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Isolation and Identification of *Salmonella spp*. Using Molecular and Phenotypic Methodes

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Abstract: *Salmonella enterica* is a frequent cause of water- and food-borne infections that can impact both human and animal hosts and result in a broad spectrum of clinical disorders. Both industrialized and developing nations continue to face a serious threat from illnesses brought on by typhoidal and non-typhoidal *Salmonella*. It can be challenging to distinguish *Salmonella enteric serovar Paratyphi*(*S. enteric serovar Paratyphi*) ,*serovar Typhi* (*S. enteric serovar Typhi*) and *serovar Typhimurium(S.enterica typhimurium*) based on clinical signs and serological tests.The current study aimed to determine the accuracy of classical diagnosis with a molecular diagnosis for detecting *Salmonella spp* isolated from patients attending Baghdad hospitals .Molecular detection was carried out for confirm species of *salmonella sp.* 78 among 114 isolates that were identified as *salmonella spp* according to morphological and biochemical tests .By using*16SrRNA* ,*flic-d* , *tym* and *flicABC* primers . However the outcomes revealed that sixty seven (58.77 %) isolates subjected to *Salmonella enteric serovar .typhi* , but none other types of *Salmonella enteric serovar* was found.

Keywords: Conventional diagnosis, PCR, , *Salmonella enterica, flic-d*

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Introduction

There are more than 2500 serovars of the bacterial pathogen *Salmonella* enterica (*S. enterica*), which is known to infect both people and animals (5). Salmonellosis in humans is caused by specific serovars of the bacteria *S. enterica*, including serovars *Typhi*, *Paratyphi*, *Typhimurium*, *Enteritidis*, and others. These serovars have an impact on the disease burden globally. Typhoid fever affects 16 million people worldwide every year, while *Salmonella* gastroenteritis affects 1.3 billion people (4). *Salmonella enterica* (*S. enterica*) is a member of the Enterobacteriaceae family and is a Gram-negative, facultative, nonspore-forming bacteria, can thrive in a wide range of temperatures, from 6 to 46 °C and are motile through peritrichous flagella (8). Over 2,500 serovars were subjected to *S.enterica* and the majority of these serovars infect a wide variety of vertebrate animals. A small number of them, including non-typhoidal Salmonella

and human-restricted typhoidal serovars like the typhoid fever-causing *S. enterica serovar Typhi* and *Paratyphi*, are host-specific (14). The non-typhoidal serovars (NTS) have been associated to zoonotic infection of a range of hosts, causing acute and self-limiting gastroenteritis that can frequently result in foodborne sickness in humans (20). In light of this, it is crucial to understand how to diagnose and type *Salmonella spp*. *Salmonella* is typically detected via biochemical and serological assays, which are conducted after selective medium-based culturing (12). The major phenotyping technique for *Salmonella spp*. is serotyping, and there are roughly 2500 recognized serotypes, according to the Kauffman-White-LeMinor scheme. All three antigens that determine the serotype are available: the flagellated antigens (H), the somatic antigen (O), and the capsule antigen (K). (11). Traditional detection methods need a great deal of effort and time. Serotyping techniques usually fail as epidemiological tools because they have low selecting power for isolates who share the same serotype or similar biochemical features. (3). In the past ten years, nucleic acid amplification by polymerase chain reaction (PCR). *Salmonella* is a pathogenic organism that exhibits a wide range of virulence characteristics that enhance the organism's pathogenicity. The design of structures approaches for host macrophage and other cell invasion and survival could include variable polysaccharides in the membrane, numerous fimbria adhesions, flagella with phase and antigenic modification capability, and numerous fimbria contractures. One of the genetic elements that exists in the bacterial chromosome as a distinct and individual entity is a pathogenicity island. (9). Bacterial flagella are thought of as locomotion organelles because they are thin, filamentous, stiff projections. Approximately 20,000 protein subunits of the flagellum (Flic), a single protein with a molecular weight of 50 to 60 KDa, make up the topmost and biggest component of a flagellum. (6). It has been hypothesized that *Salmonella* uses its flagella to escape from an intracellular position because they give the organism rigidity, motility, and the potential to participate in cell invasion (10).

Materials and methods

Location of the research

The Department performed the investigation of Biotechnology's Bacteriology and Molecular Laboratories at Baghdad University in Iraq.

Collection of specimens and isolation of *Salmonella spp*

One hundred fourteen clinical sample included, Blood samples (81), fecal sample (33) were taken from patients suffering of enteric fever after gained approval the college of science research ethics committee according to (CSEC\1021\0086) at Baghdad Medical City (National Center for Educational Laboratories, Baghdad Teaching Hospital), Al karama Teaching Hospital, Al-Yarmouk Teaching Hospital for five months, from December 2021 to April 2022. Using sterile syringes, four milliliters of fresh venous blood were

divided in half. One milliliter of blood was required for the Widal test, thus three milliliters of blood were collected. Put into a specific screw that had been installed in bact/alert 3D. If a specimen was positive, each specimen was injected intravenously into a culture of MacConkey, XLD, and SS agar (Himedia-India) using an inoculation strategy. after being immediately warmed at 37°C for between 18 and 24 hours, the inoculation cultivation dishes were stored until they were required (16). As for stool samples, it was easier to take the diarrhea sample directly and culute it on the differential media. Identification of isolated Salmonella demonstrates Red colonies with a black core were subcultured on nutritional agar (NA) (Himedial/ndia) in order to conduct (21). Gram staining identified morphological characteristics and biochemical identification included sugar fermentation, IMVC test (indole test, methyl red (MR) test Voges proskauer (VP) test and citrate test) (1).

DNA extraction and molecular Detection of *Salmonella spp*

DNA extraction

Genomic DNA was isolated from bacterial growth according to the protocol of Presto™ Mini gDNA Kit (Geneaid, Taiwan) .

Quantitation of DNA

The Quantus Fluorometer (Promega, USA) was utilized to determine the concentration of extracted DNA in order to determine the quality of samples for subsequent applications. 200l of diluted Quantifluor Dye was combined with 1 l of DNA. After 5 minutes of room-temperature incubation, DNA concentration values were measured.

Polymerase chain reaction (PCR) assay

Salmonella spp. detection was reconfirmed using PCR technology, which also helped researchers grasp the bacterium's serotyping. This method needs particular primers for the flagellin gene *(fliC-d*) for (*typhi*, *paratyphi,* and *typhmuruim*) and the *16S rRNA (ABC)* gene for *Salmonella* in general. The primer (which is a lyophilized product with a range of picomols concentrations) was dissolved in sterile deionized distilled water to produce a suspensions with a final concentration of 100 picomols/l before being diluted to 10 picomols/µl . Selected primers were made by the Macrogen Company, and the primers and these sequences are listed in (table 1).

Table (1): oligonucleotides primers sequences and there amplicon size

Primer Name	Sequence 5`- 3`	Annealing Temp. (°C)	Product Size (bp)	References
16SABC-F	CAGATGGGATTAGCTTGTTG	60	596	designed
16SABC-R	CCAAGTAGACATCGTTTACG			
Flic-d-F	ACTCAGGCTTCCCGTAACGC	60	763	(18)
Flic-d-R	GGCTAGTATTGTCCTTATCGG			
Fli15-F	CGGTGTTGCCCAGGTTGGTAAT	56	559	(2)
Tym-R	ACTCTTGCTGGCGGTGCGACTT			
FlicABC-F	GAACGAAATCAACAACAACC	56	253	designed
FlicABC-R	CTGAGAGTTGATCTGCTTCA			

Protocol for Reaction Setup and Thermal Cycling

All PCR reactions were amplified in a thermal cycler (Thermo Fisher Scientific, USA). The reaction mixture were showed in table (2) .

Table (2) the reaction mixture component in PCR technology

Master mix components	Stock	Unit	Final	Unit	Volume 1 Sample (µl)
Master Mix	2	X	1	X	10
Forward primer	10	µM	0.5	µM	1
Reverse primer	10	µM	0.5	µM	1
Nuclease free Water	50	ml			6
DNA	20-30	ng/µl			2
Total volume					20
Aliquot per single rxn	18µl of Master mix per tube and add 2µl of Template				

To identify the optimal annealing temperature of the primer, the DNA template was amplified using the identical primer pair, (Forward) (Reverse), at annealing temperatures of (55, 58, 60, 63, and 65°C) or (50, 53, 55, 58, and 60°C). PCR amplifications required 20l volumes of GoTaq Green Master Mix (2X), 1l for

each primer (10 pmol), 6l of nuclease-free water, and 2l of template DNA. Denatured at 94℃ for 4 minutes, followed by 30 denaturation cycles at 94°C for 30 seconds, annealing at 55, 58, 60, 63, or 65°C for 30 seconds, and extension at 72°C for 30 seconds (Thermal Cycler, Thermo Fisher Scientific, USA). After a 7-minute extension incubation at 72 °C, the reactions were terminated with a 10-minute incubation at 4oC. (Table – 3).

Table (3) PCR cycling

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	54 or 60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Electrophoresis of PCR Products

Following the completion of the PCR amplification in the thermal cycler, the PCR tubes were withdrawn, and 8µl of each PCR result was put onto an agarose gel well containing 1.5 percent ethidium bromide. Here, they were resolved by electrophoresis for 20 minutes. A molecular weight marker of 100 bp was employed. The amplicons were examined under ultraviolet light after electrophoresis. The amplicons were then captured on camera (19).

Results and discussion

Bacterial isolation

From overall (114) specimens, 43 (37.7%) specimen were from female and 71 (62.3%) specimen were from male. To identify bacterial cultures, numerous morphological, biochemical, and cultural tests were conducted according to methods recommended by William *et al* ., (18) .

Salmonella enterica serovar identification

Morphological and biochemical Test

Studies on morphology, biochemistry, and microscopy were used to identify *S. enterica* isolates. *Salmonella enterica* was recognized microscopically as a gram-negative cocobacili that was peritrichous, flagellated, motile and did not produce spores. *Salmonella enterica* colonies looked smooth, rounded, and convex even though *S. typhi* isolates used particular media including XLD agar, SS agar, and MacConkey agar after typical features at 37°C after, 24hrs appeared on culture medium. because of its ability to create H2S, *S. typhi* colonies on MacConkey agar emerged after 18–24 hours at 37°C as pale yellow (non-lactose ferment) colonies that were1-3 mm in diameter. *Salmonella* colonies on XLD agar also developed nicely. Growth *Salmonella spp.* On XLD and SS agar media on (figure 1).

Figure (1) : (A) strain of *Salmonella spp.* appear on SS agar smoth and Opaque which produces H2S will form black center colonies. (B) strain of *Salmonella spp.* Appear red colonies with black centers.

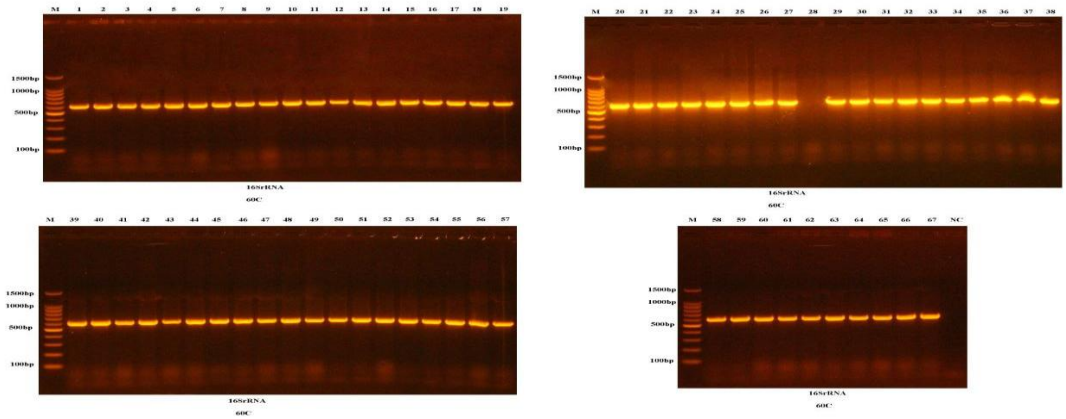
IMVC tests were employed to distinguish the *Salmonella* genus from *Citrobacter* and *Shigella*. According to the study's findings, *Salmonella* isolates tested positive for H2S , methyl red and Simmons citrate but negative for indole, Voges Proskauer. TSI agar slants, the slant and butt both changed color, turning red and yellow, respectively, showing that the butt did not experience any acidic fermentation of the glucose. The S. typhi isolates demonstrated H2S generation in TSI without gas. The outcomes (both morphological and cultural) were all identical to (13) (Table-4).

Table (4) : The biochemical tests to identify salmonella spp

Test	Oxidase	Catalase	urease	Citrate	VP	MR	Motility	Kliglar iron agar	H2S		Indole	Lactose fermentation MacConkey agar
Salmonella	-	+	-	-	-	+	+	Ak/A/+++			-	-

Detection of *Salmonella entrica serovar* genes by molecular techniques

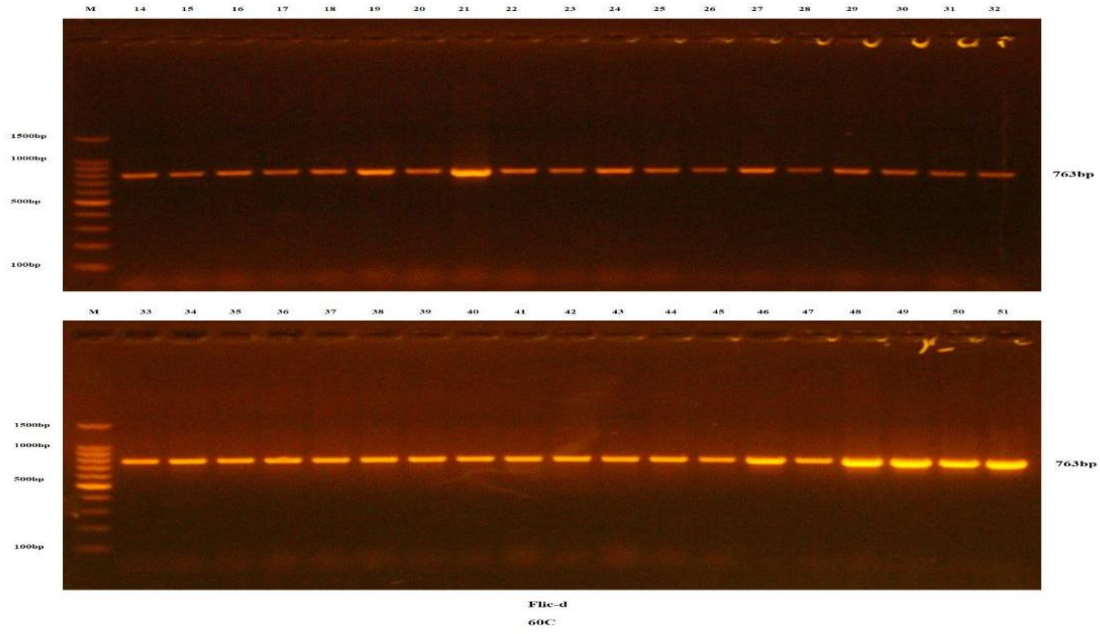
Seventy eight among 114 isolates were identified as *salmonella spp* according to morphological and biochemical test. Another approach for validating the identification of *Salmonella spp*. by a specific gene *16 SrRNA* for *ABC* gene with a molecular basis of 596 bp was the polymerase chain reaction technique, which was used to analyze all 78 clinical isolates. Only 67 samples were discovered to contain the unique *salmonella* gene that permitted for the detection of *salmonella spp*. (Figure 2).



(Figure2): PCR result of 16srRNA for ABC gene with product 596 bp on gel electrophoresis for 1hr at 70 volts/cm with ethidium bromid pigment

Detection of flagella gene of *Salmonella spp* clinical sample

All 67 clinical samples were tested by groub of flagella gene (*flic-d* , *tym* , *flicABC*) every one specific for one species . *fli-d* gene (763 bp) specifc for *Salmonella enterica serovar typhi* , *tym* gene (559 bp) specific for genus *salmonella enterica typhimurum* and *flicABC* gene (253 bp) specific for *Salmonella enterica serova paratyphi* genus. The results of PCR products showed all 67 sample (100 %) belong to *Salmonella enterica serovar typhi* have specific gene *flic-d* (763 bp).



(Figure3): PCR product of flic-d gene primer with product size 763 bp gel electrophoresis for 1hr at 70 volt/cm by using ethidium bromid pigment .

The PCR test produced negative results and revealed that none of the isolates had the genes (*tym*,*flicABC*) from the other genus of *Salmonella spp*. As Showed in (Figures 4 ,5) .

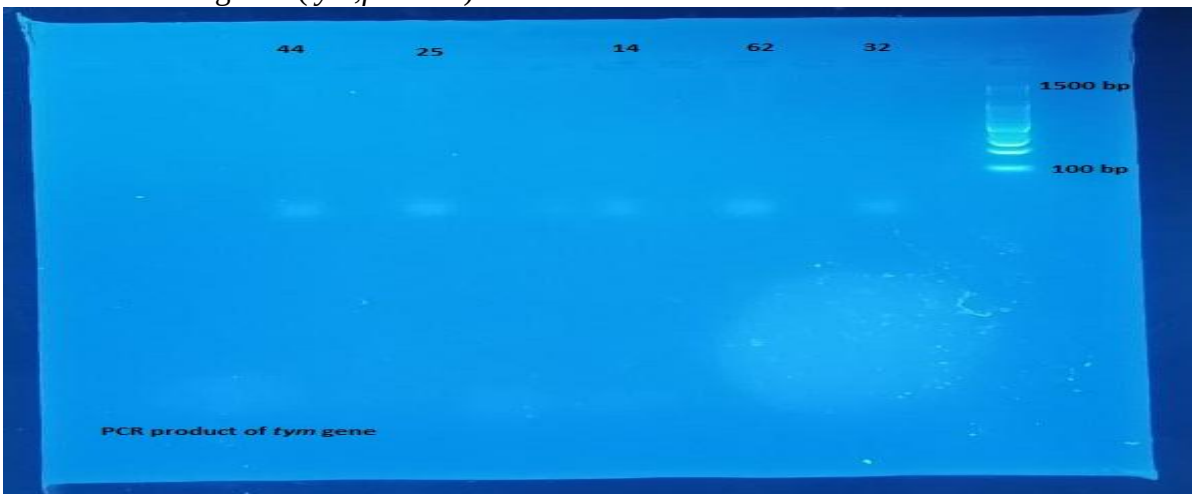


Figure (4): PCR product of *tym* gene primer with product size 559 bp gel electrophoresis for 1hr at 70 volt/cm by using green safe dye pigment

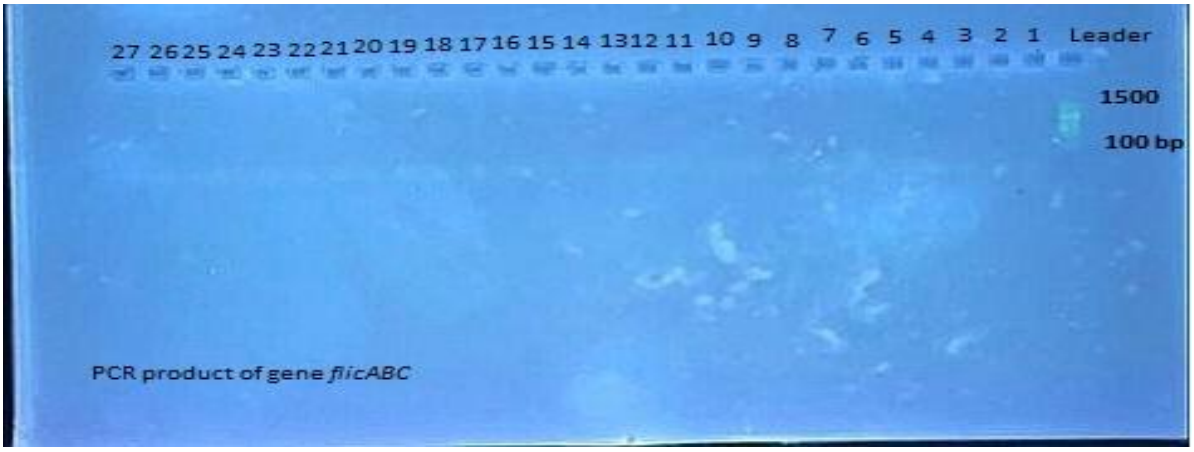


Figure (5): PCR product of *flicABC* gene primer with product size 253 bp gel electrophoresis for 1hr at 70 volt/cm by using green safe dye pigment 200 microliter

Traditional techniques used. 2.5% was the ratio. *S. typhi* Low counts of bacteria in typhoid patients may not be found by culture diagnostics, especially if the *S. typhi* patient has received antibiotics prior to testing (17). however, even at low levels, DNA from typhoid fever patients could be found using the PCR methodology. PCR detection of the *fliC* gene is a method currently utilized for identifying *salmonella spp* (23). The flagelin gene (fliC) was employed in the current work to detect *S. typhi*. (22) had previously reported on the use of fliC for detection of *S. paratyphi* using PCR technique and their method included a nonselective enrichment stage and a comprehensive DNA extraction procedure. Typhoid fever can have signs and symptoms with other common febrile illnesses, making it challenging to diagnose. Typhoid fever is often diagnosed in laboratories using serological and culture techniques (7). These techniques, however, have poor sensitivity and specificity, which makes them ineffective as diagnostic tools. Depending on the quantity of blood tested, the level of *S. typhi* bacteremia, the kind of culture medium used, the duration of the incubation period, and other factors, blood culture can only detect 45 to 70% of patients with typhoid fever. Because it requires at least two days to identify the microbe, the clinical value of the culture approach is further constrained. Additionally, a delayed presentation or past antibiotic use dramatically reduces the chance of a positive blood culture and serology diagnosis (15,25).

Conclusion

In cases of typhoid fever in an Iraqi sample of *S. typhi*, molecular approaches are more accurate in making a diagnosis than traditional tests.

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