Assiut Vet. Med. J. Vol. 61 No. 147 October 2015

MULTIDRUG RESISTANCE AND INTEGRONS IN *ESCHERICHIA COLI* ISOLATED FROM FOOD-PRODUCING ANIMALS IN SAUDI ARABIA

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ABSTRACT

Received at: 20/9/2015

Accepted: 18/10/2015

Transference of antimicrobial resistance gene determinants by integrons is one of the important factors that can contribute to the increase in multidrug-resistant bacteria. The present study aimed to investigate 56 strains of animal-derived *E. coli* strains obtained from fecal materials of slaughtered food animals for their resistance to commonly used antimicrobial agents for presence of integrons and detection of mercury reductase gene (*Mer*A) as indicative for presence of transposons. Multidrug resistance (MDR) was found in 38 (68%) of the strains as indicated by disk diffusion method. The most frequent resistance patterns was to Ampicillin, Sulfonamides, Nalidixic acid, Cephalosporin's, Tetracycline, and Aamoxicillin/Clavulanic acid. None of tested strains were resistant to Cefepime or Imipenem. The polymerase chain reaction (PCR) results showed the presence of integrons in 26 (68.4%) of MDR strains tested as well as the mercury resistance gene in 12 (46.2%) of strains with integrons, indicating the role of Tn21 transposon in dissemination of the integrons and their gene cassettes within the samples studied. Our results showed high resistance rate of *E. coli* from food animals to routine antibiotics and indicate that integrons and transposon are common among MDR isolates of food producing isolates in Saudi Arabia.

***Key words**: multidrug resistance, integrons, transposons, Animal-derived E. coli, PCR*

INTRODUCTION

Antibiotic resistance is a threat for therapy failure in human medicine. The use of antimicrobial substances in animal husbandry is considered the most important factor for debate due to its possible implications on emergence, selection and dissemination of resistant bacteria from food animals to human (Threlfall *et al*., 1993; Johnston, 1998).

One of the most recent adopted actions worldwide is to control and analyze the antibiotic resistance in individual countries, it is necessary to have adequate control systems which would integrate the data concerning antibiotic resistance of bacteria isolated from animals. The acquired resistance should be controlled not only in pathogenic bacteria, but also in the endogenous microflora of exposed animals and humans (Miles *et al*., 2006). The majority of resistance genes encoding a wide variety of resistance mechanisms are carried by mobile genetic elements such as integrons, transposons, plasmids (Iyer *et al*., 2013) which favors the transfer of MDR genotypes between commensals and pathogens, animals and humans.

Multi-drug resistance (MDR) in intestinal bacteria such as *Escherichia coli* is a major healthcare problem worldwide (Lockhart *et al.*, 2007) and known to be associated with integrons (Leverstein-van Hall *et al*., 2003). Integrons are genetic structures capable of integrating or mobilizing gene cassettes encoding antibiotic resistance determinants (Carattoli, 2001). Although integrons are not mobile, they can be transferred between bacteria by transposons or plasmids in which they are present. Accordingly, integrons are a major mechanism for the spread of multidrug resistance from both clinical isolates and normal flora of food animals, as well as in human clinical specimens (Goldstein *et al*., 2001). Three types of integrons, each with different *int* genes have been identified (intI1, intI2, and intI3) that are known to be associated with antibiotic resistance (Mazel, 2006).

Several studies have investigated prevalence of integrons in MDR *E. coli* isolates around the world

(Bass *et al*., 1999; Chang *et al*., 2000; Goldstein *et al*., 2001; Johnson *et al*., 2005; Kang *et al*., 2005; Idrees *et al*., 2011; De la Torre *et al*., 2014). However, no publicized information is available on detection of integrons in MDR isolates of *E. coli* from animals in Saudi Arabia. The aim of this study was to define the current prevalence and phenotypes of multi-drug resistant *E. coli* isolated from intestinal contents of food-producing animals at slaughtering in Saudi Arabia and to investigate associations between multi-drug resistance and existence of integrons as well as transposon.

MATERIALS and METHODS

Collection of samples

A total of seventy-nine samples of intestinal contents and/or fecal materials were collected aseptically over two months from sheep, bovines and chickens slaughtered in Taif abattoirs, Western Saudi Arabia. The samples were transported to the laboratory in ice-box.

Isolation and identification of *E. coli*

Fecal samples were resuspended in buffered peptone water (BPW). After homogenization, samples were cultured in MacConkey agar, which was incubated aerobically for 24 h at 37 ℃. The isolate identification by biochemical reactions using API 20 E system (bioMerieux, Marcy l'Etoile, France) and specific PCR amplification of the *usp*A gene (Chen and Griffiths, 1998). Isolates identified as *E. coli* were included in this study and further analysed.

Antibiotic susceptibility assay

The antibiotic resistance behavior of the isolated strains was determined on cation-adjusted Mueller-Hinton agar (Hi-Media) using disk diffusion method according to the standards and interpretive criteria described by Clinical and Laboratory Standards Institute (CLSI, 2013). The antibiotics studied were Ampicillin (AMP 25 µg), Cephalothin (CEF 30 µg), Cefotaxime (CTX 30 µg) and Ceftazidime (CAZ 30 µg), Cefepime (FEP 30 µg), Aztreonam (ATM 30 µg), and Cefoxitin (FOX 30 µg), Amoxicillin/ Clavulanic acid (AMC 20/10 µg). Imipenem (IMP 10 µg) was used to test susceptibility to carbapenems. Non-β-lactam antibiotics included Ciprofloxacin (CIP 5 µg), Sulfamethoxazole/ trimethoprim (SXT 23.75/1.25 µg), Gentamycin (GEN 10 µg), Tetracycline (TET 30 µg) and Chloramphenicol (CHL 30 µg) and Nalidixic acid (NAL 30 µg). The quality control was performed to check the quality of medium and potency of antibiotic disks before use against some sensitive ATCC reference strains, including *E. coli* ATCC 25922 (beta-lactamase negative), *E. coli* ATCC 35218 (beta-lactamase positive), and *P. aeruginosa* ATCC 27853 (MicroTrol Discs; BD Diagnostics).

Isolation of Genomic DNA

Genomic DNA was isolated from *E. coli* by boiling method (Solberg *et al*., 2006). An overnight bacterial culture (200 *µl*) was mixed with 800 *µl* of distilled water, boiled for 10 min and, after cooling, centrifuged for 5 min at 14000 rpm and the supernatant was used as the DNA template for the PCR.

Detection of integrons and transposon

All the isolates were tested for the presence of conserved integrons of classes 1, 2 and 3 by degenerated primers and PCR protocol described by White *et al*., (2001). Detection of mercuric reductase gene *mer*A, as a marker for presence of transposon Tn21, was performed by PCR amplification as recommended by Chatziefthimiou *et al*. (2007). Primers and PCR conditions are presented in table 1.

RESULTS

The *E. coli* identification

A total of 67 presumptive *E. coli*, by cultural and morphology characteristic were isolated, 56 were confirmed as *E. coli* by the specie-specific primer employing PCR assay targeting gene encoding the universal stress protein A (*usp*A). This PCR generates the 884-bp *E. coli*-specific product (Fig. 1), 32 (57.1%) of which were isolated from chicken, 15 (27%) from sheep, and 9 (16.1%) from bovine. All the 56 *E. coli* isolates tested for susceptibility to antibiotics and screened for the presence of integrons and transposon.

Antibiotic resistance phenotypes

The results of *in vitro* susceptibility testing of all of the *E. coli* isolates from different animal sources are shown in Table 2. The most frequent antibiotic resistance prevalence by strains were for Ampicillin, Sulfamethoxazol-Trimethoprim, Nalidixic acid, Tetracycline, Cephalosporins and Amoxicillin/ Clavulanic acid. The chicken feces showed the greatest resistance prevalence. On the other hand, the samples showed lower resistance prevalence to gentamicin and ciprofloxacin. It was observed that imipenem and cefepime were the most effective antibiotics, no resistances to these antibiotics were observed.

The results showed that 38 (68%) of the strains isolated from fecal material samples of different animal origins were multidrug-resistant (resistant to three or more antimicrobials), 32.1% resistant to one or two antimicrobials, and none of the strains were sensitive to all the antimicrobials studied. AMP resistance was present in approximately 78.6% of the isolates.

Incidence of integrons and transposon

All of the isolates were screened for the presence of integrase genes *intI1, intI2*, and *intI3*, resulting in 491 bp amplicons (Fig. 2). Out of 38 multiresistant isolates, 26 (68.4%) were integron-positive, while 12 (23%) were integron-negative.

The amplification of the *mer*A gene, which is considered a marker for the presence of transposon Tn*21*, resulted in a 309 bp fragment (Fig 3). Out of 26 strains carrying integrons, 12 (46.2%) were positive for *mer*A. Antibiotic resistance profiles and presence of *mer*A in integron-positive strains are presented in Table 3.

Table 1: Primers used in this study

Primer target	Primer name	Sequence (5 to 3)	PCR conditions	Product size (bp)	Reference
Universal stress protein A (*usp*A)	Ec1 Ec2	CCGATACGCTGCCAATCAGT ACGCAGACCGTAAGGGCCAGAT	initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min.	884	Chen and Griffiths, (1998)
Conserved regions for integrase encoded intI1, intI2, intI3	hep35 hep36	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT	Initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min.	491	White *et al.* (2001)
Mercury reductase (*mer*A)	A1s-n.F merA5R	TCCGCAAGTNGCVACBGTNGG CGCYGCRAGCTTYAAYCYYTC RRCCATYGT	initial denaturation step at 94°C for 5 min, 35 cycles of 94°C for 1 min, 62°C for 30 s, and 72°C for 30 s, and with a final extension for 7 min at 72°C	309	Chatziefthimiou *et al.* (2007)

Table 2: Antimicrobial resistance in *E. coli* strains isolated from different animal origins in Saudi Arabia

Antimicrobials	Isolate source			
	Chicken (n=32)	Sheep (n=15)	Bovine (n=9)	Total No. (%) (n=56)
Ampicillin (AMP)	26	11	7	44 (78.6)
Aztreonam (ATM)	3	1	0	4 (7.1)
Amoxicillin/Clavulanic acid (AMC)	8	3	0	11 (19.6)
Cefalothin (CEF)	22	8	6	36 (64.3)
Cefepime (FEP)	0	0	0	0 (0)
Cefotaxime (CTX)	6	1	0	7(12.5)
Cefoxitin (FOX)	4	1	0	5(8.9)
Ceftazidime (CAZ)	2	1	0	3 (5.4)
Chloramphincole (CHL)	7	1	0	8 (14.3)
Ciprofloxacin (CIP)	1	0	0	1 (1.8)
Gentamicin (CEN)	1	0	0	1 (1.8)
Imipenium (IMP)	0	0	0	0 (0)
Nalidixic acid (NAL)	22	6	2	30 (53.6)
Sulfamethoxizol-Trimethoprime (SXT)	29	7	3	39 (69.6)
Tetracycline (TET)	15	3	1	19 (33.9)

Table 3: Antibiotic resistance profiles and presence of *mer*A in integron-positive strains

Strain no.	Resistance pattern	Integrons	*mer*A
1	AMP, CEF, CHL, GEN, NAL, SXT	+	+
2	AMP, AMC, ATM, CEF, CTX, CAZ, FOX, NAL, TET	+	-
3	AMP, CEF, NAL, SXT, TET	+	+
5	AMP, CEF, CTX, CHL, NAL, SXT	+	-
7	AMP, NAL, SXT	+	-
8	AMP, CEF, SXT, TET	+	+

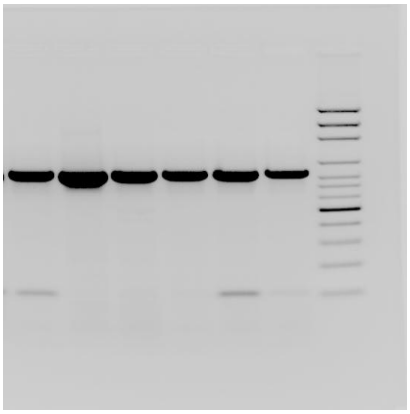

Fig. 1: The result of the PCR amplification of the DNA target gene loci of 884-bp fragment DNA region coding for universal stress protein *usp*A.

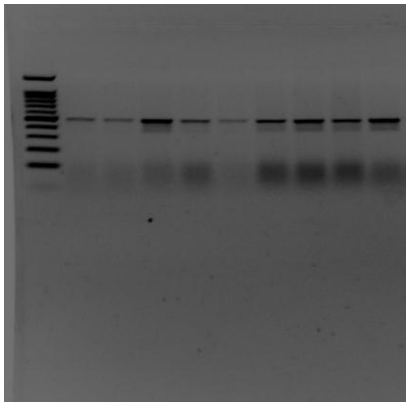

Fig. 2: The result of the PCR amplification of the DNA target gene loci of 491-bp fragment DNA coding for integrase genes intI1, intI2, and intI3.

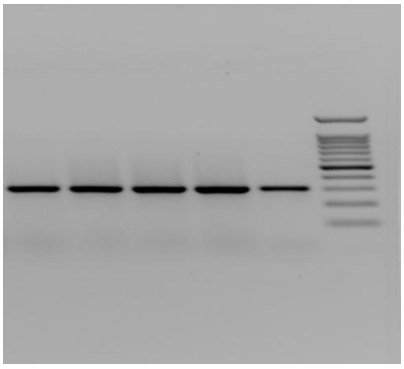

Fig. 3: The result of the PCR amplification of the DNA target gene loci of 309-bp fragment DNA region coding for mercury reductase *mer*A

DISCUSSION

Antimicrobial resistance of bacteria that colonize animals, especially those for food production, can have important implications to public health, as a source and reservoir of resistant bacteria which can be transferred to human. The present study investigated 56 animal-derived *E. coli* strains with respect to their resistance.

PCR for the specific differentiation of *E. coli* from other gram-negative bacteria, with primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein A (*usp*A) (Nystrom and Neidhardt, 1992) has been described (Chen and Griffiths, 1998). This PCR generates the 884-bp *E. coli*-specific product and allows a rapid and specific diagnosis of *E. coli* infection.

Emergence of multidrug-resistant (MDR) bacteria has become a major public health concern worldwide, in particular, intestinal bacteria of healthy food animals, resulting in transfer of antimicrobial-resistant intestinal bacteria to humans by food products. The emergence of *Escherichia coli* isolates with multiple antibiotic-resistant genotypes, has been previously reported and is considered a serious health concern (Bass *et al*., 1999). In this study, MDR *E. coli* isolates with resistance to three or more different antibiotics were common. Thirty eight isolates (68%) had MDR phenotypes. Antimicrobial resistance of commensal *E. coli* is highest in poultry, followed by sheep and veal calves. The percentage of resistance to multiple antibiotics occurring among *E. coli* isolates was lower than in other publications involving *E. coli* isolates from a variety of animal species (Lanz *et al*., 2003; Lim *et al*., 2007; Dai *et al*., 2008; Enne *et al*., 2008; Jiang *et al*., 2011; Knezevic and petrovic, 2008; Persoons *et al*., 2011, 2012).

Animal intestinal bacteria could disseminate antimicrobial-resistant genes to human intestinal bacterial flora through mobile elements like transposons and integrons (Jensen *et al*., 2008). Of all the *E. coli* isolates collected in our study, 26 (68.4%) of MDR isolates were positive for integrons. It is well known that integrons carry and transfer MDR genes in bacteria in human and animals and are therefore of public health significance (Huang *et al*., 2012; Koo and Woo, 2012). A number of studies reported the occurrence of integrons in selected populations of gram-negative bacilli (Bass *et al*., 1999; Bywater *et al*., 2004; Saenz *et al*., 2004; Johnson *et al*., 2005), and prevalence ranging from 16% to 63% were reported by (Bass *et al*., 1999; Singh *et al*., 2005).

Furthermore, many studies have shown a correlation between the presence of integrons and multi-antibiotic resistance phenotypes in enteric bacteria (Leverstein van-Hall *et al*., 2003; Fluit and Schmitz, 2004; Singh *et al*., 2005). This has also been shown in our study.

The dissemination of integrons has been attributed to the spread of an integron-containing transposon, Tn21 (Goldstein *et al*., 2001; Fluit and Schmitz 2004). Tn21, a large (19.7 kb) class II replicative transposon, carries a mercury resistance (*mer*) operon, an integron (In2) and a transposition module (Bass *et al*., 1999). In addition to drug resistance, Tn21 confers mercury resistance through its mercuric reductase gene, *mer*A (Bass *et al*., 1999). We searched for the presence of this gene in all strains containing integrons and looked into the possibility that integrons in our isolates are actually part of Tn21. In order to detect the presence of the *merA* gene encoding the enzyme responsible for mercury reduction, PCR amplification experiments using degenerate primers were performed. The gene was detected in 12 (46.2%) of the MDR strains carrying the integrons, which indicates that at least a part of integrons spread within the strains are related to Tn21. Nevertheless, as much as about 50% of the integron-positive strains did not possess *mer*A. One possible explanation for this finding is the presence of a truncated derivative or a Tn4, a Tn21 derivative that does not confer mercury resistance because Tn3 has inserted into and disrupted the *mer* locus of Tn21 (Bass *et al*., 1999). Mathew *et al*. (2009) reported identical integron amplicons in *E. coli* and *Salmonella* from a single livestock farm, suggesting horizontal transfer. Povilonis *et al*. (2010) demonstrated the existence of considerable and common pool of transferable integrons in *E. coli* and *S. enteric* present in clinical and livestock environment.

CONCLUSION

The present study showed high prevalence of integrons and transposon in isolates of animal-derived *E. coli* in Saudi Arabia and their association with multiple resistance to a range of antibiotics. These results further confirm the potential of integrons to contribute to development of resistance in *E. coli*. Their potential for transfer of antimicrobial resistance from bacteria of food animals to the human population is a cause of concern. It is, therefore, essential to continuously monitor bacterial susceptibility to antimicrobials and to study temporal trends among isolates from healthy animals.

ACKNOWLEDGEMENTS

The Authors are grateful for financial support of deanship of scientific research of Taif University, Saudi Arabia (project no. 1/435/3319).

DISCLOSURE STATEMENT

No competing financial interests exist.

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المقاومة المتعددة للأدوية والأنتجرونات في الأيشيريشيا القولونية المعزولة من الحيوانات المنتجة للغذاء بالمملكة العربية السعودية

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يعتبر انتقال المحددات الجينية المسئولة عن مقاومة مضادات الميكروبات بواسطة الأنتجرونات (عناصر وراثية قادرة علي دمج أو تحريك حوافظ جينية) من العوامل الهامة المساهمة في زيادة وانتشار البكتيريا المتعددة المقاومة للمضادات الميكروبية. تهدف هذه الدراسة في التحقيق في عدد ٥٦ سلالة من بكتيريا ايشيريشيا القولون المعزولة من براز الحيوانات المنتجة للغذاء اثناء الذبح وذلك لمقاومتها لعدد من المضادات الميكروبية شائعة الاستخدام في الحقل الطبي ووجود الأنتجرونات والتحري عن وجود الجين المسئول عن اختزال الزئبق كدليل علي وجود العناصر الوراثية القافزة (ترانسبوزون). بينت نتائج فحص الحساسية الدوائية بطريقة الانتشار القرصي تجاه مضادات الميكروبات ان ٣٨ (٦٨ %) من سلالات الأيشيريشيا القولونية تمتلك نمط المقاومة المتعددة لمضادات الميكروبات. أعلي معدل للمقاومة كان للأمبيسيلين، سلفونيميد، حمض النالديكسيك، سيفالوسبورين، تتراسيكلين، ومثبطات البيتالاكتاميز ، بينما كان اقل معدل مقاومة للسبروفلوكساسين والجنتاميسين. كانت جميع السلالات المختبرة حساسة لكل من السيفابيم والأميبينيم. اظهرت نتائج التفاعل المتسلسل المبلمر ان ٢٦ (٦٨.٤%) من الأيشيريشيا القولونية ذات النمط متعدد المقاومة لمضادات الميكروبات تحتوي الأنتجرونات وان الجين المسئول عن اختزال الزئبق كمؤشر لوجود العناصر الوراثة القافزة (ترانسبوزون) كان بنسبة ٤٦.٢% من تلك المحتوية علي الأنتجرون، مما يدل علي امكانية انتشار ونقل الحوافظ الجينية للبكتيريا المقاومة للمضادات الحيوية بالأنتجرون والعناصر الوراثية القافزة بين سلالات ايشيريشيا القولون المعزولة من الحيوانات المنتجة للغذاء بالمملكة العربية السعودية.