Fecal carriage of *Escherichia coli* harboring extended-spectrum beta-lactamase (ESBL) genes by sheep and broilers in Urmia region, Iran

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Abstract:

BACKGROUND: There is a growing concern on the impact of the presence of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from animals on public health. **OBJECTIVES:** The aim of this study was to investigate the presence of three classes of ESBL genes in *E. coli* isolates from sheep and broilers at a slaughter in Urmia region, Iran. **METHODS:** A total of 111 *E. coli* isolates were obtained from sheep (n=55) and broilers (n=56) fecal samples and the presence of $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} genes was detected by polymerase chain reaction (PCR). **RESULTS:** In general, 32 of these isolates carried $bla_{CTX\text{-}M}$, 16 bla_{TEM}, and 17 $bla_{CTX\text{-}M}$ plus bla_{TEM}. None of the isolates tested was positive for the bla_{SHV} gene. Among the 55 isolates from sheep, 33 (60%) contained one or more ESBL encoding gene; 15 (27.2%), 10 (18.2%), and 8 (14.5%) isolates were positive for $bla_{CTX\text{-}M}$, bla_{TEM}, and $bla_{CTX\text{-}M}$+bla_{TEM}, respectively. Among the 56 isolates from broilers, 32 (57.1%) isolates carried at least one ESBL encoding gene; 17 (30.4%) and 6 (10.7%) isolates were positive for $bla_{CTX\text{-}M}$ and bla_{TEM} genes, respectively, and the $bla_{CTX\text{-}M}$+ bla_{TEM} was identified in nine isolates (16.1%). **CONCLUSIONS:** This study demonstrated that sheep and broiler feces may be a reservoir of *E. coli* harboring ESBLs genes, with CTX-M being the predominant β-lactamase type. This may pose a public health risk, which requires future evaluation and control.

Introduction

Animals may be the reservoir of the resistant fecal flora. *Escherichia coli* is a common normal inhabitant of the intestinal tract of most animals (Sorum and Sunde, 2001), and can exchange genetic material with other bacterial species (Davison, 1999). Therefore, *E. coli* may serve as an important reservoir for transmissible resistance genes. Increase in the rate of β-lactam resistance in *E. coli* isolates of animal origin has been a growing problem throughout the world (Li et al., 2007). Extended-spectrum beta-lactamases (ESBLs) are one of the most important mechanisms of the bacterial β-lactams resistance. ESBLs confer resistance to penicillins, first-, second- and third-generation cephalosporins, as well as to aztreonam, but not to cefoxitin or carbapenems (Costa et al., 2009). However, several publications have reported cases of resistance of ESBL-producing organisms to the carbapenems, primarily ertapenem (Tsai et al., 2013; Woodford et al., 2007). There are more than 300 subtypes of ESBL, the most common types are the genes encoding CTX, TEM, OXA or SHV (Bush and Jacoby, 2010). In Enterobacteriaceae, ESBL-genes are mostly plasmid mediated and may be located on various types of plasmid (Fischer et al., 2014). Moreover, ESBL genes can be located on integrons, which may facilitate the spread of such genetic elements (Machado et al., 2005). ESBL-encoding genes (which mainly belong to the CTX-M type) have been reported in *E. coli* isolates from food-producing animals (Dolejska et al., 2011; Meunier et al., 2006). This fact is a significant cause of concern for human health, because it involves the transfer of resistance genes from bacteria in food to pathogens or resident bacteria of the human digestive tract (Hammerum and Heuer, 2009). In this regard, poultry (primarily broilers) have been suggested as a source of these types of resistance genes and/or the resistant bacteria (Leverstein-van Hall et al., 2011; Overdevest et al., 2011). Livestock are also considered as potential reservoirs of ESBL-producing organisms (Seiffert et al., 2013). In Iran, a high level of antibiotic consumption factor in veterinary was reported by Aalipour et al. (2014). Moreover, there is also substantial evidence on the existence of ESBL-producing bacteria of human origin (Bazzaz et al., 2009; Jabalameli et al., 2011; Malekjamshidi et al., 2010). Hosseini-Mazinani et al. (2007) concluded that *E. coli*, the predominant pathogen associated with urinary tract infections (UTI), can possess a variety of beta-lactamases that are responsible for beta-lactam resistance. Since, a potential swift route for transmission of ESBL-producing *E. coli* from food-producing animals to humans can be through contaminated meat products (Borjesson et al. 2013), therefore in a 'One Health' perspective, epidemiological studies are required to characterize bacteria from animals with respect to antibiotic resistance genes. Therefore, this study was conducted to investigate the fecal carriage of ESBL-containing *E. coli* isolates in healthy sheep and broilers at slaughterhouse level in Urmia region, Iran, and to identify the CTX-M, TEM, or SHV types of ESBLs. This study provides information about the real problem of ESBLs in food-producing animals, and valuable help to control this emerging problem and to track its future evolution.

Materials and Methods

Sampling and bacterial isolates: From December 2011 to April 2012, a total of 111 fecal samples from healthy sheep (55 samples from 6 farms) and broilers (56 samples from 9 flocks) were collected in five randomized visits to the slaughterhouses located in Urmia, a city located in the capital of West Azerbaijan Province, Iran. Samples were obtained randomly from animals raised in different production units (6-9 samples per farm) located in Urmia region and transported on ice to the laboratory immediately after being aseptically collected. Microbiological analysis was carried out within 24 h of arrival of the samples. Samples were stored at –20°C until microbiological analysis. For the primary isolation of *E. coli*, samples were inoculated on MacConkey agar medium (Merck, Germany) and incubated at 37ºC for 24 h. A dark pink colony (presumptive *E. coli*) was randomly selected and identified by subculturing on EMB (Eosin Methylene Blue) agar plates followed by classical biochemical tests (Quinn et al. 1998). One hundred and eleven isolates were identified as *E. coli* based on their colony morphology and subsequent biochemical testing. After purification, presumable *E. coli* were cryopreserved at -70°C in nutrient broth with 15% (v/v) glycerol for further analysis.

DNA extraction: *E. coli* isolates were grown overnight at 37°C on Blood agar (Merck, Germany), DNA was extracted by boiling method as earlier described with some modification (Obeng et al. 2012). Briefly, 2-3 colonies were mixed with 150 µl of distilled water and boiled for 10 min. The resulting solution was centrifuged and the 2 µl supernatant was used as the DNA template. Universal primers Eco 2083 (GCT TGA CAC TGA ACATTG AG) and Eco 2745 (GCA CTT ATC TCT TCC GCA TT) were used to confirm successful extraction of DNA from the *E. coli* isolates (Riffon et al. 2001). The cycling program involves initial denaturation at 94°C for 4 min, 35 cycles with denaturation at 94°C for 45 s, annealing at 57°C for 1 min and extension at 72°C for 2 min. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The amplified products were analysed on a 1.2% agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml) and photographed under UV transilluminator (BTS-20, Japan).

Detection of *bla* genes by PCR: Detection of $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} were carried out by single PCR using primers listed in Table 1. All the reactions were prepared by using 2 µl template DNA, 12.5 µl 2X PCR master mix (SinaClon, Iran) (0.04 U/µl Taq DNA polymerase, PCR buffer, 3 mM MgCl2, 0.4 mM of each dNTP), and 0.4 µM of each primer (CinaClon, Iran) in a volume of 25 µl. The reaction conditions for the $bla_{\text{CTX-M}}$ gene were one cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 51°C for 30 s, 72°C for 30 s, with a single final extension at 72°C for 10 min; on the other hand, the reaction conditions for the amplification of bla_{TEM} and bla_{SHV} genes separately were one cycle of 94ºC for 5 min, followed by 32 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. The control strains used for the determination of ESBL genes were *E. coli* (Persian Type Culture Collection, PTCC1533) and *Klebsiella pneumonia* (Razi Type Culture Collection, RTCC1248). Ten microliters of each mixture was separated on a 1% agarose gel in 0.5X Tris-borate-EDTA buffer at a constant voltage of 100 V for 2 h. The bands of amplified DNA were visualized by the UV transilluminator, GeneRuler$_{\text{TM}}$ 100 bp Plus DNA Ladder (Thermo Scientific, Germany) was used as a molecular size marker.

Results

A total of 111commensal *E. coli* isolates were obtained from the same number of fecal samples (55 from sheep and 56 from broilers). One or more β-lactamase genes were detected in 65 (58.5%) of the 111 isolated strains, with $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{CTX-M}}$ plus $bla_{\text{CTX-M}}$ being detected in 32 (28.8%), 16 (14.4%), and 17 (15.3%) isolates, respectively (Figs. 1, 2, 3). None of these isolates harbored bla_{SHV} gene. A summary of results of PCR tests is shown in Table 2. As shown in Table 2, among the 55 and 56 isolates obtained from sheep and broilers, 33 (60%) and 32 (57.1%) contained one or more ESBL encoding gene, respectively. Based on these results, minor differences in distribution of ESBL encoding genes were seen between sheep and broilers.

Discussion

Dissemination and increasing rate of ESBL-producing *Escherichia coli* (ESBL-EC) in healthy food-producing animals or food products has become a serious public health concern (Kluytmans et al., 2013; Leverstein-van Hall et al., 2011; Li et al., 2010). The percentage of ESBL-harboring *E. coli* was detected in 60% (33/55) in sheep isolates and 57.1% (32/56) in broiler isolates. The β-lactamase genes detected in ESBL-positive *E. coli* isolates recovered in this study were as follows: $bla_{\text{CTX-M}}$ (n= 32; 28.8%), bla_{TEM} (n=16; 14.4%) and $bla_{\text{CTX-M}}$ + bla_{TEM} (n=17; 15.3%). The data of ESBL-producing *E. coli* in fecal samples from sheep were recently reported by Geser et al. (2012) and Ramos et al. (2013), in these

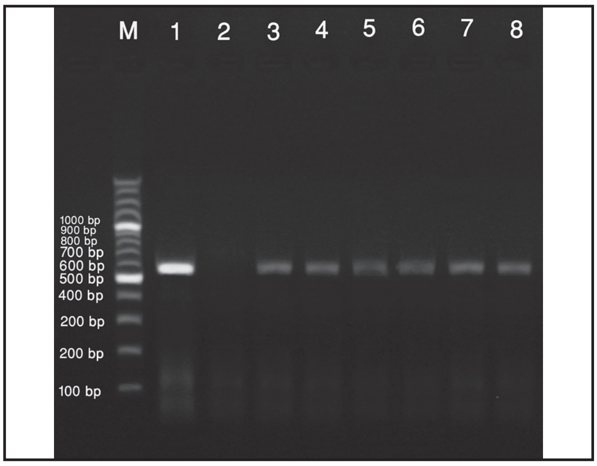

Figure 1. Agarose gel electrophoresis of PCR products with representative isolates harboring the $bla_{\text{CTX-M}}$ gene. Lane M: GeneRuler$^{\text{TM}}$ 100 bp Plus DNA Ladder, Lane 1: Positive control: *E. coli* PTCC1533, Lane 2: negative control, Lanes 3-8: Representative *E. coli* isolates with PCR products of approximately 544 bp.

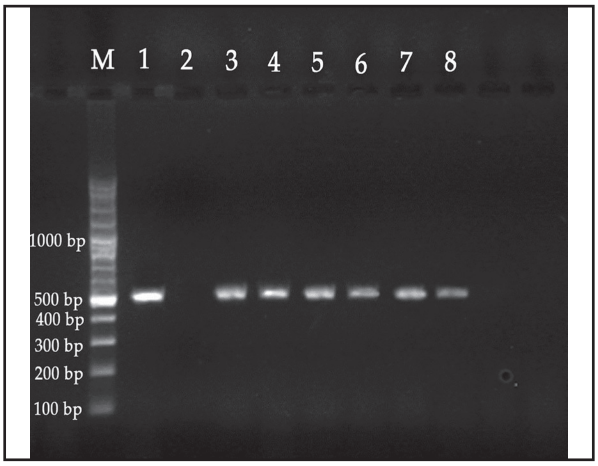

Figure 2. Agarose gel electrophoresis of PCR products with representative isolates harboring the bla_{TEM} gene. Lane M: GeneRuler$^{\text{TM}}$ 100 bp Plus DNA Ladder, Lane 1: Positive control: *E. coli* PTCC1533, Lane 2: negative control, Lanes 3-8: Representative *E. coli* isolates with PCR products of approximately 516 bp.

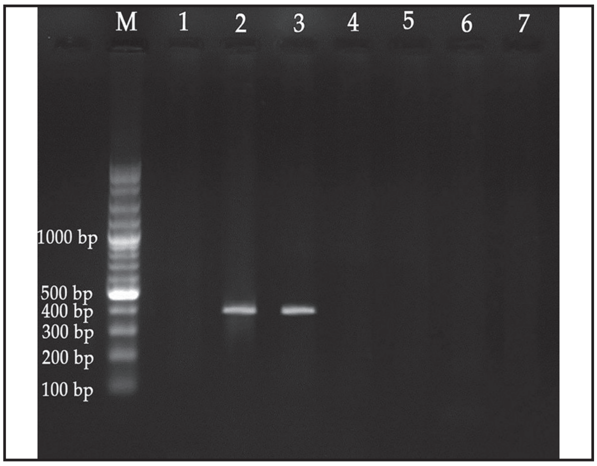

Figure 3. Agarose gel electrophoresis of PCR products with representative isolates harboring the bla_{SHV} genes. Lane M: GeneRuler$^{\text{TM}}$ 100 bp Plus DNA Ladder, Lane 1: negative control, lane 2: Positive control: *E. coli* PTCC1533, lane 3: *K. pneumonia* RTCC1248, lane 4-7: Representative *E. coli* isolates with no PCR products.

reports, lower percentages of ESBL-producing *E. coli* isolates were obtained in Portugal and in Switzerland (5.5% and 8.6%, respectively). There have also been many reports from other countries describing the distribution of ESBL-producing *E. coli* in fecal samples from broilers (Bortolaia et al., 2010; Randall et al., 2011). The wide dissemination of ESBL-harboring *E. coli* among fecal isolates from sheep and broilers is surprising, given the fact that they infrequently receive treatments with cephalosporins, which is considered one of the most important reasons for the presence and dissemination of ESBL positive isolates (Dolejska et al., 2011; Snow et al., 2012). In Iran, tetracycline class of antibiotics was the most common antibiotics sold for both livestock and poultry farms. Furthermore, fluoroquinolones are intensively prescribed for poultry (Aalipour et al., 2014). Therefore, high selective pressure generated by the massive use of tetracyclines and fluoroquinolones (which can co-select ESBL-producing strain that are often resistant to these drugs) for specific clinical conditions, prophylactic and growth promotion purposes, may contribute to this situation. Co-carriage of genes encoding broad-spectrum β-lactamases and tetracycline/quinolone resistance determinants on the same mobile genetic elements has been reported (Karisik et al., 2006; Quiroga et al., 2013). Furthermore, Moreno et al. (2008) found that *E. coli* isolates from feces of companion animals treated with enrofloxacin, produced more ESBL than those from the control group. Therefore, the impact of the use of different antibiotic classes should be taken into account for both humans and animals. However, further studies are needed to confirm the

Table 1. Primers used for PCR in this study.

Primer	Sequence (5´-3´)	Target gene	PCR product	Reference
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	bla_{CTX-M}	544 bp	(Edelstein et al, 2003)
CTX-M-R	CGATATCGTTGGTGGTGCCATA			
TEM-F	ATCAGCAATAAACCAGC	bla_{TEM}	516 bp	(Mabilat and Courvalin, 1990)
TEM-R	CCCCGAAGAACGTTTTC			
SHV-F	AGGATTGACTGCCTTTTTG	bla_{SHV}	392 bp	(Colom et al, 2003)
SHV-R	ATTTGCTGATTTCGCTCG			

Table 2. Prevalence of ESBL genes detected in the *E. coli* isolates from sheep and broilers.

Host	No.	Presence of *bla* gene				Percentage of *E. coli* positive for ESBLs (%)
		CTX-M	TEM	CTX-M + TEM	SHV	
Sheep	55	15	10	8	-	33 (60%)
Broiler	56	17	6	9	-	32 (57.1%)
Total	111	32	16	17	-	65 (58.5)

role of other factors other than antibiotics usage (e.g., environment, management and etc).

The wide dissemination of CTX-M-type ESBL among *E. coli* isolates from sheep and broilers, indicate that this ESBL type may be playing an increasing role in antibiotic resistance in the North West of Iran. This is in agreement with other publications which describe the predominance of this ESBL type from isolates of animal origin (Brinas et al., 2003; Smet et al., 2008). The rapid and extensive dissemination of CTX-M-type ESBL in veterinary settings likely depends on the combination of various factors including efficient capture and dispersal of bla_{CTX-M} gene by mobile genetic elements, association of these elements with highly successful bacterial clones and low fitness cost imposed by CTX-M production (D'Andrea et al., 2013). Studies in Iran reported that class 1 integrons are widespread among ESBL-producing isolates of K. pneumoniae and *E. coli* and suggested that appropriate surveillance and control measures are essential to prevent further dissemination of these elements among Enterobacteriaceae (Zeighami et al., 2014).

The TEM-type ESBL was found in 26.8% of the tested broiler (n=56) isolates. This appears to be much higher than that previously found (Hiroi et al., 2011), but lower than those reported in Portugal (Costa et al., 2009). For sheep, 18 out of 55 isolates (32.7%) harbored the bla_{TEM} gene. In contrast, Ramos et al. (2013) did not found TEM-type ESBL among *E. coli* isolates from 73 sheep fecal samples in Portugal. In another study carried out by Geser et al. (2012), this type of ESBL was reported only in 4 *E. coli* isolates obtained from 58 fecal samples of sheep. These discrepancies may be due to geographical variations or differences in the selection of animals studied. In this regard, some ESBLs seem to be confined to a specific geographical region, whereas others are more widely diffused (Carattoli, 2008).

The coexistence of CTX-M with TEM-type ESBL in 15.3% of the tested isolates, suggests a greater risk for the failure of β-lactam therapy. It has been demonstrated that the presence of more than one β-lactamase would raise the β-lactam resistance level and would likely expand resistance to a broader range of β-lactams (Brinas et al., 2003; Li et al., 2010).

None of the *E. coli* isolates recovered in this study harboured the bla_{SHV}. This finding was in agreement with the other studies where no SHV-type ESBL was detected in sheep (Ramos et al., 2013) and broilers (Costa et al., 2009; Randall et al., 2011). However, a few reports about bla_{SHV-12} and bla_{SHV-2} in *E. coli* from chickens/broiler have been published (Geser et al., 2012; Hiroi et al., 2011; Li et al., 2010). It should be emphasised that the isolates in this study were limited and drawn from a defined region in Iran, so large number of *E. coli* or related bacteria isolates as well as more additional geographic sites must be tested.

The high occurrence of ESBL-harboring *E. coli* in sheep and broilers is a problem of food safety and raises a potential public health concern, because antimicrobial-resistant *E. coli* can contaminate meat products during slaughter and enter the food chain (Borjesson et al., 2013). Some studies have suggested that isolates, the plasmid, and/or the genes can be transferred from broilers to humans via the meat (Leverstein-van Hall et al., 2011; Overdevest et al., 2011). It is therefore possible that such a high occurrence in feces of sheep and broilers increases the risk of contribution to the meat products ESBL load. Further studies are needed to determine the importance of the food chain for the dissemination of ESBL-producing *E. coli* among the population in Iran.

The intestinal tract of healthy sheep and broilers seems to be reservoir of ESBL-harboring *E. coli* isolates. Further investigations on a large scale are required to monitor the spread of ESBL-producing bacteria in food-producing animals, for clarifying the reservoirs of ESBL resistance genes and establishing food control programs in Iran. The present study also revealed the abundance of CTX-M- and TEM-type ESBLs among the studied isolates, so a more detailed genetic analysis will be necessary on these groups of genes.

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حمل مدفوعی اشریشیا کولای حامل ژن‌های بتا-لاکتاماز وسیع الطیف (ESBL) توسط گوسفند و جوجه‌های گوشتی در منطقه ارومیه، ایران

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چکیده

زمینه مطالعه: توجه فزاینده‌ای در مورد تأثیر حضور اکولای‌های مولد بتا-لاکتاماز وسیع الطیف جدا شده از دام‌ها بر روی بهداشت عمومی وجود دارد. **هدف:** هدف از این مطالعه بررسی حضور سه کلاس ژن ESBL در جدایه‌های اکولای بدست آمده از گوسفند و جوجه‌های گوشتی در سطح کشتار در منطقه ارومیه، ایران، می‌باشد. **روش کار:** در کل ۱۱۱ جدایه اکولای از نمونه‌های مدفوع گوسفند (n=۵۵) و جوجه‌های گوشتی (n=۵۶) بدست آمد و حضور ژن‌های bla_{TEM}، bla_{CTX-M} و bla_{SHV} توسط واکنش زنجیره‌ای پلیمراز (PCR) شناسایی شدند. **نتایج:** به طور کلی ۳۲ مورد از این جدایه‌ها bla_{CTX-M}، ۱۶ مورد bla_{TEM}، و ۱۷ مورد bla_{CTX-M} را به همراه bla_{TEM} حمل می‌نمودند. هیچ‌کدام از جدایه‌ها برای ژن bla_{SHV} مثبت نبودند. از ۵۵ جدایه بدست آمده از گوسفند، ۳۳ جدایه (۶۰٪) حاوی یک یا بیش از یک ژن کد کننده ESBL بودند؛ به طوری که ۱۵ (۲۷/۲٪)، ۱۰ (۱۸/۲٪) و ۸ جدایه (۱۴/۵٪) به ترتیب برای bla_{TEM}، bla_{CTX-M} و $bla_{CTX-M} + bla_{TEM}$ مثبت بودند. در بین ۵۶ جدایه بدست آمده از جوجه‌های گوشتی، ۳۲ جدایه (۵۷/۱٪) حداقل حامل یک ژن کد کننده ESBL بودند؛ به طوری که ۱۷ (۳۰/۴٪) و ۶ جدایه (۱۰/۷٪) به ترتیب برای ژن‌های bla_{CTX-M} و bla_{TEM} مثبت بودند و $bla_{CTX-M} + bla_{TEM}$ در ۹ جدایه (۱۶/۱٪) شناسایی گردید. **نتیجه‌گیری نهایی:** این مطالعه نشان داد که مدفوع گوسفند و جوجه‌های گوشتی ممکن است مخزن اکولای‌های حامل ژن‌های ESBL، با غالبیت بتا-لاکتاماز نوع CTX-M، باشد. این موضوع ممکن است بهداشت عمومی را با خطر مواجه سازد که نیازمند ارزیابی و کنترل آتی است.

واژه‌های کلیدی: جوجه‌های گوشتی، ESBL، اشریشیا کولای، گوسفند

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