Eurasian Journal
of Veterinary Sciences

www.eurasianjvetsci.org

RESEARCH ARTICLE

Phenotypic and molecular identification of extended-spectrum beta-lactamases in *Escherichia coli* in healthy broilers at the west region of Iran

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Received:18.11.2017, Accepted: 05.06.2018
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İran'ın batı bölgesinde sağlıklı broylerlerdeki *Escherichia coli*'lerde geniş spektrumlu beta laktamazların fenotipik ve moleküler identifikasyonu

Eurasian J Vet Sci, 2018, 34, 3, 178-184
DOI: 10.15312/EurasianJVetSci.2018.198

Öz

Amaç: İnsana yayılma olasılığı yüksek olduğu için kümes hayvanlarında genişlemiş spektrumlu beta-laktamaz (GSBL) genlerinin fekal taşıyıcılarının varlığı endişe verici bir durumdur. Bu çalışma, İran'ın batısında sağlıklı broyler bağırsağından izole edilen *Escherichia coli*'deki ESBL taşıyıcılarının sıklığını tahmin etmek için yapılmıştır.

Gereç ve Yöntem: Görünüş olarak sağlıklı broilerden toplam 300 dışkı örneği toplandı. *E. coli* suşları izole edildi, moleküler olarak doğrulandı ve disk kombinasyon yöntemi, antimikrobiyal duyarlılık testi ve ESBL-saptama polimeraz zincir reaksiyonları (PZR) kullanılarak incelendi.

Bulgular: 285 *E. coli* suşu, biyokimyasal olarak izole edildi ve hepsi, türe özgü bir PZR reaksiyonuyla doğrulandı. Fenotipik yöntemle saptanan 156 ESBL üreten *E. coli* üzerinde antimikrobiyal duyarlılık testi, bunların % 100'ünün en az bir antibiyotiğe dirençli olduğunu, bunların hepsinin de imipeneme duyarlı olduğunu ortaya koymuştur. BlaTEM, blaCTX-M ve blaSHV genlerinin prevalansı sırasıyla% 52.63,% 38.59 ve% 30.17 idi. Birkaç ESBL geninin eşzamanlı varlığı, izolatların % 52.61'inde tespit edilmiştir.

Öneri: Kümes hayvanlarında çoklu dirençli ESBL taşıyıcılarının yaygınlığı, halk sağlığına yönelik bir tehdittir. Bölgedeki kümes hayvanları endüstrisinde tutarlı izleme ve uygun antibiyotik kullanımı uygulanmalıdır.

Anahtar kelimeler: Antibiyotik direnci, ESBL üreten *Escherichia coli*, İran

Abstract

Aim: The presence of fecal carriers of extended-spectrum beta-lactamase (ESBL) genes in poultry is worrisome because it is likely to spread to a human being. This study was proposed to estimate the frequency of ESBLs carriers in *Escherichia coli* isolated from healthy broiler's gut in west of Iran.

Materials and Methods: A total of 300 fecal samples were collected from apparently healthy broiler. *E. coli* strains were isolated, confirmed molecularly and studied using disk combination method, antimicrobial susceptibility test and ESBL-detection polymerase chain reactions (PCR).

Results: Two hundred and eighty five strains of *E. coli* were isolated biochemically and all were confirmed in a species-specific PCR reaction. Antimicrobial susceptibility test on 156 ESBL-producing *E. coli* identified by phenotypic method revealed that 100 % of them were resistant to at least one antibiotic, while all were sensitive to imipenem. The prevalence of blaTEM, blaCTX-M, and blaSHV genes was 52.63%, 38.59%, and 30.17% respectively. The simultaneous existence of several ESBL genes was identified in 52.61% of the isolates.

Conclusions: The vast prevalence of ESBL carriers with multiple resistance in poultry is a threat to public health. Consistent monitoring and proper use of antibiotics in the region's poultry industry should be applied.

Keywords: Antibiotic resistance, ESBL-producing *Escherichia coli*, Iran

Introduction

Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, especially *E. coli*, cause serious problems for public health throughout the world due to its rapid emergence and widespread. ESBLs determinants are resistant to most of the beta-lactam antibiotics including the third and fourth generation of cephalosporins, and the monobactam aztreonam (Dierikx et al 2013). Bacteria harboring these genes have posed grave challenges in effective treatments due to the great importance of cephalosporins in medicine (Pitout and Laupland 2008). In addition, ESBL-producing strains show multiple resistance to typically recommended antimicrobial agents in the treatment of infections such as sulfonamides, aminoglycosides, and quinolones. This complicates the therapeutic strategies in many cases. ESBL-encoding genes are located on plasmids. This tends to complicate ESBL-producing *E. coli* epidemiology following horizontal transduction of plasmid-borne genes inside and among different strains (Bradford 2001).

High consumption of antibiotics is considered as a risk factor in the acquisition of bacteria containing ESBL. In Iran, antibiotics are prescribed at an abundance and uncontrolled. As a matter of fact, antibiotics are also sold without prescription in veterinary medicine (Aalipour et al 2010). The presence of ESBLs genes and/or resistant bacteria in poultry has been highly reported (Borjesson et al 2013; Bortolaia et al 2011; Laube et al 2013). Therefore, poultry may be a possible source of human infection through food-chain contamination or direct contact (Pitout and Laupland 2008). ESBL genes bear wide geographical differences around the world. So far, no information is available with regard to the frequency of ESBL-producing *E. coli* in the west of Iran, a region with a high human and industrial poultry farms density. Hence, the present research was aimed at studying the prevalence of ESBLs in commensal *E. coli* isolates of poultry in west of Iran.

Materials and methods

Sample screening

Three hundred fecal samples from 15 birds in 20 different industrial poultry farms were collected throughout in west of Iran, from October to December 2016. All samples were taken in sterile conditions from apparently healthy broiler and immediately after the slaughter at an abattoir. MacConkey agar (Merck, Germany) plates were inoculated by the fecal samples and incubated for 18-24 hours at 37 °C. A pink colony susceptible to *E. coli* was randomly selected and identified by biochemical tests including IMViC and TSI (Rayamajhi et al 2008).

Phenotypic detection of extended-spectrum beta-lactamase (ESBL)

ESBL determinants were identified through the method of confirmatory combination disk by using the discs of ceftazidime (30 µg), ceftazidime /clavulanic acid (30/10 µg) cefotaxime (30 µg), and cefotaxime/clavulanic acid (30/10 µg),

Table 1. Primers used in this study

Primer	Sequence (5'→3')	Amplified gene	Product size	Reference
Eco 2083	GCT TGA CAC TGA ACA TTG AG	species-specific	662 bp	(Riffon et al
Eco 2745	GCA CTT ATC TCT TCC GCA TT			2001)
TEM-F	ATCAGCAATAAACCAGC	blaTEM	516 bp	(Mabilat et al
TEM-R	CCCCGAAGAACGTTTTC			1999)
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	blaCTX-M	544 bp	(Edelstein et al
CTX-M-R	CGATATCGTTGGTGGTGCCATA			2003)
SHV-F	AGGATTGACTGCCTTTTTG	blaSHV	392 bp	(Colom et al
SHV-R	ATTTGCTGATTTCGCTCG			2003)

Table 2. PCR conditions used in this study

Target gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension
species-specific	94 °C for 4 min	94 °C for 45 s	57 °C for 1 min	72 °C for 2 min	72 °C for 10 min
BlaCTX-M	94 °C for 5 min	94 °C for 20 s	51 °C for 30 s	72 °C for 30 s	72 °C for 10 min
blaTEM and blaSHV	94 °C for 5 min	94 °C for 30 s	54 °C for 30 s	72 °C for 1 min	72 °C for 10 min

Table 3. Prevalence of ESBL genes among ESBL-producing *E. coli* and total *E. coli* isolates

Name of bla gene	No. of bla gene	Percent of bla gene among ESBL-producing E. coli isolates (n=156)	Percent of bla gene among E. coli isolates (n=285)
blaTEM	150	96.15%	52.63%
blaCTX-M	110	70.51%	38.59%
blaSHV	86	55.12%	30.17%

Table 4. Distribution of ESBL genotypes among ESBL-producing *E. coli* and total *E. coli* isolates

ESBL genotype	No. of isolates	Percent of isolates among ESBL-producing *E. coli*	Percent of isolates among total *E. coli*
blaTEM	-	-	-
blaCTX-M	4	2.56%	1.4%
blaSHV	2	1.28%	0.7%
blaTEM + blaCTX-M	66	42.30%	23.15%
blaTEM + blaSHV	44	28.20%	15.43%
blaCTX-M + blaSHV	-	-	-
blaTEM + blaCTX-M + blaSHV	40	25.64%	14.03%

(Mast Co. UK) (CLSI, 2012). The phenotypic diagnosis of the presence of ESBLs shows the increase of ≥ 5 mm in the diameter of the growth inhibitory zone of the ceftazidime/clavulanic acid and cefotaxime/clavulanic acid disks comparing with the ceftazidime and cefotaxime disks alone. Klebsiella pneumoniae ATCC 700603 was used as a positive control and *E. coli* ATCC 25922 as a negative control.

Antibiotic sensitivity testing

The Kirby-Bauer disc diffusion method was performed to determine antimicrobial susceptibility of the isolates as recommended by the Clinical and Laboratory Standards Institute (CLIS, 2012). *E. coli* ATCC 25922 was used as a quality control microorganism in the antibiogram test. The standard used antibiotic disks including amikacin (30 µg), imipenem (10 µg), chloramphenicol (25 µg), gentamicin (10 µg), nitrofurantoin (10 µg), norfloxacin (10 µg), ampicillin (10 µg), cefotaxime (30 µg), ceftiofur (10 µg), ceftriaxone (10 µg), and ceftazidime (30 µg) were prepared from Patanteb Co. Iran.

DNA extraction and molecular identification of E. coli

DNA of isolates carrying ESBLs were extracted from the overnight culture of the isolates in Luria-Bertani broth (Merck, Germany) through boiling method. Universal Eco 2083 and Eco 2745 primers were applied for molecular confirmation of the isolates (Table 1) (Riffon et al 2001). PCR reaction was prepared in a final volume of 25 µL comprising 12.5 µL 2X PCR master mix (CinnaGen, Iran), 20 ng of the template DNA and 0.7 µM of each primers. *E. coli* ATCC 25922 was used as positive control. The used thermal condition is presented in Table 2.

The *E. coli* isolates were screened in three single PCR reactions using specific primers for each of the blaTEM, blaCTX-M, and blaSHV genes (Table 1). Each PCR reaction was performed in a 25 µL volume containing 12.5 µL 2X PCR master mix (CinnaGen, Iran), 50 ng of the template DNA and 0.4 µM of each primers. The used PCR reaction conditions are represented in Table 2.

Results

Out of 300 fecal samples, 285 strains of *E. coli* were isolated and identified by the phenotypic method. In phenotypic confirmatory test, 156 (54.73%) isolates were identified as ESBL-producing E .coli, which were found on all farms. In species-specific PCR method, a 662 bp band was generated in all of the isolates as their molecular confirmation (Figure 1). blaTEM revealed itself as the dominant gene with a ratio of 150 to 156 ESBL producers (96.15%). BlaCTX-M and blaSHV were present in 110 (70.51%) and 86 (55.12%) isolates from a total of 156 ESBL-producing *E. coli*, respectively (Table 3) (Figure 2, 3, and 4). Coexistence of ESBL genes was observed in 150 (96.15%) isolates, while 6 (3.84%) isolates carried only one ESBL gene (Table 4).

Of the 156 ESBL-harboring *E. coli*, 100% of them were resistant to at least one antibiotic component. All of them

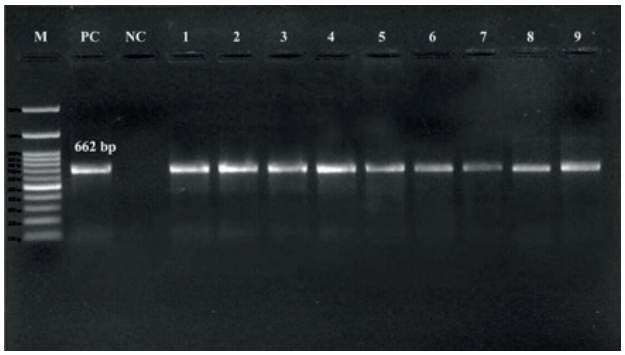

Figure 1. Agarose gel electrophoresis of PCR products with *E. coli* species-specific primers (662 bp). M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (*E. coli* ATCC 25922). NC: negative control. Lanes 1-9: field samples.

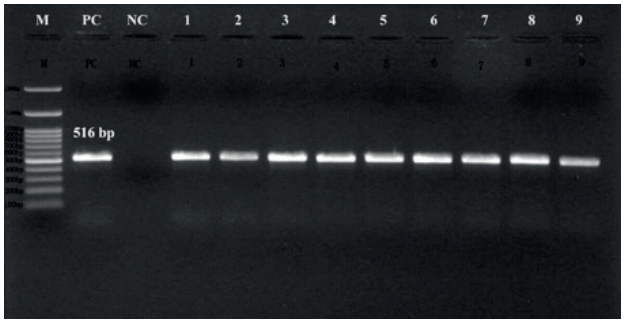

Figure 2. Agarose gel electrophoresis of blaTEM gene PCR products. Lane M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (*E. coli* PTCC 1533). NC: negative control. Lanes 1-9: Representative *E. coli* isolates with PCR products of approximately 516 bp.

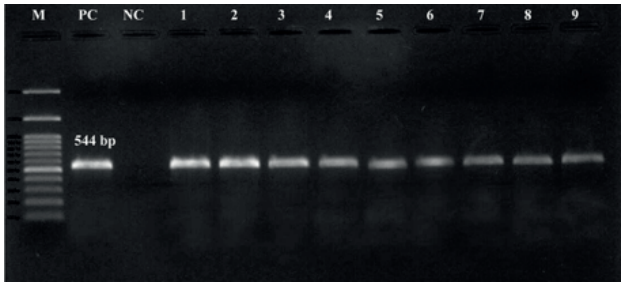

Figure 3. Agarose gel electrophoresis of blaCTX-M gene PCR products. Lane M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (*E. coli* PTCC 1533). NC: negative control. Lanes 1-9: Representative *E. coli* isolates with PCR products of approximately 544 bp.

were resistant to ampicillin, cefotaxime, and ceftriaxone, while sensitivity to imipenem was observed in 100% of the isolates. In addition, 123 (78.84%) and 96 (61.53%) isolates were resistant to ceftazidime and ceftiofur, respectively. Resistance to other antibiotics was as follows: chloramphenicol (72.43%), nitrofurantoin (69.23%), amikacin (58.97%), gentamicin (45.51%), and norfloxacin (35.25%).

Discussion

The inappropriate and/or abusive use of antibiotics in the animal husbandry and poultry industry has resulted in the expansion of antibiotic resistance. The emergence and expansion of ESBL-producing *E. coli* in healthy birds and

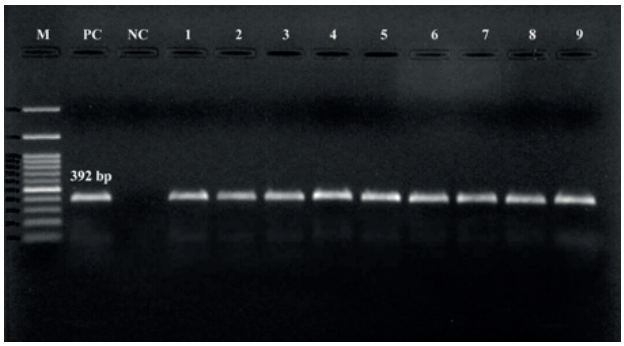

Figure 4. Agarose gel electrophoresis of blaSHV gene PCR products. Lane M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (*E. coli* PTCC 1533). NC: negative control. Lanes 1-9: Representative *E. coli* isolates with PCR products of approximately 392 bp.

animals or food products have posed a therapeutic challenge due to the rapid spread of isolates to humans (Witte 1998). The present research assesses the carrier state of extended-spectrum beta-lactamases in commensal *E. coli* isolates of poultry in Iran. Because of the consistent susceptibility of cefotaxime and ceftazidime to CTM and TEM/SHV, they were initially used as ESBL-producing *E. coli* screening test (Steward et al 2001). The results indicated a significant percentage of ESBL *E. coli* determinants (54.73%) in healthy broiler. The distribution of ESBL-producing *E. coli* in broiler has been reported previously in Iran. Among the 100 *E. coli* isolates recovered from broiler gut in northern Iran, 43% were ESBL carriers. The occurrence of blaCTX-M, blaTEM, and blaVEB were 60.3%, 37.7%, and 13.2%, respectively (Khoshbakht et al 2016). In a similar study undertaken in Iran, out of the 150 *E. coli* isolated from broiler, 18.67%, 5.33%, and 34.67% were positive for blaTEM, blaSHV, and blaCTX-M, respectively (Jafari et al 2016). Doregiraee et al (2018) isolated *E. coli* from 94% of the broiler fecal samples. 6.3% of the *E. coli* isolates were ESBL determinants, among which blaCTX-M and blaTEM had the prevalence of 25% and 39.28%. The high frequency of this group of ESBL carriers has been reported from Dutch (Dierikx et al 2013), Germany (Laube et al 2013), and China (Li et al 2016). Although it is claimed that the vast consumption of antibiotics in poultry is the main reason for the presence and broad spread of ESBL producers in normal gut flora (Snow et al 2012), other plausible reasons including vertical transmission of ESBL carriers from eggs to chicks (Laube et al 2013), over-crowded farms, and contaminated barns/storage facilities due to lack of cleansing and disinfection (Hiroi et al 2012) should have been considered in the high dispersion of ESBL-producing *E. coli* in broiler. In addition, frequent administration of tetracyclines and fluoroquinolones in the poultry industry in Iran is one of the possible causes of coselection under antibiotic pressure (Mabilat et al 1990). More investigations are needed on the role of the various factors mentioned in the dissemination of ESBLs in this region.

On the other hand, high prevalence of ESBL-producing *E. coli* isolates has been reported frequently from human

infections in Iran. Ramazanzadeh et al (2009) revealed that the frequency of *E. coli* isolated from patients in intensive care units of Sanandaj (Iran) was 64.34%. 34.85% of these isolates were ESBL carriers with the prevalence of 14.34% blaSHV, 44.27% blaTEM, 9.17% blaOXA, and 32.22% blaCTX-M genes. The frequency of ESBL-producing *E. coli* isolated from patients with urinary tract infections was evaluated by Gholipour et al (2014). A total of 107 (43.67%) out of 245 *E. coli* isolates were harbouring ESBL genes, among which blaTEM and blaSHV had the frequency of 70.47% and 29.53%, respectively. The prevalence of ESBL genes among 200 uropathogenic *E. coli* collected from inpatients and outpatients in Kermanshah, Iran, was 22%. The ESBLs CTX-M, SHV, TEM, and OXA were detected in 93.3%, 68.2%, 43.2%, and 64.5%, respectively (Norozi et al 2014). High distribution of ESBL-producing *E. coli* involved in human infections in the country is a serious public health menace. As poultry, especially broiler chickens, is one the possible contamination sources, a strict surveillance and control policy must be applied.

In the present study, the antimicrobial susceptibility testing revealed 100% resistance to ampicillin, cefotaxime, and ceftriaxone, which is consistent with the results obtained in Zambia (Chishimba et al 2016). Besides, an increased resistance tendency for chloramphenicol, nitrofurantoin, amikacin, gentamicin, and norfloxacin was observed among the ESBL determinants. Because of the 100% susceptibility of the isolates to imipenem, this agent can be prescribed as the choice drug in life-threatening infections caused by ESBL-producing *E. coli* (Chishimba et al 2016).

Despite several studies reported blaCTX-M as the most abundant ESBL-producing *E. coli* gene in broiler (Olowe et al 2015; Randall et al 2011; Ünal et al 2017), the predominant gene found in this study was blaTEM with a frequency of 52.63%, which is consistent with the research conducted by Li et al in China (2014). Meanwhile, the frequency of blaTEM in this study was more than the frequency of this gene in Japan (Hiroi et al 2014) and Portugal (Costa et al 2009). The dispersion mechanism of the blaTEM and blaSHV genes is different from the blaCTX-M gene: in such way that the above-mentioned two genes have followed the epidemic pattern and are limited to specific geographic regions due to their association with specific bacterial clones. On the contrary, the blaCTX-M genes use an allodemic pattern in which these genes are scattered simultaneously by several clones (Canton and Coque 2006). Our results indicate that the dispersion of blaCTX-M was 38.59%, which was higher than the frequency of 13% of this gene in the E. coli isolated from the poultry in Zambia (Chishimba et al 2016), but this result was relatively less than the frequency of 43.1% and 54.5% obtained in Belgium (Smet et al 2008) and Britain (Randall et al 2011), respectively. Unlike blaTEM and blaSHV genes, mobile genetic elements and plasmids carrying blaCTX-M genes play an important role in transferring these genes. It should be noted that the association of these moving factors with highly successful bacterial clones in their acquisition is effective in the wider dissemination of this group of genes (Sun et al 2010). 30.17% isolates in this study were carrying blaSHV gene. Although Costa et al (2009) and Randall et al (2011) did not report any isolate of ESBLs carrying blaSHV type, there are a few studies indicating the presence of *E. coli* producing the same gene in the poultry (Hiroi et al 2011; Li et al 2010). Like blaTEM gene, this difference may be due to geographical variations (Carattoli 2008). As a result, it can be concluded that industrial poultry population in west of Iran could be a possible source of ESBL for the human population.

In addition, the results of this study documented the simultaneous presence of several extended-spectrum beta-lactamases genes in *E. coli* isolates of healthy poultry. Similar results have been reported in other studies such as Laube et al (2013) and Randall et al (2011). In this study, the most frequent coexistence of genes has been observed for the combination of blaTEM + blaCTX-M, followed by blaTEM + blaSHV and blaTEM + blaCTX-M + blaSHV. The dominant type of ESBL-producing *E. coli* among German broiler carrying blaTEM + blaSHV simultaneously (Laube et al 2013). These findings confirm the ability of ESBL harboring plasmids for carrying more than one type of beta-lactamase gene, and hence the presence of various phenotypes (Brinas et al 2003).

Conclusion

In conclusion, this study is the first report on the molecular identification of bla (CTX-M, TEM, and SHV) genes in *E. coli* isolated from the healthy poultry gut in the west region of Iran. A large population of poultry in west of Iran has been colonized with ESBL-producing *E. coli*. The obtained results should be considered as a possible transfer risk of these bacteria in threating the human health by direct contact or the food chain. It is recommended that further studies be conducted for evaluating the role of these bacteria in human infections in the region. Also, the genetic analysis of the isolates and the determination of the phylogenetic association and similarity of the ESBL-producing *E. coli* isolates with human and poultry origin in the desired region are suggested.

Acknowledgements

The authors might want to express gratitude toward Islamic Azad University, Sanandaj Branch.

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