Bangladesh for blood culture and sensitivity test were included in this study. The positive growth *S. Typhi* was identified by gram straining, colony morphology and biochemical test. Then *Salmonella Typhi* was identified by using *Salmonella* specific antisera. Final identification was done by using *16s Rrna* by PCR. Virulence factors like hemolysin and siderophore was detected by siderophores production assay. PCR and sequencing of virulence genes were done. A total number of 50 isolated organisms were identified as *Salmonella* species of which *Salmonella Typhi* was detected in 40 (48.2%) isolates. Among 40 isolated *Salmonella Typhi,* no *Salmonella Typhi* produced hemolysin and 30 (75.0%) isolates have ability to produce siderophore. Among the isolated *Salmonella Typhi* all isolates 40 (100%) were positive for *viaB* and *staA* gene, 21 (52.5%) were positive for *iroB*. Sequencing of *viaB* and *iroB* were done for further validation. In conclusion all isolated *Salmonella Typhi* carried *viaB* and *staA, iroB* genes were responsible for *S. Typhi* pathogenicity.

CBMJ 2023 January: vol. 12 no. 01 P: 41-47

Keywords: Phenotypic characterization, genotypic characterization, Salmonella typhi, virulence genes, PCR

Introduction

Enteric fever is also known as typhoid fever, is an acute, generalized infection of the reticulo-endothelial system, intestinal lymphoid tissues and gallbladder.[1] It is a leading cause of community-acquired bloodstream infections in many low and middle socioeconomic countries.[2] It is associated with poor sanitation and unsafe food and water, particularly affects children and adolescents in developing countries of Asia, Africa and Latin America.[3] In developed countries, patients are most often ill-returned travelers or migrant workers.[4]

There are many structural and metabolic virulence factors present in the bacteria which are responsible for their survival in the host.[2]

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Lipopolysaccharides, fimbriae and capsule are the virulent factor that presents on the surface of the bacteria. These virulence factors are targeted by host immune system and the killing of bacterial cells by phagocytosis is prevented by cell wall structures and lipopolysaccharides present in cell membrane.[5]

Pathogenesis of *salmonella* infection includes adhesion to host cell, invasion and intracellular replication by *salmonella*.[6] During the course of infection, Vi capsular antigen has anti-inflammatory, antiopsonic and antiphagocytic property that protects bacteria from host immune system.[7-10] The *viaB* gene encode for the synthesis of virulence (Vi) capsule.[11]

Fimbriae are hair like structures, which present on the cell surface. These are protein in nature. It plays a major role in virulence by allowing bacteria to interact with specific host cell receptors.[12] Fimbriae operons (*tcf* and *sta*) are important *Salmonella* pathogenicity factors and *S. Typhi* harbours two intact operons which are not encoded by the *S. Typhimurium* genomes. *staA* is the first gene of fimbrial operon gene and it is very specific for detection of *Serovar Typhi*.[13]

The *IroB* gene is responsible for the synthesis and transport of enterobactin, a siderophore produced by *Salmonella* spp. and is essential for iron uptake inside the host. The specific role of *iroB* is to encode glucosyltransferase which glucosylates enterobactin.[14] Enterobactin glucosylation contributes to the virulence of the bacteria by preventing the host antimicrobial protein (lipocalin-2) from sequestering the siderophore.[15,16]

This study was done to evaluate the phenotypic and genotypic characterization of *S. Typhi* isolated from human blood with distribution of virulence genes and sequencing.

Methods

This cross-sectional study was conducted in the Department of Microbiology at tertiary care hospital Bangladesh from July 2019 to June 2020. Clinically suspected enteric fever patients irrespective of age and gender who attended in the laboratory of Department of Microbiology or OPD of Medicine in tertiary care hospital Bangladesh for blood culture and sensitivity test were included in this study. Patients or legal guardians of the patients who did not give consent were excluded from the study.

Isolation and identification of organism:

Blood was collected in a sterile blood culture bottle containing Trypticase soya broth (TSB) with sodium polyanethole sulphonate (SPS) and culture was done by conventional method. On day 7 all bottles were sub-cultured on Blood agar media & MacConkey agar media plates before being discarded as negative. Subculture plates were incubated at 37^{o}C for 24 hours in aerobic condition. The positive growth *S. Typhi* was identified by gram straining, colony morphology and biochemical test. Then identification was done by using *Salmonella* specific antisera for determination of O antigen of *S. Typhi* (MastTM Diagnostic, UK). Final identification was done by PCR.

Virulence factors (hemolysin and siderophore) detection:

1. Hemolysin production[17]

The blood agar plates were inoculated with bacterial strain, and then incubated at 37°C for 24-48 hr. Apperance of clear zone around the

bacterial colony referred to β- hemolytic or green zone referred to α- hemolytic colony.

2. Siderophores production assay[18]

Nutrient agar supplemented with 200 mM of 2, 2'-dipyridyl was used as iron-restricted agar medium. All bacterial isolates were streaked on agar plates and then incubated at 37°C for 24 hr. Any bacterial growth was considered as positive for siderophore production.

Molecular characterization of virulence genes:

PCR was done to detect *viaB*, *staA* and *iroB* genes. To prepare bacterial pellets, a loop full of 5 to 6 bacterial colonies were sub-cultured into Mueller-Hinton agar media at 37°C for 24 hours. A loop full of bacterial colonies was inoculated into a falcon tube containing trypticase soya broth. After incubating at 37°C overnight the falcon tubes were centrifuged at 4000 rpm for 10 minutes, after which the supernatant was discarded. A small amount of sterile trypticase soya broth was added into falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 microcentrifuge tubes. The microcentrifuged tubes were then centrifuged at 4000 g for 10 minutes and supernatant was discarded. The microcentrifuged tubes containing bacterial pellets were kept at -20°C as pellet until DNA extraction. Bacterial DNA was extracted by the boiling method.[19] Then mixing of master mix and primer with DNA template was done.

PCR assays were performed in a DNA thermal cycler. PCR reaction consisted of preheat at 94°C for 10 minutes followed by 36 cycles of (denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, and elongation at 72°C for one minute) followed by final extension at 72°C for 10 minutes. Then the product was held at 4°C. After amplification products were processed for gel documentation or kept at-20°C till tested. The pair of primers were used to yield PCR products depicted in (Table-I)

Table-I: List of primer of virulence gene

Genes	Sequence (5´ to 3´)		Amplicon
16s Rrna	F	AAGTACTTTCAGCGGG GAC	442
	R	TTGAGTTTTAACCTTG CGG	
Sta A	F	TGG TTA CAT GAC CGG TAG TC	537
	R	TAG CTG CCG CAA TGG TTA TG	
iro(B)	F	TGCGTATTCTGTTTGT CGGTCC	606
	R	TACGTTCCCACCATTC TTCCC	
via(B)	F	CACGCACCATCATTTC ACCG	738
	R	AACAGGCTGTAGCGAT TTAGG	

DNA sequence analysis:

For sequencing of bacterial DNA, purification of amplified PCR product was done by using DNA purification kit (FAVOGEN, Biotech Corp). Purified PCR products were sent to 1st BASE laboratories, Malaysia for sequencing by capillary method (ABI 3500). Then BLAST analysis was performed to search for homologus sequence compared with data in GenBank at www.ncbi.nlm.nih.gov.

Data analyses were done by using Microsoft Office Excel 2013 software (Microsoft, Redmond, WA, USA). This protocol was approved by The Ethical Committee of Dhaka Medical College, Dhaka.

Results

A total 323 blood samples were included in this study. Among them, 83(25.69%) samples yielded positive cultures. Among them 50 isolated organisms were identified as *Salmonella* species. Furthermore, *Salmonella Typhi* was detected in 40(48.2%) isolates in out of 50 isolates.

The identification of *Salmonella Typhi* by biochemical test and PCR were done in this study. Among 50 bacteriologically diagnosed typhoid fever cases, 40(80.0%) were positive for *Salmonella Typhi* by biochemical characteristics and PCR respectively.

The phenotypic methods to detect virulence factors in *Salmonella Typhi* were applied in this study. Among 40 isolated *Salmonella Typhi,* no *Salmonella Typhi* produced hemolysin and 30(75.0%) isolates have ability to produce siderophore.

The virulence genes among isolated *Salmonella Typhi* in blood sample were recorded by PCR. Among the isolated *Salmonella Typhi* all isolates 40 (100%) were positive for *viaB* and *staA* gene, 21 (52.5%) were positive for *iroB* (Table-II and Fig. 1-3).

Table-II: Virulence genes among isolated *S. Typhi* by PCR (n= 40)

Gene	Frequency	Percent
viaB	40	100.0
iroB	21	52.5
staA	40	100.0

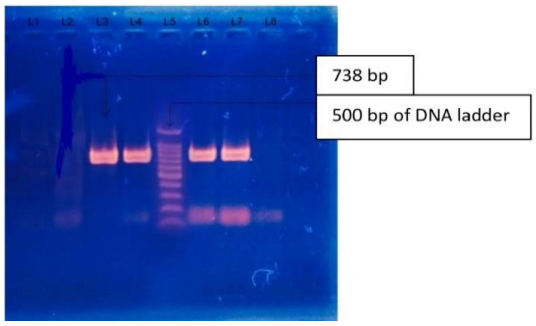

Fig. 1: Photograph of gel electrophoresis of negative control without DNA (TE buffer) (lane 1), negative control *Escherichia coli* ATCC 25922 (lane 2), amplified DNA of 738 bp for *viaB* gene (lane 3, lane 4, lane 6 and lane 7), hundred bp DNA ladder (lane 5), negative sample (lane 8).

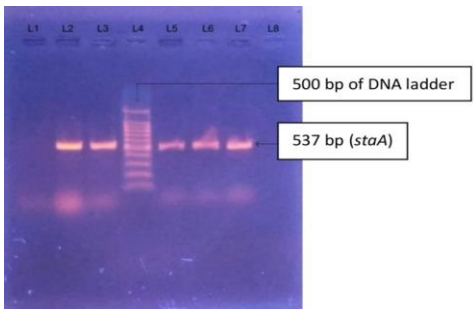

Fig. 2: Photograph of gel electrophoresis of negative control without DNA (TE buffer) (lane 1), amplified DNA of 537 bp for *staA* gene (lane 2, lane 3, lane 5, lane 6, lane 7), hundred bp DNA ladder (lane 4), negative control *Escherichia coli* ATCC 25922 (lane 8).

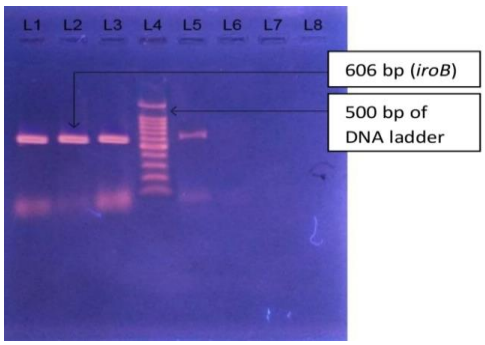

Fig. 3: Photograph of gel electrophoresis of amplified DNA of 606 bp for *iroB* gene (lane 1, lane 2, lane 3 and lane 5), hundred bp DNA ladder (lane 4), negative control without DNA (TE buffer) (lane 6), negative control *Escherichia coli* ATCC 25922 (lane 7), negative sample (lane 8).

In this study, DNA sequence of amplified PCR product of *viaB* gene which was 95% identical with *S. Typhi* strain CMCST_CEPR_1 chromosome which is available in GenBank (accession number gi|1861334377|CP053702.1) and DNA sequence of the amplified PCR product of iroB gene which is 97% identical with *Salmonella Typhi* strain 311189_208186 chromosome which is available in the GenBank (accession number gi|1510427450|CP029949.1).

Discussion

Typhoid is a public health concern in many areas around the globe, particularly in developing countries where sanitary conditions may be poor.[20] The rate of morbidity of typhoid fever more than 21 million cases and some of those cases lead to death per year worldwide.[21] In this present study, among 323 enteric fever suspected cases, a total of 83(25.69%) were culture positive. Among them 50 (15.47%) were positive for *Salmonella* spp. which was confirmed by biochemical test and specific antisera and 33 (10.21%) were other organisms. Among 50 cultures positive *Salmonella* species 40(80.0%) isolates were *Salmonella Typhi* and 10(20%) were *Salmonella Paratyphi*. In a study by Akter *et al.*[22], the *Salmonella Typhi* was 77.68%, whereas *Salmonella ParaTyphi* was 22.32% isolates. Saha[23] has reported that *Salmonella Typhi* and *Salmonella ParaTyphi* ratio was 4:1. *Salmonella Typhi* was found in 48.19% of samples which was almost similar with the study of Dahhan[24] who found 44.5% *Salmonella Typhi* among the culture positive sample. The findings of these studies were consistent with the present study.

The results of virulence factors of *Salmonella Typhi* isolates, showed no *Salmonella Typhi* produced hemolysin and 30(75.0%) isolates had ability to produce siderophore. These results were similar by Keller *et al*.[25] that found 72.2% of *Salmonella Typhi* that are able to produce siderophore. Al-Dahhan *et al.*[24] also did not find any hemolysin producing *Salmonella Typhi* in their study, which is similar to my study.

In the present study, PCR was done to detect some virulence factor genes in *S. Typhi*. Among the isolated *S. Typhi* all isolates (100%) were positive for *viaB,* 21 (52.50%) were positive for *iroB,* 100% were positive for *staA* gene. Dahhan *et al.*[24] reported that, 100% of *Salmonella Typhi* producer isolates carrying *tviA* and *staA* genes which was similar with my study. In our study, DNA sequence of amplified PCR product and translated nucleotide base sequence of *viaB, iroB* showed point mutation.

Conclusion

To summarize, *Salmonella Typhi* has the ability to produce siderophore and hemolysin is not produced by any of the isolates, in this study. The most common isolated virulence gene is the *viaB* and *staA* genes. It could be an important diagnostic tool for rapid identification of typhoid fever.

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