

Antibiotic susceptibility and high prevalence of extended spectrum beta-lactamase producing *Escherichia coli* in Iranian broilers

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SUMMARY

Extended-spectrum β -lactamase (ESBL) producing *Escherichia coli* have rapidly spread worldwide and cause serious threats for public health. The study was conducted to determine the antibiotic resistance and characterization of ESBL producing *E. coli* strains isolated from broilers in Northern Iran. Antibiotic susceptibility test was done for a total of 100 isolates of *E. coli*, recovered from 240 broiler fecal samples at the slaughterhouse stage. ESBL production was screened using double-disc synergy test (DDST) and presence of four ESBL genes including bla_{PER} , bla_{VEB} , bla_{TEM} and bla_{CTX-M} was tested using PCR. Among 100 strains isolated from broilers, 53% were identified as ESBL-producing *E. coli*. All (100%) ESBL positive isolates were typed according to the presence of one or two ESBL-associated genes. The most prevalent gene among ESBLs was CTX-M (60.3%) and the PER gene was not present among isolates. All isolates in this study were resistant to colistin and nalidixic acid but were 100% sensitive to cefalexin and furazolidone. The results demonstrated the high prevalence of antibiotic resistant and ESBL producing *E. coli* among broilers which representing the risk of increasing these strains in human infections associated with food animals.

Keywords: *E. coli*, Antibiotic susceptibility, ESBL genes, PCR, Broilers.

RÉSUMÉ

Sensibilité aux antibiotiques et prévalence élevée d' *Escherichia coli* porteurs de bêta lactamases à large spectre chez les poulets en Iran.

Des *Escherichia coli* porteurs de bêta-lactamases à large spectre (Extended-spectrum β -lactamase, ESBL) se propagent rapidement dans le monde entier et causent de graves menaces pour la santé publique. Cette étude a été menée afin de déterminer la résistance aux antibiotiques et caractériser la production d'ESBL par des *E. coli* isolés de poulets de chair dans le nord de l'Iran. Un test de sensibilité aux antibiotiques a été fait sur un total de 100 isolats obtenus à partir de 240 échantillons fécaux de poulets de chair à l'abattoir. La production d'ESBL a été criblée en utilisant un test de synergie à deux disques et en recherchant par PCR la présence de quatre gènes de résistance, dont bla_{PER} , bla_{VEB} , bla_{TEM} et bla_{CTX-M} . Parmi les 100 souches isolées de poulets de chair, 53% ont été identifiées comme *E. coli* producteurs d'ESBL. Tous les isolats producteurs d'ESBL ont été typés pour la présence d'un ou deux gènes. Le gène le plus répandu était CTX-M (60,3%) alors que le gène PER n'était pas présent parmi les isolats. Tous les isolats de cette étude étaient résistants à la colistine et l'acide nalidixique, mais étaient 100% sensibles à la céfalexine et furazolidone. Ces résultats ont démontré la haute prévalence des souches résistantes aux antibiotiques *E. coli* producteur d'ESBL chez les poulets de chair en Iran.

Mots-clés : *E. coli*, sensibilité, antibiotiques, résistance, bêta-lactamase, PCR, poulet.

Introduction

E. coli strains can cause a lot of bacterial infections including gastroenteritis, urinary tract infections, neonatal meningitis, hemolytic uremic syndrome, mastitis and septicemia and for this reason they are considered as the most common pathogen of bacterial diseases worldwide. One of the serious concerns of clinical and veterinary medicine is antibiotic resistance observed from different bacteria [1]. Bacterial strains use several mechanisms to acquire resistance against common used antimicrobials, among them, production of extended spectrum β -lactamases (ESBLs) considered as the main mechanism [22]. ESBLs were first reported in 1983 and have been identified worldwide in a number of different organisms such as *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Morganella morganii*, *Serratia marcescens*, *Shigella dysenteriae*, *Pseudomonas aeruginosa* and *Salmonella* species [2]. These enzymes cause resistance to extended spectrum cephalosporins and monobactams but

do not affect carbapenems and cephamycins [2, 11]. There are different kind of ESBLs namely SHV, TEM, CTX-M, VEB and PER. The TEM1 enzyme was first found in *E. coli* isolates and now is considered as the most common β -lactamase produced by *Enterobacteriaceae* [22]. The CTX-M family is considered as a rapidly growing family of ESBLs that tends to hydrolyze cefotaxime rather than ceftazidime [19]. *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates produce VEB-1 ESBL and it was first reported from Thailand and the widespread presence of VEB-1 in mentioned bacteria suggests that this gene may exist in numerous gram negative species [8, 10]. The PER family, first reported in *P. aeruginosa* and manifests ESBL like activities. Although the predominance of ESBLs is not known, it is clearly increasing in different origins so, the aim of this study was to investigate the antibiotic susceptibility, prevalence of ESBL producing strains and occurrence of ESBL associated genes (TEM, CTX-M, VEB and PER) among *E. coli* isolates recovered from broiler fecal samples in northern Iran.

Materials and methods

SAMPLING PROTOCOL AND BACTERIAL STRAINS

During January to March 2014, a total of 240 fecal samples were collected from cloaca of healthy slaughtering broilers in three slaughterhouses in ten visits. Fecal samples were collected in TSB broth tubes using sterile swabs and taken to the microbiological laboratory, School of Veterinary Medicine, Amol University of Special Modern Technologies, in less than 6 hrs. Fecal swabs were cultured on MacConkey and EMB agar (Himedia, India) and incubated at 37°C for 18-24 h. Indole, methyl red, Voges-Proskauer reaction and Simons citrate tests were performed with the colonies that showed growth features of *E. coli*. Identified isolates were subjected to the antimicrobial and molecular procedures.

ANTIMICROBIAL DRUG SUSCEPTIBILITY TESTING AND ESBL DETECTION

Antimicrobial drug susceptibility was assessed by disc-diffusion method on Mueller-Hinton (MH) agar plates (Merck, Germany), according to the antibiogram standard methods [5]. Tested antimicrobial agents were as followed: Colistin (10µg), ciprofloxacin (5µg), nalidixic acid (30µg), ampicillin (10µg), sulfamethoxazole-Trimethoprim (30µg), gentamicin (10µg), cephalotin (30 µg), cefexime (30 µg), cefalexin (30µg), cefotaxime (30µg), Furazolidone (30µg), Florfenicol (30µg), Erythromycin (15µg) and Nitrofurantoin (50µg). Quality control was carried out using the reference strain *E. coli* ATCC 25922. To screen ESBL production a double-disc synergy test (DDST) was used as a standard

disc-diffusion assay on MH agar. For this purpose, Discs containing ceftazidime (30 mg) and cefotaxime (30 mg) were placed at a distance of 30 mm (centre to centre) around a disc containing cefotaxime/clavulanic acid (20/10 mg) and ceftazidime/clavulanic acid (20/10 mg). Isolates that showed difference in the inhibition zone more than five mm, were considered as ESBL producing strains [5].

DNA PREPARATION

A loopful colony of each isolate on agar plate was picked and then suspended in 200 µl of distilled deionized water. After vortexing, the suspension was boiled for 5 min, and 50 µl of the supernatant was collected after spinning for 10 min at 14,000 rpm in a micro centrifuge. The DNA concentration of boiled extracts was determined with spectrophotometer [16].

PCR ASSAY

In the present study, PCR amplifications were performed in a final volume of 25 µL in PCR tubes. The PCR reaction mixtures consisted of 2 µL of the DNA template, 2.5 µL 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), (CinnaGen, Iran), 1 µL dNTPs (50 µM), (CinnaGen, Iran), 1 µL (1U Ampli Taq DNA polymerase), (CinnaGen, Iran), 1 µL (25 pmol) from the forward and reverse primers (CinnaGen, Iran), of both primer pairs (Table I) and the volume of the reaction mixture was completed to 25 µL using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under the following specifications: Initial denaturation at 94°C for

Primer	Sequence (5' to 3')	Target gene	Annealing temperature	Product size (bp)	Reference
CTX-1-un-F CTX-1-un-R	CATGTGCAGYACCAGTAA CCGRATATCRTTGGTGGTG	<i>bla</i> _{CTX-M-1} cluster	42°C	544	[21]
PERF PERR	ATGAATGTCATTATAAAAAGC AATTTGGGCTTAGGGCAGAA	<i>bla</i> _{PER}	47°C	925	[10]
VEBF VEBR	CGACTTCCATTCCCAGATGC GGACTCTGCAACAAATACGC	<i>bla</i> _{VEB}	51°C	643	[17]
OT3 OT4	ATGAGTATTCAACATTTCGG TTACCAATGCTTAATCAGTGA	<i>bla</i> _{TEM}	46°C	858	[7]

TABLE I: Nucleotide sequences used as primers in the PCR reactions.

Strains (No)	Antibiotic resistant pattern (%)													
	CL	CP	NA	AM	SXT	GM	CF	CFM	CN	CTX	FR	FF	E	FM
Total (100)	100 (100)	39 (39)	100 (100)	90 (90)	86 (86)	36 (36)	100 (100)	0 (0)	23 (23)	0 (0)	0 (0)	72 (72)	87 (87)	20 (20)
ESBLs (53)	53 (100)	30 (56.6)	53 (100)	53 (100)	42 (79.2)	19 (35.8)	53 (100)	0 (0)	20 (37.7)	0 (0)	0 (0)	33 (62.2)	50 (94.3)	28 (52.8)

CL, Colistin; CP, Ciprofloxacin; NA, Nalidixic acid; AM, Ampicillin; SXT, Sulfamethoxazole-trimethoprim; GM, Gentamycin; CF, Cephalotin; CFM, Cefexime; CN, Cefalexin; CTX, Cefotaxime; FR, Furazolidone; FF, Florfenicol; E, Erythromycin; FM, Nitrofurantoin.

TABLE II: Antimicrobial resistance of *E. coli* strains isolated from broiler fecal samples.

5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing as shown in Table I for 1 min and extension at 72°C for 1 min. Final extension was carried out at 72°C for 7 min and the PCR products were stored in the thermal cycler at 4°C until they were collected. Then amplified products were separated by electrophoresis in 1.2% agarose gel stained with ethidium bromide. Visualization was undertaken using a UV transilluminator and the 100 bp, DNA ladder was used as molecular size marker.

Results

In this study a total of 100 *E. coli* isolates were recovered and distinguished from 240 randomly collected broiler fecal samples. The isolated organisms were identified for their antibiotic resistance and presence of *PER*, *VEB*, *CTX-M* and *TEM* ESBL-associated genes. Antimicrobial resistance pattern of isolated organisms are shown in table II. All 100 isolates in this study were resistant to colistin and nalidixic acid but were 100% sensitive to cefalexin and furazolidone. Among the isolates, 90, 87, 86 and 72% showed relatively high level of resistance to ampicillin, erythromycin, sulfamethaxazole and florfenicol respectively. Fifty three percent of all *E. coli* isolates were ESBL producing. PCR analysis was performed and agarose gel electrophoresis of the mentioned genes obtained from ESBL producing *E. coli* are shown in Figures 1, 2 and 3. The rate of the distribution of ESBL genes is presented in table III. The occurrence of the *CTX-M*, *TEM* and *VEB* genes among ESBL isolates were, 60.3% (32/53), 37.7% (20/53) and 13.2% (7/53) respectively. The *PER* gene was not present. Among ESBL strains 6 (11.2%) showed multiple presence of ESBL associated genes (Table III).

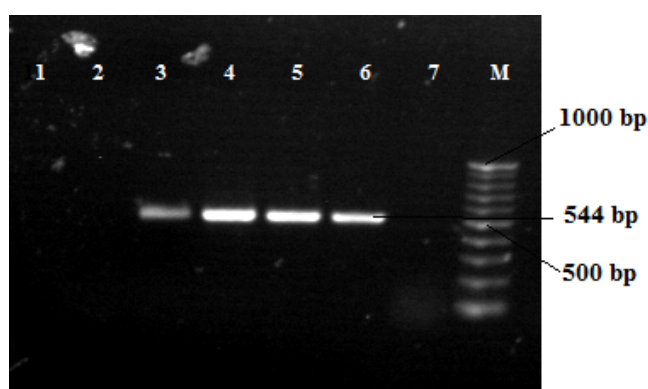


FIGURE 1: Agarose gel electrophoresis of CTX-M PCR products. Lanes 1-2: Negative samples; Lanes 3-6: Positive samples; Lane 7: negative control; Lane M: the 100 bp DNA marker.

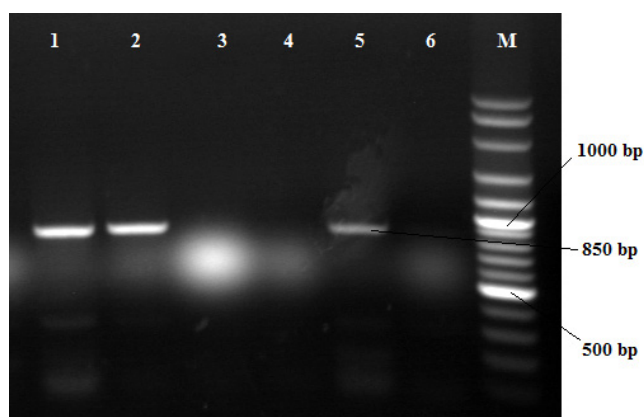


FIGURE 2: Agarose gel electrophoresis of blaTEM PCR products. Lanes 1-2: Positive samples; Lanes 3-4: Negative samples; Lane 5-6: Positive and negative controls; Lane M: the 100 bp DNA marker.

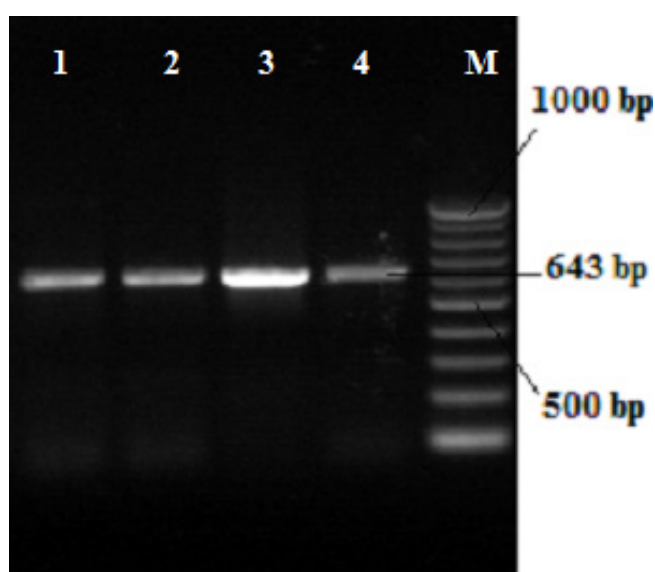


FIGURE 3: Agarose gel electrophoresis of VEB gene PCR products. Lanes 1-4: Positive samples; Lane M: the 100 bp DNA marker.

Discussion

It has been proved that among gram negative pathogens such as *Enterobacteriaceae* family, resistance to an extended spectrum β -lactamase is widely associated with ESBLs. For this reason accurate identification and isolation of such microorganisms is a great challenge for clinical associated laboratories that can use both phenotypic and genotypic tests for detection of genes related with β -lactamase production [4, 13]. ESBL producing *E. coli* has been widely isolated from different animals. The first CTX-M producing *E. coli* in healthy chickens and cattle was reported in 2001 in Spain and Japan respectively [18, 23]. Afterward this organism has been isolated from wide range of animals and their related

Number of isolates	ESBL producing (%)	Positives for ESBL genes (%)					
		PER	VEB	CTX-M	CTX-M+VEB	CTX-M+TEM	TEM
100	53 (43)	0 (0)	4 (7.5)	26 (49)	3 (5.6)	3 (5.6)	17 (32)

TABLE III: Occurrence of ESBL producing *E. coli* and ESBL-associated genes among broiler fecal isolates.

products including chicken meat samples in Tunisia, cattle and poultry samples in France, broiler chickens in Japan and Belgium [12, 15, 24, 25]. ESBL *E. coli* has become widespread in humans since their first appearance in 1989 and it is important to take attention to possibility relation of this bacterium between animals and human [18]. Refer to published reports, the prevalence of ESBLs producing *E. coli* is in a range of 5% in Japan to 50% in other Asian countries and in Europe, the prevalence varies from 3% in Sweden to 34% in Portugal [3, 4, 26]. There are some reports on prevalence of *E. coli* carrying ESBLs from pets and wild animals but to our knowledge, there are limited investigations on prevalence of ESBLs producing *E. coli* from chickens and poultry fecal samples. CORTES *et al.* [6] after isolation and characterization of potentially pathogenic antimicrobial resistant *E. coli* strains from chicken and pig farms in Spain reported that isolates from poultry and pig farms differ with respect to ESBL associated genes, serotypes and virulence genes and in their study the isolates from poultry are potentially more pathogenic for humans than those from pigs. In another study GIRLICH *et al.* [9] after detection of genes encoding extended spectrum β -lactamase CTX-M-1 in 12 *E. coli* isolates, reported a widespread diffusion of CTX-M-1 in *E. coli* in food producing poultry in France. RANDALL *et al.* [18] reported that the prevalence of *E. coli* carrying ESBLs from broiler chickens and turkeys in Great Britain between 2006 and 2009 were 54.5% in the broiler abattoirs and 3.6% in individual broiler fecal samples and they concluded that poultry derived CTX-M *E. coli* in Great Britain are different from major CTX-M sequence types causing disease in humans. To the best of our knowledge, epidemiologic data and characterization of ESBL producing *E. coli* in Iran are not documented completely. According to results of present study, high occurrence of ESBL *E. coli* strains was obtained (53%) relatively. Although this result is lower than those of RANDALL *et al.* [18] those ESBLs producing *E. coli* was 54.5% of the broiler samples. This study reports a high dissemination of CTX-M gene (60.3%) in ESBL producing *E. coli* strains. There are several studies that reports CTX-M as the main gene responsible for antibiotic resistance of ESBLs *E. coli* [6, 18]. The VEB β -lactamase gene was detected in 7.5% (4/53) of ESBL positive *E. coli* strains. Interestingly, the PER gene was not detected among 100 *E. coli* isolates. As can be seen in Table III the presence of the TEM gene was more frequent in comparison with PER and VEB genes and although we did not investigate the presence of other ESBL-associated genes like SHV which were predominant in past decades, it seems that in this sampling area the CTX-M and TEM genes manifested essential role in creating antibiotic resistance of ESBLs *E. coli*. Three (5.6%) of total 53 ESBL positive samples had both CTX-M and VEB and three (5.6%), had CTX-M and TEM together and it seems that they formed relatively high portion of isolated *E. coli* strains. The epidemiological distribution of ESBLs associated genes is different. It has been documented that PER-1 are detected more in isolates in Turkey, France and Italy [20]. VEB-1 and VEB-2 are identified in strains from Southeast Asia and CTX-M gene, the dominant non-TEM

ESBL among *Enterobacteriaceae*, have been detected in wide epidemiological area and have disseminated in all countries as a result of epidemic plasmids and/or particular epidemic strains [14, 20]. Results of antimicrobial susceptibility testing showed that isolates from broiler samples manifested high resistance to colistin, nalidixic acid and cephalotin with 100% resistance. As presented in Table II, tested isolates were completely resistant to first generation cephalosporin (cephalotin) and interestingly showed full susceptibility to third generation cephalosporins (cephotaxime and cefexime). Resistance to these antibiotics can be related to other ESBL-associated genes. All cefalexin resistant isolates were ESBL and there were no significant differences between ESBLs and non-ESBL isolates in antibiotic susceptibility testing. These results revealed that replacing of first generation cephalosporins with third generation ones can help to reduce the ESBLs producing *E. coli* infections in this region of Iran. In conclusion, we report the relatively high prevalence and molecular properties of ESBL genes and the epidemiological characteristics of ESBLs producing *E. coli* in Iran. Data would suggest that *bla*_{CTX-M} to be the most prevalent ESBLs gene and antibiotic susceptibility testing showed that isolates had high rate of resistance against the colistin, nalidixic acid and cephalotin.

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