Detection of Extended Spectrum Beta-lactamase gene production by *E. coli* isolated from human and broiler in Sulemania province/ Iraq

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Abstract

A total of 311 *Escherichia coli* isolated from 460 healthy chickens at marketing age, and 56 *E. coli* isolated were obtained from 124 adult workers in broiler farms between April and October 2013 -. Isolated *E. coli* were identified by cultural, biochemical, and Api E 20 test. Antimicrobial susceptibility testing was determined by using the modified Kirby-Bauer's disc diffusion method. The antibiotic sensitivity result showed that the isolates were highly resistant to Beta-lactam antibiotics. Among the 311 isolated *E. coli* from healthy chickens 264 (84.9%) produced Beta-lactamase, while only 35 (62. 5%) of the 56 human isolates were found to produce the enzyme. All of the 264 β-lactamase producer *E. coli* isolated from healthy chickens and 35 β-lactamase producer *E. coli* isolated from human were tested for extended spectrum -β-lactamase (ESBL) production, it was found that only 201 (76.1%) and 19 (54.3%), were ESBL producers of *E. coli* isolated from healthy chickens and human respectively. Plasmids were detected in all ESBL producer- *E. coli* (201) isolated from healthy chickens, which were highly resistant to Beta-lactam antibiotics, while plasmid was detected only in 15 (78.9%) from 19 ESBLs producer *E. coli* isolated from human. By transformation the extracted plasmids into *E. coli* DH5α; it was found that 165 (82.1%) Beta-lactamase genes from 201 isolates from healthy chickens were carried by plasmid, while 10 (66.6%) Beta-lactamase genes from 15 plasmid isolated from human were carried by plasmid. The majority of these ESBL genes (69.7%) were *bla*$_{TEM}$ and (10.9%) were *bla*CTX-M genes in healthy chickens, while in human the majority of ESBL genes were (60%) *bla*$_{TEM}$ and (20. %) were *bla*$_{CTX-M}$ genes. Eleven isolated from healthy chickens and one isolated from human did not contain *bla*$_{TEM}$, *bla*$_{SHV}$ or *bla*$_{CTX-M}$.

Keywords: - *E. coli* ; healthy chicken; human; Beta-lactamase; ESBL gene, plasmid isolation.

I. Introduction:

In humans, *Escherichia coli* can cause a variety of intestinal and extra-intestinal infections, such as diarrhea, urinary tract infection, meningitis, peritonitis, septicemia, and gram-negative bacterial pneumonia. The enteric *E. coli* are classified on the basis of virulence properties into enterotoxigenic, enteropathogenic, enteroinvasive, vero-toxigenic, enterohemorrhagic, and entero aggregative *E. coli* (found only in humans). All are causative agents of diarrhea. *E. coli* is a commensal bacterium in the intestine of poultry, cattle, and pigs that are used for food production, and food of animal origin can be contaminated with *E. coli* during slaughter of the animals. *E. coli* from meat has mostly been associated with intestinal pathogenic *E. coli* (e.g., enteropathogenic, enterotoxigenic, and verotoxigenic *E. coli*), but recently, *E. coli* of animal origin has been shown to be also associated with extra-intestinal infections, such as urinary tract infections [1]. In many cases, the origin of *E. coli* that

causes infection in humans remains unknown, and the significance of the animal reservoir of antimicrobial-resistant *E. coli* has not been quantified. However, treatment options in humans are compromised if the causative bacteria are already resistant to commonly used antimicrobial agents. Antimicrobial-resistant *E. coli* may be selected in the intestines of patients, especially immune-compromised patients (e.g., patients with cancer or who have undergone transplantation), who are often treated with several antimicrobial agents. Because of heavy use of antimicrobial agents in food animal production, bacteria originating from food animals frequently carry resistance to a range of antimicrobial agents, including those commonly used in humans. This article describes findings regarding *E. coli* from the animal reservoir that carry resistance to antimicrobial agents regarded as critically or highly important for human therapy and the spread of these bacteria or associated antimicrobial resistance genes from animals to humans, [2].

The predominant characteristic of β-lactam resistance in *E. coli* and other gram-negative bacteria is the production of β-lactamases, which are encoded on chromosomes or by plasmids [3]. The relatively narrow-spectrum β-lactamases are often called penicillinases or cephalosporinases. Others have a much broader spectrum, such as extended-spectrum β-lactamases (ESBLs), and can hydrolyze many different β-lactams. The majority of the ESBLs belong to the ambler class A and the Bush group 2be, and they are mainly composed of the TEM, SHV, OXA, and CTX-M enzymes [4]. Many studies have demonstrated the presence of ESBL-producing *E. coli* in animals and meat, most likely caused by the use of the third-generation cephalosporin ceftiofur in food animals. Since the late 1990s, ESBL-producing *E. coli* have been detected in retail meat and production animals in Europe, Asia, Africa, and the United States [5]. At 2006, Blanc *et al.* [6], detected CTX-M-14–, CMY-2–, SHV-2–, and TEM-52–producing *E. coli* isolates in animals in Spain. In 2006, Jensen *et al.* [7], isolated the first ESBL-producing *E. coli* isolates from meat in Denmark; the TEM-52-producing *E. coli* isolated from imported German beef.

As part of a national surveillance of antimicrobial susceptibility in Japan, ESBL-producing *E. coli* isolates were detected in fecal samples from broiler chickens; both CTX-M- and CMY-2-producing *E. coli* isolates were detected among the isolates [8].Yan *et al.* (2004)[9], isolated ESBL-producing *E. coli* from animal feces and retail ground meats and isolated ESBL-producing *E. coli* in specimens from outpatients with urinary tract infections in Taiwan. Similar CMY-2- encoding plasmids were found among *E. coli* isolates from humans and ground pork, indicating a community spread of the bla>CMY-2 gene.

In contrast to human antibiotic use, antibiotic use in the poultry industry is very high in the Sulemani/Iraq, [10]. The prevalence of ESBL-producing *E. coli* in broiler farms in Sulemania was 86.7% [11]. For these reasons the poultry industry has been considered a potential reservoir of ESBL-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated meat. The aim of this study was to determine the distribution of ESBL genes, plasmids and strain genotypes in *E. coli* obtained from chicken meat in the Sulemania and defined these as 'poultry associated' (PA). Subsequently, we quantified the proportion of *E. coli* isolates with PA related ESBL genes, plasmids and strains in a large and representative sample of clinical *E. coli* isolates from Sulemania workers in broiler farms.

II. Materials & Methods

A. *Sample from broiler farms*

Cloacal swabs were collected from 460 apparently healthy chickens at marketing age;

these samples were collected randomly from 92 broiler farms distributed around Sulemania province/Iraq, between April and October 2013.

Samples were transported in icebox within one hour of collection to the Microbiology laboratory of the college of Veterinary Medicine, University of Sulemania, where they were processed.

B. *Samples from human*

All workers or caretakers (human) in broiler farms were asked to participate in personal testing for ESBL producing *E. coli* carriage. Only 124 workers agreed to send urine samples.

C. *Bacteriological isolation*

Homogenized samples were inoculated onto MacConkey agar (LAB 2, idg®, Lancashire) plates and incubated at 37 °C for 24 hrs. The isolates of *E. coli* were identified by observing gross colony morphology using Eosin Methylene Blue (EMB) agar (LAB 61, idg®, Lancashire), Gram's staining properties and motility were tested as described by Calnek [12].

D. *Identification of isolates*

Isolates were subjected to different biochemical tests such as sugar fermentation test, Indole production test, Methyl-Red and Voges- Proskauer (MR-VP) test, Api E20 test following the standard methods described by Cowan [13]. These *E. coli* isolates were transferred to 2 ml Luria broth and incubated 37°C for 18–24 hours. One millilitre (1 ml) of this culture was added to 0.8 ml of sterile 80% glycerol in a sterile tube, vortexed and stored at -80°C [14], until used for β-Lactamase production tests and antimicrobial susceptibility tests.

E. *Antimicrobial susceptibility testing*

Susceptibility of all the isolates to different antibiotics was determined by the disk diffusion method, as recommended by the National Committee for Clinical Laboratory Standards. Antibiotics used in this study are Penicillin G (PEN 10 U), Ampicillin (AMP 10µg), Amoxicillin (AMX 10µg),, Ceftiofur (CEE 30µg), Ceftriaxone (CRO 30µg), Cefoperazone (CFP 75µg), Cefepime (FEP 30µg), Ceftazidime (CAZ 30µg), Cefpodoxime (CPD 10µg), Cefoperazone/ Tazobactam (CFP/TAZ 30/10 µg), Ceftiofur/ Sulbactam (CEE/SAM 30/10 µg), Amoxicillin-Clavulanic acid (AMC 20/10µg), Gentamicin (GEN10µg), Amikacin (AMK 30µg) , Florfenicol (F 30µg), Doxycycline (DOX 30µg), Trimethoprim -Sulfamethoxazole/ (SXT 1.25/ 23.75µg), Enrofloxacin (ENR 5µg), and Colstin (CT 10 µg). Isolates were sub-cultured onto Miller's LB agar and incubated for 18–24 h before being transferred to 5 ml sterile 0.9% saline to match the '0.5' MacFarland standard (BioMerieux SA, Marcy Etoile, France). A sterile cotton-tipped swab was used to streak air-dried Mueller-Hinton plates within 15 min of adjustment of turbidity. Subsequently, antimicrobial discs (Oxoid, UK), were added and plates were incubated aerobically at 35 ± 2°C for 16–18 h. The diameter of the zones of inhibition surrounding the antimicrobial discs was measured to the nearest mm. Isolates were deemed resistant only when the zone of inhibition was less than or equal to the resistance breakpoint recommended by the Clinical Laboratory Standards Institute guidelines [15].

F. *Detection of beta-lactamase production*

The production of β-lactamase enzymes of resistant isolated *E. coli* was tested by using the broth method. Two colonies of each of the test bacteria were picked from an overnight nutrient agar culture plate and inoculated into 1ml of sterile nutrient broth. Inoculated broths were appropriately labeled and incubated at 37°C for 18 hours. After incubation, four drops of nitrocefin® solution (Calbiochem, Germany), prepared as directed by the manufacturer, were added to the broth culture and observed for color change within 30 minutes. Nitrocefin ® is a chromogenic

cephalosporin that changes color from yellow to red on hydrolysis. Beta-lactamase production was inferred when the broth turned red within 30 minutes of addition of reagent.

G. *Identification of ESBL producers*

Extended spectrum Beta-lactamase (ESBL) producers were screened by a phenotypic confirmatory test using Cefotaxime (30µg), Ceftazidime (30µg), Cefotaxime /Clavulanate (2:1) (30/10 µg), and Ceftazidime /Clavulanate (2:1) (30/10 µg), according to CLSI recommendations [15]. These discs were placed on the surface of Mueller-Hinton agar that was inoculated with the standardized inoculums of the test bacteria. After 24 hours incubation at 37°C, the inhibition zones produced were measured. Each test was performed in duplicate and mean zone size was recorded to the nearest whole millimeter [16]. All ESBL-producing isolates were collected for further investigation.

H. *Plasmid profiling and transformations*

All isolates resistant to Ampcillin (CLSI interpretive zone ≤ 13 mm) were screened for plasmid content according to the alkaline-lysis mini Plasmid Qiagen kit, and resolved on 0.8% agarose gels and stained with ethidium bromide. The stained gel was examined under UV light to look for the presence of plasmid bands. Competent cells of *E. coli* DH5- alpha were prepared as mentioned by Sambrook & Russell [17]. A 5-10 µg of plasmid DNA were used. Competent cells cannot grow on antibiotic plates without a plasmid carrying the resistance gene so the negative control plate should not grow colonies. As a positive control we used 1-5 µg of a plasmid (e. g. ptz57) that have previously transformed this batch of competent cells efficiently. Transformants were analyzed on LB media containing 30, 50 or 100µg/ml ampicillin (Sigma-Aldrich) after incubation at 37°C for 18–24 hours.

I. *Characterization of genes encoding β-lactamases.*

Presence of beta-lactamase genes (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) was tested by PCR amplification, with using the primers listed in table (1). All PCR amplicons were verified by gel electrophoresis on a 1.0% (w/v) agarose gel and stained with ethidium bromide (0.5 µg ml−1).

Table 1: Primers used for PCR amplification of *bla* genes

Target	Primer name	Primer sequence (5′→3′)	Product size (bp)	GenBank accession no.
bla$_{TEM}$	TEM-F	TCG CCG CAT ACA CTA TTC TCA GAA TGA	445	AF332513
	TEM-R	ACG CTC ACC GGC TCC AGA TTT AT		
bla$_{SHV}$	SHV-F	ATG CGT TAT ATT CGC CTG TG	747	AY036620
	SHV-R	TGC TTT GTT ATT CGG GCC AA		
bla$_{CTX-M}$	CTX-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	AF252622
	CTX-R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

III.Results

A total of 311 *Escherichia coli* isolated from 460 healthy chickens at marketing age, and 56 *E. coli* isolates obtained from 124 human, were subjected to studies (between April and Octo-ber 2013). Isolated *E. coli* were gram negative bacilli and the results of gross colony morpho-logy on EMB agar and MacConkey agar, Gra-m's staining and motility tests are summarized in table (2).

Table 2: Identifying characteristics of *E. coli* isolated from human and healthy chickens.

Bacteria isolates	Morphology	Staining properties	Colony characterization		Motility
			MacConkey agar	EMB ager	
E. coli	Short rod, single, pair or in short chain	Gram negative	Bright pink or red color	Yellow green metallic sheen	+

For biochemical characterization, a series of biochemical tests selective for *E. coli* were performed with the suspected Gram-negative rod shaped bacteria. All the isolates fermented the five basic sugars producing acid and gas. All the isolates were Methyl Red was positive, Voges-Proskauer test was negative and Indole test was positive and its identification was confirmed by using Api E 20 system.

Among the 367 isolated *E. coli* from healthy chickens (311) and human (56), penicillin-G, ampicillin, amoxicillin, ceftiofur, ceftriaxone, cefoperazone, cefepime, ceftazidime and cefpodoxime had the highest number of resistant isolates.

Both the Healthy chickens and human isolates were sensitive to the Beta-Lactam inhibitors as follows: cefoperazone/ tazobactam (97.75%), ceftiofur/ sulbactam (90.35%) and amoxicillin/ clavulanic acid 77.8% for the healthy chickens, while the human isolates had 100% sensitivity for cefoperazone/ tazobactam and ceftiofur/ sulbactam and 71.4% of the isolates were sensitive to Amoxicillin/ clavulanic acid. Other results are stated in figure (1). Out of 311 isolated *E. coli* from healthy chickens only 264 (84.9%) produced Beta-lactamase while only 47 (15.1%) showed absence of the enzyme, while only 35 (62. 5%) of the human isolates were found to produce the enzyme Beta-lactamase from 56 isolated E. coli.

Out of 264 producers of β-lactamase, *E. coli* isolated from healthy chickens were tested for ESBL production, among them only 201 (76.1%) were ESBLs producers, while only 19 (54.3%) were ESBL producers from 35 β-lactamase producer of *E. coli* isolates from human.

Plasmids were detected in all ESBL producer- *E. coli* (201) isolated from healthy chickens which were highly resistant to Beta-lactam antibiotics, while plasmid was detected only in 15 (78.9%) from 19 ESBLs producer *E. coli* isolated from human . By transformation the extracted plasmids into *E. coli* DH5α; it was found that 165 (82.1%) Beta-lactamase gene from 201 plasmid isolated from healthy chickens were carried by plasmid, while 10 (66.6%) Beta-lactamase gene from 15 plasmid isolated from human were carried by plasmid. Among ESBL-producing *E. coli* isolated from healthy chickens and human were shown in table (3).

The majority of these ESBL genes (69.7%) were bla_{TEM} and (10.9%) were $bla_{\text{CTX-M}}$ genes in healthy chickens, while in human the majority (60%) of ESBL genes were bla_{TEM} and (20. %) were $bla_{\text{CTX-M}}$ genes. Eleven isolated from healthy chickens and one isolated from human did not contain bla_{TEM}, bla_{SHV} or $\text{bla}_{\text{CTX-M}}$ (figure 2).

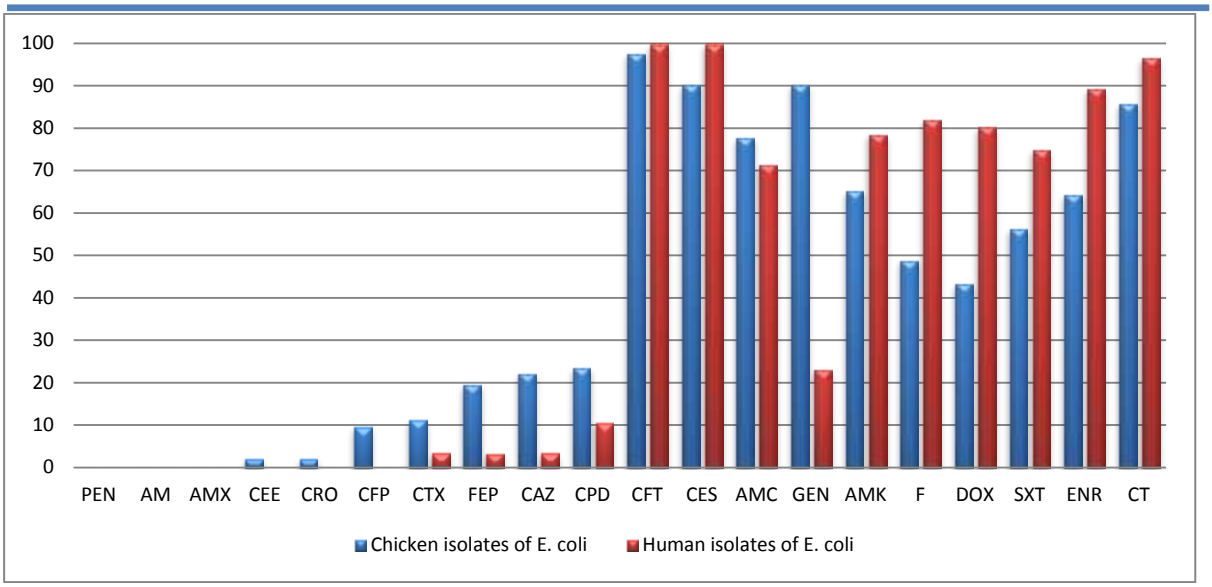

Fig. 1: Percentage of Antibiotic Susceptibility Pattern of the (311) healthy chickens and (56) human *E.coli* Isolates. PEN:Penicillin G (10 U), AMP: Ampicillin (10µg), AMX: Amoxicillin (10µg), CEE : Ceftiofur (30µg), CRO: Ceftriaxone (30µg), CFP: Cefoperazone (75µg), FEP: Cefepime (30µg), CAZ : Ceftazidime (30µg), CPD: Cefpodoxime (10µg), CFT: Cefoperazone/ Tazobactam (30/10 µg), CES : Ceftiofur/ Sulbactam (30/10 µg), AMC: Amoxicillin-Clavulanic acid (20/10µg), GEN: Gentamicin (10µg), AMK : Amikacin (30µg) , F : Florfenicol (30µg), DOX : Doxycycline (30µg), SXT : Trimethoprim –Sulfamethoxazole (1.25/ 23.75µg), ENR : Enrofloxacin (5µg), CT : Colstin (10 µg)

Table 3: Molecular characterization of bla genes among ESBL-producing *E. coli* isolates (165 Healthy chickens and 10 human)

***bla* gene**	**Healthy chickens(165)**	**Human (10)**
TEM	115 (69.7%)	6 (60%)
CTX-M	18 (10.9%)	2 (20%)
SHV	0 (0%)	0 (0%)
TEM-1 and CTX-M	21 (12.7%)	1 (10%)
Non-TEM, non- SHV and non-CTX-M	11 (6.7%)	1 (10%)

Fig. 2: Agarose gel electrophoresis showing the PCR amplified products of the ESBL gene.
Lanes M: DNA molecular weight marker (100 bp -3000 bp)
Lanes 1: Negative of bla_{SHV} gene.
Lanes 2 and 5: positive with the bla_{CTX-M} gene
Lanes 3 and 4: positive with the bla_{TEM} gene

IV. Discussion

Bacterial organisms producing extended spectrum B-lactamases (ESBLs) in human populations have been studied for over three decades and their presence in animal population has also been recently reported [18]. With the extensive use of β-lactam antibiotics in poultry such as amoxicillin and cephalosporins, especially extended-spectrum cephal-osporins, ESBL-mediated resistance in Gram-negative bacilli has become increasingly critical, and therapeutic options for such infections are becoming limited [2, 19]. It is important to detect ESBL producers in order to know the ESBL prevalence in animal-associated bacteria and to limit the spread of these Multi-drug resistance (MDR) organisms in veterinary settings [20, 21].

Because of the intensive use of antimicrobial agents in food animal production, meat is frequently contaminated with antimicrobial-resistant *E. coli*. Humans can be colonized with *E. coli* of animal origin, and because of resistance to commonly used antimicrobial agents, these bacteria may cause infections for which limited therapeutic options are available. This may lead to treatment failure and can have serious consequences for the patient. Furthermore, *E. coli* of animal origin may act as a donor of antimicrobial resistance genes for other pathogenic *E. coli*. Thus, the intensive use of antimicrobial agents in food animals may add to the burden of antimicrobial resistance in humans. Bacteria from the animal reservoir that carry resistance to antimicrobial agents that are regarded as highly or critically important in human therapy (e.g., aminoglycosides, fluoroquinolones, and third and fourth-generation cephalosp-orins) are of especially great concern.

In the present study, ESBL/Ampc - producing *E. coli* isolates were found in cloacal swabs from healthy chickens at all 92 Sulemani broiler farms tested. This is similar to the results of studies performed in Netherlands, Belgium and Spain, where

cloacal swabs tested positive for ESBL/ AmpC-producing *E. coli* isolated at 5/5 and 10/10 farms, respectively [22, 23]. It was found that, within any flock, the prevalence of birds carrying ESBL/AmpC-producing *E. coli* was very high. The studies performed in Belgium and Spain, which used less selective methods, found a lower prevalence in positive flocks (27%–75% based on 89–100 samples per farm in Belgium and 10%–100% based on 10 samples per farm in Spain). Data collected at slaughterhouses in Denmark and Sweden using selective pre-enrichment with cephalosporins at concentrations selective for ESBL/ AmpC producers 18 suggest that the prevalence among individual broilers in these northern countries was 27% and 34%, respectively, and was lower in the Netherlands. Likewise, broiler meat produced in Denmark is much less contaminated (3.3%–8.6%) with ESBL/ AmpC-producing isolates than imported broiler meat (36%–50%) and in comparison with data reported from the Netherlands [24, 25]. This clearly indicates differences in the prevalence of ESBL/AmpC-producing isolates in different countries.

In 2013 another survey showed that the prevalence of ESBL-producing animal-associated bacteria in Sulemania was 86.7 % [11]. These results indicate that the incidence of ESBLs in bacteria isolated from broilers in Sulemania was high. Compared with other countries, Sulemania uses more antibiotics per kg live weight of food-producing animals [10].

As long ago as 1976, Levy *et al*., [26], showed that resistant bacteria and plasmids bearing resistant genes could be transferred from chicken to chicken and from chicken to humans. Our data confirm the hypothesis that Sulemania farmers are at higher risk than the general Sulemania population of carrying ESBL/AmpC-producing isolates in their gastrointestinal tract, because the prevalence in healthy chickens was 76.1%, compared with 54.3% in human [24]. Colonization could have resulted from contact with the broiler isolates; Levy *et al.* [26], suggested that colonization occurs through inhalation into the nasal passages and thence via the sputum to the gastrointestinal tract.

There are indications that resistant bacteria can be transferred from animals to humans via the food chain [27, 28]. Therefore, a high prevalence of animals carrying ESBL/AmpC-producing isolates is not desirable [29].

In our study it was found that among 264 β-lactamase producer of *E. coli* isolated from healthy chickens only 201 (76.1%) were ESBLs producers, while only 19 (54.3%) were ESBLs producers from 35 β-lactamase producer of *E. coli* isolated from human. Plasmids were detected in all ESBLs producer- *E. coli* (201) isolated from healthy chickens which were highly resistant to Beta-lactam antibiotics, while plasmid was detected only in 15 (78.9%) from 19 ESBLs producers of *E. coli* isolated from human. By transformation the extracted plasmids into *E. coli* DH5α was found that 165 (82.1%) Beta-lactamase genes from 201 plasmid isolated from Healthy chickens were carried by plasmid, while 10 (66.6%) Beta-lactamase gene from 15 plasmid isolated from human were carried by plasmid.

The majority of these ESBL genes (69.7%) were *bla*TEM and (10.9%) were *bla*CTX-M genes in healthy chickens, while in human the majority of ESBL genes were (60%) *bla*TEM and (20. %) were *bla*CTX-M genes (figure 4). Eleven isolates from healthy chickens and one isolate from human did not contain *bla*TEM, *bla*SHV or *bla*CTX-M, see; table 3, further studies will be needed to look for other ESBL genes in these isolates. None of the isolates contained the *bla*SHV gene. These findings demonstrate that *bla*TEM and *bla*CTX-M genes are the two dominant types in ESBL producing healthy chickens and human isolates and that the *bla*TEM type β-lactamase is playing an increasing role in antibiotic resistance in Sulemania. These findings are suggestive for transmission of ESBL-producing *E. coli* from

poultry to humans, most likely through the food chain.

Our findings suggest a relationship between contamination of chicken meat with drug-resistant bacteria and appearance of ESBL genes in humans in the Sulemania/ Iraq. This relationship was further supported by genomic comparison of strains detected in chicken meat with those detected in human urine specimens.

This indicated that TEM-1 is the most common β-lactamase among *E. coli* isolated from chickens in Sulemania.

TEM-1, the most prevalent β-lactamase - encoded enzyme in human clinical isolates worldwide [30, 31, 32, 33], is not classified as an ESBL. However, several TEM-1 derivatives confer ESBL properties [34].

V. Conclusion:

We have reported the first extensive study of the prevalence and molecular characterization of ESBL-producing E. coli isolated from chickens in Sulemania Province, Iraq. This study clearly indicated that TEM-1 and CTX-M are widespread in this region. The results of this study should increase awareness of the potential risk that these bacteria pose to human health (by transmission to humans via direct contact or via the food chain). The effects of measures to reduce antibiotic use in broiler production should be investigated. Further investigation is needed to assess the role of recirculation of strains on farms and to evaluate the effect of improved infection control at farms and hatcheries. This approach will hopefully lead to a reduction in the burden of multidrug-resistant isolates in broilers, the food chain and the farm environment.

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