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Molecular Detection of *InvA and Sop* Genes in *Salmonella* spp. Isolates from Stool Samples of some Iraqi Patients

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Abstract: *Salmonella typhimurium* is the main cause of gastrointestinal diseases for the human population and one of the most dangerous foodborne bacteria. This study aims to detect the occurrence of different important resistance genes in *Salmonella typhimurium.* Stool samples were collected from some hospitals in Baghdad-Iraq from different patients for bacterial isolation. 110 samples aggregate and identified by morphological tests and affirmed using the vitek-2 system. Antibiotic sensitivity tests of fourteen isolates belonging to clinical samples were spread by agar diffusion method. The result showed that *Salmonella* showed the diameters of the inhibition zones for 7 antibiotics and the antimicrobial Susceptibility rate of 14 *Salmonella* Isolates while a genomic DNA kit was used to extract bacterial genome from isolates then screened for virulence and resistance genes mainly *InvA and Sop* performed by polymerase chain reaction (PCR). In addition, the study showed that *InvA* and *Sop* genes are found in all the 24 isolate which means even asymptomatic patients had resistance genes.

Keywords: *Salmonella*, resistance genes, PCR, antibiotic susceptibility, bacterial resistance.

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Introduction

Salmonella is a prevailing genus of *Enterobacteriaceae* and a foodborne disease that can cause food poisoning (1). *Salmonella* is Gram-negative, rod-shaped, facultatively anaerobic, and motile as pathogenic bacteria within the *Enterobacteriaceae* family (2). Most isolates that cause diseases in humans and animals belong to *S. enterica*. These species are divided into more than 2,600 serovars capable of causing illnesses in humans and animals (3). In humans, *S. enterica* serovars *Typhi and Paratyphi* cause severe systemic typhoid fever (4). In contrast, nontyphoidal *Salmonella* (NTS), such as *Salmonella* ser. *typhimurium* and *Salmonella* ser. *Enteritidis*, are commonly associated with gastroenteritis, the most common clinical presentation of *Salmonella* infections (5). in many people. The illness can also appear as a febrile invasive disease, often without diarrhea, with bacteremia, meningitis, or focal infections that can be fatal if left untreated or improperly treated (6). *Salmonella* is non-fastidious, as outside the living hosts it can grow and multiply in a variety of environments.

Salmonella is heat-sensitive and is frequently killed at temperatures of 70°C or above (7). It varies in structure and sugar composition, thus providing discrimination of *Salmonella* serogroups, such as O antigen (8). On the other hand, H antigens are strongly immunogenic and induce antibody formation rapidly and in high titers following infection or immunization The flagellar antigen is dual, occurring in one of the two phases (9). Bacterial pathogenicity is governed by virulence factors. The pathogenicity island aims to investigate these gene clusters, closely associated with bacterial pathogenicity and virulence factors (10). Many virulence genes have been linked to the pathogenicity of *Salmonella*, and the severity of infection depends mainly on the presence or absence of the *InvA, Stn, Hila, and SpvC* genes. These virulence genes are located either chromosomally or plasmidically and encode products that help NTS interact with the host cells (11). The invasion gene (*invA*) is located on the pathogenicity island 1 (SPI1) and has been widely studied for its ability to promote virulence and as a biomarker for the detection of *Salmonella typhimurium* (12). The *sop* gene is important for the adhesion of host cells. They are also involved in bacterial invasion (13) while *spvr* plays a role in the multiplication of *Salmonella* intracellularly, and survival of *Salmonella* within macrophages (14) as *invA* initiates internalization for invasion of deep tissues (15) as well as the *sop* gene found to cause salmonellosis and by further pathogenicity pathway to human (16).

Material and Method

Sample Collection

The present study started collection through the period extending from November 2022 until May 2023, a total of one hundred ten different stool samples from patients randomly were collected from various ages and occupations and both sexes attended medical city and AL-Karama General Hospital in Baghdad-Iraq using sterile tube contained tetrathionate broth and transmitted to the laboratory with cooling box. Microscopic examination was done for the characterization of stool specimens (mucoid, watery, soft, and solid) while a small portion of each 24 stool samples using a sterile swab incubated for 24h at 37 ºC suspended in tetrathionate broth. The sample was transferred to 10 mL tetrathionate (x2) broth and incubated for 24h at 37 °C. A loop full of tetrathionate broth positive growth, transferred to 100 mL single tetrathionate broth, incubated 24 or 48h in ± 42 °C Then streaked on XLD at 37°C 24h. Biochemical tests (vitek2) and Serological conformation (Send to central public health laboratory CPHL).

Molecular method

DNA extraction

The DNA was obtained from *Salmonella* (*typhimurium*) isolates using the genomic DNA extraction kit (Genaid, Taiwan) the purity and concentration of the extraction DNA were determined using Primers of different *Salmonella* resistance genes were designed and were selected for this study; these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmoles/ µL as recommended by the provider and

stored in a deep freezer until used in PCR amplification. Primers table and PCR requirements detailed below respectively:

Table (1): Primers of target gene used in study

Target Gene	Primer Sequence F (5-3) forward	Primer Sequence R (3-5) reverse	Product size (bp)	Reference
InvA	GAGCGGAGGACAAATCCATA	ATGCCCGGTAAACAGATGAG	**238**	Primer design
Sop	TACCAGCACATCCTCAGTGC	CGGACCACTCGGTAAACTGT	**215**	Primer design

Table (2): Program of PCR thermocycling condition.

No.	Steps	TEMP.(°C)	Time (m: s)	Cycle
1	Initial Denaturation	95	05:00	1
2	Denaturation	95	00:20	45
3	Annealing	45-65	00:20	
4	Extension	72	00:20	
5	Final Extension	72	10:00	1

Detection of virulence gene from *S.typhimurium* by conventional PCR:

The mixture of the reaction was set up in the PCR after DNA extraction was done and then PCR amplification was done using microcentrifuge tubes inside a thermocycler. Depending on the optimization protocol reaction mixture (20 µl) follows: (10 µl) master mix, (4 µl) free nuclease water, and (2 µl F+R) set primers of each gene detected with (4 µl) of DNA nucleotides. After the mixture volume was prepared and set in the thermocycling device amplification began according to the program described below:

Table (3): Reaction of PCR technique components.

Master mix components	Stock	volume
Master mix	25	10 µL
Forward primer	10	1 µL
Reverse primer	10	1 µL
Nuclease free water		4 µL
DNA		4 µL
Total volume		20 µL

After amplification, each aliquot was removed from reaction mixture and checked by using gel electrophoresis at (100volt/Amp for 60min) in gel composed of 1% agarose gel mixed in 100ml of TAE buffer and then visualized under UV using gel image analysis system.

Results and discussion

Isolation and Identification

All the hundred ten stool specimens were cultured on XLD media, there were fifty-six specimens gave positive culture, and fifty-four specimens with no growth on culture media. Fifty-six of suspected bacterial growths on XLD media were characterized by the appearance of transparent or colorless colonies with a blackish color in the middle due to H2S gas formation. Founded the appearance of *Salmonella* on XLD media as shown in Figure (1).

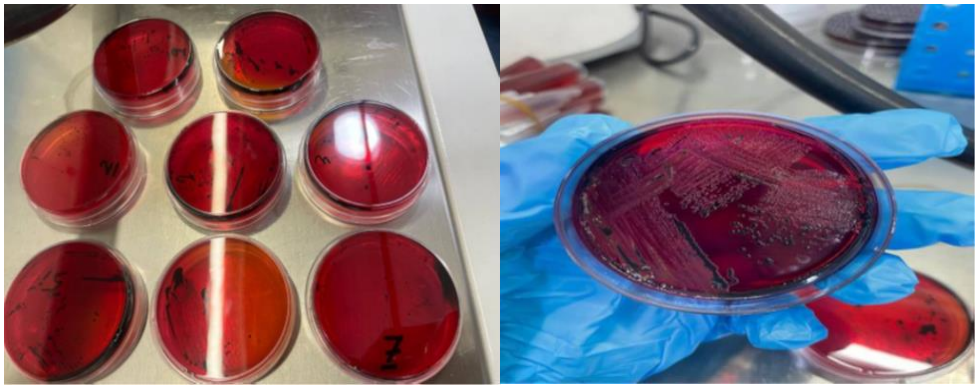

Figure (1): Growth of *salmonella* isolation on Xld agar.

Biochemical Identification Using VITEK2 System

Biochemical analyses were conducted on 24 clinical isolates that grew on the selection media XLD to diagnose by using the vitek2 system by the identification card for gram-negative strains (IDGN). This system was used in many previous studies and gave good results for identification and confirmation with conventional methods. All the (24) bacterial isolates of the current study had been identified using the Vitek2.

Antibiotic Susceptibility of *Salmonella* Isolates:

The Susceptibilities of fourteen isolates to seven antibiotics by Kirby Bauer disk diffusion method. The zone of inhibition diameter was measured, and the results were interpreted based on the guidelines of the Clinical and Laboratory Standards Institute. Antimicrobial Susceptibility table rate of 14 *Salmonella* Isolates against 7 antibiotics shown in Table 4:

Antibiotic susceptibility test of fourteen isolates belonging to clinical samples were screened by agar diffusion method, the result indicated that *Salmonella typhimurium* showed the diameters of the inhibition zones for 7 antibiotics and the antimicrobial Susceptibility rate of 14 *Salmonella* Isolates.

Table (4): Antimicrobial Susceptibility for *salmonella* isolates.

Sample	CAZ	SXT	C	AMP	ATM	TE	CIP
1	I	S	I	R	S	S	I
2	S	S	S	R	S	S	R
3	S	S	I	R	S	I	S
4	S	S	I	I	R	S	I
5	S	S	S	R	S	S	S
6	S	S	S	R	S	S	S
7	I	S	S	R	S	I	S
8	R	R	R	R	I	R	I
10	R	S	R	R	R	R	R
11	I	S	I	R	S	I	I
12	S	S	I	R	S	S	S
18	R	S	S	R	R	S	R
20	R	S	S	R	I	S	I
24	R	S	S	R	R	S	R

R= Resistance I=Intermediate S= Sensitive

Six isolates were sensitive to Ceftazidime (CAZ), two isolates were resistant to Chloramphenicol(C) (100%), with all sensitive except one to trimethoprim (SXT), resistance from all except one for ampicillin (AMP), while just eight sensitive and four resistances for aztreonam (ATM), furthermore, there are nine samples sensitive and two resistances for tetracycline (TE) and five sensitive for ciprofloxacin (CIP). These results were closely resembling those obtained by Habeeb; Z. S. (2009) (17) who tested different serogroups of *Salmonella* to a variety of antibiotics while the present study showed different percentages shown the Table 5 below:

Table (5): The percentage of 7 antibiotic susceptibility.

Gene	CIP	TE	ATM	AMP	C	SXT	CAZ
Resistant	35.7%	14.2%	28.6%	92.8%	14.3%	7.1%	35.8%
Sensitive	21.5%	64.2%	57.1%	0%	50%	92.9%	42.9%
Intermediate	42.8%	21.5%	14.3%	7.2%	35.7%	0%	21.3%

These outcomes agree with the results of (18), and in disagreement with Kazem, (19) who indicated a sensitivity of *Salmonella* to people with typhoid fever in the city of Diwaniyah.

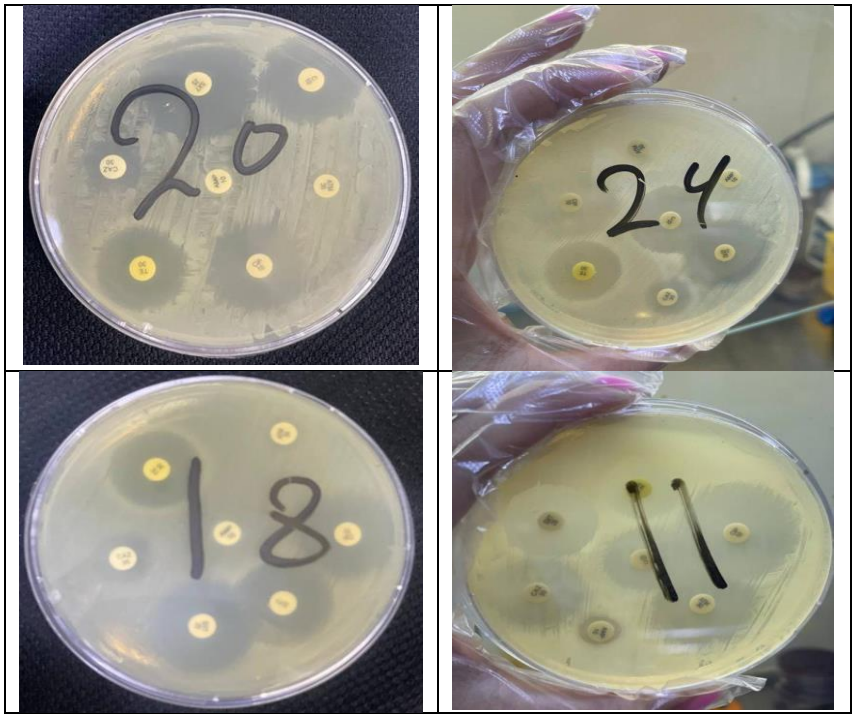

Figure (2): Shows antibiotic sensitivity test.

Serotyping for suspected *salmonella* spp. Isolation

Present study performed serotype test in central health public laboratory CPHL-Baghdad and the results revealed out of 24 samples only 14 samples strains serotype were detected this might be attributed to invalid, poor storing circumstances or due to activation protocol trial error at some point for the rest out of the total number been reactivated. The test results of our

study showed the common *Salmonella* strains which were one Salmonella hato, one *Salmonella Enteritidis*, and the rest of the strains were *Salmonella* typhimurium.

Molecular identification of some virulence gene in *Salmonella:*

Molecular identification method is one of the most important techniques to detect for *Salmonella* genes. In this study PCR technic was used to detect the genes. Depending on specific primers for the *invA* gene, which are specific for the diagnosis of *Salmonella* spp. All the 24(100%) *Salmonella* typhimurium, isolates give positive results for the invA gene.

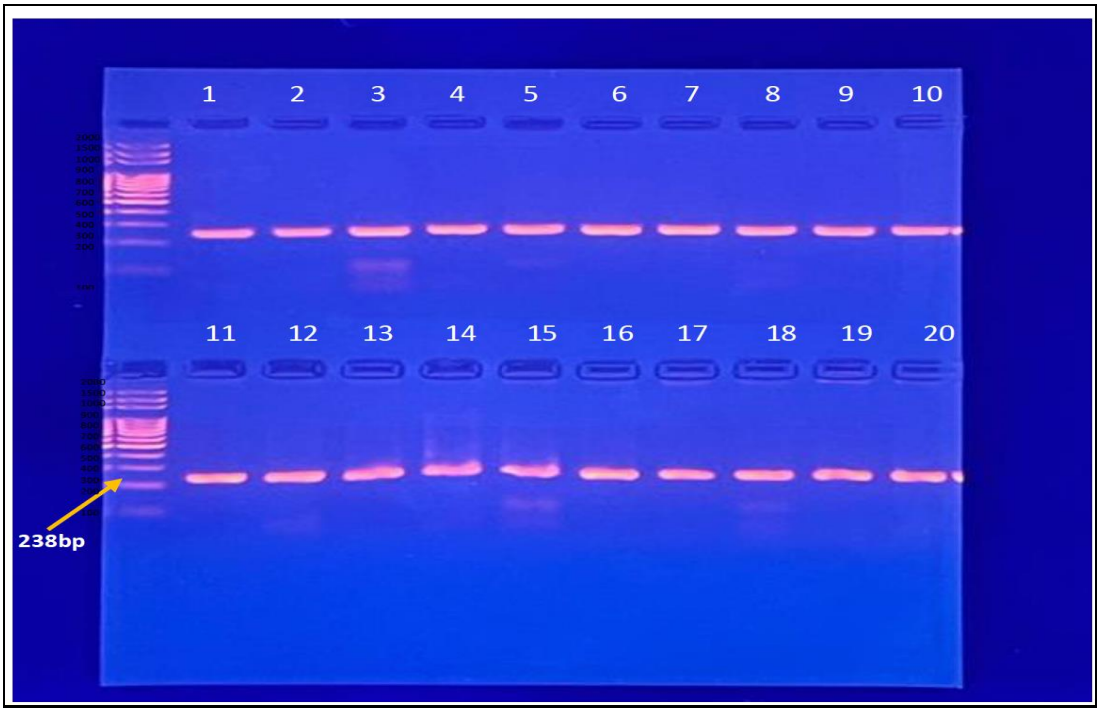

Figure (3): The PCR Product results of the *invA* gene of *Salmonella* isolate on agarose gel electrophoresis for 40 minutes at 50 volts/cm on leadder size 2000 bp.

The current results were agreed with Abbas, F. (2022) (20) who concluded that *invA* was one of the most predominant genes existing in all 24 Sample according to the result of 50 samples which was collected from Baghdad hospitals, Iraq from patients suffering from food poisoning.

In addition, the results of this study indicate that 24 in all *Salmonella* spp. were positive for *sop* gene which was detected by gel electrophoresis as shown in figure (4) which is higher as compared to 21. Elkenany, R.; Elsayed, M. M.; Zakaria, A. I.; El-sayed, S. A. E. S. and Rizk, M. A. (2019) (21) which shows a lack of sop genes, could be attributed to epidemiological conditions or varying serotypes. On the other hand, many results were higher than our study such as mentioned by Zou, M.; Keelara, S. and Thakur, S. (2012) (22) prevailing at about 99% including sop gene dominancy with other ones as well Farahani *et al.*, (23) appeared with the same results while, Ammar *et al*. (24) detected about (41%) which is similar to the present study.

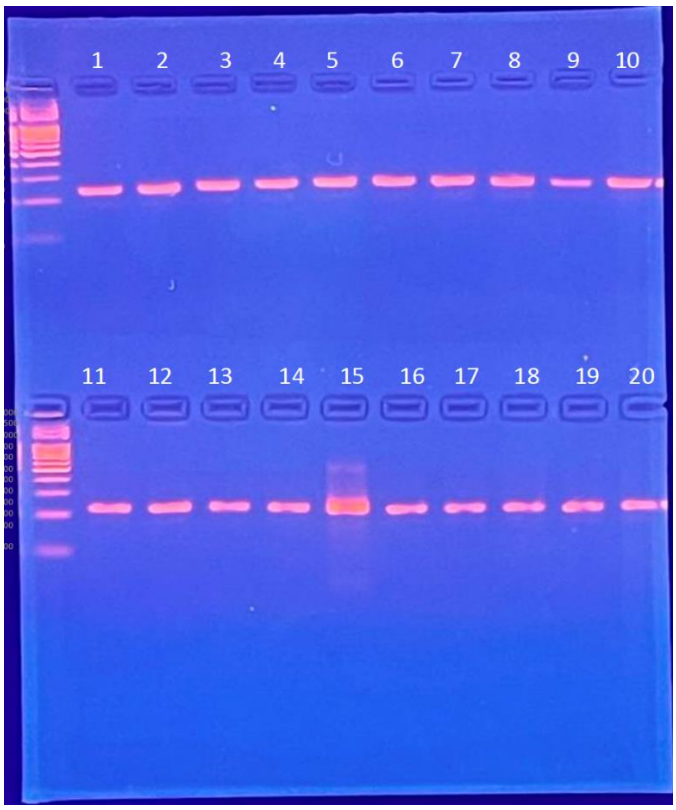

Figure (4): The PCR Product results seen by 2% agarose gel electrophoresis stained with ethidium bromid pigment started the amplification of (215) bp of *sop* gene of *salmonella* isolates, for 40 minutes at 50 volts/cm on leadder size 2000 bp.

Conclusion

Salmonella colonies on XLD agar shows blackish color in the middle The *Salmonella* isolates in this study showed a high degree of genetic diversity between the bla gene. Have the potential for β-lactam antibiotic resistance. The detection of drug resistance genes in this study, blaTEM has a high Carrying rate of β-lactam antibiotic resistance genes, which should be taken into consideration. PCR based assay proved to be a good and perfect method for diagnosis of *Salmonella* when using specific primers. It has several advantages include speed which can reduced the diagnosis time, high sensitivity and specific. The results of PCR technique were in fit with results of traditional methods.

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