

Phenotypically and genotypically estimation of virulence factors in *Salmonella* serovar *typhi* isolated from patients with enteric fever in Al-Najaf, Iraq

LIQAA SALIM ZGHAIR, ZAHRAA YOSIF MOTAWEQ*, HAIDER CHAYAD LAFTA

Department of Biology, Faculty of Science, University of Kufa. Kufa, Iraq. *email: zahraa.mutawak@uokufa.edu.iq, liqaas.buhan@uokufa.edu.iq

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Abstract. Zghair LS, Motaweq ZY, Lafta HC. 2022. Phenotypically and genotypically estimation of virulence factors in *Salmonella* serovar *typhi* isolated from patients with enteric fever in Al-Najaf, Iraq. *Nusantara Bioscience* 14: 128-133. *Salmonella* serovar *typhi*, often known as enteric fever, causes typhoid fever and has been a major human infectious disease for centuries, surviving in poor sanitation and overcrowding. Only 64 (58.1%) Gram-negative bacteria were found from the 110 total specimens, with 46 (41.8%) Gram-positive bacteria. The 64 samples were divided into 42 (65.6%) males and 22 (34.4%) females. This work presents the isolation and identification of 64 *Salmonella typhi* isolates obtained from specimens. In addition, the flagellin gene was found in 64 isolates of probable typhoid fever patients (*fliC-d*). In this study, phenotypic techniques were used to detect several virulence factors. The results showed that small colonies of L-form bacteria grow on the edges of a petri dish when one of the β -lactam antibiotics (a class of antibiotics that includes penicillin) is given to wild-type bacteria, showing 52 (81.3%) of isolates could produce L-form and observed in 44 (68.75%). The ability to generate CFA/I and CFA/II was found in 68.7% of isolates. The large percentage of CFA produced showed CFA/III production, 64 (100%).

Keywords: CFA, L-form, *Salmonella* serovar *typhi*, typhoid fever, virulence factors

INTRODUCTION

Salmonella is a Gram-negative bacterium belonging to the Enterobacteriaceae family, with rod-shaped bacteria. The genus *Salmonella* flagellated (flagella peritrichous-found everywhere around the cell body) is facultative-anaerobes and non-spore formation predominantly motile with cell diameters ranging from: 0.7 to 1.5 μm , lengths from 2.0 to 5.0 μm . Two genera exist, *S. enterica* and *S. bongori*, divided into six subspecies, including over 2,600 serotypes (Fàbrega and Vila 2013; Gal-Mor et al. 2014; LeLièvre et al. 2019).

As a result, increasing the chances of discovering carriers is crucial to limit the harm they bring to populations. A sensitive, specific, and quick diagnostic technique for identifying typhoid patients and carriers would be ideal. *Salmonella* species that are pathogenic attack non-phagocytic gut epithelium by delivering a specific set of effectors via finely tuned hardware involving the Type 3 secretion system (T3SS), which plays a crucial role in *Salmonella* pathogenesis (Que et al. 2013). The *S. typhi* utilizes two T3SSs: *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). SPI-1 is a gene cluster with a 40-kb district that includes 39 genes encoding T3SS-1, its chaperones, effector proteins, and transcriptional controllers that regulate the expression of multiple destructiveness genes both inside and outside SPI-1 (Zhang et al. 2018).

Surface K antigens are the smallest common antigens discovered in *Salmonella* species and are heat-sensitive polysaccharides located on the bacterial capsule surface.

Antigens of virulence (Vi) are a subclass of K antigen, Dublin, *Paratyphi C*, and *Typhi* are the only three pathogenic serovars (Wattiau et al. 2011). The capsular Vi antigen is a linear homopolymer of alpha 1-4. They are coupled to galactose aminouronic acid, which is variably acetylated at the C3 site. One of the main traits differentiating *S. typhi* from non typhoid *Salmonella* (NTS) is the production of a polysaccharide capsule named the Vi antigen. The Vi capsule reduces phagocytosis while promoting serum resistance, most likely by preventing antibodies from attacking the O-antigen (Hart et al. 2016). This work aimed to identify the phenotype of pathogenicity factors in *Salmonella* serovar *typhi* represented by capsule, L-form bacterium, and Colonization Factor Antigen type.

MATERIALS AND METHODS

The research was carried out at the Bacteriology and Molecular Laboratories, Department of Biology, Faculty of Sciences, Kufa University, Iraq.

Clinical specimens and patients

Blood samples (110) were taken from patients suffering from enteric fever at AL-Sadder Medical City and AL-Furat General Hospital/Al-Najaf-Iraq for three months, from August 2021 to November 2021. Furthermore, using sterile syringes, four milliliters of fresh venous blood were taken and separated into two halves. For the Widal test, one milliliter of blood was used, with three milliliters of blood delivered into a special screw, placed in a bact/alert 3D

apparatus incubated at 37°C for a week. If a positive sample was found, each specimen was inoculated using a direct method of inoculation on a culture of selective media, namely MacConkey, XLD, and SS agar. The inoculation cultivation dishes were incubated overnight at 37°C for 18 to 24 hours, then stored until needed (Cheesbrough 2010). Identification of *S. typhi* isolates by Microscopic Properties, Cultural Characteristics, and Biochemical Tests. GN ID cards were used to confirm *S. typhi* isolates using the automated VITEK-2 compact system. Furthermore, it was performed on each bacterial isolate to complete the final identification. The GN ID card is based on well-established biochemical (64 reactions) methodologies and newly created substrates that measure many metabolic processes (German BioMerieux Company).

Molecular diagnostic methods

Purification and extraction of DNA

The extracted *S. typhi* DNA was prepared using the boiling technique. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 100°C for 15 minutes in the water bath. Then immediately frozen at -20°C for one hour, centrifugation at 14,000 x g for 10 min, and the supernatant was conserved for the amplification operation (Yang et al. 2008). The concentration and purity of DNA can be determined by Williams et al. (2007).

Polymerase Chain Reaction (PCR) assay

Thermo cycle PCR was used to re-confirm *S. typhi* diagnosis; this technique requires specific primers for the *fliC-d* gene, including sequence information. The primer of flagellin gene *fliC-d-F*: 5'-ACTCAGGCTTCCCGTAA CGC-3' and *fliC-d-R*: 5'-GGCTAGTATTGTCCTTATCGG-3', in the product size 763 bp (Levy et al. 2008). Five µl of master mix, five µl of template DNA mixed with 2.5µl each set of primers in a suitable PCR tube, the rest of the total volume was attained to 25 µl by sterile nuclease-free water, the mixture vortexing well. The PCR for the *fliC-d* gene included a primary denaturation step, denaturation, annealing, and extension at 94°C for 4 minutes. Next, at 40 cycles at 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, respectively. The reaction mixture was kept at 4°C until employed after the last extension step at 72°C for roughly 10 minutes (Levy et al. 2008). Then, all PCR products were examined using 1% agarose gel electrophoresis and 3µl of ethidium bromide dye. The specific cover on the electrophoresis tank was closed, and the electric current was matched (70 volts for 1.5-2 h). Finally, the gel documentation system was used to identify the electrophoresis data.

Phenotypic detection of some virulence factors

Detection of capsule (vi antigens) production

A loopful of suspected culture was mixed with a loopful of nigrosin stain on a clean and dry slide, allowing it to air dry at room temperature. Next, the slide was gently cleaned with water before being stained for 2 minutes by methylene blue stain and left to air dry at room temperature. Next, the slide was softly washed with water and viewed using an oil laboratory microscope. The nigrosin stain gives the

unstained capsule a dark backdrop, while the methylene blue stain gives the cells a blue tint (Harley and Prescott 2002).

L-form detection of S. typhi

According to multiple studies, tiny colonies of L-form bacteria grow on the plate's edges when one of the β - lactam antibiotics (which includes penicillin) is administered to wild-type bacteria in a petri dish. Penicillin treatment not only selects L-forms (which are penicillin-resistant) but also stimulates L-form growth (Casadesús 2007). Penicillin discs (10µ) were used according to the Kirby-Bauer disc diffusion method. The method included the following steps : (i) A isolate of previously discovered bacteria was improved by mixing a growth from an isolated colony with 5 ml of sterile normal saline at a cell density comparable to the turbidity of McFarland tube No. (0.5), equivalent to 1.5x10⁸ cells/ml of bacteria. Inoculums were obtained using a sterile cotton swab and streaked over Muller Hinton agar medium. With heated, sterilized forceps, the antibiotic discs were placed on the surface of the medium at evenly spaced intervals. Incubate the plate at 37°C for 18 hours (Perilla et al. 2003). (ii) According to the Domingue methods, the agar containing the bacteria was cut out from the edges of the plate as discs, then transported to the variant broth incubated at 35°C for 7-10 days. Then by using a cotton swab immersed in bacterial suspension, spread the bacteria on variant agar; after incubating for 18 hours at 35°C, the L-form or cell wall deficient bacteria were grown as fried egg colonies on variant agar. (iii) After staining with Gram stain, the bacterial colonies appear spherical or ovoid shapes and agglutinated (Domingue et al. 1979).

Haemagglutination

It was detected via the presence of clumping of erythrocytes, caused via fimbriae of *S. typhi* when D-mannose is present. The assay was carried out using the mannose-sensitive and mannose-resistant haemagglutination assays and the bacterial Haemagglutination assay-slide method. The *S. typhi* was inoculated on nutrient broth at 37°C for 48 hrs. Blood from human (O) and different animal blood types was collected under sterile conditions into Alsever's solution at a ratio of 2 volumes: 1 volume of blood and stored in the refrigerator. Each type of blood was taken, and NS was washed three times and formed up to a 3% suspension in NS. (AL-Khafagee 2018). The slide was rocked at room temperature for 5 minutes after one drop of RBC suspension was introduced to a drop of broth culture. The presence of clumping was viewed as a sign of haemagglutination. The absence of haemagglutination revealed mannose-sensitive by a comparable series of tests in which a drop of 2% W/V D-mannose and a drop of broth culture were added to the red cells. The presence of 3% haemagglutination in the presence of 2% W/V D- mannose was used to detect mannose-resistant haemagglutination. This technique was also used to identify the type of fimbria in crude oil (Vagarali et al. 2008).

RESULTS AND DISCUSSION

Salmonella typhi isolates identification

A total of 110 blood samples from typhoid patients were collected during the present study period. The first identification of *S. typhi* isolates has been based on morphological, biochemical, and microscopical studies. Gram-negative bacilli were microscopically identified as *S. typhi*, which are peritrichous flagellated, motile, and non-spore-producing bacteria. Although the morphological of *S. typhi* isolates utilized special media such as XLD (Xylose Lysine Deoxycholate) agar, SS (*Salmonella* Shigella) agar, and MacConkey agar, appeared on culture medium once appeared the typical characteristics at 37°C after 18-24h. *S. typhi* colonies appeared smooth, rounded, convex, non-hemolytic, and grey-white color on blood agar. However, *S. typhi* colonies on MacConkey agar looked pale yellow (non-lactose ferment), 1-3 mm in diameter, and after 18-24 hours at 37°C, as well as good development of *S. typhi* colonies on XLD agar emerged gray hue with black center colonies due to its ability to create H₂S.

The TSI, Sugars, Oxidase, Indole, Ureases, and Simmons citrate assays indicated the biochemical results of *S. typhi* isolates. In the TSI slants test, the slant and butt turned AKL/ACID red and yellow, suggesting non-fermentation of glucose on the slant and acid generation with H₂S in the bottom. Further tests of *S. typhi* isolates yielded negative results for oxidase, indole generation, urease generation, and citrate use. The identification of *S. typhi* isolates using the VITEK-2 GN ID Cards System comprised various biochemical assays. The results indicated *S. typhi* with cards IDing a wide range of good isolates (percentage from 95 to 99%). On MacConkey agar, there are 64 *S. typhi* colonies.

Typhoid fever is caused by *S. enterica* serovar *Typhi*, an acute systemic sickness that causes a significant proportion of illness and mortality, especially in impoverished nations. The *S. enterica* serovar *Typhi* infections are common among travelers returning from disease-endemic areas in Europe (Fabrizio et al. 2009).

Confirmation of *S. typhi* by PCR amplification of *FliC-d*

Salmonella typhi clinical isolates were tested using the polymerase chain reaction technique to confirm the identification of *S. typhi* with a specific gene with 763 bp. The observations are that most *S. typhi* isolates carry the *fliC-d* gene, which is typical of *S. typhi*. Furthermore, the PCR showed a total of 64 (100%) positive results from blood (Figure 1). This finding is in agreement with Khan et al. (2012), who found that out of 80 suspected typhoid fever cases, the flagellin gene (*fliC-d*) was detected by PCR in 56 (70%) cases, which matches the results of a previous study in Bangladesh, where PCR was positive in 88.7% of suspected typhoid fever cases.

The result obtained by the VITEK-2 system is the same obtained by the PCR technique. Furthermore, these results are comparable to those of Ali (2015), who discovered that the positive result from the Vitek 2 compact system and the PCR technique was 65 (32.5%).

Of 64 patients, 42 (65.6%) were male, and 22 (34.4%) were female. Therefore, males were estimated to be infected at a higher rate than females. This result is compatible with study results in the Al-Musaib District (Al-Khafaji et al. 2006) and Diyala District (Saleh 2013). That could be because most males were out-doored and could be seen as food-eating and handling or contact with other patients from this perspective (Flayyih 2017). The patients' age rate is from (1-60) years old; the patients' ages are distributed in Figure 2.

The lowest incidence was among the (31-40) and (41-50) were 4.7% and 6.3%, respectively. On the other hand, the percentage of the age group (21-30) was 7.8%, while the highest incidence was among the (0-10) 46.9%. The disease affects age groups (51-60) and >60 at 12.5%. These findings are dissimilar to Al-Sultany's (2003) previous research, which obtained the most infective age between (16-20) at 23.5%. The results also correlated with Flayyih (2017). The age range (of 11-20) had the highest occurrence, according to the researchers (54%), while Ali (2015) found that the majority of participants in his study were aged (51-60) years. These results also compare with Prince (2002), who found the ages (35-10) the most infective, as in Table 1.

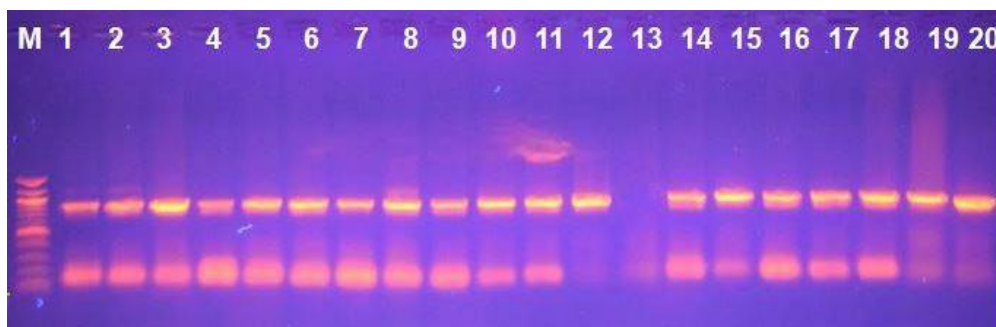


Figure 1. PCR product of *fliC-d* gene primers with product 763 bp gel electrophoresis Lane (M): DNA molecular size marker (100-bp ladder), Lanes (1-20): positive *fliC-d* gene results

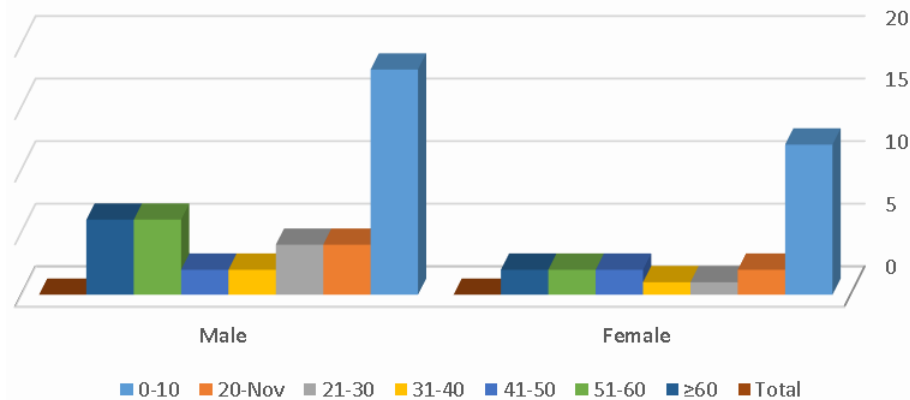


Figure 2. Age rate from female and male *S. typhi* patients

Table 1. Prevalence of *Salmonella* serovar *typhi* according to the age groups

Age group	Total no. (%)	Female	Male
0-10	30 (46.9)	12	18
11-20	6 (9.4)	2	4
21-30	5 (7.8)	1	4
31-40	3 (4.7)	1	2
41-50	4 (6.3)	2	2
51-60	8 (12.5)	2	6
≥60	8 (12.5)	2	6
Total	64 (100)	22 (34.4)	42 (65.6)

Salmonella typhi virulence factors detection

S. Typhi's ability to create a large number of all virulence factors.

Capsule detection

The encapsulated isolates of *S. typhi* were detected using the nigrosin stain. The findings revealed that 56 isolates (87.5%) were encapsulated isolates with tiny polysaccharide capsules.

The *S. typhi*, unlike most other *S. enterica* serovars, may produce a carbohydrate capsule known as Vi-CPS antigen. The production of this antigen, which is influenced by environmental inputs, is critical for extracellular survival and protection against neutrophil oxidative bursts. TNF- α response in human macrophages is likewise reduced following absorption. According to current thinking, Vi-CPS is implicated in immune evasion during infection in the human host, hence critical during infection (Eed et al. 2011).

Hemolysin production

The results of the *S. typhi* isolates' virulence factors revealed that none of the *S. typhi* isolates produced hemolysin in a blood agar medium. These results agree with the findings of Flayyih (2017).

Colonization Factor Antigen (CFA):

Figure 3 shows that 44 (68.75%) isolates have CFA/I and CFA/II. Because of the significant production of CFA,

64 (100%) of *S. typhi* isolates were found to produce this component. CFA/I and CFA/II were also identified in *S. typhi* isolates, albeit in lower percentages than CFA/III. These results disagree with the findings of Ali (2015). According to the findings, 31% of isolates could produce CFA/III, 15% could produce CFA/II, and 92% of *S. typhi* isolates could produce CFA I.

Fimbriae are thought to play an essential function in epithelial cell adhesion. Fimbriae mediate bacterial colonization and host-cell communication by binding to specific host receptors. Fimbrial adhesins control the bacterial pathogen's fate in the host as well as the progression of the disease process. Type-1 fimbriae are also significant in determining the organism's pathogenicity. Jaroni's (2014) experiments revealed that a mannose-resistant haemagglutinin was required for *Salmonella* to attach to target cells. Fimbriae 1 is thought to play a crucial function in adhesion to epithelial cell surfaces (which facilitates bacterial colonization) and determining *S. typhi* pathogenicity.

The adherence of bacteria in mucous surfaces or epithelial cells of the gastrointestinal tract revealed a link between mannose-sensitive hemagglutinin (MSHA) or type 1 fimbria and bacterial pathogenicity. Among the isolates, CFA/II exhibited the lowest prevalence. This factor produces agglutination in chicken blood and helps bacteria bind to unique and complex carbohydrate receptors on small intestinal epithelial cells (Hamid and Jain, 2008).

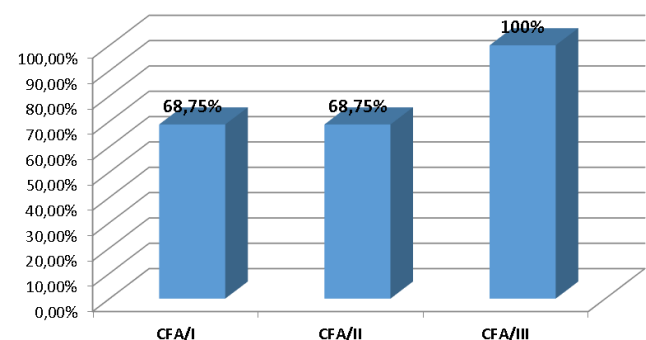


Figure 3. Types of colonization factor antigen in *Salmonella* serovar *typhi*

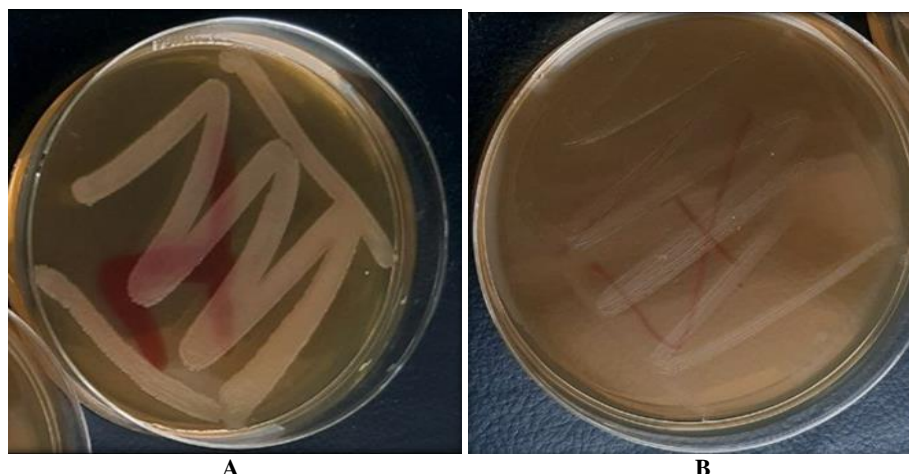


Figure 4. L. form of *Salmonella typhi* isolates after incubation at 37°C for 24 hrs. A. L. form cell on variant agar B. control cell on variant agar

Table 2. Showed isolates bacteria to produce L. form

Num	L-form	Total
1-64	positive	52
	negative	12
total		64

L. form detection

According to multiple studies, tiny colonies of L-form bacteria grow on the plate's edges when one of the β -lactam antibiotics is administered to wild-type bacteria in a petri dish. Likewise, penicillin treatment not only selects for L-forms (which are penicillin-resistant) but also causes L-form growth (Casadesús 2007).

Table 2 showed that 52 (81.3%) isolates could produce L-form. This result agrees with Al-Sultany's (2003) findings, which found that 82.3% of isolates could lose their cell wall and produce L-form after culturing on special media prepared for this target. After staining with Gram stain and examining with a light microscope, the bacterial colonies appear as spherical or ovoid shapes and agglutinated (Kalaivani et al. 2014), as in Figure 4.

In conclusion, this study found that about 100% of *S. serovars Typhi* isolated from the blood of enteric fever patients had many virulence factors by phenotypic tests.

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