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Research Article

# Genotypic Study of Some Virulence Factors in *Salmonella typhi* Carrier Associated with Gall Bladder Chronic Infection

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**ABSTRACT**

Introduction: A total of (50) clinical specimens were collected from patients suffering from gall bladder attending to AL-Sadder Medical City and AL-Furat General Hospital include (gall bladder tissue, stool and blood) during the period from December 2016 to September 2017 from different age and sex. Methodology: The identification of *Salmonella typhi* isolates were depended on colonial morphology and biochemical tests as a primary identification. The final identification was performed with the automated VITEK-2 compact system. Result: According to the results obtained by the VITEK tests, thirty five clinical isolates of *Salmonella typhi* were obtained. This study revealed that totally 35 (70%) positive result include that 23 (65.7%) isolates was positive from gall bladder tissue, 11 (31.4%) from stool and 1 (2.8%) from blood. The study investigated the virulence factors encoding genes of *Salmonella typhi*, which play a major role in enterococcus pathogenicity such as *Vi* (R1), *Vi* (R2), *ViaB* , *InvA, SpvC,* and FimA. These genes have important role in invasion and adhesion. Conclusion: The study showed that the virulence factors of *Salmonella typhi*, which play a major role in their pathogenicity among gall bladder chronic infection.

**Keywords**: *Salmonella typhi,* VITEK-2 compact system, *Vi*(R1), *Vi*(R2), *ViaB*, *InvA, SpvC* and *Fim* A.

## INTRODUCTION

The gallbladder is a small organ located on the right side of the abdomen, just below the liver. The gallbladder's main function is to store bile made by the liver and secrete it into the small intestine to help digestion. Bile is made of water, cholesterol, fats, bile salts (natural detergents that break up fat), and a pigment called bilirubin[1].

Propagation of *S. typhi* infection is due to its ability to enter a dormant state through the formation of a biofilm in the human gallbladder (typhoid carriers), enabling it to evade the immune system and do not show any symptoms. The only reservoir for *S. typhi* which is transmitted via contaminated water or food[2].

Because *S. typhi* is a human-specific pathogen, these carriers serve as a critical reservoir for further spread of the disease through bacterial shedding in feces, which is a sporadic and intermittent event[3].

Particularly in areas of high endemicity, the carrier state is linked to the presence of gallstones, as approximately 80 to 90% of chronically infected carriers have this gallbladder abnormality[4].

*Salmonella typhi* can form biofilms on the surfaces of cholesterol gallstones in the gallbladders of mouse and human carriers and on the gallbladder epithelium of mouse carriers[5]. This biofilm formation has been demonstrated to be a mechanism of persistence and chronic colonization in the gallbladder[6].

The production of Vi by *serovar typhi* is a distinguishing feature of the bacterium, and agglutination using Vi antisera is a routine procedure for the identification of *S.typhi* in research and diagnostic laboratories[7].

Flagella provide the *S. typhi* with motility and may play a role in cell entry, are down regulated inside cells, and it has been suggested that they are used for escape from an intracellular site[8].

## METHODOLOGY

*Identification of bacteria*

A total of (50) clinical specimens were collected from patients suffering from gall bladder infection attending to AL-Sadder Medical City and AL-Furat General Hospital include (gall bladder tissue, stool and blood) during the period from December 2016 to September 2017 from different age and sex. A single colonies were isolated from primary positive cultures and identified according to the criteria of[9] using specific media such as MacConkey agar, XLD and S-S agar which identified by morphological, biochemical tests, the automated VITEK-2 compact system (VITEK -2 GN-ID kit was used in bacterial diagnosis (BioMérieux –France).

*Molecular Study:*

*Extraction of Genomic DNA*

Genomic DNA was extracted by using a commercial extraction system (Genomic DNA promega Kit).

*PCR Assay*

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Table 1: Sequences and Product size of each primers.

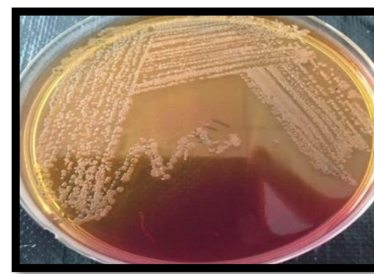
| Primer type | | Primer sequence (5'-3') | Product size(bp) | References |
|---|---|---|---|---|
| *Vi* (R1) | F | GTTATTTCAGCATAAGGAG | | (10) |
| | R | ACTTGTCCGTGTTTTACTC | 599 | |
| *Vi*( R2) | F | GTGAACCTAAATCGCTACAG | | (10) |
| | R | CTTCCATACCACTTTCCG | 307 | |
| *ViaB* | F | TGTCGAGCAGATGGATGAGCAT | | (11) |
| | R | ACGGCTGAAGGTTACGGACCGA | 516 | |
| *InvA* | F | ACAGTGCTCGTTTACGACCTGAAT | | (12) |
| | R | AGACGACTGGTACTGATCGATAAT | 234 | |
| *SpvC* | F | ACTCCTTGCACAACCAAATGCGGA | | (13) |
| | R | TGTCTTCTGCATTTCGCCACCATCA | 570 | |
| *Fim A* | F | CCT TTC TCC ATC GTC CTG AA | | (14) |
| | R | TGG TGT TAT CTG CCT GAC CA | 85 | |

Table 2: PCR programs that apply in the thermocycler.

| Gene Name | Temperature (°C) / Time | | | | | Cycles Number |
|---|---|---|---|---|---|---|
| | Initial Denaturation | Cycling Conditions | | | Final Extension | |
| | | Denaturation | Annealing | Extension | | |
| *Vi –R1* | 94/3 min | 95/5 min | 49/30 s | 72/30 s | 72/5 min | 35 |
| *Vi-R2* | 94/3 min | 95/5 min | 56/30 s | 72/30 s | 72/5 min | 35 |
| *Via B* | 95/2 min | 95/1min | 58/1 min | 75/1 min | 72/10 min | 30 |
| *InvA* | 94/2 min | 95/1 min | 58/1 min | 75/1 min | 72/10 min | 35 |
| *SpvC* | 94/2 min | 95/1 min | 58/1 min | 75/1 min | 72/10 min | 35 |
| *Fim A* | 95/5 min | 94/1min | 58/30 s | 72/1 min | 72/5 min | 20 |
| | 95/5 min | 94/1min | 58/30 s | 72/5 min | 72/5 min | 20 |

A: Growth of *S. typhi* on XLD agar

B: Growth of *S. typhi* on S-S agar



C: Growth of *S. typhi* on macConkey agar.

Figure 1: *S. typhi* colonies on different culture media

The PCR assay was performed to detect the virulence factors encoded genes such as *Vi* (R1), *Vi* (R2),*ViaB* , *InvA, SpvC* and *FimA*.

*Primers Selection:*

The primer in this study was synthesized by Bioneer company (Korea), as in table (1).

*PCR Programs*

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in table (2).

*Detection of DNA Content by Agarose Gel Electrophoresis*

Gel electrophoresis was used for detection of DNA by UV transilluminator[15]. Each well is loaded with 7µl of DNA specimen and standard molecular weight of DNA ladder (marker) is loaded in a first well. Electrophoreses run at 80 volt/cm for 1hr. Gel was visualized with UV transilluminator and photographed by using digital Camera[16].

## RESULTS AND DISCUSSION

*Salmonella typhi* Identification

Microscopically *S. typhi* appeared gram negative bacilli, peritrichous flagellated, motile, non sporulating forming bacteria, encapsulated with small polysaccharide capsule. Biochemically all *Salmonella enterica serovar typhi* isolates gave negative result for haemolytic on blood agar and showed pink colour colonies with black centre on XLD agar as in figure 1(A).

All *S. typhi* isolates were able to ferment-arabinose but not xylose, the isolates were aerobic and facultative anaerobic, it was grown on simple laboratory media in temperature optimally at 37C° and required enrichment medium such as amino acids or vitamins. *Salmonella typhi* were isolated from different clinical specimens using specific media such as MacConkey agar, XLD and S-S agar.

Figure 2: Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Salmonella typhi* isolates that amplified with *Vi (R1)* gene primers with product 599 bp for 1 hr. at 80volt/cm.

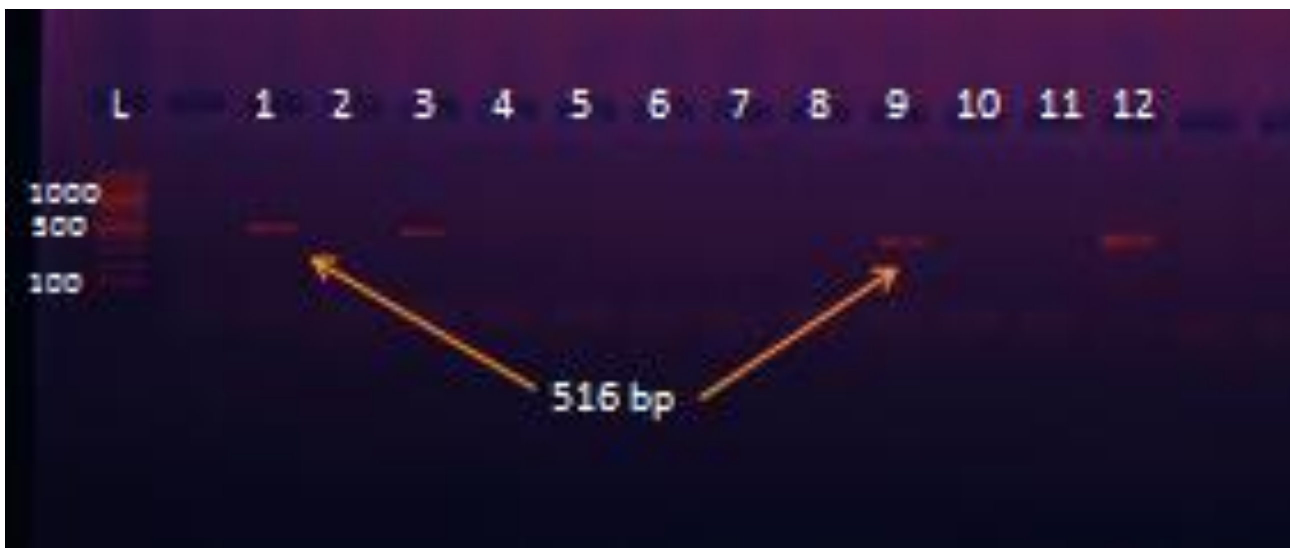



Figure 3: Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Salmonella typhi* isolates that amplified with *ViaB* gene primers with product 516 bp for 1 hr. at 80volt/cm.

The result obtained showed that the *S. typhi* appeared on S-S agar is highly selective medium formulated to inhibit the growth of most *E. coli* form organisms and permit the growth of species of *Salmonella* and *Shigella* from environmental and clinical specimens as in figure 1(B).

The present study revealed that *S. typhi* colonies of most strains were moderately large 2-3 mm in diameter after 24 hr. at 37C°. The colonies of *Salmonella typhi* on MacConkey agar appeared after 18-24 hour at 37C˚ were pale yellow, 1-3 mm in diameter and easily distinguished from the pink red colonies of lactose fermenting commensally coliform bacilli e.g.

*Escherichia coli* colonies (none lactose fermentation), slightly mucoid less than *klebsiella* with regular edges as in figure 1(C).

IMVC tests was used to differentiated genus *S. typhi* from *Shigella* and *Citrobacter*. The results showed, that *S. typhi* isolates were gave negative result to simmons citrate, voges proskauer, oxidase tests and positive to $H_2S$ production and catalase test, all isolates of *S. typhi* were positive to methyle red test . In triple sugar iron slants, both the butt and slant turned into yellow and red colour respectively indicating the fermentation of glucose alone and no production of acid in the butt.

The *S. typhi* isolates showed production of hydrogen sulphide and no gas production in TSI. Isolates were negative for oxidase test, indole production, urease production and citrate utilization. All the result (morphology and cultural) were identical with[17].

The results obtained by VITEK system revealed that totally 35 (70%) positive result include that 23 (65.7%) isolates was positive from gall bladder tissue, 11 (31.4%) from stool and 1 (2.8%) from blood. This results similar with the results of (18) who found the positive result was 65 (32.5 %) from both Vitek system and PCR technique.

*Monoplex PCR Detection of Virulence Genes*

Figure 4: Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Salmonella typhi* isolates that amplified with *InvA* gene primers with product 234 bp for 1 hr. at 80volt/cm.

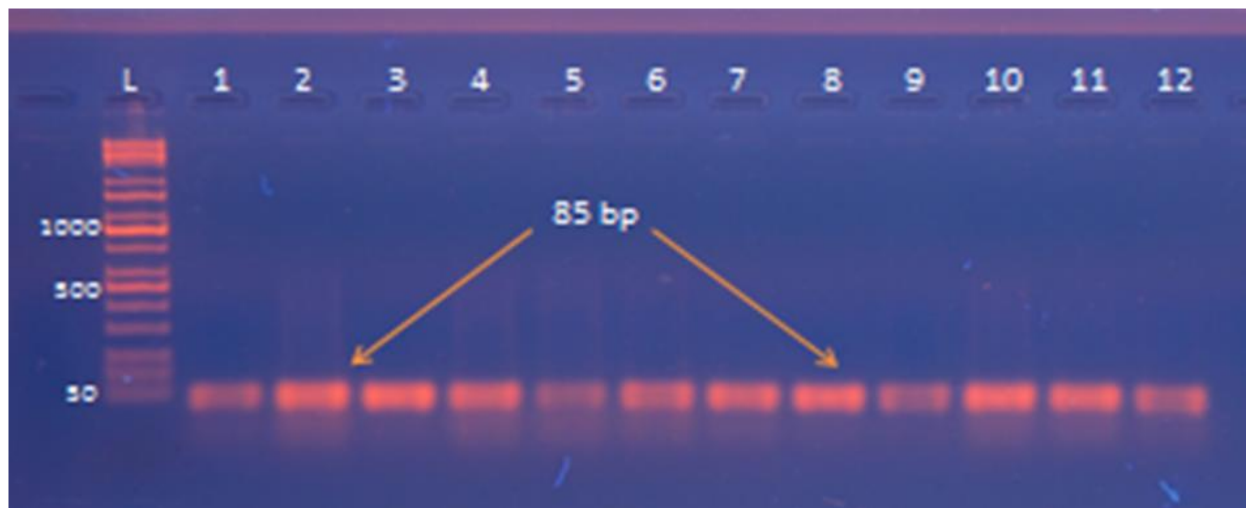



Figure 5: Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Salmonella typhi* isolates that amplified with *FimA* gene primers with product 85 bp for 1 hr. at 80volt/cm.

The finding of monoplex PCR of virulence factors encoding genes showed that 35 (100%) isolate of *S. typhi* carried *InvA* gene on total DNA but it gave negative result (100%) for *Vi* (R2), *SpvC* gene and 31 (88.5%) for *ViaB*. While gave positive result 19(54.2%) 30(85.7%), for *Vi*(R1) and *Fim A* respectively. *S. typhi* isolates were showed possessing these genes which responsible for *S. typhi* pathogenicity, table (3)

The results of monoplex PCR of virulence factors genes clarify that only 19 (54.2%) isolates of *S. typhi* carrying *Vi*(R1) gene and 35 (100%) was negative for *Vi*(R2). This results disagree with the finding of (10), that found in nested PCR technique just one of the 200 samples (0.5%) was positive for the *S. typhi* capsular gene *Vi* (R2).

The prevalence of carriers in some countries like India was reportedly as high as 17.4% to 79% (19). Birhaneselassie and colleagues reported positive results in one out of 107 stool specimens taken from food handlers; they used bacteriological characteristics, biochemical and serologic methods to spot the organism[20].

The Vi antigen of *Salmonella typhi,* the agent of human typhoid fever, This polysaccharide is a linear homopolymer of *a*- 1,4 2-deoxy-2-N-acetylgalactosamine uronic acid variably O-acetylated at the C3 position. All strains of *S. typhi* and *Salmonella paratyphi* C, as well as a few strains of *Salmonella dulblin* and *Citrobacter freumdii,* are capable of expressing Vi antigen[21].

Unlike most other serovars of *Salmonella enterica*, *S. typhi* can express a carbohydrate capsule commonly known as Vi-CPS antigen. The expression of this antigen related to environmental signals, is important for extra-cellular survival and protection against the oxidative burst of neutrophils. It also reduces the response of TNF-alpha in human macrophages after uptake. The current view is that Vi-CPS is involved in immune evasion during infection in the human host, and therefore, is of vital importance during infection[22].

Studies have reported that specific nested-PCR amplification of the *S. typhi* genes such as flagellar or capsular genes is more sensitive than assaying anti-Vi antibodies titers in serum for detection of *S. typhi* carriage[23].

Disabling of *Vi* gene in some patients is another reason for the negative result of typhoid carriers[24]. Our findings

Table 3: Prevalence and percentage of virulence factors genes of 35 isolates of *S. typhi.*

| Primers \ Sample (35) | Positive | | Negative | |
|---|---|---|---|---|
| | No. | % | No. | % |
| *Vi* (R1) | 19 | 54.2 | 16 | 45.7 |
| *Vi* (R2) | 0 | 0 | 35 | 100 |
| *ViaB* | 4 | 11.4 | 31 | 88.5 |
| *InvA* | 35 | 100 | 0 | 0 |
| *SpvC* | 0 | 0 | 35 | 100 |
| *Fim A* | 30 | 85.7 | 5 | 14.2 |

are consistent with other studies that reported *S. typhi* culture results. Andargie and colleagues[25] and Hamze and colleagues (26) reported no positive results for typhoid carriers, figure (2).

Expression of Vi antigen is controlled by two widely separated loci, *viaA* and *viaB,* located at 43 and 92 min on the chromosome of *S. typhi,* respectively. Functional *via A* genes are present not only in Vi-expressing strains of *Salmonella* and *Citrobacter,* but also in *E. coli*. In contrast, the *viaB* locus is specific to Vi-expressing strains.

The *viaB* locus of *S.typhi* appears to contain at least two regions : one involved in biosynthesis of vi antigen and the other required for translocation of the polysacharide of the cell surface[21].

The results of monoplex PCR of virulence factors genes clarify that only 4 (11.4%) isolates of *S. typhi* carrying *viaB* gene and (88.5%) was negative, This results was disagree with the findings of[18] that found 100% of isolates had carrying this gene, table (3) and figure (3).

In mPCR study, the *inv*, *spv* genes and Vi antigen coded by *via*B virulence genes were used as the basis of identification of *S. enterica serovar typhi* from the clinical cases of typhoid fever in humans. The mPCR result depicted in this study established that these three genes are highly conserved among the isolates of *S. typhi* and could be very useful marker genes for the rapid detection of only *S. typhi* isolates[27]. *Salmonella* carries the *invA* gene, which is not present in any other bacterial species, it would show that the bacteria in the sample contains an *invA* gene, and is most likely to be *Salmonella*[28].

The results showed that all *S. typhi* isolates tested were positive for virulence genes *invA* but it was negative for *spvC* gene, in the current study, the finding table (3), is revealed that *S. typhi* isolates carry almost genes in percentage 100 % for each gene which has important role in bacterial pathogensity, exception *spvC* gene was gave negative result for all isolates. These results was exactly agree with[18].

The *invA* gene encodes an invasion protein and has been reported to be present in most strains of *Salmonella typhi* isolated from animals and humans. In this study, a newly developed multiplex PCR reaction, which detects virulence genes (*invA* and *spvC*) is reported[29].

Although, the pathogenesis of *S. typhi* has been mediated by several virulence factors, the role of *inv*A gene was significant as this gene helped *S. typhi* for adhesion and invasion to the host epithelial cells[30], figure (4).

The *fimA* gene seems to be unique at least in the region of our primers, to *Salmonella* strains and can differentiate between *Salmonella* and non- S*almonella spp*. This is very useful in the diagnosis of *Salmonella* organisms at the genus level but not at the species level. The lack of nonspecific bands during amplification plus the lack of false-positive results makes this method unique. Designing the primers to amplify only *Salmonella* DNA with a small fragment makes it useful for attachment to microtiter plates or synthesis on a synthesizer for *Salmonella* detection. The PCR technique provides a new strategy for rapid and sensitive detection of *Salmonella* strains. Bacterial adherence is generally believed to be a prerequisite for infection, and there is evidence that many bacteria have surface appendages, such as fimbriae or pili, that mediate binding to specific receptors on the epithelial cell surface, to urinary tract or intestinal mucus[14]. Only type 1 fimbriae have been implicated in *Salmonella* pathogenicity. The results of PCR amplification found that 30 isolates (85.7%) contain *fim*A gene as in table (3), figures (5). These results was agree with the finding of[14] that *fimA* were contained in 34(89.7%) of isolates .

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