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Molecular Detection of Some *Salmonella spp.* in Chicken from Local Markets in Baghdad

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Abstract: *Salmonella* species stand as notably significant foodborne pathogens worldwide. The objective of this research was to compare the outcomes obtained from standard microbiological methods and the Polymerase Chain Reaction (PCR) technique for detecting *Salmonella spp*. Each isolate was subsequently subjected to validation through various cultural media, including Tetrathionate Broth Base as enrichment media and desoxycholate citrate agar, SS agar, and XLD agar, which were utilized to distinguish colonies. Moreover, the antibiotic susceptibility profile was assessed, revealing resistance to Penicillins , fluoroquinolones, and third-generation Cephalosporins while displaying sensitivity to aminoglycosides, Sulfonamides, and carbapenems. Polymerase Chain Reaction has been employed to identify the presence of *Salmonella*, specifically the *Typhimurium* strain, implicated in cases of foodborne diseases within chicken markets in Iraq. *Salmonella* identity was accomplished by detecting the *invA* gene, which is indicative of the *Salmonella* genus. Additionally, the *stm* gene specific to *Salmonella Typhimurium* was targeted using PCR, allowing identification of *Salmonella* spp. and specifically *Salmonella Typhimurium*. Furthermore, the outcomes unveiled that PCR identified *Salmonella* spp. presence in 20% of the specimens. Paralleling the findings of conventional microbiological techniques. The PCR approach successfully detected *Salmonella Typhimurium* in 15 out of the 50 samples, accounting for 32% of the total.

Keywords: Antibiotic resistance, *inv* A gene, PCR, *Salmonella* spices, *Salmonella Typhimurium*.

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Introduction

Salmonella species stand as notably significant foodborne pathogens worldwide (1). Within Canada, *Salmonella Enteritidis* and *Typhimurium* have emerged as prominent culprits of foodborne infections, collectively contributing to approximately 80% of all documented human *Salmonella* infections worldwide (2). Notably, *S. Typhimurium* causes severe inflammatory and intestinal ailments in young chicks (3), although clinical manifestations may not consistently manifest in older avian populations (4). In these circumstances, mature hens become carriers of *S. Typhimurium*, amplifying intestinal colonization and subsequently shedding bacteria, infiltrating various environments (5). The presence of *Salmonella* carriers among avian populations further accentuates the issue of contaminated poultry products within slaughterhouses, underscoring the imperative to integrate biofilm strategies into the value chain mechanisms (6). Although the conventional approach to *Salmonella* detection involves cultivating the bacteria in a culture medium followed by testing, this method is recognized as the national standard, and it comes with drawbacks such as being time-consuming and costly. Consequently, techniques like (PCR) have gained widespread adoption for swift *Salmonella* identification, emerging as a

pivotal testing method that targets the specific *inv* A gene associated with Salmonella. PCR, the foremost DNA amplification technique, employs genus-specific primers to pinpoint distinct genes, offering a dependable and rapid means of identifying clinical samples with enhanced precision and sensitivity (6). PCR methods exhibit versatility in identifying an extensive array of organisms through specific primers that amplify minute gene segments. Notably, the *inv* A gene has been harnessed to enable efficient and rapid diagnosis of *Salmonella* in chicken-derived food, serving as a valuable tool due to its involvement in epithelial cell invasion, activation, and macrophage mortality. As the *inv* A gene remains a conserved pathogenic trait across virtually all *Salmonella* species, it presents a robust avenue for *Salmonella* identification through diverse PCR techniques (7).

The primary objective of this research was to compare the outcomes obtained from standard microbiological methods and the Polymerase Chain Reaction (PCR) technique for detecting *Salmonella* spp. The investigation involved the utilization of the virulence gene invasion A (*inv* A) to identify the *Salmonella* genus, while the *stm* gene was employed to discern the specific species, namely *Salmonella Typhimurium*. Additionally, an evaluation of antibiotic resistance among the *Salmonella* isolates was also determined in the study.

Materials and methods

Samples collection

First, A total of 50 specimens were collected from butcher shops located in the Al-shaab, AL-huraia, al-Mansour, and Hay-Aljameah districts of Baghdad, Iraq, during the period spanning from March to September 2022, in this period, samples were collected in Baghdad at ambient temperature, which varied between 26 and 47 ^{O}C. The study were designed for confirming the presence of *Salmonella spp*. especially *S. Typhimurium* in butcher shops and chicken market because increasing in cases of food poisoning associated with the consumption of poultry meat. The samples comprised sections of chicken cages, cutting boards and knives previously employed in processing chicken meat. Samples were obtained using sterilized swabs, carefully preserving their integrity during transportation through suitable media. These samples were promptly sent to the microbiology laboratory at Baghdad University for analysis.

Culture and biochemical tests

The collected samples were subjected to cultivation on Tetrathionate Broth Base media, serving as enrichment media, and were allowed to incubate at 37 °C for 24 hours. Subsequent colonies were identified using biochemical assays on desoxycholate citrate agar, and SS agar. Moreover, XLD agar was used to identify colonies maintained at 37 °C for another 24 hours. Additionally, the biochemical test employed to verify the existence of characteristic *Salmonella* bacteria. The biochemical identification tests used were Oxidase, Catalase, Urease, Citrate, Voges-Proskauer, Methyl Red, Motility, Indole, and Lactose Fermentation. A Gram stain technique was employed to scrutinize potential colonies further, enabling the morphological evaluation of staining features (8).

Disk-diffusion assay

Mueller-Hinton agar plates with bacteria were inoculated, and various antibiotic disks, including those containing Penicillins (Ticarcillin), fluoroquinolones (Ceftazidime), third-generation Cephalosporins (Ceftazidime), aminoglycosides (Amikacin, Gentamicin), Sulfonamides (Trimethoprim-sulfamethoxazole), and

carbapenems (Imipenem, Meropenem), were carefully positioned on the surface of the agar. The prepared dishes were then incubated at 37 ^{O}C for twenty-four hours (9, 10). The experiments were carried out, and their outcomes were recorded.

DNA extraction

The current study employed a DNA extraction kit (Promega, USA) to extract DNA from cultures, following the manufacturer's instructions. The purity and quantity of the extracted DNA were evaluated through spectrophotometric analyses. The collected DNA samples underwent PCR amplification, focusing on detecting the *inv* A and *stm* genes (11, 12).

Polymerase cain reaction assay

The *inv* A gene was utilized to identify *Salmonella* genus strains as outlined in Table (1), while the *stm* gene was employed to identify *Salmonella Typhimurium* species isolates (13,14). Within each PCR operation, a mixture comprising 2.5 µl of 10X PCR buffer, 2mM MgCl2, 0.2mM dNTPs, 1 unit of Taq DNA polymerase enzyme (bioneer, Korea), 0.5 µl of each primer (primer details provided in Table 1), and 3 µl of DNA was prepared. The final volume was adjusted to 25 µl using high-purity deionized water.

For the *inv* A gene, the first denaturation stage was conducted at 94°C for five minutes, followed by 35 cycles encompassing a one-minute denaturation at 94°C, a two-minute annealing phase at 62°C, and a two-minute extension at 72°C. The amplification cycles for both procedures are delineated in Table 1. The PCR results were seen through electrophoresis on a 1.2% agarose gel and subsequent Ethidium bromide staining. Additionally, the other PCR reaction conditions included denaturation at 94^{o}C 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 62°C for 2 minutes, and extension at 72°C for two minutes, culminating in a final extension of 10 minutes at 72°C (Table 2, 3).

Table (1): The primers that have been used targeting *Salmonella* spp.

Primer	DNA sequence (5′ → 3′)	Amplified product	Size (bp)	Reference
inv A	F-GTGAAATTATCGCCACGTTCGGGCAA	*Salmonella genus*	*284*	13, 15
	R-TCATCGCACCGTCAAAGGAACC			
stm 4497	F-GGAATCAATGCCCGCCAATG	*Salmonella Typhimurium*	*523*	14, 16
	R-CGTGCTTGAATACCGCCTGTC			

Table (2) : PCR programs that have been used targeting *inv A* gene

Steps	Temperature ^{o} C	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	62°C	2 min
Extention	72°C	2 min
Final extention	72°C	7 min
Cycles number	35 cycles	

Table (3): PCR programs that have been used targeting *stm* gene

Steps	Temperature ^{o} C	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	62°C	2 min
Extention	72°C	2 min
Final extention	72°C	10min
Cycles number	35 cycles	

Result and discussion

Culture and biochemical tests

The colonies observed on desoxycholate citrate agar exhibited a smooth, bright appearance, circular in shape, and possessed a translucent pink color, complemented by a central black spot. (Figure 1) displays colony isolation on desoxycholate citrate agar.Meanwhile, the colonies on Salmonella-Shigella agar displayed a rounded configuration, pale yellowish concave, and measured approximately 2-3 mm in diameter, featuring a black central point. On XLD agar, the colonies were tiny, smooth, concave, red, and had a black middle. The IMVC tests used to differentiate the *Salmonella* genus of *Citrobacter* and *Shigella*, the research's results indicate that *Salmonella* samples had positive results for methyl red, and Motility, but negative results for indole, Voges Proskauer test (Table 4). Generally, The FDA recommends culturing on XLD involving pre-enrichment followed by selective enrichment in tetrathionate (TT) broth to isolate *Salmonella* from food samples (17). Among the array of *Salmonella* selective media frequently employed, the noteworthy candidates encompass SS Agar, Bismuth Sulphite Agar, Xylose Lisin Deoxycholate (XLD) Agar, Brilliant Green, and Hektoen Enteric (HE) Medium.Notably, *Salmonella* colonies exhibited a distinct black hue on desoxycholate citrate agar, SS agar, and XLD agar, attributed to the production of H2S (Figure 1). As mentioned earlier, these media are commercially available, characterized by their distinct ingredient compositions (13). In cases of typhoid fever found in an Iraqi sample of S. typhi, molecular techniques yield better diagnostic outcomes than standard tests (14).

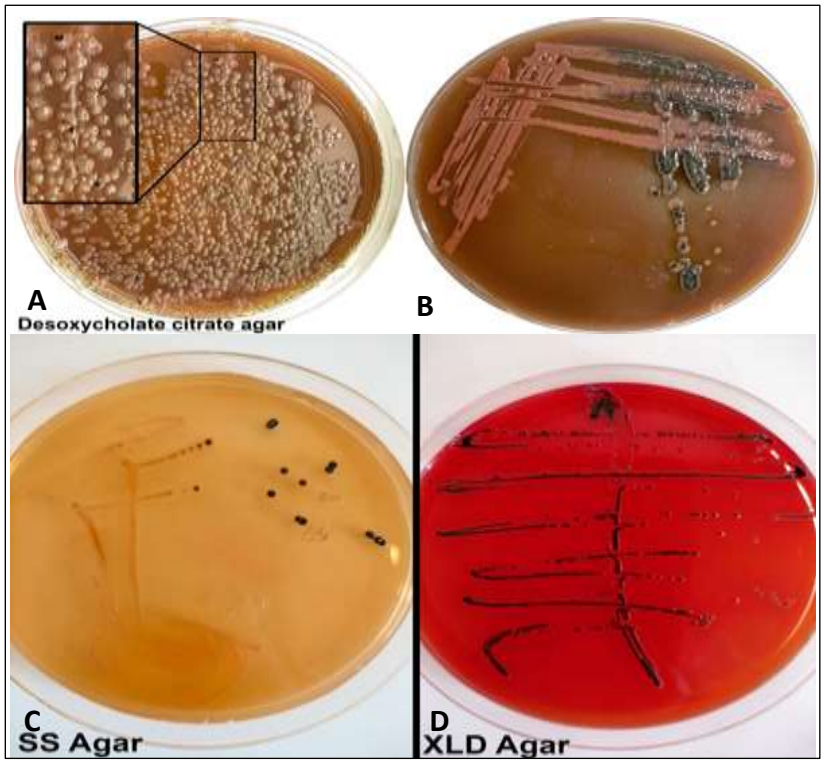

Figure (1): (A) Shows the initial isolated of colonies on Desoxycholate citrate agar. (B) Shows single colony, it was smooth, bright, and round, with a black center. (C) Colonies of *Salmonella* spp. on SS agar, the colonies appear smooth, convex, and pale with a black center and *Salmonella* spp. (D) the colonies grow on XLD medium, where clusters are silky smooth flat and crimson in shade, with a black core.

Table (4): Biochemical assays for the identification of *Salmonella* spp.

Test	Oxidase	Catalase	urease	Citrate	Voges proskauer	methyl red	Motility	Indole	Lactose fermentation
Salmonella	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve

Generally, the findings of this present study showed the prevalent resistance of all strains to Penicillins (Ticarcillin), fluoroquinolones (Ceftazidime), and third-generation Cephalosporins (Ceftazidime). Conversely, a noticeable sensitivity was evident towards aminoglycosides (Amikacin, Gentamicin), Sulfonamides (Trimethoprim-sulfamethoxazole), and carbapenems (Imipenem, Meropenem), The findings demonstrate the existence of many forms of antibiotic resistance in *Salmonella* bacteria, suggesting a strong likelihood that chickens are being excessively administered antibiotics in their diet as a method of illness control, as illustrated in (Table 5). The present study's findings were in line with a parallel study that revealed the resistance of *S. Typhimurium* to Penicillins and Ceftazidime. Notably, the evaluation of various classes of antimicrobial medications indicated a more robust resistance to Ceftazidime in comparison to ceftriaxone (18). On the other hand, congruent with prior research, the current study demonstrated the sensitivity of *Salmonella spp*. to imipenem, meropenem, minocycline, and trimethoprim/ sulfamethoxazole(19). Research findings have indicated that aminoglycoside antibiotics such as Gentamicin and amikacin efficacy in treating typhoid symptoms remains limited due to challenges in penetrating cells, rendering them less effective against intracellular bacteria like *Salmonella enterica serovar Typhi* (20) (Table 2). In response to these challenges, Fluoroquinolones, particularly ciprofloxacin, have emerged as viable alternatives for combating *salmonella* infections (21). Nonetheless, recent studies have noted a decline in ciprofloxacin susceptibility and, more recently, the development of fluoroquinolone resistance, prompting an increased utilization of third-generation cephalosporins in enteric fever (22). Recent research shows that the increasing number of antibiotic-resistant *S. Typhi* strains underscores the direct correlation between antibiotic usage and the proliferation of resistance. Consequently, a more strategic approach is essential to mitigate multidrug resistance and its propagation(23). Moreover, the presence of antimicrobial resistance in *S. Typhimurium* strains emphasizes the critical need to discover more about the relationship between antibiotics and the presence of virulence genes (24).

Table (5): Antibiotic susceptibility pattern against *Salmonella* spp isolates

Classes of antibiotic	Antibiotic	Antimicrobial activity
Penicillins	Ticarcillin	R 93%
Aminoglycosides	Amikacin	S 100%
	Gentamicin	S26%
Fluoroquinolone	Ciprofloxacin	R 15%
Cephalosporins 3rd generation	Ceftazidime	R 100%
Carbapenems	Imipenem	S 3%

Polymerase chain reaction assays

The PCR approach focused DNA fragments corresponding to segments within previously identified *Salmonella* genes. These genes encompass *inv* A (284 bp) and *stm* (523 bp), both originating from *Salmonella Typhimurium* refer to (Figures 2, 3).

Out of the 50 samples derived from butchered chicken meat from various markets within Baghdad city, mere 20 samples yielded positive results for *Salmonella* presence. The screening of these same samples through PCR analysis confirmed the existence of *Salmonella* spp. in 20 instances, as evidenced by the particular *inv* A gene primers. Furthermore, among the 50 chicken samples collected from diverse markets across different areas of Baghdad, 15 isolates demonstrated the presence of the *stm* gene. This particular gene indicated the isolation of *Salmonella Typhimurium* within these samples (Table 6). Ultimately, the samples were diagnosed using VITEK technique following molecular detection to verify the final result that presence of *salmonella*. In vitro, DNA amplification by the PCR method is more potent than traditional microbial investigations. Its distinct advantage lies in its heightened specificity and sensitivity, rendering it a robust tool for precisely detecting harmful microorganisms across a broad spectrum of food components (24). In addition, the PCR approach demonstrates swifter and more accurate outcomes, enabling the precise measurement of DNA (25). An earlier study conducted in 2015 indicated a higher incidence of *Salmonella* isolation from chicken meat as opposed to beef meat (9.5% versus 4.7%), involving various serovars, including *S. Typhimurium*, *S. enteritidis* and *S. Livingstone*, with an overall rate of 16.6% (19). Another study underscored the utility of the *inv* A gene as a rapid identification technique for various *Salmonella* spp., with results achievable within no more than twelve hours (26). Research utilized modifications to the *inv* A gene primers, which have since been regarded as standard *Salmonella* primers within the scientific community(27). These findings have illuminated the possibility of identifying *Salmonella* at the genus level by applying universal primers (27). The outcomes of this study are harmonious with the discoveries of recent research, who detected the *inv* A gene within each isolate sourced from chicken specimens(28). Another study examined the presence of the *inv* A gene, encoding a protein situated within the barriers of bacterial cells. Given that the *inv* A gene generates a protein essential for penetrating donor epithelial cells, the Salmonella-specific primer proved adept at swiftly and accurately identifying Salmonella spp. (29). Because *inv* A is a factor in the outer membrane composition of *Salmonella* spp., it allows *S. Typhimurium* to infect chicken epithelial cells and induce macrophage apoptosis (7)

The potential for *Salmonella* contamination in chicken exists throughout the entire spectrum, encompassing production, preparation, transportation, sales, storage, and cooking stages (14). Our study's conclusions align with numerous studies that have reported an upsurge in *S. Typhimurium* prevalence within poultry-derived meat from diverse origins. Earlier research highlighted the *inv* A and *stm* genes as virulence determinants chosen by *S. Typhimurium* to confirm its pathogenicity (30). Notably, *stm* represents a gene encoding a cytoplasmic protein with an unknown function unique to *Salmonella*

Typhimurium. Consequently, the *stm* gene has been earmarked as a distinctive identifier for detecting *S. Typhimurium* (11).

Table (6): The number of samples taken from various chicken markets in some Baghdad, region and shown the presence of *inv* A and *stm* genes in the detection samples

Samples	No. of sample	No. of *inv A* positive isolates	No. of *stm* positive isolates
Chicken, cages, cutting boards and knives	50	20 *Salmonella* genus	15 *Salmonella Typhimurium*

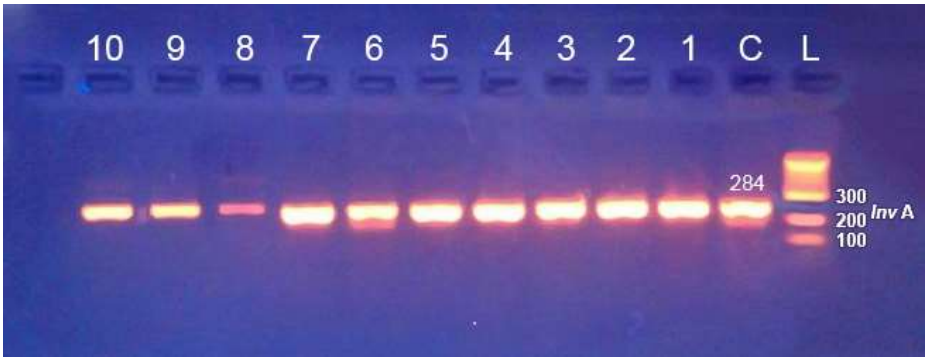

Figure (2): The findings of the replication of *Salmonella* spp. *inv* A gene (284 bp) were fractionated on 1.5% agarose Gel electrophoresis dyed with Ethidium Bromide. M: 100bp as a ladder indicator the first band is control positive.

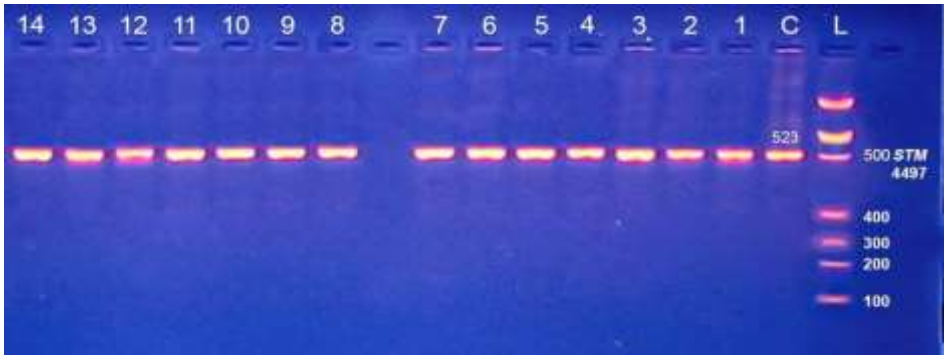

Figure (3): The findings of the replication of the *Salmonella* spp. stm gene (523bp) were split using 1.5% agarose Gel electrophoresis dyed with Ethidium Bromide M: 100bp as a ladder indicator (the first band is control positive).

Conclusion

The PCR assay has been validated as a successful approach for the precise and sensitive identification of *Salmonella* in poultry and meat products, offering less time-consuming results than traditional methods. Using the PCR technique, the presence of the *inv* A and *stm* genes within laboratory settings provides valuable tools for confirming the presence of *Salmonella spp*. and *S. Typhimurium*, respectively. Nevertheless, in cases where PCR-based procedures are not immediately accessible, using *Salmonella*-specific media remains a valuable alternative within veterinary diagnostic laboratories, especially for detecting *Salmonella* in samples extracted from butchered chicken cutting boards and knives. Notably, the *Salmonella* isolates exhibited resistance to Ticarcillin, Ceftazidime, and Ceftazidime yet demonstrated sensitivity to Amikacin, Gentamicin, Trimethroprim-sulfamethoxazole, Imipenem, and

Meropenem. These findings indicated the diverse range of antibiotic responses observed within the *Salmonella* strains examined.

Ethical approval

In Iraq, the Ministry of Higher Education and Scientific Research approved the ethical study committee for scientific research.

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