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ORIGINAL ARTICLE

Study of Some Resistance Genes of *Salmonella* from Asymptomatic Patients in Baghdad Government

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Abstract

Aim The current study aimed to isolate and diagnose *Salmonella* typhi bacteria from people with typhoid fever and their role in pathogenicity and to determine the antimicrobial susceptibility and virulence-associated genes.

Methodology In all, 110 human stool samples from Iraqi patients were collected between November 2022 and the end of April 2023. The colony morphology method was used to identify the isolates. The antimicrobial susceptibility test was conducted using the disc diffusion method and the VITEK-2 Compact system.

Results The antibiotic sensitivity test was conducted, as the current study showed that all isolates were 100% resistant to Ampicillin (AM) and Pincillin (P) of the β-lactams group, and Cefotaxime (CTX) of the Cephalosporines. The resistance was 93.7% for amoxicillin-clavulanic acid, while the resistance was weak or non-existent for the group of Carbapenem antibiotics, which was 9.37% for imipenem (IPM) and non-existent for meropenem (MEM), meaning that the bacteria are fully sensitive. The results also showed that the resistance to some antibiotics of the Tetracycline group was low compared to the Cephalosporine group and the β-lactams group. In Tetracycline (TE), it was 15.6%, and Doxycycline antibody was 21.8%. Aztreonam (ATM), which belongs to the Monobactams group, showed a high resistance of 96.8%, as well as high resistance also appeared by 93.7% to Ciprofloxacin (CIP) and 56.1% to Chloramphenicol (C), while the resistance was low by 18.7% to Levofloxacin (LEV).

Conclusion Serotype typhi was the most common serotype of *Salmonella* enteric that caused enteric fever during the course of the study. The majority of the *Salmonella* isolates exhibited an intermediate level of sensitivity to ciprofloxacin, indicating a heightened resistance to the drug used in the treatment of enteric fever.

Keywords: *Salmonella*; Resistance genes; PCR, Antibiotic susceptibility, Bacterial resistance

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1 Introduction

Typhoid is a common infection in areas with low economic development and inadequate public health facilities. Estimating the global occurrence is difficult because the current gold standard for diagnosis is the culture of *Salmonella* enterica serovar Typhi from a clinical sample. An estimated 200,000 people die globally each year, but there are far more new cases—more than 20 million—than deaths [1]. A genus of rod-shaped, Gram-negative bacteria belonging to the *Enterobacteriaceae* family is called *Salmonella*. It con-

sists of up of two species: *S. enterica* and *S. bongori*. *Salmonella* species can be divided into two primary serotype groups based on their capacity to cause particular human pathologies: typhoidal and non-typhoidal [1].

Zoonotic means that non-typhoidal serotypes (NTS) can spread from animal to animal as well as from human to human. Typhoidal serotypes can only be passed from person to person. They can cause bacterial infections of the intestinal tract, foodborne infections, and sometimes bloodstream infections (typhoid fever). They can also invade organs and secrete endotoxins (the septic form), which can spread throughout the body. They typically only infiltrate the digestive system and produce *salmonellosis* [2, 3].

About 20 million new cases of typhoid disease are caused by S. typhi infections worldwide each year. These infections are particularly common in low- and middle-income countries, developing nations, and places where typhoidal *Salmonella* is endemic, have poor sanitation, and lack access to clean water and food [4]. Iraq has serious problems with S. typhi infections related to public health, especially in light of the rise in antibiotic-resistant *Salmonella* species worldwide. *Salmonella* is known to possess distinct virulence genes associated with adhesion and host cell invasion. Inv, spv, stn, fim, pap, pef, viz, and sop are some of these genes. They also take part in the survival and propagation of bacteria [5].

Salmonella has a variety of virulence factors that are linked to pathogenicity and the severity of infection based on the presence of virulence genes like enterotoxin (stn), which encodes a protein that causes acute diarrhea, and invasion A (invA), which helps the bacteria invade the host cell [6–8]. The aim of the current study is to detection of some antibiotic's resistance gene and their role in pathogenicity.

The antibiotic sensitivity test was conducted, as the current study showed that all isolates were 100% resistant to Ampicillin (AM) and Pincillin (P) of the β-lactams group, and Cefotaxime (CTX) of the Cephalosporines. The resistance was 93.7% for amoxicillin-clavulanic acid, while the resistance was weak or non-existent for the group of Carbapenem antibiotics, which was 9.37% for imipenem (IPM) and non-existent for meropenem (MEM), meaning that the bacteria are fully sensitive. The results also showed that the resistance to some antibiotics of the Tetracycline group was low compared to the Cephalosporine group and the β-lactams group. In Tetracycline (TE), it was 15.6%, and Doxycycline antibody was 21.8%. Aztreonam (ATM), which belongs to the Monobactams group, showed a high resistance of 96.8%, as well as high resistance also appeared by 93.7% to Ciprofloxacin (CIP) and 56.1% to Chloramphenicol (C), while the resistance was low by 18.7% to Levofloxacin (LEV)

2 Material and methods

2.1 Isolation and identification of *Salmonella* isolates

A total of 110 stool samples were collected from Iraqi patients suffering from diarrhea with age ranges (10 - 60) years from Medical City and al-Karama Hospital in Baghdad. From the beginning of November 2022 till the end of April 2023. All samples were cultured on the Xylose lysine deoxycholate (XLD) and incubated at 37 °C for 24 h. They were further identified using the Vitek-2 system (BioMe'rieux, France).

2.2 Antimicrobial susceptibility

Salmonella isolates were subjected to an automated VITEK-2 Compact system to determine their susceptibility to seven antimicrobial agents based on Minimum Inhibitory Concentration (MIC), which includes Ampicillin, Ampicillin, Aztreonam, Tetracycline, Ciprofloxacin, Ceftazidime, Trimethoprim.

2.3 Molecular identification

To extract DNA from the isolates, a genomic DNA kit (Genaid, Taiwan) was applied, according to the manufacturer's recommendations in the leaflet. Briefly, a volume of 200 μl of each suspension was suspended equal to 180 μl of GT, 10 μl of proteinase K was added to it with 200 μl of lysis solution, and it was incubated for 10 min at 70°C. to lysis the samples, 5 μl of RNase are added and incubate 5 min at room temp. After the incubation period, 200 μl of absolute ethanol was added. Afterward, 400 μl of W1 buffer was added to the samples that were washed three times and centrifuged. 100 μl of elution buffer was added and incubated for 3min to elute the DNA.

2.4 Molecular detection of invA, blaOXA, and blaSHV Genes in S. species

Specific primer sequences of three virulence genes, including invA, blaOXA, and blaSHV, were amplified by the PCR, Table 1. Primers were utilized for the PCR amplification using a 25 μl volume for the reaction. A final volume of 20μl was used for the polymerase chain reaction (PCR) amplification, containing 4μl of DNA template, 1μl of each **primer listed in tables (3.9)**, and 14μl of nuclease-free water. The components were distributed at a rate of 16μl per sample. The samples were then transported to a thermal cycle using the

amplification program, which consisted of an initial denaturation at 95°C for 5 minutes, 35 cycles with a denaturation at 94°C for 40s, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Table 1: Sequence of primers used for gene identification.

Gene	Primer Sequence F (5-3) forward	Primer Sequence R (3-5) reverse	Product size (bp)
invA	GAGCGGAGG ACAAATCCATA	ATGCCCGGTA AACAGATGAG	238
blaOXA	ACGATAGTTGT GGCAGACGAAC	ATYCTGTTTGGC GTATCRATATTC	602
blaSHV	GAACAGCTGG AGCGAAAGAT	CCTCATTCA GTTCCGTTTCC	250

2.5 Statistical analysis

The Graph Pad Prism 8 software was used to analyze the data for *S. enterica* in the biological test replication. The significance level was established at $P \leq 0.05$ in order to detect significant differences.

3 Results

3.1 Isolation and identification

One hundred ten stool specimens were cultured on XLD media; 56 of the specimens showed positive with *Salmonella*, and 54 showed no growth at all. The production of H2S gas resulted in the production of translucent or colorless colonies with a black in the middle of the bacterial growth on XLD media, as seen in Figure 1. Further confirmation of all isolates was done by the VITEK 2 System. *Salmonella* culture features vary depending on the type of media used. For example, it can appear as a transparent, colorless colony on MacConkey agar, but it can also appear as a tiny, spherical, smooth, and black colony on SS agar and a pinky translucent zone on BG agar [9].

3.2 Serological Identification of *Salmonella* spp.

The phenotypic results of *Salmonella* spp. on the XLD agar showed that out of 110 stool samples, (14 samples) were confirmed as S. typhimurium, (one sample) as *S. enteritidis*, and (one sample) as *S. hato*. followed by the biochemical Vitek-2 system (BioMe'rieux, France).

3.3 Antibiotic Susceptibility of *Salmonella* Isolates

Results indicated that *Salmonella* spp, as in Table 2, showed the diameters of the inhibition zones for seven antibiotics in 14 *Salmonella* Isolates.

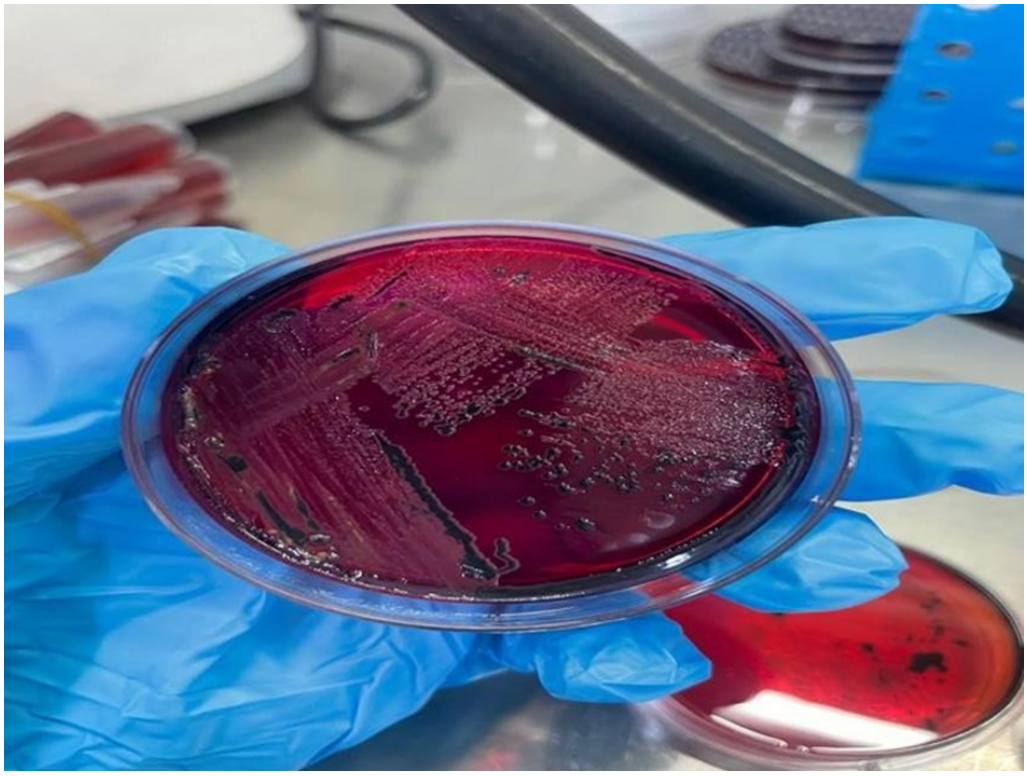

Figure 1: *Salmonella* colony on XLD agar shows a blackish color in the middle.

Table 2: Antimicrobial susceptibility rate of *Salmonella* isolates.

Susceptibility	CIP	TE	ATM	AMP	C	SXT	CAZ
Resistance	35.7%	14.2%	28.6%	92.8%	14.3%	7.1%	35.8%
Susceptible	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%

The six isolates were sensitive to Ceftazidime (CAZ) and showed two resistances for Chloramphenicol(C), 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 sensitive samples: two resistances for Tetracycline (TE) and five sensitive ones for Ciprofloxacin (CIP).

3.4 Molecular identification of virulence-associated genes among *Salmonella* serovars and Resistance Genes

Salmonella detection and typing are becoming more and more dependent on molecular techniques. PCR, which uses certain primers to separate *Salmonella* species from other species, is one method of *Salmonella* molecular detection. This involved amplifying a conserved section of the invA gene, which is specific for all *Salmonella* ssp, depending on particular invA gene primers that are unique for *Salmonella* diagnosis, as seen in Figure 2.

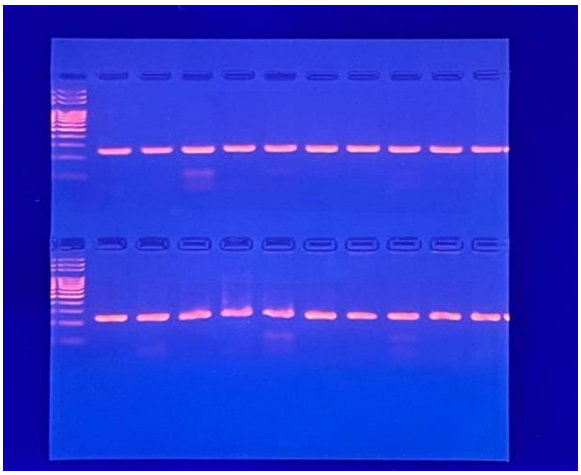

Figure 2: The PCR Product results seen by 2% agarose gel electrophoresis stained with ethidium bromide pigment started the amplification of (238) bp of invA gene of *Salmonella* isolated for 40 minutes at 50 volts/cm.

Regarding the identification of virulence genes, the PCR test revealed that every single one of the 14 *S. enterica* isolates possessed a minimum of one gene linked to the production of biofilms. The invA was the most predominant gene in all 14 *S. enterica* isolates. Also, this study's data showed no detection of Sulphydryl Variable (SHV) and Oxacillinase (OXA) genes in the current *Salmonella* isolates.

4 Discussion

Typhoid fever continues to be one of the main causes of illness and death despite recent advancements in public health and sanitation [10]. Although antimicrobial resistance occurs naturally, overprescribing and inappropriate antibiotic use in humans and animals hasten its development. Antibiotic use has increased due to the absence of national health regulations.

Ampicillin and *Ceftriaxone* are two common antibiotics used as first-line treatments in hospitals and the community. Overuse of these drugs exacerbates resistance and plays a significant role in the spread of pathogens that are resistant to drugs, such as S. typhi. Countries, geographical regions, and even hospitals or centers exhibit notable variations in S. typhi profiles, antibiotic sensitivity patterns, and prevalence. Soon after the identification of *Salmonella* isolates from stool samples, the multi-drug resistance of the isolates was determined in the current study [11]. Data from this study revealed that the prevalent resistance of S. typhi was against Ampicillin, which is considered the first-line therapy for enteric fever. These outcomes are in agreement with the results conducted by MA AL-Mazini [12]. On the other hand, bacterial isolates were sensitive to four antibiotics, which include Chloramphenicol (C), Aztreonam (ATM), Tetracycline (TE), and Trimethoprim (SXT).

These data share the same results as those of another study that revealed almost the same results [13]. Both Ciprofloxacin (CIP) and Ceftazidime (CAZ) antibiotics showed that they have an equal proportion of sensitivity and resistance to bacterial isolates. It can be concluded that antimicrobial resistance has developed among *Salmonella* bacteria as a result of the careless use of antibiotics. *Salmonella* demonstrated widespread ampicillin resistance, which led to the use of alternative antimicrobials such as trimethoprim.

A further diagnosis was conducted by using PCR for isolates that were previously confirmed as *Salmonella* by morphology and biochemical tests in addition to the viteck 2 system through amplifying a conserved region of the (invA) gene, which is consid-

ered specific for all *Salmonella* ssp, depending on specific primers for (invA) gene, as can be seen in Figure 3.

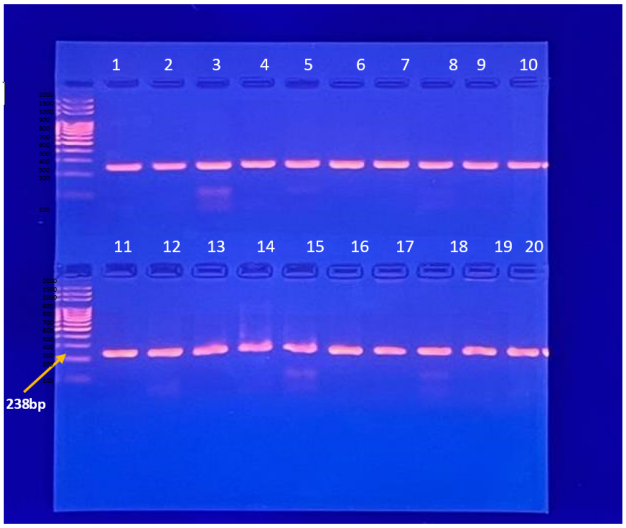

Figure 3: The PCR Product results seen by 2% agarose gel electrophoresis stained with ethidium bromid pigment started the amplification of (238) bp of invA gene of *Salmonella* isolated, for 40 minutes at 50 volts/cm.

The current data was in agreement with the previous study [14], which detected virulence genes in *S. enterica* that were collected from stool samples in Baghdad hospitals, Iraq. In total, 50 swab stool samples were collected from patients suffering from food poisoning. The results showed that invA was one of the most predominant genes in all *S. enterica* isolates.

Salmonella spp. can be diagnosed using the invA gene, which is thought to be the international standard for characterizing the species and contains sequences unique to it at the level of genus. Many species of *Salmonella* have been documented to contaminate animal products, yet a serovar may dominate a region for many years before being supplanted by another. *S. Typhimurium* and *Salmonella* Enteritidis are the most prevalent and clinically relevant serovars with the invA virulence gene that causes *salmonellosis* worldwide [15]. This is also consistent with another study that noted PCR results on non-*Salmonella* strains such as *E. coli*, *Klebsiella*, Proteus, and Shigella were negative for the invA gene [16].

Furthermore, the identification of *Salmonella*-specific by PCR with primers for the invA gene is considered fast, sensitive, and specific in detecting *Salmonella* in many clinical samples [17]. The current isolates of *Salmonella* did not contain the blaSHV or blaOXA-2 genes. For example, it was reported that the prevalence of the blaOXA gene was 6.6% among different resistance genes in Egypt. This could be attributed to environmental similarities between Egypt and Iraq, where *Salmonella* is regarded as an endemic nosocomial bacteria because of the lower social attention to healthcare [18]. Furthermore, a new study from Iraq revealed that the genes blaOXA and blaSHV were absent from several *Salmonella* serotype samples. This finding suggests that a small number of *Salmonella* serovars may not have plasmid-transferred copies of these genes, which could reduce susceptibility to antibiotics [19].

5 Conclusion

Salmonella colonies on XLD agar show a blackish color in the middle. *Serotype* typhi was the most common *serotype* of *Salmonella* enteric that caused enteric fever during the course of the study. The majority of the *Salmonella* isolates exhibited an intermediate level of sensitivity to *Ciprofloxacin*, indicating a heightened resistance to the drug used in the treatment of enteric fever.

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Conflict of Interest: No conflicts of interest exist between the authors and the publication of this work.
Ethical consideration: The study received ethical approval from the Institute of Genetic Engineering and Biotechnology, University of Baghdad.

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