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Bacteriological and Molecular Study of *Salmonella Typhi* that Isolated From Typhoid Patient in Al-Muthanna City

Alaa K. Hameed [1], Ilham A. Bunyan [2]

Abstract

In this investigation, blood samples from 150 typhoid fever patients who visited Al-Hussain Teaching Hospital in Al-Muthanna City between April 2023 and February 2024 were obtained Testing for culture and biochemistry was used to identify the subject, and the Vitek2 system was used to confirm the results. The Vitek2 technique revealed that 27 (18%) of the isolates tested positive for Salmonella enteric serovar typhi. The investigation of some virulence factors were also performed of all isolated of S. typhi. All isolates have not ability to produce hemolysin in blood agar, while all isolates can produce sidrophores (100%) when investigation on M9 media supplanted with dipyridyl.

27 (100%) of the isolates under examination were positive for colonization factor antigen 3 (CFAIII). Tryptic soy broth mixed with 1 percent glucose was used in microtiter (biofilm test) experiments to measure the production of "biofilms quantitatively". The examined isolates gave positive results were 11(40.74%) were produce biofilm strongly, 9(33.33%) were minor production of biofilm, while 7(25.92%) were not produce biofilm.

The initial step of the molecular analysis process was the extraction of genomic DNA from all of the Salmonella typhi isolates using an approach that worked well for this purpose. Second, the (flicA) gene was detected by "PCR technique" and compared to an allelic ladder, yielding a (578 bp) result. It was discovered that 25 (92.59%) isolates of the positive sample contained the (flicA) gene. Also, molecular method showed Wzy gene detected with product molecular weight (451bp) the result showed that 19(70.37%) isolates were positive for this gene.

Key words: Salmonella typhi, colonization factor antigen 3 (CFAIII), Siderophore, flicA gene, Wzy gene.

[1] Department of Pathological analyzes, College of Science, Al-Qasim Green University, Babylon 51013, Iraq

[2] University of Babylon in Iraq, College of Medicine

Introduction

The most serious foodborne pathogen is "*Salmonella enterica", which is commonly connected to poultry product contamination*. Approximately 93 million gastroenteritis cases and 155,000 fatalities are caused by *Salmonella* each year in the world [1]. typhoid fever *which caused by Salmonella enterica* serotype Typhi (*S* Typhi) is common in places with inadequate infrastructure for water, sanitation, and hygiene (WaSH). If treatment is not received, 10%−15% of patients experience serious problems. This is mostly due to insufficient diagnostic techniques and the prevalence of antibiotic-resistant bacteria, which make clinical management and prognosis more difficult. Through the organism's fecal shedding, both acutely infected individuals and asymptomatic chronic carriers contribute to the ongoing transmission of the infection. Typhoid fever's high morbidity and mortality rates in low- and middle-income nations highlight the necessity of an integrated control strategy, which could eventually result in the disease's eradication in the twenty-first century. [2].

Children, the elderly, and immunocompromised patients are more likely to be vulnerable and experience more severe clinical signs, such as sepsis [2-3]. In other circumstances, the infection may cause the host to remain in a long-term, asymptomatic condition of carriage. [4]. Salmonella Typhimurium and Salmonella Enteritidis are the most prevalent serotypes of Salmonella that infect people. [5-6]. People living in low- and middle-income nations are particularly vulnerable to contracting typhoid fever [7-8].

The infection mostly spreads through oral consumption, and flagellar mobility is crucial to the infection process. The ability to travel across mucosa and other viscous environments, find the surface of the host cell, and stick to the cell membrane in order to infect, is provided by the flagella. [10-11]. *The two flagellin genes in salmonella, fljB and fliC, are expressed autonomously at a frequency of 10−3–10−4 per cell every generation, generating either FljB or FliC for filament production*. Phase variation is the term for this. Studies have shown that, in comparison to FljB-expressing bacteria,Bacteria that expressed FLIC exhibit a notable advantage for invading the majority of epithelial cell lines of murine and human origin. Additionally, variations in swimming patterns close to surfaces have been noted, with FliC-expressing bacteria more commonly exhibiting a "stop" behavior [1**2**].

Material and methods

Specimens

One hundred and fifty specimens include blood were collected from different ages individuals with typhoid fever who was admitted to Al-Hussain Teaching Hospital and outpatient clinic in Al-Muthanna city during the duration (April 2023- February 2024).

Detection of isolates:

Sterilized syringes were used to draw 10 milliliters of fresh venous blood from probable typhoid patients. The sample was then placed in a special crew-cupped culture bottle containing 100 milliliters ofBHI broth (brain heart infusion broth) and cultured for three days at 37 o C before being kept in a Bact Alert 3D apparatus for a week. Then the positive sample cultured on selective and differential media "MacConkey, Blood, XLD and Salmonella and Shigella agar" and incubation at 37 °C for 24 h. The isolates were identified using traditional culture methods, the VITEK 2 Compact System, biochemical tests, and Gram stain.

Bacterial Virulence factors detection

Production of Hemolysin

In order to produce hemolysin, blood agar medium was inoculated with bacterial isolate at 37 °C for 24 to 48 hours. Complete hemolysis was indicated by the formation of a clear zone surrounding the colonies.

Partial hemolysis (α-hemolysis) is defined as hemolysis (β-hemolysis) or a greenish zone surrounding the colonies; non-hemolysis (γ-hemolysis) is defined as no change. [13].

Siderophores production:

Following autoclaving sterilization and cooling to 50°C, 2.25 mg/L of filter-sterilized glucose and 200 μmol/L of dipyridyl were added to the M9 medium that had been prepared. Following the organisms' inoculation into this medium, a 24-hour incubation period at 37°C was observed. The outcomes were observed whether or not there was organism growth [14].

Colonization factor antigen (CFA):

The purpose of this test was to determine whether bacterial isolates could produce the colonization factors antigen (CAF). (CAF-III) generated can be found in the following ways:

- To create the RBC suspension, human blood specimens (group A) were placed in a tube containing EDTA, mixed with a 1:1 phosphate buffer solution, and centrifuged for five minutes at 8000 rpm. After discarding the supernatant, the sediment containing the red blood cells was three times cleaned with PBS before the cells were suspended up to 3%.
- Using the bacterial growth for each strain from TSA (tryptic soy broth), a bacterial suspension was made in normal saline. Tannic acid (20%) is prepared in order to measure RBC agglutination. After adding one drop of bacterial broth, one drop of RBC suspension, and one drop of tannic acid on a clean slide, observe the human RBC type A blood group agglutination at room temperature for one to two minutes. [15].

The tissue culture plate method (TCP) is used to produce biofilms.

The TCP assay, which was first published by Christensen et al. in 1985, is the most popular and was once thought to be a gold standard for identifying biofilm formation.

The TCP approach, as previously published by Christensen et al. [16], was used in the current study to screen every isolate for the capacity to generate biofilm. However, the length of incubation was changed to 24 hours in accordance with [17].

Fresh agar plate colonies were diluted (1) in (100) with fresh medium after being inoculated in tryptic soy broth and incubated for eighteen hours at 37°C in a stationary condition. (0.2 ml) aliquots of the diluted cultures were placed into each well of 96 sterile polystyrene well-flat bottom tissue culture plates. Broth was used as a control to ensure medium sterility and non-specific binding. The tissue culture plates were incubated at 37°C for eighteen and twenty-four hours.

The plates were gently tapped to release the contents from each well after incubation. To eliminate "planktonic" germs that float freely, 0.2 ml of phosphate buffer saline (pH 7.2) was used to clean the wells four times. The adherent "sessile" organisms in the biofilms generated on the plate were preserved with 2% sodium acetate and stained with 0.1% w/v crystal violet. After thoroughly cleaning the excess discoloration with deionized water, the plates were set aside to dry. Adherent bacterial cells were typically uniformly stained with crystal violet and developed biofilm on all side wells. Using a micro ELISA auto reader set to 570 nm in wavelength, the optical density (OD) of the adhering bacteria that had been stained was measured (OD570 nm).

The experiment was carried out in triplicate and three times. After that, the data was averaged, and Table 1 was used to interpret the findings. [17].

Table (1): Bacterial adhesion classification using the TCP method

Mean of OD value at "630nm"	Biofilm production
$<OD_C$	No - adherence
$OD_C < OD \leq 2 \times OD_C$	weakly adherence

$2 \times OD_C < OD \leq 4 \times OD_C$	Minor adherence
$4 \times OD_C < OD$	Strongly adherence

Molecular Study

Detection of genes using PCR method:

For every gene, the PCR mixture was produced in a total volume of (20) µl. as illustration in Table (2).

Table (2): PCR mixture volume

Reagents	Volume
DNA template	2 µl
Free water	7.5 µl
Master mix	8 µl
Primer mix	2 µl
MgCl	0.5 µl
Total	20 µl

Primer sequences:

Table (3) sequence of primers

Genes	Sequence.	Product	References
Flic A	F:GCTTAATGTCCAAGATGCCTC R:GAGCAACGCCAGTACCATCTG	(587bp)	Kumar *et al*., 2006
Wzy	F:GAAATTATGCCATCTTGGCGA GCG R:CATGTGAAGCCTGAAGGCAAACTC	451bp	Patel *et al*.,2012

PCR Thermocycling Conditions:

PCR tubes were put on the PCR apparatus, and the appropriate conditions for the PCR cycle program were set up, as shown in Table (4).

Table (4): Conditions of PCR Thermocycling

Gene	Initial Denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extinction
Flic A	94C° for 5 min	35	94C° for 1 min	55C°for 1min	72C°for 1min	72C°for 5min
Wzy	94C° for 5 min	35	94˚C for 30 s	59˚C for 30s	72˚C for 5 min	72˚C for 5 min

Statistical Analysis:

SPSS 23 was used to do statistical analysis. The Pearson Chi-square test and the ANOVA test were used to ascertain the statistical differences between the groups. It was determined that the likelihood of ($P \leq 0.01$) was statistically significant**.** [18].

Result and Discussion

Identification of Bacterial Isolates

Samples of blood were drawn from typhoid fever patients. from Al Hussain Teaching Hospital and outpatient clinic through the duration (April 2023- February 2024). The present result demonstrated that from a total of 150 blood samples 27(18%) were bacterial culture positive and confirmed by Vitek 2 system as illustrated in figure 1.

According to a study by Kazem [19], 51 samples out of 120 isolates tested positive, meaning that 27% of them were S. typhi-related. where as another study done by Salim and Motaweq [20] in Najaf city found that out of 110 specimens Only 64 (58.1%) Gram-negative bacteria.

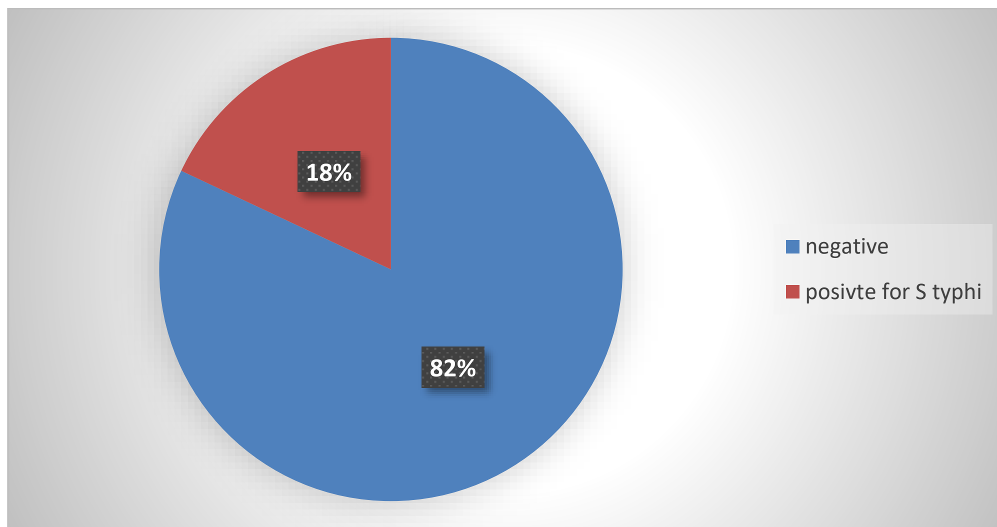

Figure (1): Distribution of *S typhi* according to culturing

Virulence factors of *Salmonella typhi*:

3.3.1 Colonization Factor Antigens:

The colonization of the human mucosa was mediated by CFA, which are specific antigenically fibrillae. An important adherence component that may not have been detected yet contributed to the pathogenesis and the host's immune response. [21].

27 (100%) of the isolates under examination were positive for colonization factor antigen 3 (CFAIII). Table (3-2) presented these results. According to a study by Salim, l., & Motaweq [20], 100% of the isolates (64) generate CFAIII.

Salmonella enterica serovar typhi clearly has a high ability to adhere to epithelial cells, as evidenced by isolates that produce positive results in an agglutination test with (group A) human RBCs in the presence of tannic acid. These results are identical with those reported by [22].

Coli surface antigen, or CFA III, is a groups of fourteen genes together form the cof operon colonization factor antigens (CFAs). The etiology and pathophysiology of disease are significantly influenced by these genes, which promote adhesion to and colonization of host epithelial cells. [23].

3.3.2 Hemolysin and Sidrophores production:

This study examined the potential of "Salmonella typhi" isolates to produce several virulence factors. The findings clearly demonstrate that not all S. typhi isolates can produce hemolysin on blood agar 27(100%). These results agree with SALIM, L., & MOTAWEQ [20] who discovered that in a blood agar medium, none of the S. Typhi isolates generated hemolysin. and also These study agree with the results of Flayyih [24] also found that all isolates not produce hemolysin.

Every isolate of Salmonella typhi was subjected to an M9 media inoculation in order to investigate the possibility of Sidrophore as a substitute mechanism for iron uptake. As a result, all 27 isolates (100%) developed on the medium. The results showed in Table (4).

The outer membrane receptor proteins found on gram-negative bacteria are able to identify particular iron (III)-sidrophore complexes on the cell surface. An energy-dependent mechanism made up Sidrophore receptor protein, actively transports ferric Sidrophore complexes across cell membranes; this property was investigated by cultivating the material on M9 media. The Sidrophore consider importance virulence factors for *salmonella typhi*, which is iron source[25].

Siderophores are low molecular weight iron chelators that help them meet their iron needs. Almost all species need iron, and in places where iron is scarce, highly efficient iron acquisition systems are used to

scavenge iron from the surrounding environment. These systems involve the release and uptake of siderophores, which are extracellular ferric chelators. [26].

Table (4) Distribution of Hemolysin and Sidrophore production by *Salmonella typhi.*

Virulence factors	***Salmonella typhi*** **n= 100**	
	No. and (%) of positive isolates	**No. and (%) of negative isolates**
Hemolysin	0(0%)	27(100%)
Sidrophore	27(100%)	0(0%)
CFA =III	27(100%)	0(0%)

Biofilm formation of *Salmonella typhi*:

A semi-quantitative microtiter plate test was used to measure the production of biofilm on a polymetric surface (biofilm assay).For each isolate, this experiment was run in triplicate to improve the assay's accuracyBased on the average optical density (OD) value at 590 nm, the following outcomes were observed: > 0.240, < 0.120, and < 0.120, respectively. Fifteen (93.75%) of the tested isolates yielded positive results. This result agrees with results obtained by Abida *et al*., [27] who found that 29(96.6%) isolates from *S. typhi* produce biofilm due to ability to adhere to any collection of microorganisms when the cells are sticky to one another and frequently to a surface. Often, an extracellular polymeric matrix that the adhering cells self-produce surrounds them.

The primary constituents of the polymeric aggregate referred to as biofilm extracellular polymeric material, or slime, include extracellular DNA, proteins, and polysaccharides. Biofilms are commonly found in industrial, medicinal, and natural settings and can form on both living and non-living surfaces.

In contrast, planktonic cells are single cells with the ability to swim or float in a liquid medium; they are not physiologically similar to the microbial cells that form biofilms. Microbes can build a biofilm in response to a variety of stimuli, including cellular recognition of specific or non-specific attachment sites on a surface, nutritional signals, or certain environmental conditions. [28].

Table 5 shows that of the isolates that were investigated, 24 (88.88%), 11 (40.74%), and 9 (33.33%) were found to be strongly produce biofilm, while 7 (25.92%) were found to be not produce biofilm. This variation in biofilm formation ability agrees with Verstraeten *et al*., [29] who found that 12(20%) were moderate biofilm, 7(11.6%) strong biofilm and 2(3.3%) weak biofilm due to variation motility required for both pathogenesis and biofilm formation.

The bacteria found in the biofilm are referred to as sessile bacteria because they have different phenotypes from planktonic bacteria and are in a stationary or inactive development phase [30].

Table (5): Biofilm formation among *S. typhi*

Case	O.D*	Biofilm Response**	Biofilm Response
case-1	1.0	weakly adherent	-
case-2	2.5	moderately adherent	++
case-3	2.5	moderately adherent	++
case-4	5.2	strongly adherent	+++
case-5	3.8	moderately adherent	++
case-6	3.9	moderately adherent	++
case-7	2.5	moderately adherent	++
case-8	3.1	moderately adherent	++
case-9	5.7	strongly adherent	+++
case-10	2.5	moderately adherent	++
case-11	3.1	moderately adherent	++

case-12	2.2	weakly adherent	-
case-13	7.2	strongly adherent	+++
case-14	2.1	moderately adherent	++
case-15	9.2	strongly adherent	+++
case-16	10.4	strongly adherent	+++
Case17	11.8	strongly adherent	+++
Case18	1.2	weakly adherent	-
Case-19	5.5	strongly adherent	+++
Case20	6.5	strongly adherent	+++
Case21	6.2	strongly adherent	+++
Case22	1.5	weakly adherent	-
Case23	5.1	Strongly adherent	+++
Case24	7.5	strongly adherent	+++
Case25		weakly adherent	-
Case26		weakly adherent	-
Case27		weakly adherent	-

***O. D fold   O.D increment compared to control**

Table (6) mean of OD value of Biofilm production

Mean of OD value at 630nm	Biofilm formation
$<OD_C$	Not –adherence
$OD_C < OD \leq 2 \times OD_C$	weakly adherence
$2 \times OD_C < OD \leq 4 \times OD_C$	Minor adherence
$4 \times OD_C < OD$	Strongly adherence

Molecular Study

Detection (*flic A*) gene and WZY polymerase gene:

The fliC gene with a product size of 578 pb was discovered using the "PCR technique" as indicated in Figure (2). PCR was utilized in this investigation to identify the fliC gene in 27 isolates, and the results showed that 25 (92.59%) of the isolates tested positive for this gene. Also PCR was used to detect Wzy gene with product molecular weight (451bp) the result showed that 19(70.37%) isolates were positive for this gene as shown in figure (3).

Similar to the findings reported by Hassuny et al. [31] in Iraq, our data showed 100% PCR positivity. This result is related to Khan et al. [32], who discovered that flagellin gene (fliC) was detected in 80% of suspected typhoid fever cases. A study conducted in Bangladesh reported that 88.7% of suspected cases of typhoid fever had positive PCR results [32].

Astudy conducted by Mina *et al* [33] found that The *fliC* gene was detected in 21 isolates out of 23 biochemically identified bacterial genomic DNA. ALSO study done by Kazem [19] showed that FLICd gene detected in 100% of isolates.

There are three main components to a flagellum: the filament, which is the longest and outermost section, is made up of about 20,000 protein subunits of a single protein called flagellin (Flic), which has a molecular weight of between 50 and 60 KDa. The production of the H (flagellar) antigen is encoded by the gene fliC-d. This antigen serves as the foundation for "Salmonella's Kauffman-White classification system". [34].

There are more than 100 Salmonella serovars that carry the fliC gene. We searched for a different or additional diagnostic method because blood cultures for S. typhi in patients clinically diagnosed with typhoid fever in the community had a low culture-positivity rate. [35].

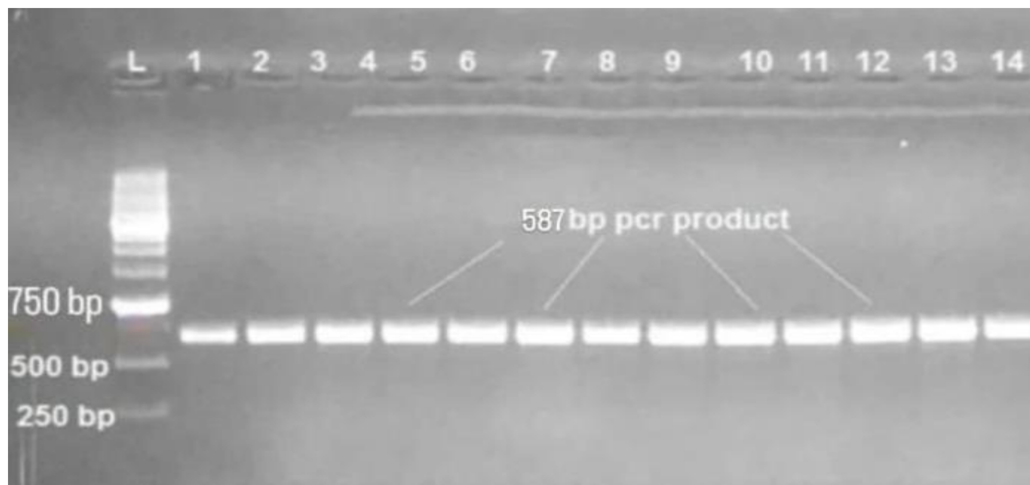

Figure (2): PCR results of *Salmonella sp.* Flic gene. Lane 1-7 shows some positive genes with a product size of 587 bp.

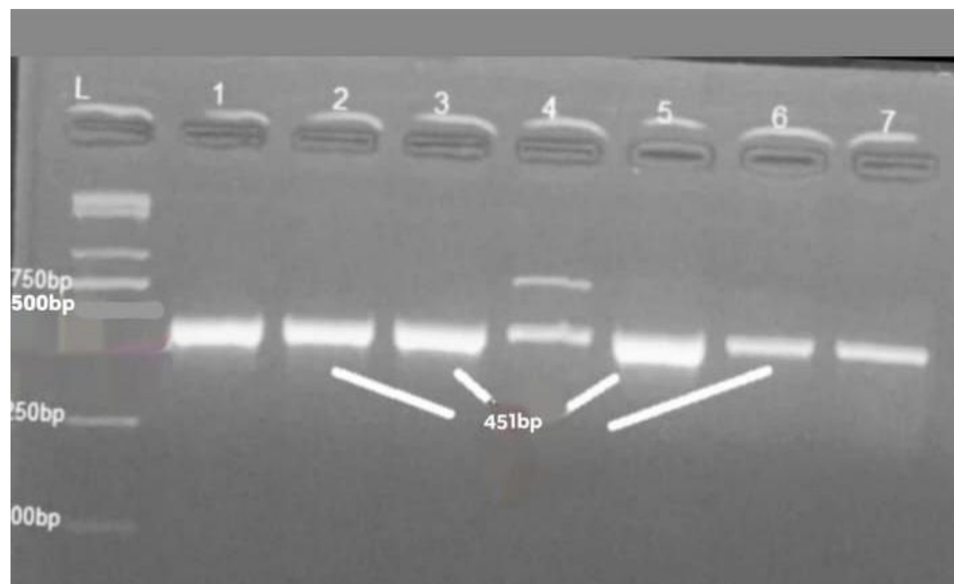

Figure 3: PCR results of *Salmonella sp.* Wzy gene. Lane 1-7 shows some positive genes with a product size of 451 bp.

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