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Shiga toxin and beta-lactamases genes in *Escherichia coli* phylotypes isolated from carcasses of broiler chickens slaughtered in Iran

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ABSTRACT

Two hundred and four *Escherichia coli* strains were isolated from external and visceral cavity surfaces of 102 slaughtered broiler carcasses. The isolates were screened to determine the phylogenetic background and presence of Shiga toxins (*stx*$_1$, *stx*$_2$), intimin (*eae*) and beta-lactamase (*bla*$_{TEM}$, *bla*$_{SHV}$) genes. Phylotyping results revealed that the *E. coli* isolates segregated in four phylogenetic groups A (56.86%), B1 (19.12%), B2 (4.90%) and D (19.12%). PCR assays revealed that 13 isolates (6.37%) from 12 carcasses were positive for *eae* (12 isolates) and/or *stx*$_2$ (2) genes. The *eae* positive isolates belonged to phylogenetic groups A (A_0, A_1), B1, B2 ($B2_2$) and D (D_2). Two *stx*$_2$ positive and seven *eae* positive isolates were recovered from visceral cavity surface, whereas only 5 *eae* positive isolates were from the external surface of the carcasses. On the other hand, thirty one *E. coli* strains isolated from visceral cavity and external surface of 26 carcasses carried the *bla*$_{TEM}$ (27) and *bla*$_{SHV}$ (4) genes and belonged to different phylo-groups. This study suggests that broiler carcasses could be considered as an important source of EPEC and STEC pathotypes in southeast of Iran; as well as the examined antibiotic resistance genes, which were carried by some isolates and could be transferred to pathogens through the food chain.

1. Introduction

Escherichia coli (*E. coli*) strains are a part of intestinal normal microflora of many animals, including humans and birds (Brzuszkiewicz et al., 2011). Most *E. coli* strains are harmless commensals; however, some strains have evolved pathogenic mechanisms to cause enteric/diarrheagenic infections in humans and animals (Clements et al., 2012). The diarrheagenic *E. coli* are divided into seven pathotypes including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adhering *E. coli* (DAEC) (Nunes et al., 2012). Some STEC strains are regarded as emerging food-borne pathogens of significant clinical and public health concern (Kawano et al., 2012), which are the leading cause of several human illnesses ranging from symptom-free carriage to hemorrhagic colitis and even life-threatening sequelae such as hemolytic uremic syndrome (HUS) (Bandyopadhyay et al., 2011). The virulence factors contributing to STEC pathogenesis include the production of two phage-encoded toxins, Shiga toxin 1 (STX_1) and/or Shiga toxin 2 (STX_2) (Döpfer et al., 2012). Another important virulence factor is the outer membrane protein, intimin, which is responsible for attaching and effacing (AE) lesions in the enterocytes. Intimin is encoded by the *eae* gene located on a chromosomal pathogenicity island named the locus for enterocyte effacement (LEE) (Bentancor et al., 2012). EPEC pathotype is defined as intimin-containing diarrheagenic *E. coli* isolates that possess the ability to form AE lesions, but not possess genes coding Shiga toxins (Bhat et al., 2008).

STEC have a low minimal infectious dose and may survive in a range of foods and also in the harsh environment of the gastrointestinal tract (Rode et al., 2012). Although ruminants, especially cattle are the principal reservoir of STEC strains, some of these pathogens have been detected from the fecal samples of healthy birds (Ghanbarpour and Daneshdoost, 2012). Transmission to humans mainly occurs by contaminated foods of animal origin or cross contamination due to inadequate food manipulation. The occurrence of STEC contamination in chicken meat can be related to the evisceration process, mainly to the rupture of the animal intestine (Alonso et al., 2012).

Resistance to antimicrobial agents is a matter of great concern in the current antimicrobial resistance scenario (Escudero et al., 2010). Transfer of antimicrobial resistant strains of *E. coli* from chickens to the food chain is a well-recognized phenomenon (Obeng et al., 2012). Production of beta-lactamases confers resistance to the majority of the commonly used beta-lactam antimicrobials (Ryu et al., 2012). This resistance has been observed in strains originating from all animal species, especially in the isolates from intensive broiler productions

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(Depoorter et al., 2012). Beta-lactamases have been observed in virtually all the species of Enterobacteriaceae family and are often mediated by bla_{TEM} and bla_{SHV} genes (Monstein et al., 2007; Sharma et al., 2010).

Phylogenetic analysis of the *E. coli* species has revealed that the majority of strains belong to four phylogenetic groups: A, B1, B2, and D. There is increasing evidence that the different phylogenetic groups play a distinct ecological role (Chaudhuri and Henderson, 2012; Escobar-Páramo et al., 2004).

The purposes of this study were to determine (i) the presence of Shiga toxins and intimin genes, (ii) genotypic detection of some beta-lactamases, and (iii) phylogenetic distribution of *E. coli* isolates from broiler carcasses, collected during the slaughtering process in the south-east of Iran.

2. Materials and methods

2.1. Sampling and microbial isolates

From October 2009 to March 2010, samples from 102 healthy broiler carcasses were obtained during slaughter in an abattoir in the Kerman province (southeastern), Iran. The sampled broiler carcasses were aged between 45 and 58 days and originated from seven different flocks. Swab samples were collected from the external and visceral cavity surfaces of each carcass. The external swab samples were obtained from the skin of neck, breast, wing, leg and back area of carcasses after defeathering. Visceral cavity swab samples were obtained from the same carcasses after evisceration. The swab samples were placed directly in tubes containing Stuart transport medium (Oxoid, Hampshire, England) and transferred to the laboratory for immediate processing. Each sample was streaked on Mac Conkey agar plates (Biolife Laboratories, Milan, Italy) and incubated at 37 °C for 24 h. Bacterial colonies showing *E. coli* characteristics were submitted to Gram staining and were confirmed to be *E. coli* by using the biochemical API 20E identification system (BioMérieux, Marcy l'Etoile, France). The confirmed *E. coli* isolates were stored in Luria–Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at −80 °C. One confirmed isolate was chosen from each sample; therefore, the PCR assays were undertaken on 204 isolates (102 from visceral cavity and 102 from external surface).

2.2. Phylotyping assay

Several strains from the ECOR collection were used as controls for phylogenetic grouping: ECOR58 (B1 group), ECOR50 (D group), ECOR62 (B2 group) and *E. coli* strain MG1655 as a positive control for phylogenetic ECOR group A. The triplex PCR method described by Clermont et al. (2000) was used to assign the *E. coli* isolates. The presence/absence of the three PCR products (*chuA*, *yjaA* and *tspE4.C2*) is used to assign an unknown isolate to one of the phylo-groups. The Clermont method has the potential to yield seven distinct phylogenetic groups and subgroups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1 and D_2). The phylotype of each isolate was determined as described previously (Escobar-Páramo et al., 2004; Gordon et al., 2008).

2.3. PCR assays

Freshly grown over night cultures of *E. coli* isolates and reference strains were used for DNA extraction by boiling. Sakaï reference strain was used as positive control for *eae*, stx_1 and stx_2 genes and *E. coli* strain MG1655 was used as a negative control for these genes. Reference *E. coli* strains ATCC 35218 and *Klebsiella* 700603 were used as positive control for bla_{TEM} and bla_{SHV} genes respectively. *E. coli* strain ATCC 25922 was used as negative control for β-lactamase genes. DNA extracts from *E. coli* isolates and reference strains were tested by PCR assays for the presence of the genes encoding β-lactamase as described by Sharma et al. (2010) and stx_1, stx_2 and *eae*, genes as described by Paton and Paton (1998). Specific primers (TAG Copenhagen, Denmark) used for amplification of the genes are presented in Table 1. PCR-amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide.

3. Results

From the 102 broiler carcasses, 204 *E. coli* isolates were obtained and confirmed for molecular examinations. PCR phylotyping revealed that the 204 *E. coli* isolates segregated in phylogenetic groups A (56.86%), B1 (19.12%), B2 (4.90%) and D (19.12%). The results showed that the isolates belong to 7 phylogenetic subgroups, including 75 isolates (36.76%) to A_0, 41 isolates (20.10%) to A_1, 39 (19.12%) isolates to B1, 5 isolates (2.45%) to $B2_2$, 5 isolates (2.45%) to $B2_3$, 30 isolates (14.71%) to D_1 and 9 isolates (4.41%) to subgroup D_2 (Table 2).

Among the 102 sampled carcasses, 12 carcasses (11.76%) were positive for stx_2 and/or *eae* genes, whereas 13 (6.37%) isolates were positive for these genes. Out of positive *E. coli* isolates, 11 isolates from 10 carcasses were positive for *eae* gene. Only one isolate from a carcass possessed stx_2 and one isolate from another carcass possessed both stx_2 and *eae* genes. None of the isolates was positive for stx_1 gene. The positive isolates for *eae* and stx_2 genes were distributed in 4 phylogenetic groups and 5 phylogenetic subgroups (Table 3).

Among the 204 investigated isolates, 27 isolates (13.24%) from 22 carcasses and 4 isolates (1.96%) from 4 other carcasses were positive for the bla_{TEM} and bla_{SHV} genes respectively. These positive isolates belonged to 4 phylogroups and 6 phylo-subgroups (A_0, A_1, B1, $B2_2$, D_1 and D_2). Twenty-seven positive isolates for bla_{TEM} gene belonged to A (14 isolates), B1 (6), B2 (2) and D (5) phylo-groups. All the four bla_{SHV} positive isolates belong to A phylo-group (Table 3). According to the results, all the positive isolates had only one of the β-lactamase encoding genes (bla_{TEM} or bla_{SHV}).

PCR assays for phylotyping of 102 external isolates indicated that the isolates are distributed in phylo-groups: 64 (62.75%) isolates in A, 19 (18.63%) in B1, 7 (6.86%) isolates in B2 and 12 (10.76%) isolates in D. These isolates fell into 7 phylo-subgroups (Table 2). Among 102 external isolates, 5 were positive for *eae* gene, which belonged to A_0 (1 isolate), A_1 (2), and $B2_2$ (2) phylo-subgroups. None of the isolates possessed stx_2 gene. According to PCR assays, 18 external isolates possessed β-lactamase encoding genes, of which 15 bla_{TEM} positive isolates segregated in A_0 (8 isolates), A_1 (1), B1 (4), $B2_2$ (1) and D_1 (1) phylo-subgroups. The three other isolates were positive for bla_{SHV} gene, which belonged to A_0 (1) and A_1 (2) phylo-subgroups (Table 3).

One hundred and two visceral cavity isolates fell into four phylogenetic groups, including 50.98% (52 isolates) into A, 19.61% (20) into B1, 2.94% (3) into B2 and 26.47% (27) into D group. The isolates were distributed in 4 and six phylo-groups and subgroups respectively (Table 2). Among the visceral cavity isolates, 7 isolates were positive

Table 1
The specific primers used in this study.

Gene	Primer sequence (5′–3′)	Product size (bp)	References
bla_{TEM}	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080	Sharma et al. (2010)
bla_{SHV}	GGGTAATTCTTATTTGTCGC TTAGCGTTGCCAGTGCTC	928	Sharma et al. (2010)
stx_1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	Paton and Paton (1998)
stx_2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	Paton and Paton (1998)
eae	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	Paton and Paton (1998)
chuA	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	Clermont et al. (2000)
yjaA	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	Clermont et al. (2000)
tspE4C2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	Clermont et al. (2000)

Table 2
Distribution of visceral cavity and external *E. coli* isolates in detected phylo-groups/subgroups.

Phylo-group	A no. (%)		B1 no. (%)	B2 no. (%)		D no. (%)		Total no. (%)
Phylo-subgroup	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_1	
Visceral cavity isolates	32 (31.37)	20 (19.61)	20 (19.61)	1 (0.98)	2 (1.96)	20 (19.61)	7 (6.86)	102 (100.00)
External isolates	43 (42.16)	21 (20.59)	19 (18.63)	4 (3.92)	3 (2.94)	10 (9.80)	2 (1.96)	102 (100.00)
Total phylo-subgroup	75 (36.76)	41 (20.10)	39 (19.12)	5 (2.45)	5 (2.45)	30 (14.71)	9 (4.41)	204 (100.00)
Total phylo-group	116 (56.86)		39 (19.12)	10 (4.90)		39 (19.12)		204 (100.00)

for *eae* and 2 were positive for stx_2. Of the seven *eae* positive isolates, 3 isolates were from A phylo-group (Table 3). One of the stx_2 positive isolates possessed *eae* (from D_2 phylo-subgroup) and another one possessed bla_{TEM} gene (from A_1 phylo-subgroup) simultaneously (Table 3). Of the 102 visceral cavity isolates, 12 isolates carried bla_{TEM} gene, which belonged to A_0 (4 isolates), A_1 (1), B1 (2), $B2_2$ (1), D_1 (2) and D_2 (2) phylo-subgroups. The only bla_{SHV} positive isolate from the visceral cavity surface belonged to A phylo-group (Table 3).

4. Discussion

Human infection with STEC strains occurs after consumption of contaminated food or contact with an infected animal or human. Therefore, identification of the sources of infection is an important step towards decreasing the prevalence of this pathogen and thus decreasing the risk of infection of human (Pedersen et al., 2006). Cattle and sheep are thought to be the major reservoir of STEC, since the species often carry STEC in their intestinal flora and serve as a source of food contamination (Martins da Costa et al., 2011). There are some studies about the presence and prevalence of STEC and EPEC in healthy poultry feces and product. The results of the present study revealed the presence of two stx_2 positive isolates, which were considered as STEC pathotype. On the other hand, 11% of the examined external and visceral cavity isolates were only positive for the *eae* gene, which were considered as EPEC strains. Moreover, one of the isolates possessed both stx_2 and *eae* genes which is considered as highly pathogenic. This isolate belonged to phylo-group D and phylo-subgroup D_2. Salehi (2012) in Iran has evaluated the presence of virulence factors in 290 fecal *E. coli* isolates from healthy broilers and found out that 9 and 3 isolates possessed *eae* and stx_2 genes respectively, which is similar to the results of the current study. Several studies also revealed that EPEC contamination predominated over STEC contamination in chicken meat and feces (Alonso et al., 2012; Kagambega et al., 2012; Kobayashi et al., 2002; Oh et al., 2012).

STEC and EPEC strains are important pathogens related to public health and there are several reports of these strains' contamination in chicken meat (Chinen et al., 2009; Drugdová et al., 2010). Retail poultry products are routinely heavily contaminated with avian fecal *E. coli* isolates (Johnson et al., 2003). Farooq et al. (2009) in India found that 4.24% of the investigated avian species isolates were STEC and 15.56% were EPEC. Another study on avian fecal *E. coli* isolates revealed that 4.5% and 1.8% of isolates were positive for *stx2* and *eae* genes respectively (Ghanbarpour et al., 2011).

According to the results, *eae* positive (STEC) and stx_2 positive (EPEC) isolates belonged to A, B and D phylogenetic groups. A phylogenetic analysis indicated that EHEC/*eae* + STEC isolates from animal origin fell into phylogenetic groups A (35.5%) and B1 (38.7%), and EPEC strains belonged to groups B1 (69.2%), and B2 (30.8%) (Tramuta et al., 2008). Ghanbarpour et al., 2011 reported that commensal *stx2* positive *E. coli* isolates belong to B1 and D phylogenetic groups, whereas the *eaeA* positive isolates fell into group A. In USA the majority of the STEC strains, which were initially isolated from the ruminants, carried the stx_{1c} and/or stx_{2d}, *ehxA*, and *saa* genes and fell into *E. coli* phylogenetic group B1 (Ishii et al., 2007). Moreover, some data referring to the relationships between the phylogenetic background of *E. coli* and the presence of *stx* and *eae* genes in animals reveal partial similarities to the results of the current study (Girardeau et al., 2005).

To understand the role of commensals in the acquisition and maintenance of various virulence genes, it is essential to investigate the evolutionary origin of these strains (Baldy-Chudzik et al., 2008). According to the results of the current study, the isolates fell into 4 phylogenetic groups, with the majority classified in the A phylo-group. Several studies indicated that commensal strains fall into groups A and B1, whereas extraintestinal *E. coli* (ExPEC) belong mainly to group B2 and diarrheagenic strains fall into groups A, B1, and D (Chapman et al., 2006; Gordon et al., 2008). genotyping of *E. coli* from environmental and animal samples in USA showed that chicken intestinal tract isolates were distributed over all the four phylo-groups. In agreement with the results, a survey of 40 isolates recovered from fecal samples of 20

Table 3
Details of positive isolates for stx_1, stx_2, *eae*, bla_{TEM} and bla_{SHV} according to phylogenetic background.

Isolates from visceral cavity surface of the carcasses							Isolates from external surface of the carcasses					
No	Phylotype	genes					No	Phylotype	genes			
		stx_1	stx_2	*eae*	bla_{TEM}	bla_{SHV}			stx_1	stx_2	*eae*	bla_{TEM}	bla_{SHV}
4	A (A_0)[a]	−	−	−	+	−	8	A (A_0)	−	−	−	+	−
2	D (D_2)	−	−	−	+	−	4	B1 (B1)	−	−	−	+	−
2	D (D_1)	−	−	−	+	−	2	A (A_1)	−	−	−	−	+
2	A (A_0)	−	−	+	−	−	2	B2 ($B2_2$)	−	−	+	−	−
2	B1 (B1)	−	−	−	+	−	2	A (A_1)	−	−	+	−	−
2	B1 (B1)	−	−	+	−	−	1	B2 ($B2_2$)	−	−	−	+	−
1	A (A_0)	−	−	−	−	+	1	D (D_1)	−	−	−	+	−
1	B2 ($B2_2$)	−	−	−	+	−	1	A (A_0)	−	−	−	−	+
1	D (D_2)	−	−	+	−	−	1	A (A_0)	−	−	+	−	−
1	D (D_2)	−	+	+	−	−	1	A (A_1)	−	−	−	+	−
1	A (A_1)	−	−	+	−	−							
1	A (A_1)	−	+	−	+	−							
Positive isolates (no.)		0	2	7	12	1	Positive isolates (no.)		0	0	5	15	3
Positive isolates (%)		0.00	1.96	6.86	11.76	0.98	Positive isolates (%)		0.00	0.00	4.90	14.71	2.94

[a] Phylo-group/phylo-subgroup.

chickens reared in Maryland showed that 50% were phylotype A (Higgins et al., 2007). A phylogenetic typing of broiler fecal and meat isolates in Slovakia revealed that all fecal isolates from healthy chicken broilers fell into pathogenic group D (62.5%) and group B2 (37.5%), while 53% of meat-associated strains belonged to commensal phylogenetic groups A and B1 (Drugdová et al., 2010). It is believed that although *E. coli* commensal strains are not completely associated with the occurrence of disease, they carry some of the virulence genes (Baldy-Chudzik et al., 2008).

One of the purposes of this study was to determine the presence and prevalence of bla_{TEM} and bla_{SHV} genes and phylogenetic background of the positive isolates. Several antibacterial agents are widely used as feed additives in the poultry industry to treat, prevent infections and promote growth in Iran. Widespread antibiotic prescription in the field of veterinary medicine may be one of the factors causing the presence of antibiotic-resistant bacteria to reach epidemic proportions in recent years (Saei et al., 2012). In Portugal, analyses of *E. coli* isolates from uncooked chicken carcasses, feces of healthy chickens and feces of healthy swine samples demonstrated that ESBL-producing isolates belonged to phylogenetic groups A, D or B1 (Machado et al., 2008). A study on human enterohemorrhagic *E. coli* (EHEC) infections in France showed that all of the ESBL-producing isolates were allocated to one of the four phylogenetic groups A, B1, B2, and D. The results indicated that 55.4% of these isolates belonged to group A, 25.5% to group D, 15.6% to group B1 and only one strain was found in group B2 (Valat et al., 2012). It is believed that antibiotic resistant human isolates were overall more similar to poultry isolates than to antibiotic-susceptible human isolates (Johnson et al., 2007).

In conclusion, the results of this study indicate that *E. coli* isolates, which originated from the external and visceral cavity surfaces of broiler carcasses, belong to different phylo-groups/subgroups, and contain intimin, Shiga toxin and beta-lactamase coding genes. This study mentions that broiler carcasses could be considered as an important source of EPEC and STEC pathotypes in the southeast of Iran. Considering the presence of *eae* positive isolates, further investigations on the screening of poultry originated *eae*-positive *E. coli* isolates could help to elucidate the role and importance of these isolates. On the other hand, although EPEC and STEC isolates tended to carry few antibiotic resistance genes, some isolates possessed the examined antibiotic resistance genes, which could be spread and transferred to pathogens through the food chain. Regarding the public health and zoonotic importance of EPEC and STEC and antibiotic-resistant *E. coli* isolates, continuous researches on genotypic characterization of isolates from poultry sources can clarify the risk of human infections from broiler carcasses.

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