



# IDENTIFICATION AND LABORATORY DIAGNOSIS OF *SALMONELLA TYPHI* ISOLATES FROM PATIENT SUFFERING FROM TYPHOID FEVER IN IRAQ

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## Abstract

*Salmonella typhi* causes enteric fever, which is a serious public health problem in both developing and developed countries. *Salmonella* enteric subspecies, is considered a significant food-borne pathogen responsible for animal and human illness. Current study was designed for isolating and identifying of *Salmonella typhi* from blood samples of patient with typhoid fever. This study was conducted on 95 patients of both genders suffering from typhoid fever. They were aged 1 to 60 years. The blood samples were collected from hospitals of different localities in Iraq during the period from January 2019 to July 2019. Blood samples were cultured directly on brain heart infusion broth. After that the isolates were subcultured on blood agar, MacConkey agar, XLD agar, S.S agar. biochemical tests, combined with Vitek2 system were employed to identify the *Salmonella typhi*. The results of this study have shown that only fifty isolates were given typical morphological characteristics and biochemical tests related to *Salmonella typhi*. The highest incidence of *Salmonella typhi* in children was among the 1-10 years old age group, whereas the highest incidence in adults was among the 21-30 years old age group. It has been found that the incidence was higher among females (52%) compared to 48% in the males.

**Key words:** *Salmonella typhi*, Enteric fever, Salmonellosis, vitek2 system

## Introduction

*Salmonella* is a genus of the Enterobacteriaceae family that is Gram-negative, oxidase-negative, catalase-positive, non-spore rodforming. They are also optional anaerobic. Almost all of the *Salmonella* species are motile via peritrichous flagella (Ryan *et al.*, 2017). The *Salmonella*, enteric subspecies, is now a significant food-borne pathogen responsible for animal and human illness (Kagirita *et al.*, 2017). *Salmonella Typhi* is the etiological agent of typhoid fever, while paratyphoid fever is caused by *Salmonella Paratyphi* A, B and C. Since the clinical symptoms of paratyphoid fever are indistinguishable from typhoid fever, the term enteric fever is used collectively for both fevers and both *Salmonella Typhi* and *Salmonella Paratyphi* are referred as typhoid *Salmonella* (Connor and Schwartz, 2005). Typhoid is a

systemic infection induced by *Salmonella Typhi* and *Salmonella Paratyphi* A, human-restricted bacteria transferred faecorally. Typhoid *Salmonella* and *Salmonella Paratyphi* A are clonal and their restricted genetic diversity has prevented the identification of long term transmission networks in regions with a heavy disease burden (Baker *et al.*, 2011). *Salmonella* are the most complicated of all Enterobacteriaceae with more than 2399 serovars with a wide host range including humans, livestock and birds. Salmonellae cause a range of manifestations of disease which include acute and chronic enteritis. *Salmonella* produces a range of putative virulence determinants, which include haemagglutinins, adhesions, invasions, fimbriae, exotoxins and endotoxins (Refaiet *et al.*, 2017). *Salmonella* pathogenesis are also affected by the virulence plasmids, that contain virulence genes. *Salmonella Typhimurium*, *Salmonella Dublin* and *Salmonella Enteritidis* virulence plasmids have been

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documented to be accountable for the systematic spread of infection in the mesenteric lymph nodes, spleen and liver. It has been indicated that virulence plasmids are frequently discovered in *Salmonella* isolated from human or animal organs or blood, rather than in fecal, food, or environmental specimen (Refai *et al.*, 2017). *Salmonella* virulence factors, such as adhesion, invasion, and toxin genes are grouped in particular areas of the chromosome recognized as “Salmonella pathogenicity islands” (SPI). The SPI encode for Type III Secretion System (T3SS) that is accountable for the ability of these microorganisms to inject effector proteins into the cytosol of host cells and to alter host cells signaling cascades to the benefit of bacteria. Once inside the cell, these effects can change cellular functions including cytoskeleton structure, membrane transport, signal transduction, and cytokine expression. These alterations allow the invasion and continuity of the bacterium in the infected cell (Dos Santos *et al.*, 2019).

## Materials and Methods

### Collection of clinical specimens

Specimens have been collected from 95 patients of various ages and genders suffering from Typhoid fever attended to different Hospitals in Imam Al- Hussein Medical city and Karbala Teaching hospital for children and Public Health laboratory in Karbala governorate and Merjan Teaching hospital in Al-Hilla governorate and Fatima Al-zahra Teaching for women and children in Baghdad, Iraq during the period from January/2019 to July /2019. Approximately five *mL* blood samples were collected under aseptic precautions from each patient who gave the consent. The blood sample was inoculated aseptically into sterile bottle containing 50 *mL* of sterilized brain heart infusion (BHI) broth and then incubated at (37°C) for (7) days. Regularly blood cultures were examined to check the turbidity and color change that referred to microbial growth. If positive sample, subcultures from each positive blood bottle, loopfuls of colonies were transferred to selective media namely MacConkey, Blood, XLD and SS agar, to be incubated at (37°C) for 24 hrs. (Nahab *et al.*, 2018). After incubation positive cultures were further analyzed by subjecting the colonies to biochemical tests and VITEK 2 system until more species were identified. Upon completion of diagnosis the pure colony was stored in brain heart broth with 15% glycerol in deep freezing.

### Identification of bacteria

#### Morphological characteristics

Morphological colonies characteristics were recorded

on the media used for primary identification as blood agar, Mac Conkey agar, XLD agar and SS agar (Carroll *et al.*, 2014).

#### Microscopical examination

Grams stain was used to examine the isolated bacteria for studying the microscopic properties, depending on the color and shape and the existence of a single or series (Isenberg and Garcia, 2004).

#### Biochemical tests

Important biochemical tests have been done such as Oxidase test, Indol test, Urease test, Citrate utilization, Kligler test, Motility test and Catalase test were conducted according to (MacFaddin, 2004, Garrity, 2005, Forbes *et al.*, 2007).

#### Identification of bacteria by VITEK 2 system

The VITEK 2 is an automated microbiology device using growth-based technology for bacterial isolate identification and susceptibility testing, available in three formats (VITEK 2 compact, VITEK 2 and VITEK 2 XL) which differ in increasing levels of capacity and automation. All three systems have identical colorimetric reagent cards, which are automatically incubated and interpreted. VITEK 2 identification method depends on series of biochemical tests performed together by using VITEK cards; each card contains 47 wells representing the biochemical tests and metabolic reactions to identify of tested bacteria. A sterile swab or applicator stick was used to transfer a sufficient number of pure culture colonies and to suspend the microorganism in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 × 75 mm clear plastic (polystyrene) test tube. The turbidity was set to (0.50-0.63) McFarland and measured using the DensiChek turbidity meter.

#### Serological test of bacteria isolation

The Serological tests (Anti-salmonella H test) (Sifin/Germany) reagents were intended for use in checking the H-antigens of salmonella strains in accordance with (Kauffmann-White scheme) using slide agglutination in Central public health laboratory (CPHL) in Baghdad. Small amount of bacterial culture from a 16-20 hrs. old subculture (nutrient or Kligler) agar transferred to a clean glass slide and mix well with a drop (25µl) of anti-salmonella H reagent (test serum), mixed slowly with a sterile stick. The result is a homogenous, slightly milky suspension, the slide placed on a dark surface. Positive and negative controls were tested in similar way on the same slide.

#### Statistical Analysis

The Statistical Analysis System -SAS (2012) program

was used in analyzing the data to study the effect of different factors on the studied percentages. The significant differences between percentages were compared with the Chi-Square ( $\chi^2$ ).

Confidentiality of the patients was maintained at all times.

### Results and Discussion

This study was conducted on 95 specimens of blood samples, during the period January/2019 to July/2019. The results have shown that only 50 isolates (52.63%) were given typical morphological characteristics and biochemical tests related to *Salmonella typhi* among the total of 95 blood samples that were obtained from cases of typhoid fever, while the remainder (45 cases) have given negative growth (47.37%) as shown in table 1. Hence, *Salmonella entericaserovar Typhi* was considered to be the main cause of enteric fever. The clinical diagnosis depended on the existence of some symptoms such as fever, headache, anorexia, nausea and vomiting, abdominal discomfort with diarrhea or constipation for (6-18) days.

Results of isolation in this study agreed with Shrestha *et al.*, (2016) who referred that out of 620 blood samples, 83(13.38%) were found as positive culture for *Salmonella* spp. and *S.typhi* was the predominantly (57.83%) isolated bacteria, followed by *S. Para typhi* A (42.26%). In this study, out of a total of 50 isolates that related to *S. typhi*, 26 isolates (52%) were isolated from females and 24 isolates (48%) were from males see Fig. 1. The high ratio of typhoid fever isolated from females agreed with Rasul *et al.*, (2017), who collected 382 blood samples from both genders. 52.62% of females isolates compared to 47.38% of them were from males. The higher incidence of typhoid fever in women was attributed to the lack of awareness and improper hygienic condition and to the absence of education and using contaminated drinking water (Khan *et al.*, 2017). In addition to that most of the females who live in rural areas perform most of the house chores and collection of water from contaminated water bodies such as wells and streams,

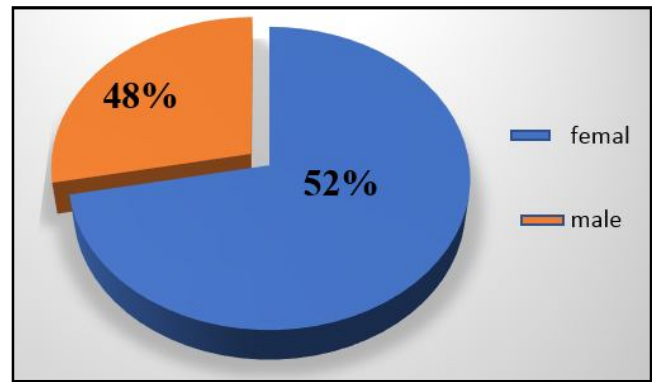


Fig. 1: Percentage of infected patients according to sex.

making them highly exposed to the infection (Joshua *et al.*, 2017).

The results have also revealed that the higher incidence of infection with *S.typhi* in children the 13(26%) between (1-10) years and in the adults 13(26%) between (21-30) years as indicated in table 2. In children, the highest occurrence of infection with *S.typhi* was concurrent with the findings of Sharvani *et al.*, (2017), who isolated 167 strains of *S.typhi* from total of 319 *Salmonella* isolates the have analyzed. The prevalence of *S.typhi* is 51 (30.5 %). The higher percentage of children infection by *S.typhi* could be due to that the undeveloped immune system in growing children makes them susceptible to this enteric pathogen (Afroz Pokharel *et al.*, 2014). In fact the higher incidence of infection among the children pointed out to the need for a vaccine that could be used in routine childhood immunization programs targeting children under 2 years in endemic countries to acquire long-term immunity.

The infection incidence in adults with *S.typhi* seem to agree with (Alsaffar *et al.*, 2020). Who collected 40 blood samples from patients with typhoid fever, 30 isolates from females and 10 specimen from males. The highest percentage of infection occurred at age between (21-30) years with (55%). The high prevalence of infections at this age group may be due to that this working age group who are exposed to infection precocious in the community, Other possible causes include their

Table 1: Ratio of isolation of *Salmonella typhi* from typhoid fever cases in different cities in Iraq.

Cities	Positive (growth)		Negative (no growth)	
	No. of samples	%	No. of samples	%
Karbala city	42	84%	35	77.78%
Al-Hilla city	6	12%	7	15.56%
Baghdad city	2	4%	3	6.67%
Total No.	50	100%	45	100%

Table 2: Age distribution of *Salmonella Typhi*.

Age group (Years)	No. of samples	No. of positive samples	of positive % samples
10-1	25	13	26%
20-11	23	10	20%
30-21	21	13	26%
40-31	9	7	14%
50-41	10	4	8%
60-51	7	3	6%
Total No.	95	50	100%

consumption of unhygienic food and water in working places, and colleges along with rise number of social aggregation and polluted food the major reason of infection in young adults. Typhoid fever is affecting people unheeding of age and gender, popular among infants, young babies and adults in Southeast Asia (Velema *et al.*, 1997; Malik and Malik, 2001; Tran *et al.*, 2005).

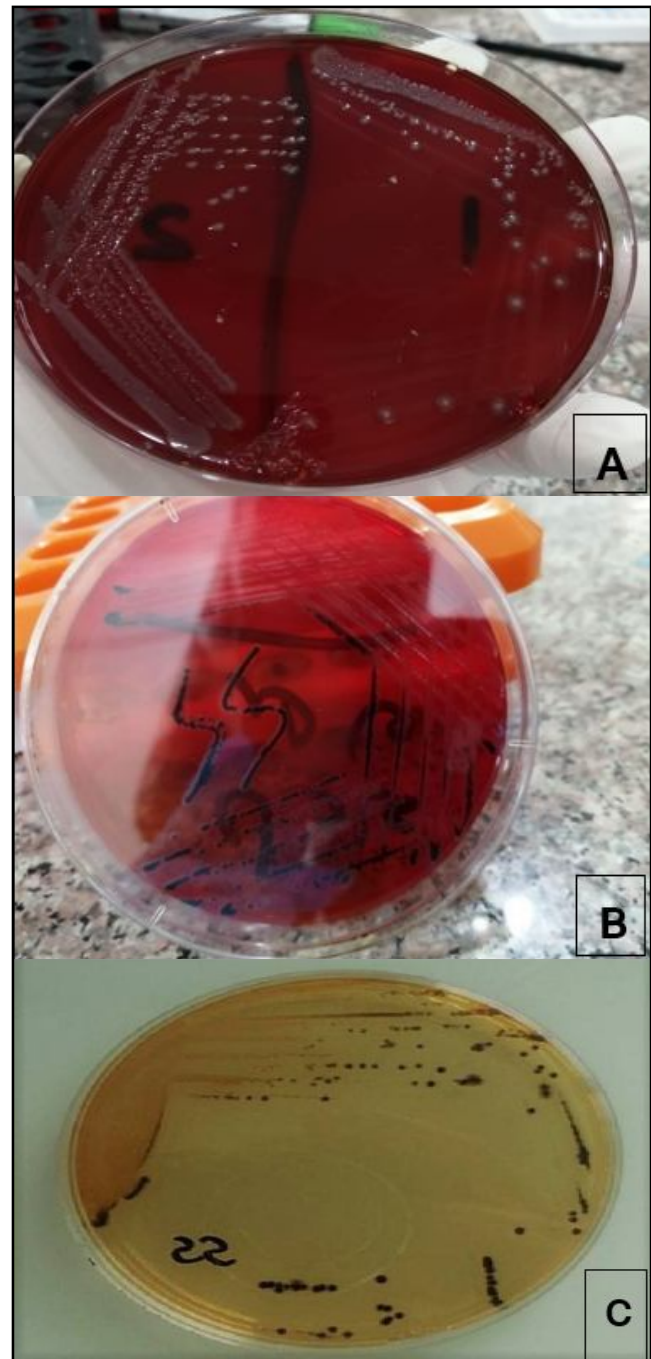
### Identification of bacterial isolates

#### Cultural characterization

There are many methods of isolation and diagnosis of *Salmonella* in use world-wide (Reissbrod, 1995). The results of the present study have depended on morphological characteristic and biochemical tests for identification of *Salmonella* spp. Primary identification of bacterial isolates were achieved after being incubated aerobically on MacConkey agar, blood agar, SS agar and XLD agar plates at 37°C for 24-48 hrs. These tests have revealed the typical feature described by similar studies (Quinn *et al.*, 2004; Patrick *et al.*, 2005; Pakzad *et al.*, 2007). Those samples were directly streaked on (MacConky agar) and incubated at 37°C for 24 hrs. Colonies appearing pale or almost colorless on MacConky agar due to their inability to ferment lactose which leading to no acid production and, no change in pH allowing to no changes in the neutral red which is considered as indicator for color change of the media. The morphology of the bacteria was appearing as circular and wet with smooth convex surface and entire edge on this medium (Abu-Resha *et al.*, 2019). On the enrichment blood agar medium, the tested bacterium formed colonies with variable sizes ranging from (2-3 mm) after 24 hrs. of incubation. Depending upon conventional cultural procedures, the expected *S.typhi* isolates were further tested for their hemolytic activity on blood agar medium. After 24 hrs. of incubation the colonies appeared as circular and whitely to colorless colonies without hemolysis of blood (Nalbantsoy *et al.*, 2010). For certifiable diagnosis loopfuls colonies were further streaked on (XLD) agar dishes and incubated at 37°C for 24 hrs. The colonies appear smooth and red in color with black colony center (McFadden, 2000) as described in Fig. 2. The black center of colonies on (XLD) medium is due to ability of *Salmonella* to metabolize thiosulfate in media to produce hydrogen sulfide and allows them to be differentiated from the similarly colored *Shigella* colonies on this medium (Nye *et al.*, 2002). On other hand *Salmonella* on SS agar medium, the isolates formed smooth and colorless colonies usually with black spot due to H<sub>2</sub>S production by reaction with the iron in the medium. All the results (morphology and cultural) were identical with Nat *et al.*, 2010; Abu-Resha *et al.*, 2019.

### Microscopic characterization

Identification of the suspected isolated colonies by gram stain has depended on the morphological properties (size, shape, arrangement). The bacteria appeared under oil immersion lens (100x) as Gram-negative, pink short rod coccobacilli, non-spore forming and flagellated Fig. 3. These microscopical characteristics are in conformity with those characteristics being reported for *Salmonella* by another study (Muthiadin *et al.*, 2015).



**Fig. 2:** Growth of *Salmonella typhi* on (A) Blood agar, (B) XLD agar and (C) SS agar.

### Biochemical characterization of *S.typhi* isolates

According to the results of morphological and microscopic characteristics, bacterial isolates were initially subjected to a number of biochemical tests as show in table 3 such they were gave positive results in catalase test, but it gave negative results in oxidase test. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide ( $H_2O_2$ ) to water and gaseous oxygen that prevent the assemblage of toxic metabolites (Warren *et al.*, 2000). In Kligler Iron Agar (KIA) test, it differentiates the genera of Enterobacteriaceae from each other based on their carbohydrate fermentation patterns and  $H_2S$  production. *S.typhi* isolates have ability to ferment glucose only on Kligler iron agar (KIA) and give alkaline slant so they gave alkaline slant with acid bottom with  $H_2S$  production but no gas (Carroll *et al.*, 2010). In the indole test, *S.typhi* isolates gave negative results due to the inability of bacteria to hydrolyze tryptophan to indole and production of tryptophanase enzyme, so that when Kovac's reagent was added to a broth free of indole, a red ring did not appear at the top of the broth. Utilization of citrate is one of several important physiological test used to diagnose members of Enterobacteriaceae, *S.typhi* showed negative reactions for citrate, so that the citrate in simmon citrate medium was important to detect weather the bacteria isolates capable to grow on it as a unique carbon and energy source. Additionally, Simmon's medium also contains bromothymol blue as a *pH*



**Fig. 3:** Microscopical appearance of *Salmonella typhi* as Gram-negative short bacilli.

**Table 3:** Results of biochemical tests for *Salmonella typhi* isolates used in the study.

Biochemical test	Results
Catalase	+
Motility	+
Indole	-
Oxidase	-
Citrate Utilization	-
Urease	-
Glucose and lactose Fermentation on Kilgler Iron agar ( KIA)	(alkaline (red) slant and acid (yellow) butt, with production of $H_2S$ blackening of agar) No gas

indicator.

Whereas *S.typhi* was tested negative with urease. Urease enzyme catalyzes the breakdown of urea and the bacteria that can produce this enzyme is able to detoxify the waste products and to command metabolic energy from its utilization which could change the medium color from yellow to purple-pink, indicating urease positive test.

In the motility test, *S. typhi* isolates were motile. The movement of the growth away from the stab line or a hazy appearance through the semisolid medium appear that the bacteria are motile.

Our results of biochemical tests, seem to agree with (Mortazavi, *et al.*, 2010; Brenner *et al.*, 2005; Tindall *et al.*, 2005).

### Identification of bacterial isolates by VITEK system

Further Identification of bacterial isolates was also conducted by VITEK 2 system, which was installed at Al-Rasoul medical labs in Karbala city, by using the GN (Gram Negative) card, which contained forty seven biochemical tests and one negative control well. The current results have demonstrated that only (50) isolates from blood as *S. typhi* with ID message confidence level ranging between (95-99%). The results of VITEK 2 system were in agreement with that obtained from the biochemical tests applied for the pathogenic isolates that are shown in table 4. In general , the VITEK 2 system is an easy instrument to handle. It provides a rapid (4 to 15 hrs.) and relatively accurate means for the identification of most bacterial species to be accurately detected (Garcia Garrote *et al.*, 2000).

Funke *et al.*, (1998) found that VITEK- 2 system is a promising, highly automated tool for the rapid identification of gram-negative bacilli from human clinical specimens and could identify members of the family Enterobacteriaceae such as *S.Typhi*. Several studies indicate the importance of using this technology in determining the genus of Salmonella (Szabo *et al.*, 2008; Priego *et al.*, 2009; Martiny *et al.*, 2012).

### Serological test (Slide Agglutination test)

Serological identification of *Salmonella* are based on H- antigens according to the Kauffmann–White scheme. Slid agglutination test was used by Anti Salmonella H test reagents (Sifin/Germany) as the results showed that the bacteria belonging to the genus S. Typhi gave a positive result for the agglutination test react through one min. indicate that positive reaction, as it is characterized by serological diagnosis performed easily and speed of the results obtained this agreed with (Pfeifer

*et al.*, 2009; Abdullahi, 2010).

## Conclusion

In conclusion, it was found that 50 out of 95 (52.63%) had positive blood culture and the isolates belong to *salmonella typhi*, whereas (45) isolates (47.37%) gave negative results. The incidence of *S. typhi* was generally higher in females than in males and the incidence of *S. typhi* was considerably higher in children in age (1-10) years and in young adults (21-30) years.

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