DETECTION OF *INV*A GENE AND THE EMERGENCE OF EXTENDED-SPECTRUM β-LACTAMASE (ESBL) GENES IN *SALMONELLA ENTERICA* ISOLATED FROM DIFFERENT SOURCES IN ERBIL CITY, IRAQ

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Abstract. *Salmonella enterica* is a significant foodborne disease, originating from contaminated eggs and meat from poultry being the predominant cause of illness and a serious global public health problem. The aim of this study to determine of antimicrobial resistance, *Salmonella enterica* virulence (*inv*A) gene and extended spectrum bata-lactamase (ESBL) production in food and hospitalized patients. In the present study, a total (of 630) samples were collected from different specimens. Conventional protocols used to isolate *Salmonella enterica*. Typical black colonies were verified using the VITEK®2 automated system., subsequently antimicrobial susceptibility test (AST) for isolated strains was conducted. In addition, all *Salmonella enterica* isolates were examined for the presence of the *inv*A gene, as well as phenotype and genetic ESBL production genes (targeting three different resistance genes., bla_{TEM}, bla_{SHV} and bla_{CTX-M}) using polymerase chain reaction (PCR). Identification of 60 samples among (630) via traditional method were black colony, in which 52 positive samples were obtained using VITEK®2 system, while 55 positive samples were reidentified by applying the PCR technique. The highest resistant rate via AST that was recorded against Gentamycin antibiotic, and the result was (94.6%). The disc placement method revealed that all 55 *Salmonella enterica* isolates showed (ESBL) production. PCR assay revealed that the most frequently identified genes were bla_{TEM}, which was present in (100%) of strains. Genotype analysis of isolates for the presence of the *inv*A gene, in which 37 positive PCR products were sequenced and all sequences (n = 37) were uploaded to GenBank. TEM resistance gene was the most prevalent ESBL type among *Salmonella* isolates and 37 isolates (n = 37) of *Salmonella* were identified.
Keywords: *Salmonella enterica, foodborne diseases, meat, antimicrobial resistance, virulence gene*

Introduction

Salmonella is one of the most prevalent causes of diarrhea worldwide, affecting millions (Lapierre et al., 2020). *S. enterica* causes food poisoning and infects the human gastrointestinal system. Animal products and faces are these microorganisms principal sources, which makes sterilization important (Bialucha et al., 2020). *S. enterica* is regarded globally as the leading cause of foodborne illnesses. This bacterium is present within human gastrointestinal tracts and animals and is detectable in contaminated food (Besharati et al., 2020). *S. enterica* is a significant zoonotic foodborne pathogen that persists on poultry farms around the globe (Yu et al., 2021). *S. enterica* can be isolated from various animal species, Gram-negative, rod-shaped, and Enterobacteriaceae bacterium. The intestinal tract is the primary reservoir of these zoonotic bacteria, and intensive animal production promotes their colonization (Ghoddusi et al., 2015). Enteric pathogens like *S. enterica* can tolerate low pH conditions and threaten food safety when raw poultry is marinated (Kiprotich et al., 2020). *Salmonella* is a zoonotic pathogen, some *Salmonella* subspecies enterica serovars are economically significant poultry-

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specific pathogens in many countries with a developing poultry industry (Abdelwahab et al., 2019).

S. enterica is the most prevalent pathogen transmitted by food, it is transmissible between chickens to humans via contaminated poultry products (Wang et al., 2019). Although the zoonotic potential of these bacteria remains uncertain, considering their role as a reservoir for antibiotic resistance and as a potential threat to human health due to close contact between animals and humans (Dor et al., 2020). *S. enterica* is widespread in the natural environment and can grow under ambient temperature conditions. *S. enterica* causes severe systemic infections with high morbidity and mortality rates; it increases in humans if medical care is not provided (Li et al., 2019). *S. enterica* control in food production chains is critical for ensuring food safety and minimizing the risk of foodborne illness (Dos Santos Bersot et al., 2019). *S. enterica* presence throughout the digestive tract of healthy poultry has been identified as the most predominant risk factor for human infection; it can contaminate carcasses following slaughter, spreading the pathogen to customers (Borges et al., 2019). Detection of bacterial isolates and their antimicrobial susceptibility profiles identified by using the VITEK® 2 system (Suyat et al., 2020).

Salmonella enterica antibiotic resistance has been a worldwide problem over the past several decades. However, there is limited data on *S. enterica* among various breeds of breeder chickens (Shi et al., 2023). The fast spread of multidrug-resistant *S. enterica* threatens global health, based on phenotypic testing, *S. enterica* strains were MDR and ESBL producers. The presence of bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{TEM} genes were detected by molecular analysis of ESBL-producing strains, confirming the prevalence of ESBL-producing *S. enterica* strains in poultry meat (Gambino et al., 2022). PCR technique was utilized to detect the *inv*A gene and antibiotic-resistant gene makers. This is knowledge concerning the suitability of the *inv*A gene primer set as a PCR target for identifying *Salmonella* (Anjorin et al., 2021). *S. enterica* was detected by amplifying nucleotide sequences within the *inv*A gene for diagnostic purposes (Rahn et al., 1992). Gram-negative pathogens that produce (ESBL) are a leading cause of β-lactam antibiotic resistance. Since their discovery in the early 1980s, they have become increasingly popular in hospital and community-associated Enterobacterales (Castanheira et al., 2021). (ESBLs) are considered to be one of the most important role of antibiotic resistance mechanisms (Ehlers et al., 2009). Members of Enterobacteriaceae are known to produce (ESBL) which hydrolyze the beta-lactam group of antibiotics (Saeed et al., 2023).

The molecular identification of the bacterial isolates was verified by the presence of the *inv*A gene that regarded as the standard diagnostic target for *S. enterica* (Elhariri et al., 2020). This unique PCR method confirmed in the amplification of the *inv*A gene, it is currently considered an international benchmark for detecting the *Salmonella* genus. This gene encodes a protein in the bacterial inner membrane that invades the host's epithelial cells (Shanmugasamy et al., 2011). The *inv*A is a common molecular target gene for *S. enterica* detection methods. It is recommended as a target for PCR validation of putative *Salmonella* isolates by the U.S. Food and Drug Administration Bacteriological Analytical Handbook (Buehler et al., 2019). This research aims to investigate the antimicrobial susceptibility and existence of ESBL-producing *S. enterica* between human and poultry products, the presence and evaluation of the virulence genes (*inv*A) underline the public health importance of the current serovars by analyzing the genetic relation between the chicken isolates and their relation to human isolates.

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Materials and methods

Sample collection

A total of 630 samples were collected in different sources, fresh human stool (n = 130), cloacal swab from apparently healthy layer chicken in private poultry layering farm (n = 200), eggshell (n = 150) and chicken meat (n = 150). Under aseptic conditions, the samples were randomly collected from different sources in Erbil city, Iraq, from August, 2021 to February, 2022. Samples were labelled, then the samples were transformed into a cool box and examined immediately after arriving at the laboratory department of Veterinary directorate of Erbil.

Isolation and identification of S. enterica

Salmonella. enterica was isolated using the standard cultivation method suggested by ISO 6579-1 (Mooijman, 2018). Swab samples (human stool, cloaca and egg) aseptically were premoistened with buffered peptone water (Neogen), then the swabs put it into sterile tube which contain 10 ml buffered peptone water (Sabry et al., 2020), and 25.0 g of meat samples were inserted into a stomacher bag which contained 250 mL of buffered peptone water. According to the sample type, if needed, homogenization was carried out under sterile conditions; concisely, the pre-enriched samples were incubated for 18 h at 37°C. After that, 0.1 mL aliquots of the pre-enriched broth were inoculated into tubes containing 10 mL of Rappaport Vassiliadis (R.V.) enrichment broth (Neogen) and were incubated at 42°C for 24 h, A loopful of enrichment broth was plated on Xylose Lysine Deoxycholate agar (XLD) (Neogen) and *Salmonella Shigella* (SS) agar (Neogen), then incubated at 37°C for 24 h.

Suspect black colonies were biochemically identified using the VITEK®2 compact system (bioMerieux, France) and applying the Gram-negative card (G.N.) Lot NO (2411765503) (bioMerieux) following the manufacturer's instructions. This system is intended to identify most Gram-negative fermenting and nonfermenting bacteria accurately. Three to five new black colonies of each sample were transferred into two tubes containing 3 mL of normal saline, and DensiCHEK Plus used to correct the turbidity to 0.5 McFarland standard solution. After that, the suspension was inoculated into the VITEK®2, applying a Gram-negative identification card. Meanwhile, from Media Diagnostic Center (MDC) we obtained the *S. enterica* ATCC14028 strain, that was used as a (positive control) quality control for the rest of experiments (Patil et al., 2022). After that polymerase chain reaction (PCR) technique was applied to confirm the test result of the VITEK®2 compact (Var et al., 2018).

Antimicrobial susceptible test

Testing the antibacterial susceptibility of the single black colony for 55 isolated *S. enterica* strains was performed by using VITEK 2 system and used AST-N419 card lot (0441910204) (bioMerieux). The tested antimicrobials were Ampicillin/Sulbactam (AMS); Piperacillin/Tazobactam (TZP); Cefotaxime (C.Z.); Ceftazidime (CAZ); Ceftazidime/Avibactam (CAZA); Ceftolozane/Tazobactam (C/T); Cefepime (CEPN); Imipenem (IPM); Meropenem (MRP); Amikacin (AMK); Gentamicin (G.M.); Ciprofloxacin (CIP); Tigecycline (T.G.); and Trimethoprim/Sulfamethoxazole (SXT), the turbidity was corrected to 0.5 McFarland standard according to the manufacturer's specifications. The categorization as resistant (R), intermediate (I) and sensitive (S) was

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based on “Clinical and Laboratory Standards Institute (CLSI)”-established criteria (Lozano-Leon et al., 2022).

Phenotypic ESBL production determination by double-disc test

Using the Kirby-Bauer disk diffusion method, screening ESBL by double-disc test (DDT) method was conducted for 55 *S. enterica* strains were inoculated on Mueller-Hinton agar (Oxoid). Morphologically two to four bacterial single colonies were mixed with sterile normal saline. The turbidity was adjusted to match 0.5 McFarland standards. Dilution was prepared uniformly and directly dispensed and spread on the surface of the Mueller-Hinton agar plate by using a sterile cotton swab under sterile conditions. The following four antimicrobial discs were placed on the inoculated Muller-Hinton Agar plate, which took place in a safety cabinet: by applying cefotaxime (CTX) and ceftazidime (CAZ) alone and in combination with clavulanic acid (CTX 30 mg, CTX/clavulanic acid, 30/10 μg) and (CAZ 30 μg, CAZ/clavulanic acid, 30/10 μg) (Wen et al., 2022). After 24 h, incubation, the results were interpreted according to the guidelines of the CLSI (Humphries et al., 2021). The positive ESBL production of isolates when zones of inhibition were increased by 5 mm or more on the discs with clavulanic acid than that observed on the corresponding disc without clavulanic acid (Lee et al., 2016).

DNA extraction

DNA from bacterial colonies of an overnight culture grown on XLD agar were extracted using (AddPrep Genomic DNA Extraction Kit Lot No (G202202D)/South Korea) according to the protocol recommended by the manufacturer (AddBio); this process took place in a safety cabinet to prevent contamination. The quantity and quality were determined visually by Gel electrophoresis and NanoDrop spectrophotometer and finally stored in the freezer (-20°C) for downstream work.

Molecular detection of invA gene and multiplex PCR analysis of β-lactamase genes

Alpha thermal cycler (PCR_{max}, UK) machine was used for amplification and detection of *inv*A genes in 60 isolated *Salmonella,* specific primer sequence used as an important target gene for detection and confirmation of *S. enterica*. The oligonucleotides primer sequence specific for detecting *inv*A and we used of the Add Tag master mix kit (Lot NO: 1905A) (Shanmugasamy et al., 2011) (Skyberg et al., 2006), the PCR run method program for amplification was summarized in (*Table 1*). For identification of the *inv*A gene, the PCR mixture reaction was 10.0 μL of (2×) add tag master mix, 1.0 μL of each primer (forward and reverse), 2.0 μL of DNA, and to complete the total volume of reaction 20 μL to 1×; we added 6.0 μL of PCR grade water under specific PCR run method.

After confirmation of isolates, the essay was performed to detect of three genes encoding ESBL antibiotics resistance genes, ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) of ESBL genes; and Multiplex PCR master mix kit (GeNet Bio, Lot No. G721604A) was used, The oligonucleotides three set primer sequence specific for detecting $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes as described by the PCR mixture reaction was 25 μL of 2× Multiplex master mix, 3.0 μL of DNA template were used and 1.5 μL of each set primers Forward and Reverse, to complete the total volume 50 μL we applied 14 μL the PCR grade water and run method summarized in (*Table 1*). *S. enterica* ATCC (14028) strain was used as

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a (positive control) for both PCR essays, and one reaction as negative control (PCR reaction without DNA template) to be aware of the contamination. The amplicon of the PCR product was visualized on a 1% agarose gel stained with Safe Gel Stain Dye (Lot NO. SGD2201C). A molecular weight marker of 100 bp ladder (GeneDireX Lot. NO. DM23110070) was applied to determine the size of the PCR amplicons was carried out at 80 V for 45 min (Delaney et al., 2018) the band visualized and documented by blue light trance illuminator (GeneDireX). PCR products were sequenced (Sanger sequencing method) by Macrogen-Seoul, South Korea.

Table 1. *Primer sequences and PCR condition for gene amplification of Salmonella virulence invA and ESBL genes*

Target	Primers	Sequences	Condition	Cycle	Amplicon size (bp)	References
invA	*inv*A	GTG AAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	95℃ for 5 min, 95℃ for 35 s, 60C for 40 s, 72℃ for 35 s, 72℃ for 5 min	35	284	Rahn et al. (1992)
bla_{SHV}	bla_{SHV}	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	95℃ for 5 min, 94℃ for 30 s, 60℃ for 30 s, 72℃ for 2 min, 72℃ for 5 min	35	747	Monstein et al. (2007)
bla_{TEM}	bla_{TEM}	TCGCCGCATACACTATTCTCAGAA TGA ACGCTCACCGGCTCCAGATTTAT			445	
bla_{CTX}	bla_{CTX}	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTAR GTSACCAGAAYCAGCGG			593	

Results

Isolation and identification

Sixty isolates of *S. enterica* were isolated from microbiologic analysis of six hundred and thirty (630) collected samples in different sources, in which (60) samples colony of bacteria appeared black on XLD and S.S. agar. For further confirmation biochemical test VITEK®2 compact automated system and GN VITEK®2 card was an identification test used; 60 samples were tested, of which 52 were positive test results for *S. enterica* and eight were negative results (*Table 2*). Still, for more confirmation, we applied PCR to detect Genetic and molecular diagnosis for 60 isolates, using *inv*A target gene set primer of which 55 samples were positive for *Salmonella*, and five were negative (*Table 2*). Detection by VITEK®2 is a biochemical test relatively identical to PCR detection except for three samples negatives in VITEK®2 had the difference with molecular detection in which three samples more were confirmed positive in a total of 60 samples.

Table 2. *Occurrence of S. enterica among different sources diarrheic hospitalized patient, layer poultry and poultry product*

Species	Examined no.	PCR positive no. (%)	Virulence *inv*A gene	Vitek result
Human	130	11 (8.46%)	All isolates were positive for *inv*A	11
Cloaca	200	9 (4.50%)		9
Chicken breast meat	150	22 (14.66%)		19
Eggshell	150	13 (8.66%)		13
Total	630	55		52

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Antibiotic susceptibility Test AST and ESBL screening

AST-N419 VITEK®2 card as an antibiotic susceptibility pattern applied to 55 *S. enterica* isolates for their susceptibility to 14 antibiotics. The results showed that the highest resistance rate was recorded against Gentamycin antibiotic, and the result was (94.6%). Meanwhile, ceftazidime/Avibactam and Tigecycline (2.7% and 2.7%) were the lowest resistance. The other isolates showed a variation in the result against the remaining antibiotics, as revealed in (*Table 3*). The percentage resistance of Amikacin 91.8%, Ceftazidime 70.2%, trimethoprim/sulfamethoxazole 24.3%, Ciprofloxacin 21.6%, Ampicillin/Sulbactam was 13.5%, Cefotaxime 8.1%, Imipenem 5.5, Ceftazidime 0%, Ceftolozane/Tazobactam 0%, Cefepime 0, Meropenem 0%. Among all isolates depicted intermediate resistance against Ciprofloxacin and Tigecycline were (32.4%), (13.3%) respectively.

Table 3. *Antibiogram of S. enterica isolates (n = 55) displaying specific phenotype profile (resistance, intermediate and sensitive) against 14 types of antibiotics using VITEK 2 compact system AST card*

Antibiotics	**Susceptibility test rates**		
	R no. (%)	**I no. (%)**	**S. no. (%)**
Ampicillin/Sulbactam	5(13.5)	0	32(86.5)
Ceftazidime	0	0	37(100)
Cefotaxime	3(8.1)	0	34(91.9)
Ceftazidime	26(70.2)	0	11(29.8)
Ceftazidime/Avibactam	1(2.7)	0	36(97.3)
Ceftolozane/Tazobactam	0	0	37(100)
Cefepime	0	0	37(100)
Imipenem	2(5.4)	0	35(94.6)
Meropenem	0	0	37(100)
Amikacin	34(91.8)	0	3(8.2)
Gentamicin	35(94.6)	0	2(5.4)
Ciprofloxacin	8(21.6)	12(32.4)	17(46)
Tigecycline	1(2.7)	5(13.3)	31(84)
Trimethoprim/sulfamethoxazole	9(24.3)	0	28(75.7)

R: Resistance; I: Intermediate; S: Susceptible

The representative biochemical characterization of 55 isolates from various sources, and evidence of phenotyping ESBL production was observed for isolates, and an increase in the zones of inhibition was recorded. In the confirmatory test, a > 5 mm increase in zone diameters for the combined discs of ceftazidime and cefotaxime with clavulanic acid versus the zone diameter of the agent when tested alone was observed (*Fig. 1*).

Detection and sequence analysis of invA gene

The 60 positive samples traditional tested results were evaluated for more confirmation by PCR, as shown in *Figure 2*. The PCR-positive result of *S. enterica* was in the humane stool (11), cloaca (13), meat products (22), and egg (9), over all PCR essay revealed 55 sample positives. After PCR amplification, the positive results of PCR product according to the hosts (n = 37) isolates were selected and sequenced (10 human stools, 5 eggs, 8 cloaca and 14 breast chicken meat) (*Table 4*) by (Sanger sequencing method) (Macrogen, South Korea). Nucleotide sequence analysis was done using the BLAST software (NCBI, Bethesda, MD, USA) available from the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi.).

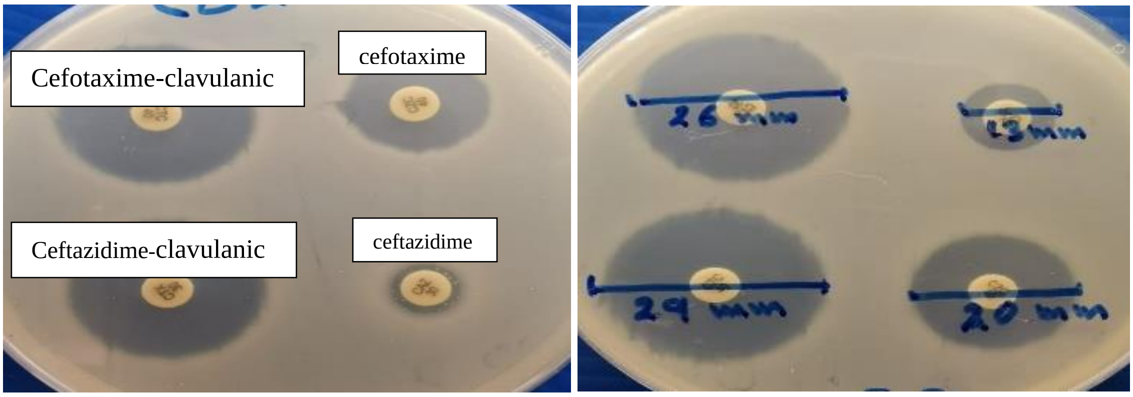

Figure 1. *Phenotyping expression confirmatory test for ESBL formation with a > 5 mm increase in zone diameters for the combination of ceftazidime and cefotaxime with clavulanic acid compared to the agent's zone diameter when tested alone*

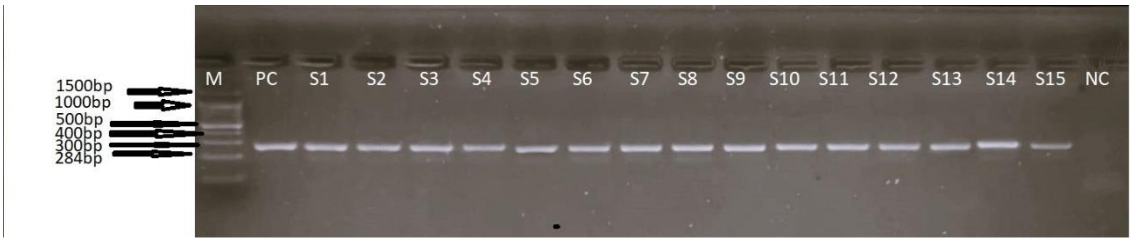

Figure 2. *Genetic and molecular identification of S. enterica by PCR targeting invA virulence gene with PCR amplification of 284 bp. M: DNA ladder, PC: positive control, S1-S15: Positive samples, and NC: Negative control*

BioEdit v7.0.5 was applied to evaluate the quality of sequenced data using NCBI BLAST and in the website the sequences were compared. Align a laboratory or query sequence with another biological sequence to determine greater similarity and nucleotide diversity with other targets. The submission procedure was guided by BankIt, a web-based tool featuring wizards. The GenBank database was intended for newly determined and annotated sequence data in Iraq. All sequences (n = 37) were uploaded to GenBank, then the accessions number were obtained (*Table 4*). The isolates showed *S. Enteritidis* (n = 5), *S. Typhimurium* (n = 1), *S. arizonae* (n = 1), *S. Muenchen* (n = 1), *S. Agona* (n = 2), *S. Abeokuta* (n = 5), *S. Hadar* (n = 2), *S. Adjame* (n = 1), *S. Poona* (n = 1), *S. Kentucky* (n = 2), S. *Gallinarum* (n = 1), S. *Senftenberg* (n = 1), S. *Tennessee* (n = 1), *S. Berta* (n = 1), *S. Dublin* (n = 1), *S. Anatum* (n = 2), *S. Heidelberg* (n = 1), *S. Infantis* (1), *S. Muenster* (n = 1), *S. enterica* (n = 1), *S. Albany* (n = 1), *S. Bredeney* (n = 2), *S. Paratyphi* B (n = 1), *S. Salamae* (n = 1).

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Table 4. *ESBL genes and invA gene detection results of the isolated S. enterica serovars*

Isolates	ID	Accession no	Sources	*inv*A	ESBLs gene detection
S. Enteritidis	EBLSE1	ON391757	Human stool	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Typhimurium	EBLSE2	ON391758	Human stool	+	bla_{TEM}, bla_{SHV}
S. arizonae	EBLSE3	ON391759	Human stool	+	bla_{TEM}, bla_{SHV},
S. Muenchen	EBLSE4	OQ023872	Human stool	+	bla_{TEM}
S. Agona	EBLSE5	ON553609	Human stool	+	bla_{TEM}
S. Abeokuta	EBLSE6	ON599013	Human stool	+	bla_{TEM}, bla_{SHV}
S. Abeokuta	EBLSE7	OQ440903	Human stool	+	bla_{TEM}, bla_{SHV}
S. Hadar	EBLSE8	ON599014	Human stool	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Abeokuta	EBLSE9	OQ440904	Human stool	+	bla_{TEM}
S. Agona	EBLSE10	OQ440906	Human stool	+	bla_{TEM}, bla_{SHV}
S. Enteritidis	EBLSE11	ON783807	Eggshell	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Abeokuta	EBLSE12	OQ440905	Eggshell	+	bla_{TEM}, bla_{SHV}
S. Hadar	EBLSE13	OQ440907	Eggshell	+	bla_{TEM}
S. Adjame	EBLSE14	ON783808	Eggshell	+	bla_{TEM}, bla_{SHV}
S. Poona	EBLSE15	ON783809	Eggshell	+	bla_{TEM}, bla_{CTX-M}
S. Abeokuta	EBLSE16	ON783810	Cloaca	+	bla_{TEM}
S. Enteritidis	EBLSE17	OQ434461	Cloaca	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Enteritidis	EBLSE18	OQ434462	Cloaca	+	bla_{TEM}, bla_{SHV}
S. Kentucky	EBLSE19	ON783811	Cloaca	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Gallinarum	EBLSE20	ON783812	Cloaca	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Senftenberg	EBLSE21	ON783813	Cloaca	+	bla_{TEM}
S. Tennessee	EBLSE22	ON783814	Cloaca	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Enteritidis	EBLSE23	OQ434463	Cloaca	+	bla_{TEM}, bla_{SHV}
S. Berta	EBLSE24	OP596331	Chicken breast	+	bla_{TEM},
S. Dublin	EBLSE25	OP596332	Chicken breast	+	bla_{TEM}
S. Anatum	EBLSE26	OP596333	Chicken breast	+	bla_{TEM}, bla_{SHV}
S. Heidelberg	EBLSE27	OP596334	Chicken breast	+	bla_{TEM}
S. Anatum	EBLSE28	OP596335	Chicken breast	+	bla_{TEM}, bla_{SHV}
S. Infantis	EBLSE29	OP596336	Chicken breast	+	bla_{TEM} bla_{CTX-M}
S. Muenster	EBLSE30	OP599920	Chicken breast	+	bla_{TEM},
S. enterica	EBLSE31	OP599921	Chicken breast	+	bla_{TEM}, bla_{SHV}
S. Kentucky	EBLSE32	OQ440908	Chicken breast	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Albany	EBLSE33	OP599922	Chicken breast	+	bla_{TEM}
S. Bredeney	EBLSE34	OP599923	Chicken breast	+	bla_{TEM}, bla_{CTX-M}
S. Infantis	EBLSE35	OQ440910	Chicken breast	+	bla_{TEM}, bla_{SHV}
S. Paratyphi B	EBLSE36	OP599924	Chicken breast	+	bla_{TEM}, bla_{SHV}, bla_{CTX-M}
S. Salamae	EBLSE37	OQ440909	Chicken breast	+	bla_{TEM}

Detection of ESBL genes

The genes encoding beta-lactamase activity in 37 ESBL-producing isolates were identified using PCR (*Fig. 3*). All examined bacteria had Extended Spectrum β-Lactamase genes, corroborating the phenotypic results of ESBL production experiments. The most commonly discovered genes were bla_{TEM}, which was present in all isolates

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(100%). Meanwhile, both genes of *bla*$_{SHV}$ and *bla*$_{CTX-M}$ were presented in (51.8%) and (33.31%) of strains, respectively (*Table 4*). Furthermore, all strains were harbored the *bla*$_{TEM}$ gene specifically, while *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ were harbored on nine strains. Moreover, the *bla*$_{TEM}$ and bla$_{SHV}$ together were harbored on thirteen strains; likewise, the *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes were harbored on three strains together (*Table 4; Fig. 4*).

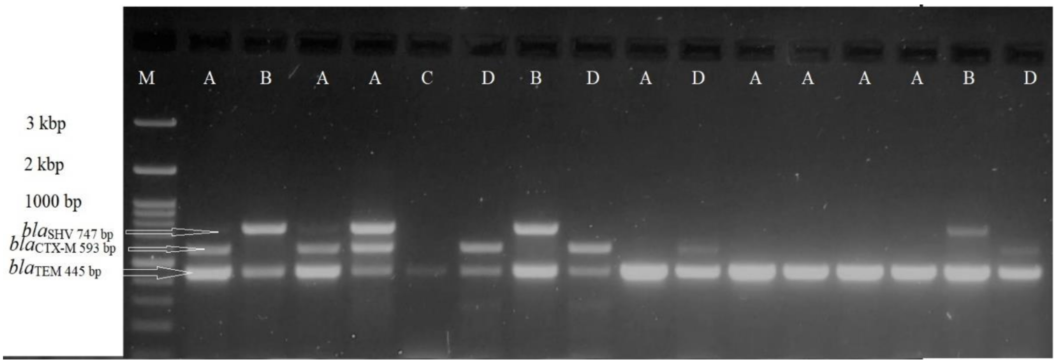

Figure 3. *Gel image displaying the PCR product bla$_{TEM}$, bla$_{SHV}$ and bla$_{CTX-M}$ genes in which lane A (bla$_{TEM}$, bla$_{SHV}$ and bla$_{CTX-M}$), lane B (bla$_{TEM}$ and bla$_{SHV}$), lane C (bla$_{TEM}$) and lane D (bla$_{TEM}$ and bla$_{CTX-M}$)*

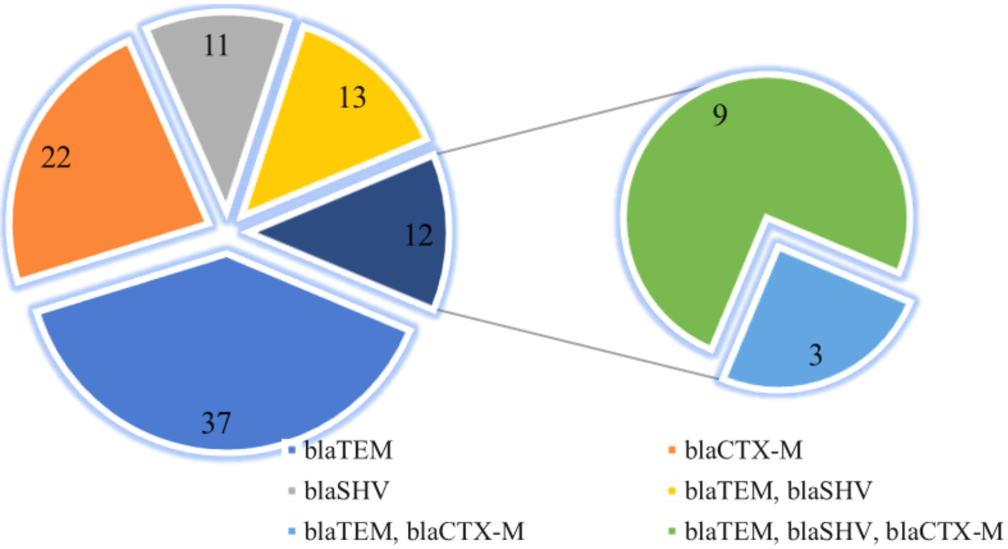

Figure 4. *Distribution of extended spectrum β-Lactam (ESBL) genes among (37) positive isolates of S. enterica*

Discussion

Salmonellosis is a prevalent infectious illness in humans caused by *Salmonella* spp., whose occurrence has increased in recent years, with product of avian origin serving as a common transmission vector (Lozano-Villegas et al., 2023). In the present study Black colonies on XLD agar and S.S. agar were suspected colonies of *S. enterica*, *Citrobacter* spp. and *Proteus* spp. due to the production of H_2S. Consequentially, the identification depending on the culture medium is inaccurate, consistent with the earlier study (Park et al., 2012). Further confirmation of *Salmonella* detection by VITEK®2 as

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a biochemical test was slightly identical to PCR detection except for three samples negatives in VITEK®2 had the difference with molecular detection in which three samples were more confirmed positive in a total of 60 samples, which means PCR assay is more accurate than VITEK®2. The same level of accuracy existed in the previous studies (Salman et al., 2021), which showed both PCR and VITEK®2 are the best methods of *S. enterica* isolation and are accurate.

VITEK®2 automated system was applied for the antibiotic susceptibility pattern of 55 *S. enterica* isolates for their susceptibility to 14 antibiotics. The results showed that the highest resistance rate was recorded against Gentamycin antibiotic, and the result was (94.6%). Meanwhile, ceftazidime/Avibactam and Tigecycline (2.7% and 2.7%) were the lowest resistance. The other isolates showed a variation in the result against the remaining antibiotics, as revealed in (*Table 3*). Among all isolates (32.4%), (13.3%) of our isolates depicted intermediate resistance against Ciprofloxacin and Tigecycline accordingly., However, another study (Dégi et al., 2021), reported that trimethoprim/sulfamethoxazole (68.75%), ampicillin (62.5%), ampicillin/sulbactam (56.25%), gentamicin (56.25%), nitrofurantoin (50%), and amikacin (31.25). Whereas other antimicrobials showed no resistance: ciprofloxacin, ertapenem, imipenem, levofloxacin, piperacillin/tazobactam, and tobramycin.

In this study, we showed that the isolation of *S. enterica* by PCR from the diarrheic hospitalized patient were (8.46%, 11/130), cloaca of apparently healthy layer chickens (4.50%, 9/200), chicken breast meat (14.66%, 22/150) and egg shell (8.66%, 13/150). All the isolates were verified by PCR for identification of the *salmonella*-specific *inv*A gene, which is the gold standard gene for *S. enterica* diagnosis. Our findings were also supported by the studies (Tiwari et al., 2022), who reported the existence of *inv*A genes as a diagnostic tool for detecting *S. enterica* isolates. Also, the PCR approach was used to detect the *inv*A gene in 30 isolates, and all isolates were positive for this gene (Fatta et al., 2020).

Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae have been identified more often and pose a substantial hazard to public health (Tan et al., 2023). Thirty-seven *S. enterica*-isolates suspected of producing ESBL were subjected to a further confirmatory test by PCR, which demonstrated that all isolates 37 (100%) produced ESBL, our finding was greater than the previous study (Sabry et al., 2020), who reported that 16 (80%) *S. enterica* possessed ESBL in Egypt and also the majority of bacteria had the ESBL genes bla_{TEM} and bla_{SHV}, while the minority included $bla_{\text{CTXM-M}}$. In our study, most ESBLs in isolated *S. enterica* were (bla_{TEM}) predominant. However, the other researcher reported that the bulk of ESBLs in *S enterica* serovars are bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$., and $bla_{\text{CTX-M}}$ appear to be predominant (Ahamed Riyaaz et al., 2018). Researchers from different countries also reported other findings where $bla_{\text{CTX-M}}$ was discovered from 100% *Salmonella* isolates.

The detected *S. enterica* were of the species enterica and exhibited a wide variety of serotypes; from the thirty-seven *S. enterica* isolates, twenty-four different serovars were identified, namely *S. Enteritidis* (n = 5) and *S. Abeokuta* (n = 5) were the most prevalent serotype. In comparison, S. *Hadar* (n = 2), *S. Kentucky* (n = 2), *S. Infantis* (n = 2), *S. Agona* (n = 2) and *S. Anatum* (n = 2) were double isolated. The rest serovars were represented by a single isolate *S. Typhimurium* (n = 1), *S. arizonae* (n = 1), *S. Muenchen* (n = 1), *S. Adjame* (n = 1), *S. Poona* (n = 1), *S. Gallinarum* (n = 1), *S. Senftenberg* (n = 1), S. Tennessee (n = 1), S. Berta (n = 1), *S. Dublin* (n = 1), *S. Heidelberg* (n = 1), *S. Bredeney* (1), *S. Muenster* (n = 1), *S. enterica* (n = 1), *S. Albany* (n = 1), *S. Paratyphi*

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B (n = 1), *S. Salamae* (n = 1). In our research, we detected *S. Enteritidis* (n = 5) and *S. Abeokuta* (n = 5) were significantly predominant, slightly differing from previous findings, indicating a prevalence of *S. enterica* isolates: Typhimurium and Kentucky (El-Sharkawy et al., 2017). A recent assessment of serotypes in several nations revealed that *S. Typhimurium* and *S. Enteritidis* were the most often isolated serovars from meat and poultry products, including beef (Nikiema et al., 2021) (Oueslati et al., 2023). In an earlier Brazil trial, *S. Enteritidis* was the most prevalent *S. enterica* serovar on poultry (Paiao et al., 2013). In other researchers showed that the *inv*A virulence gene has the potential to serve as a single-gene marker for molecular serotyping of *Salmonella* via phylogenetic analysis (Pavon and Rivera, 2021). In spite of the limited data from this study, we found differences in the prevalence of *Salmonella* strains in different sources of Erbil Province, or as predicted, most trees (*Fig. 5*) shown. All trees showed the 37 isolates of bacteria grouped in different cluster with high similarity into same genus and species of NCBI GenBank after blast. In these findings, more steps should be made to prevent *Salmonella* from harming chicken flocks and endangering public health in Erbil.

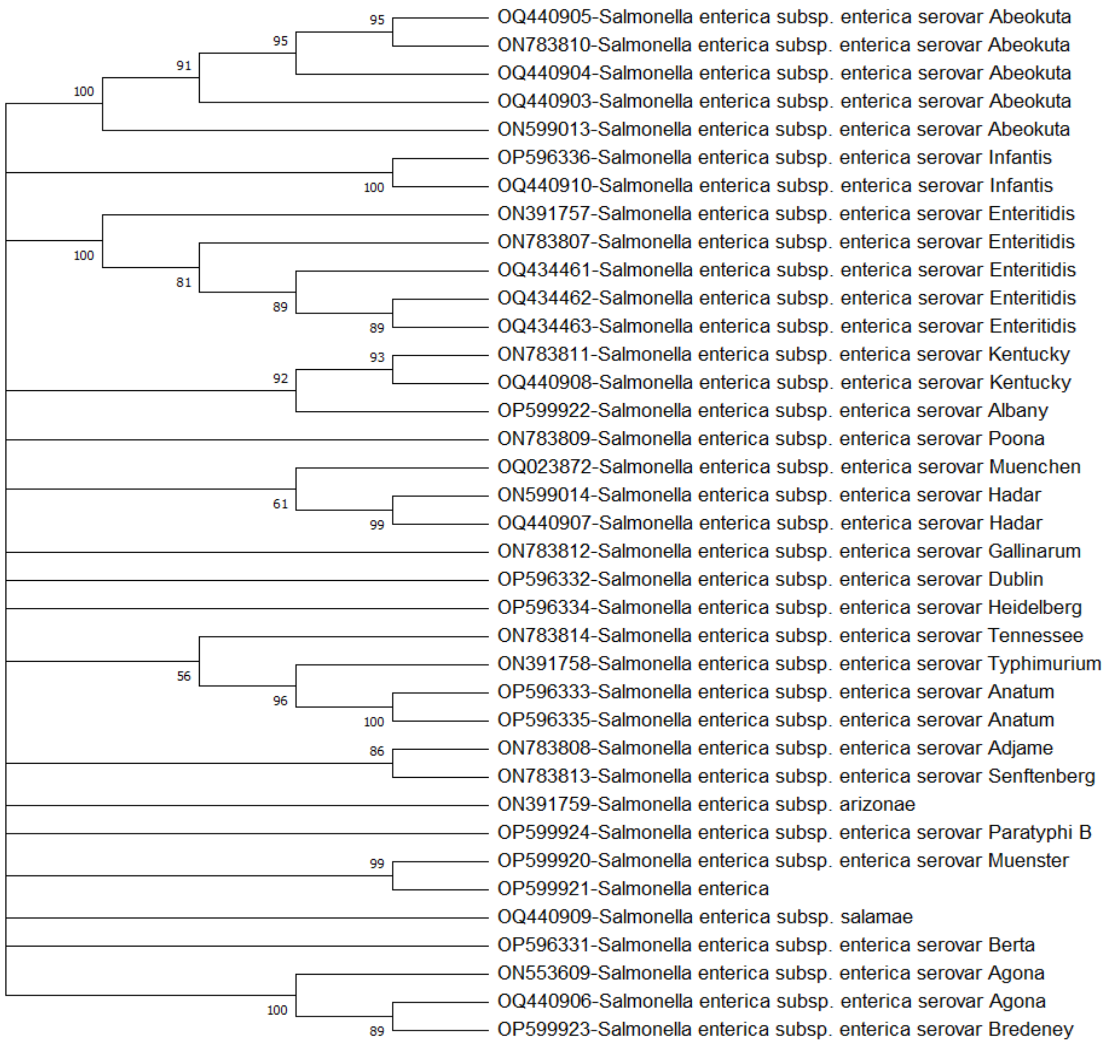

***Figure 5.** Employing Maximum Likelihood model with bootstrap of MEGA 11 program, show phylogenetic positioning of each salmonella species and sub species of 37 isolates with similar GenBank sequences of invA partial gene that available in GenBank*

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Conclusions

Our study combined enrichment/molecular (conventional PCR) method as a screening assay to isolate of *S. enterica*, their antimicrobial susceptibility profiles and detection of ESBLs genes in majority of *Salmonella* isolates in Erbil/Iraq from different sources (diarrheic hospitalized patient, chicken breast meat, eggshell and cloaca). Overall, the prevalence (8.73%) of *Salmonella* spp. was observed, and 24 strains were identified.

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