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Phenotypic and Genotypic Characterization of *Salmonella Typhi* Virulence Factors Isolated From Patients with Typhoid Fever in Najaf Province /Iraq

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Abstract: *Salmonella entericaserovar Typhi remains a major public health problem in many developing countries .The severity and pathogenesis of the disease depending on virulence factors. Previously, different virulence factors have been detected phenotypically and serologically for clinical and epidemiological investigation of salmonella. Beyond the phenotypic characterization, a reliable molecular methods are require. Therefore, this study was attempted To use different molecular methods for investigation the present of tviA , staA , and nested staA genes by monoplex –PCR. Sixty five S.typhi bacteril isolates that been isolated and diagnosed depending on the culture charecters and the present of 16SrRNA with 441bp. The results of monoplex PCR of virulence factors genes clarify that all isolates (100%) of S. typhi producers isolates carrying tviA, staA and nested staA gene that predominance gene among other genes under study, which was found in all bacterial isolates producers respectively. S. typhi isolates were showed possessing these genes which responsible for S. typhipathogenicity.*

Keywords: *tviA., staA and nested staA, virulence factors, Salmonella typhi, typhoid fever, PCR.*

1. INTRODUCTION

Typhoid fever is an acute, potentially fatal systemic illness caused by *Salmonella enterica*serovar Typhi and Paratyphi, pathogens specific only to humans. Most of the burden of the disease occurs in the developing world where it still remains an important cause of morbidity and mortality (Crump *et al.*, 2004). Multiple survival strategies allow *S. typhi* to cause epidemic outbreaks of typhoid fever in many developing countries (Wain *et al.*, 2008). A number of factors contribute to emergence and spread of this disease in endemic areas such as India, Pnegladish and Pakistan. Some of the identified risk factors include eating unhygienic food Luby *et al.*, (2014), drinking contaminated water, having a close contact or relative with recent typhoid fever, poor housing with inadequate facilities for personal hygiene Gasem et al., (2002). Therefore, Salmonella represents a major health risk according to the World Health Organization Propagation of S. typhi infection (WHO, 2006).

Bacteria have evolved numerous structural and metabolic virulence factors that enhance their survival rate in the host .The surface structures of S. typhi such as lipopolysaccharides, fimbriae and capsule are affect the virulence of the bacteria, and also are key targets of the host immune system, resulting in selective pressure to generate genetic polymorphisms coding for antigenic diversity .The cell wall structures and lipopolysaccharides (LPS) present in the cell membrane prevent killing of the bacterial cells by phagocytosis (Robert , 2012).

Different bacterial species use similar infection strategies due to the acquisition of diverse pathogenicity islands. pathogenicity island, a region should carry genes encoding one or more virulence factors such as adhesins, toxins, and invasins. Pathogenicity islands are located on the bacterial chromosome or on a plasmid and carry functional genes for DNA recombination such as integrase, transposase, or part of an insertion element (Wassenaar and Gaastra,. 2001).Due to the importance and the role of virulence factors in the pathogenicity of S.typhi, the present study was designed to detect the plasmid profile and virulence encoding genes of S. typhi i*solated* by nested PCR from typhoid patients such as *tvi*A.*via*B, and nested *sta*A.

2. MATERIAL & METHODS

2.1. Patients and Clinical Specimens

The study was carried out in the Biology department, collage of science, Kufa University, Iraq. during the period from December 2013 to September 2014 of Al Najaf provenance.250 clinical suspicion patients with typhoid fever who attended to different hospitals (Al-sader medical city, AlZahrra hospital for Maternity and children and general health laboratory). Three Clinical specimens (blood, urine, stool) were collected from each patient on the same day. Five mlvenous blood samples were collected from typhoid patients by sterile syringes which divided into two parts: 2ml of blood was used for Widaland the other part of blood was delivered into screw cupped tubes containing 10 ml of tetrathionete broth (Lewis, *et al*., 2001).

2.2. Bacterial Isolates and Culture Conditions

The blood samples were inoculated (Blood culture by lytic centrifugation method) onto blood agar and MacConkey agar media. The inoculated culture plates were immediately placed in an incubator at 37C for 24 - 48 hours, then keep until used. (Richard, et *al.*, 2007; Cheesbrough, 2010).Urine and stool samples had been inoculated on the suitable culture media. The sediment of urine sample were cultured directly on above mention culture media by sterile loop, stool samples were cultured by sterile cotton swabs. All plates were incubated aerobically at 37˚ C for 24 hr. (Collee, *et. al*., 1996).

2.3. Identification of Bacteria

A single colonies were isolated from primary positive cultures and identified morphology and biochemically according to the standard criteria of MacFaddin (2000) and Collee *et. al*. (2011).

2.4. Serological Characterization of S.Typhiisolates

Detection of typhoid antibodies in blood sample was doing by widal test using O and H (Fisher-Garantie) antigens according to Saha *et al.,* (1996).

2.5. Identification by Vitec 2 Compact system

It was done on each bacterial isolate to complete the final identification by use the vitec compact apparatus (Hettich,Germany), The Vitek 2 Compact then analyses the card in (4-8) hours and generatesa printable report index (1). This report is also stored in the resident memory.

2.6. Molecular Identification

16S rRNA gene for confirmation the identification of *S.typhi* accordance to Sambrook and Russell (2001). The genomic DNA of each strain was obtained through the wizard genomic DNA purification kit (Promega kit). Gel electrophoresis was used for detection of DNA by UV transilluminator. The PCR assay was performed to detect the (16S rRNA) gene for confirmation the identification of *S .typhi* shown in table-1. This primer synthesized by Alpha DNA Company, Canada (table-2).

2.7. Virulence Factors Detection

1. Hemolysin production

   The blood agar plates were inoculated with bacterial test, and then incubated at 37˚C for 24-48 hr. Appearance of clear zone around the bacterial colony referred to β –hemolytic or green zone referred to α- hemolytic (Baron *et al*., 1994).
2. Detection of protease production (Elsner*et al*., 2000).
3. Siderophore production (El-Sanousi *et al* ., 1987).
4. Colonization Factor Antigen (CFA/I,II,III)(Al-Zaag, 1994) .
5. Detection of β- lactamase production (Konemane*t al*., 1992) .

2.8. Sample Preparation Step

Bacterial suspension was prepared when isolate was inoculated in Luria broth (LB) media (incubated at 37C˚ for 24 hr.), the turbidity was adjusted for obtaining approximately $1x10^9$ CFU/ml, and then 1ml was transferred of suspension to Eppendrof tube and was centrifuged at 14000 rpm for 1 min., then was discarded the supernatant, and 200µl of GT buffer was added to deposit, re-suspended the cell pellet by shaking vigorously and then was incubated at room temperature for 5 min.

2.9. Extraction of DNA

Genomic DNA was extracted by using a commercial purification system (Genomic DNA Minni Kit Bioneer). Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is indicated by ratio of OD 260-280 which is in the range of 1.8±0.2 for pure DNA. PCR program that apply in the thermocyler table3.5. The PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel (Sambrook and Russell 2001).

2.10. Polymerase Chain Reaction (PCR) Technique

a. Primers

In this study, monoplex PCR was done to detect the 16DrRNA and a number of genes that encode for virulence factors in clinical isolates of *S. typhi*. monoplex PCR was used to detect for, *tviA*, *staA*, and nested *staA*genes. (Alpha DNA company, Canada) with 570 bp, 560 bp and nested 442 bp respectively (table-1). Multiplex PCR was done to detect for genes which encode *spvC, invA, viaB* with599 (*spvC*), 516(*invA*), and 243(*viaB*) bp fragments of genes respectively (table 1) in clinical isolates of *S. typhi*.

b. Amplification Reaction

Polymerase chain reaction mixture was set up in a total volume of 50µl which included: 5µl of PCR premix (contains: *Taq* DNA polymerase, MgCl2, dNTPs, KCl, stabilizer tracking dye and tris-HCl), 1.5µl of each primer (final concentration was 10 picomol /µl) and 1 µl of DNA template. Then volume was completed with sterile D.W., negative control contained all components except DNA template which was replaced by D.W was used. PCR reaction tubes were blended and finally were placed into thermocycler instrument. Conditions of polymerase chain reactions are listed in table (2). In multiplex reaction the same components and steps that were used in monoplex have been done except type of primers were used as 1.5 µl (final concentration was 10 picomol / µl) of each primer from three.

Table1. *Primers Sequence were used in PCR.*

Target gene	Primer	Sequence	Reference
16 s rRNA	16s RNA	F: 5 –AAGTACTTTCAGCGGGGAC - ^{3}442 R 5-TTGAGTTTTAACCTTGCGG-3 - ^{3}442	Muhammed, *et al*.,(2005)
Sta A	Sta A	F1 5'-TGG TTA CAT GAC CGG TAG TC-3' 537 R1 5'-TAG CTG CCG CAA TGG TTA TG-3' 537	Townsend, *et al.*,(2001)
StaA	StaAnesred	F2 5'-CAT CGG CAC GAA CGT AAG AC-3' 377 R2 5'-TC AAG CGA CTG ATG GTG ACG -3' 377	Prakash, *et al.*,(2005)
tviA	tviA	5-GTTATTTCAGCATAAGGAG-3 599 5- ACTTGTCCGTGTTTTACTC-3 599	Hashimoto, *et al.)*(1995

Table2. *Amplification Conditions of genes were used by PCR reactions.*

Gene **Uniplex genes**	**Initial denaturation**	**No.of cycles**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
16s rRNA	94 Cº for 1 min.	40	95 Cº for 1min.	55 Cº for 1 min	72 Cº for 2 min.	72Cº for 10 min.
TviA	95 Cº for 5 min.	30	94 Cº for 30 sec.	55 Cº for 30 sec.	72 Cº for 2 min.	72Cº for 5 min.
*Sta*A	94 Cº for 5 min.	35	94 Cº for 1 min.	60 Cº for 1min.	72 Cº for 1 min.	72Cº for 7 min.
nested *sta*A	94 Cº for 5 min.	35	94 Cº for 1 min.	64 Cº for 1 min.	72 Cº for 1 min	72Cº for 5 min.

3. RESULTS & DISCUSSION

The results of serological method (widal test) showed that 200 out of 250 patients clinically suspicion to have typhoid fever gave positive results. Morphological and culture characters showed 44.5% (79) of S.typhi isolates, while vitec compact system diagnosed 32.5% (65) S.typhi isolates. Polymerase

chain reaction technique of the *S. typhi* clinical isolates revealed one fragment with 441 bp that represented the 16S rRNA gene. The results show that almost isolates of *S. typhi*, carrying *16S r RNA* gene that is characteristic of *S. typhi* as shown in (figure 1).

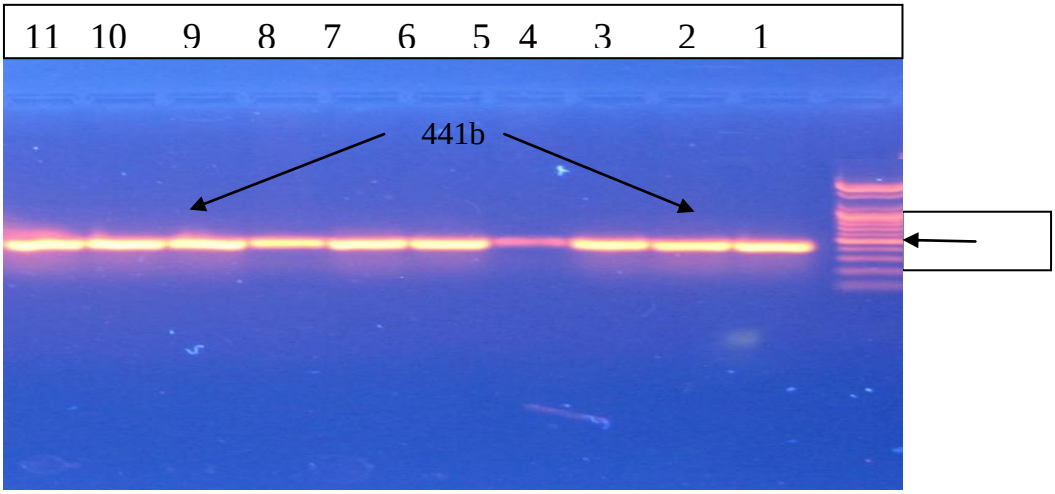


Figure1. Agarose gel electrophoresis of *16S RNA* gene (441 bp) of the *S. typhi* using.

Lane (1), DNA marker (100bp ladder).

Lane (2, to, 11) No. of amplify of 16Sr RNA gene in S. typhi isolates

In the present study, PCR was used to detect *16 S RNA* gene as specific of *S. typhi*, among 79 bacteriology diagnosed typhoid fever cases the PCR showed (65, 32%) positive result(figure1). The result in this finding is similar to study was done in Delhi, India, that used blood samples from 100 clinically suspected cases of typhoid fever, and found 100% of culture positive and 50% of culture cases positive by PCR (Kumar *et al.*, 2002).

3.1. Virulence Factors of *S. Typhi*

The results of virulence factors of *S. typhi* isolates, show that all *S. typhi* not produced hemolysin and extracellular protease. All isolates of *S. typhi* had Vi- polysaccharide capsule surrounding the bacterial cell (100%). From the results, it was appeared *that S.typhi* isolates have the ability to produce siderophore 60 (93%) as shown in (table 2).

S typhi have evolved numerous structural and metabolic virulence factors that enhance their survival rate in the host. Vi-Capsular formation has long been recognized as a protective mechanism for bacteria. Encapsulated strains of many bacteria are more virulent and more resistant to phagocytosis and intracellular killing than are nonencapsulated strains (Jasmine, *et al.*, 2012). As shown in the results all *S.typhi* isolates are able to producing a siderophores.

Table2. *Virulence factors of S. typhi species*

Virulence factors		*S. typhi* n= 100	
		No.& (%) of positive isolates	No.& (%)of negative Isolates
Hemolysin		0 (0%)	65 (100%)
Vi-Capsular		65(100%)	0(0%)
β –lactamase –producers		50(77%)	15(23%)
Siderophore		60(92%)	5 (8%)
CFA	CFA / I	22 (31%)	43(69%)
	CFA/ II	10 (15%)	55(85%)
	CFA/III	60(92%)	5(8%)
Protease		0 (0%)	65 (100%)

These results are partially agreement with those obtained by Keller *et al., (*1998) that found 72.2% of *S.typhi* that are able to produce siderophore, and all *S. typhi* isolates studied produced bioactive enterochelin but not aerobactin. The enterochelin influences either the extent of bacterial translocation from the intestinal tract, the extent of bacterial multiplication in tissues following translocation, or

both. Thus, enterochelin secretion in vivo could be an important step in the stages of the infection cycle (Khaled, *et al.*, 2013).

Table (2) shows 31% of isolates were had ability to produce CFA / I, while the ability of isolates for produce CFA/II were less 10 (15%). The high production of CFA, was appeared in produce of CFA / III, (92%) of *S. typhi*isolates were production this factor and CFA/II also were found in isolates of *S.typhi* but in percentage less than that in CFA/ III. Keller *et al.*,(1998) they found mannose – sensitive hemagglutinin (MSHA) or type 1 fimbriae in most strains of *S.typhi*. The relationship between this factor and pathogenisity of bacteria was established from adherence of bacteria in mucous surfaces or epithelial cells of gastric tract. CFA/II was appeared that the lower presence among isolates. This factor causes agglutination of chicken blood, and act to adhere of bacteria with specific and complex carbohydrate receptors of epithelial cells of small intestine (Nowsheen and Jain, 2008).

The β–lactamase–producing isolates of *S. typhi* will be illustrated in (table 4.3). It was found that, 50/65(77%) of *S. typhi* produced β –lactamase. The results revealed, that all isolates of *S. typhi* have higher resistance to penicillins (amoxicillin, ampicillin), this may be due to the production of β–lactamases or failed reaching antibiotics to the target (PBPs) (Harwood, *et al*., 2000). In relation to cephalosporins, most isolates of *S. typhi* are resistant to cephalothin and the resistance to these antibiotics can be attributed to poor permeation of bacteria by the drug or lack of BPBs or degradation of drug by β-lactamases (Brooks, *et al*., 2007).

3.2. Genotypic Detection of *S typhi* Virulence Factors

In this study, PCR assay was done for amplification of some virulence genes by using ,(monoplex and multiplex) kit .A total of 65 isolates tested with the primers of genes tv*iA, viaB, spvC, invA, and stA with nested stA*genes carrying on plasmid and genomic DNA of all *S typhi* isolates with specific forward and reverse primers; resulted fragments of the predicted size at 599 bp, 516 bp, 570 bp ,243and 560 bp with nested 442 bp respectively**.**

The results of monoplex PCR of virulence factors genes clarify that all isolates (100%) of *S. typhi* producers isolates carrying *tvi*^{A}gene. (Figure 3A).

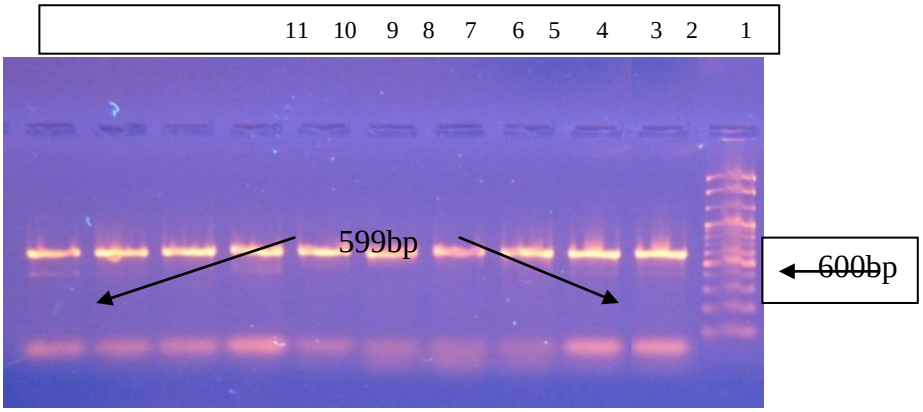
A crucial role is ascribed to the TviA regulatory protein, encoded on the *S. typhi*–specific SPI-7 (viaB locus), which down regulates flagellin production and enhances Vi antigen attachment to the cell surface. The tviA protein therefore serves as a regulatory switch affecting the ability of the host to recognize *S. typhi* as an intruder at crucial stages of the spread in humans (Su, *et al.*,2010).

Also, the results was agreement with those reported by Iversen & Forsythe (2004) that *S .typhi* were contained Vi aid an adherence to surfaces including other cell types the results of PCR amplification found that 65 isolates (100%) contain *sta*A and nested *staA* gene as in(table 4), (figure3B,C)

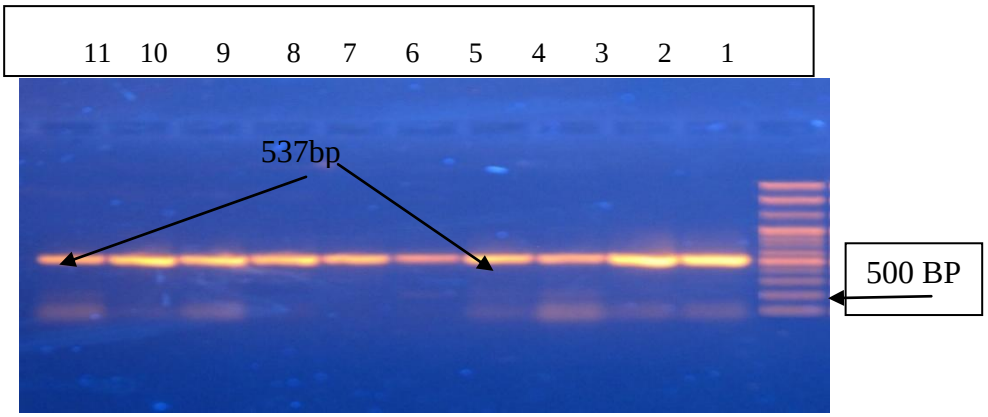
Fimbriae are small hair-like proteinaceous structures present on the cell surface that play a critical role in virulence by allowing bacteria to interact with specific host cell receptors (Chantal, *et al*.,2007). Townsend *et al*., (2001) demonstrated that 13 known putative operons for fimbriae, *bcf, csg (agf), fim, saf, sef,* ***sta****, stb, stc, std, ste, stg, sth,* and *tcf,* as well as *pil*coding for the type IV pili in the genome sequence of serovarTyphi, and putative fimbrial gene *staA,* found within the *sta*operon, has been reported to be specific to *S. typhi* .

Fimbriae operons (*tcf*and *sta*) are believed to be important *Salmonella* pathogenicity factors and *S. typhi* harbors two intact operons which are not encoded by the *S. typhi* genome. Moreover, fimbrial proteins *staA* and *steD,* as well as the gene encoded within the Type IV pilus cluster of SPI-7, play a major role in the pathogenesis of the *S. typhi* serotype (Sheikh, *et al*., 2011; Chandra, *et al.*, 2013).

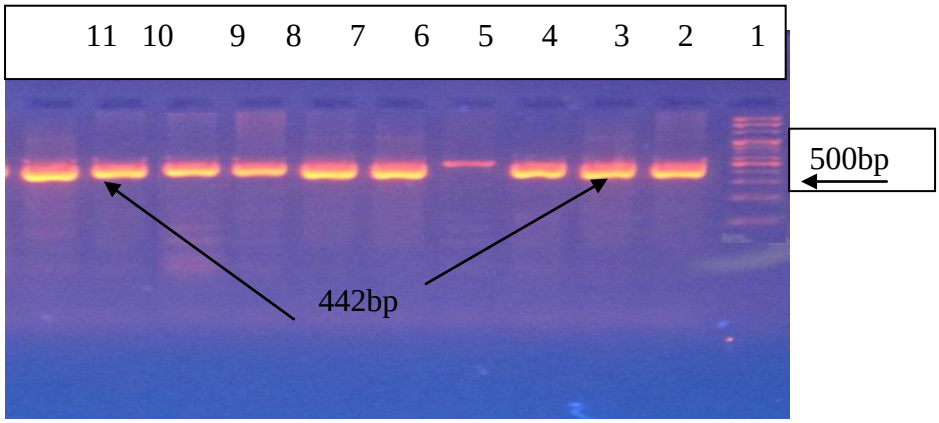
The s*taA* gene sequence is unique to serotype Typhi; therefore, this gene was targeted for amplification using a nested PCR protocol. The observations made in the present study suggest that PCR based on *staA*can be used very efficiently to detect *S. typhi,* especially in clinical specimens, using a nested protocol. In the present study, nested PCR was found to be very specific and sensitive in detecting *S. typhi.* Pathogenicity islands are located on the bacterial chromosome or on a plasmid and carry functional genes for DNA recombination such as integrase, transposase, or part of an insertion element (Su Y, *et al*., 2010; Khan, *et al*., 2012) .Fimbrial operon *staA*gene as a PCR target, and observed it to be very specific for the detection of serovarTyphi (Grace, *et al.*, 2010).



***A-tvi*Agene(599 bp.).**



B-sta A gene(537 bp.).



C-nested sta A (442 bp.)

Figure3. *PCR amplified products of the A-(tviA), B-(staA), C-(nested staA) of S. typhiusing the designed primers with expected size*

Lane (1), DNA marker (100bp ladder).

Lane (2, to, 11) were positive result for tviAgene in S. typhi isolates,

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