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Detection of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from diarrhoiec dogs in Iran

Detección de virulencia y genes de resistencia antimicrobiana en aislados de *Escherichia coli* provenientes de perros en Irán

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ABSTRACT. This study was conducted to investigate the presence of some virulence and antimicrobial resistance genes in *E. coli* isolates from diarrhoiec dogs in Iran. Seventy dogs were randomly selected by direct sampling. Rectal swabs were collected and cultured for isolation and identification of *E. coli* following standard methods. Polymerase chain reaction (PCR) was used to detect 5 virulence genes and 12 antibacterial resistance genes in 14 of the isolates. From the 70 rectal swabs cultured, 33 (47.1%) gave positive growth of *E. coli*. Out of 14 isolates tested for the presence of virulence genes, 9 (64.3%) were positive for PCR of *stx* (1), 5 (35.7%) were positive for *stx* (2), 7 (50%) were positive for *eae*, and 1 (7.1%) isolate was positive for *cnf* (1). Out of the 14 isolates tested for the presence of antibacterial resistance genes, 9 (64.3%) were positive for CITM gene, 6 (42.9%) were positive for *aad* (A1) and *bla* (SHV), 5 (35.7%) were positive for *tet* (A), *dfr* (A1) and *cat* (1), 4 (28.6%) were positive for *aac (3)*-IV, 3 (21.4%) were positive for both *tet* (B), *sul* (1) and *cml* (A), while 1 (7.1%) of the isolate was positive for *ere*. The results showed that enterohaemorrhagic *E. coli* (EHEC), shiga toxigenic *E. coli* (STEC) and necrotoxic *E. coli* (NTEC) strains harboring several antibacterial resistance genes could be involved in canine diarrhoea in Iran.

Key words: antimicrobial resistance, diarrhoea, dogs, *Eschericha coli*, gene, virulence.

RESUMEN. El objetivo de este studio fue investigar la presencia de algunos genes de virulencia y resistencia a los antimicrobianos en *E. coli* aislados de perros diarreicos en Irán. Setenta perros fueron seleccionados al azar y muestreados directamente. Se recogieron hisopos rectales y se cultivaron para el aislamiento e identificación de *E. coli* siguiendo métodos estándar. Se utilizó la reacción en cadena de la polimerasa (PCR) para detectar cinco genes de virulencia y 12 genes de resistencia a antibacterianos en 14 de los aislamientos. De 70 hisopos rectales cultivados, 33 (47,1%) dieron positivos al crecimiento de *E. coli*. De 14 cepas analizadas para detectar la presencia de genes de virulencia, nueve (64,3%) fueron positivas para PCR de *stx* (1), cinco (35,7%) fueron positivos para *stx* (2), siete (50%) fueron positivos para *eae*, y uno (7,1%) fue positivo para aislar *cnf* (1). De los 14 aislamientos probados para determinar la presencia de genes de resistencia antibacterial, nueve (64,3%) fueron positivos para el gen CITM, seis (42,9%) fueron positivos para aad (A1) y *bla* (SHV), cinco (35,7%) fueron positivos para *tet* (A), *dfr* (A1) y *cat* (1), cuatro (28,6%) fueron positivos para *aac* (3) -IV, tres (21,4%) fueron positivos para ambos *tet* (B), *sul* (1) y *cml* (A), mientras que uno (7,1%) del aislado fue positivo para *ere*. Los resultados mostraron que cepas de *E. coli* enterohemorrágica (EHEC), *E. coli* shiga toxigénica (STEC) y *E. coli* necrotóxica (NTEC) que albergan varios genes que codifican para la resistencia antimicrobiana podrían estar involucrados en la diarrea canina en Irán.

Palabras clave: resistencia antimicrobiana, diarrea, perros, *Escherichia coli*, genes, virulencia.

INTRODUCTION

Escherichia coli, a member of the family Enterobacteriaceae, constitute part of normal commensal bacterial flora of animals and humans (Nataro and Kaper 2003, Rahimi *et al* 2012, Puno-Sarmiento *et al* 2013, Tajbakhsh *et al* 2016). *E. coli* have been implicated severally in clinical cases of diarrhoea in dogs (Beutin 1999, Morato *et al* 2009, Paula and Marin 2009, Puno-Sarmiento *et al* 2013). But mere isolation of

E. coli from diarrhoiec faeces is not enough to regard such isolate as a diarrhoeagenic strain. Diarrhoeagenic *E. coli* isolate may belong to the enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), entroaggregative *E. coli* (EAEC), necrotoxic *E. coli* (NTEC), enterotoxigenic/shiga-like toxin producing *E. coli* (STEC) or diffusely adherent *E. coli* (DAEC) strain/ pathotypes, depending on the type of virulent factor(s) elaborated and the type of lesion produced (Bien *et al* 2011, De Rycke *et al* 1999, Puno-Sarmiento *et al* 2013, Salvadoris *et al* 2003). Nevertheless, canine diarrhoea may not primarily be caused by *E. coli*, although pathogenic strains of *E. coli* has been widely incriminated in cases of diarrhoea in humans and animals (Aslani *et al* 2008, Salvadoris *et al* 2003, Shahrani *et al* 2014). In many clinical conditions of dogs such as canine distemper, parvoviral enteritis, coronavirus infection, helminthosis, etc and a myriad of non-infectious and toxic conditions, the integrity of intestinal mucosa is altered resulting in enteritis and diarrhoea (Hammermueler *et al* 1995, Torkan *et al* 2015). In these

conditions, secondary opportunistic infections by pathogenic *E. coli* following immune depression and their subsequent discharge in diarrhoeic faeces may occur. Diarrhoeagenic *E. coli* strains have been reported to harbour genes which encode virulent factors responsible for their pathogenicity (Aslani *et al* 2008, Bien *et al* 2011, Shahrani *et al* 2014). Virulent factors often possessed by pathogenic *E. coli* strains and used for their classification into pathotypes include: Shiga-like/Shiga toxin (stx) encoded by Shiga toxinogenic (*stx*) genes 1 and 2 (*stx*1 and *stx*2), cytotoxic necrotizing factor (cnf) encoded by cytotoxic necrotizing factor genes 1 and 2 (*cnf*1 and *cnf*2), and intimin encoded by *E. coli* attaching and effacing (*eae*) gene (De Rycke *et al* 1999, Landraud *et al* 2000, Salvadoris *et al* 2003, Bentancor *et al* 2007, Puno-Sarmiento *et al* 2013). These virulent factors have been widely reported to be associated with diarrhoea in humans and animals (Randall *et al* 2004, Aslani *et al* 2008, Kavitha *et al* 2010).

Treatment of companion animals especially dogs with antibacterial agents such as β-lactams, fluoroquinolones, potentiated sulfonamides, etc., in suspected cases of bacterial infection, is often practiced by veterinary clinicians and non-veterinarians, especially in countries where there are no strict regulations for the use of these drugs in animals (Bradford 2001, Guardabassi *et al* 2004, Abatcha *et al* 2014, Torkan *et al* 2015). This resulted in increased detection of antibacterial-resistant *E. coli* both pathogenic and non-pathogenic strains, in companion animals worldwide (Hammermueler *et al* 1995, Bradford 2001, Guardabassi *et al* 2004, Ewers *et al* 2012). *E. coli* develop resistance following prolonged exposure to antibacterial agents especially in sub-therapeutic doses by acquisition of antibacterial resistance genes from other resident commensal or transient pathogens colonising the individual or the environment. Various antimicrobial resistance determinants including multidrug resistance genes encoding for extended-spectrum β-lactamases have been described in *E. coli* isolates from companion animals (Bradford 2001, Costa *et al* 2008, Ewers *et al* 2010, Shaheen *et al* 2011, Tajbakhsh *et al* 2015). Antimicrobial resistance genes spread easily among bacterial organisms by mobile genetic elements like plasmids, and transposons (Salvadoris *et al* 2003, Randall *et al* 2004).

Faecal shedding of *E. coli* by companion animals constitutes an important source of environmental contamination (Morato *et al* 2009). Animals with clinical conditions such as diarrhoea usually have immune suppression which favours increased faecal shedding of *E. coli* (de Almeida *et al* 2012). Diarrhoeic animals defecate frequently and uncontrollably, thus they tend to spread *E. coli* more than the non-diarrhoeic ones. Because both pathogenic and non-pathogenic E. coli isolates are potential reservoirs of antimicrobial resistance genes, their presence in diarrhoeic faeces of dogs pose serious threat to public health following zoonotic transmission; dog owners/handlers, children and veterinarians, are more at risk since they have direct close contact with these animals (Hammermueler *et al* 1995, Paula and Marin 2009). In many parts of the world, compromise/ complications during antibacterial therapy in dog owners were traced to acquisition of antibacterial resistance genes from *E. coli* colonizing companion animals (Warren *et al* 2001, Abatcha *et al* 2014).

Isolation of diarrhoeagenic antimicrobial-resistant *E. coli* from dogs with or without diarrhoea and/or their handlers have been reported in countries such as Italy (Carattoli *et al* 2005), Portugal (Costa *et al* 2008, Bien *et al* 2011), Poland (Rzewuska *et al* 2015), Brazil (de Almeida *et al* 2012, Paula and Marin 2008, Paula and Marin 2009, Siqueira *et al* 2009, Puno-Sarmiento *et al* 2013), the Netherlands (Ewers *et al* 2010, Ewers *et al* 2012), Argentina (Bentancor *et al* 2007), America (Shaheen *et al* 2011), and Egypt (Ali and Metwaly 2015, Yunis *et al* 2015). In the available literature, studies on pathogenic *E. coli* in diarrhoeic and/or healthy dogs in Iran include the reports of Zahrei Salehi *et al* (2011) and Koochakzadeh *et al* (2014). These studies detected STEC and EPEC strains in dogs with or without diarrhoea, but neither of them assessed antimicrobial resistance genotypes of the isolates. Other E. coli pathotypes have been isolated from diarrhoeic and non diarrhoeic animals elsewhere (Bentancor *et al* 2007, Kavitha *et al* 2010). Zahrei Salehi *et al* (2011) only determined the phenotypic resistance profile (antibiogram) of the isolates. But phenotypic resistance is determined by the genotype (Morrison *et al* 2015). Moreover, Aslani *et al* (2008) characterised the virulence genes and antibiogram of *E. coli* isolates from diarrhoeic humans in Iran. The findings of the study showed that the *E. coli* isolates are diarrhoeagenic strains that can cause zoonotic infections. Therefore, further investigations are needed regarding the pathogenic potential of *E. coli* isolates from dogs reared in Iran and their capacity as reservoirs of antimicrobial resistance genes. Characterisation of the virulence and antibacterial resistance determinants in the *E. coli* isolates is necessary for empirical treatment of infections associated with these organisms. The objective of this study was to isolate and detect some virulence and antimicrobial resistance genes in *E. coli* isolates from dogs with diarrhoea presented to the Islamic Azad University Veterinary Teaching Hospital (IAUVTH), Iran.

MATERIAL AND METHODS

SAMPLING

This cross-sectional study was conducted between February and April, 2014. By directed sampling, a total of 70 diarrhoeic dogs of varied breeds, sex and ages (puppies and adults) presented to IAUVTHI for diagnosis and treatment were randomly selected. Prior to administration of any drug, rectal swab was collected from the dogs using sterile swab sticks. The swabs were transported aseptically in ice-packs to Microbiology Laboratory, Islamic Azad

Table 1. PCR primers used for detection of virulence genes.
Cebadores de PCR utilizados para la detección de genes de virulencia.

Virulence factor	Target virulence gene	Primers Sequence	Amplicon size (base pair)	Annealing temperature (ºC)	Reference
Shiga-like toxin	*Stx* (1)	F: 5´- CAGTTAATGTGGTGGCGAAGG- 3´ R: 5´- CACCAGACAATGTAACCGCTG- 3´	348	56	(Cebula *et al* 1995)
	Stx (2)	F: 5´- ATCCTATTCCCGGGAGTTTACG- 3´ R: 5´- GCGTCATCGTATACACAGGAGC- 3´	584		
Attaching and effacing factor	eae	F: 5´- TGCGGCACAACAGGCGGCGA- 3´ R: 5´- CGGTCGCCGCACCAGGATTC- 3´	629	56	(Heuvelink *et al* 1995)
Cytotoxic necrotizing factor	*Cnf* (1)	F: 5´- GGGGGAAGTACAGAAGAATTA- 3´ R: 5´- TTGCCGTCCACTCTCACCAGT- 3´	1111	56	(Toro *et al* 2005)
	Cnf (2)	F: 5´- TATCATACGGCAGGAGGAAGCACC- 3´ R: 5´- GTCACAATAGACAATAATTTTCCG- 3´	1240		

University of Shahrekord Branch, Iran and processed within 6 hours of collection.

ISOLATION AND IDENTIFICATION OF *E. coli* ISOLATES

The rectal swabs were cultured on Mac Conkey agar[1] and incubated at 37 °C for 24 hours aerobically. On each plate that produced growth, three lactose-fermenting (pinkish) colonies were purified by sub-culturing on fresh Mac Conkey agar and incubated at 37 °C for 24 hours. Characterization and identification of the isolates as *E. coli* was done by subjecting the purified isolates to Gram staining, oxidase, indole, citrate, urease, methyl-red and triple sugar iron tests and they were further evaluated for production of characteristic greenish metallic sheen by inoculating on eosin methylene blue agar[2] following standard procedures.

ANTIMICROBIAL RESISTANCE AND VIRULENCE GENOTYPE OF THE *E. coli* ISOLATES

DNA of 14 isolates was extracted using bacteria DNA extraction kit[3] following the manufacturer's instructions. Using Ependorf Mastercycler[4], the presence of the following 5 virulence genes: Shiga-like toxin genes *stx* (1) and *stx* (2), attaching and effacing gene *eae*, and cytotoxic necrotizing factor genes *cnf*(1) and *cnf*(2) was investigated in the *E. coli* isolates using primers that have been described by other authors (table 1).

Table 1 shows the list of primers, annealing temperatures and predicted sizes used for the detection of virulence genes of *E. coli* isolated. Positive controls from the collection of

the Islamic Azad University of Shahrekord Branch, Iran were included in each PCR reaction. Sterile distilled water was used as the negative controls. The analysis of the PCR products was performed in 1.5% horizontal agarose gel electrophoresis stained with ethidium bromide under UV light. The isolates were categorised based on the virulence genes they carried. The isolate that carried both *stx* and *eae* genes was considered as enterohaemorrhagic *E. coli* (EHEC) strain. The one that was PCR positive for only *cnf* gene was regarded as necrotoxic *E. coli* (NTEC) strain, while those that were PCR positive for only *stx* gene was considered Shiga-like toxin producing *E. coli* (STEC) strain.

The presence of the following 12 antimicrobial resistance genes: streptomycin – *aad* (A1), tetracycline – *tet* (A), *tet* (B), trimethoprim – *dfr* (A1), fluorquinolone - *qnr*, gentamicin – *aac* (3)- (IV), sulfonamide – *sul* (1), cephalothin – *bla* (SHV), ampicillin - CITM, erythromycin – *ere* (A), and chloramphenicol – *cat* (1) and *cml* (A) was investigated in 14 of the *E. coli* isolates by PCR using primers that have been described by other authors (table 2), annealing temperatures and predicted sizes of amplified products for primers (table 2). The positive and negative controls were sourced from and used as aforementioned in each PCR reaction. Analysis of the PCR products was performed as above.

STATISTICAL ANALYSIS

Data generated were subjected to descriptive statistics using Microsoft Excel version 2010 (Microsoft, USA) and expressed in percentages.

RESULTS

OCCURRENCE OF VIRULENCE GENES IN *E. coli* ISOLATES FROM DIARRRHOIEC DOGS

Out of 70 rectal swabs cultured, 33 (47.1%) gave positive growth of *E. coli*. Out of 14 isolates tested for the presence of virulence genes, 9 (64.3%) were positive for PCR of

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[1]   MCA; Oxoid Basingstoke, United Kingdom

[2]   EMB; Basingstoke, United Kingdom

[3]   Cinagen, Tehran, Iran

[4]   Ependorf, Hamburg, Germany

Table 2. PCR primers used for detection of antimicrobial resistance genes.
Cebadores de PCR utilizados para la detección de genes de resistencia a los antimicrobianos.

Antimicrobial agent	Target resistance gene	Primers Sequence	Amplicon size (base pair)	Annealing temperature (ºC)	Reference
Streptomycin	*aad* (A1)	F: 5´- TATCCAGCTAAGCGCGAACT- 3´ R: 5´-ATTTGCCGACTACCTTGGTC- 3´	447	58	(Puno-Sarmiento *et al* 2013)
Tetracycline	*tet* (A)	F: 5´- GGTTCACTCGAACGACGTCA- 3´ R: 5´-CTGTCCGACAAGTTGCATGA- 3´	577	57	
	tet (B)	F: 5´- CCTCAGCTTCTCAACGCGTG- 3´ R: 5´-GCACCTTGCTGATGACTCTT- 3´	634	56	(Puno-Sarmiento *et al* 2013)
Trimethoprim	*dfr* (A1)	F: 5´- GGAGTGCCAAAGGTGAACAGC- 3´ R: 5´- GAGGCGAAGTCTTGGGTAAAAAC- 3´	367	45	(Torkan *et al* 2015)
Fluoroquinolone	qnr	F: 5´- GGGTATGGATATTATTGATAAAG- 3´ R: 5´-CTAATCCGGCAGCACTATTTA- 3´	670	50	(Li 2005)
Gentamicin	*aac (3)*- (IV)	F: 5´- CTTCAGGATGGCAAGTTGGT- 3´ R: 5´-TCATCTCGTTCTCCGCTCAT- 3´	286	55	(Van *et al* 2008)
Sulfonamide	*sul* (1)	F: 5´- TTCGGCATTCTGAATCTCAC- 3´ R: 5´-ATGATCTAACCCTCGGTCTC- 3´	822	47	(Van *et al* 2008)
Cephalothin	*bla* (SHV)	F: 5´- TCGCCTGTGTATTATCTCCC- 3´ R: 5´-CGCAGATAAATCACCACAATG- 3´	768	52	(Van *et al* 2008)
Ampicillin	CITM	F: 5´- TGGCCAGAACTGACAGGCAAA- 3´ R: 5´-TTTCTCCTGAACGTGGCTGGC- 3´	462	47	(Van *et al* 2008)
Erythromycin	ere	F: 5´- GCCGGTGCTCATGAACTTGAG- 3´ R: 5´-CGACTCTATTCGATCAGAGGC- 3´	419	52	(Van *et al* 2008)
Chloramphenicol	*cat* (1)	F: 5´- AGTTGCTCAATGTACCTATAACC- 3´ R: 5´- TTGTAATTCATTAAGCATTCTGCC- 3´	547	55	(Van *et al* 2008)
	cml (A)	F: 5´- CCGCCACGGTGTTGTTGTTATC- 3´ R: 5´-CACCTTGCCTGCCCATCATTAG- 3´	698	55	

stx (1), 5 (35.7%) were positive for *stx* (2), 7 (50%) were positive for *eae*, and 1 (7.1%) isolate was positive for *cnf* (1) (figure 1). None of the isolate was positive for PCR of *cnf* (2). Among the 14 isolates, 7 (50%) were positive for both *stx* and *eae* (EHEC), 6 (42.9%) were positive for only *stx* (STEC) while 1 (7.1%) was positive for *cnf* (1) (NTEC) (figure 2).

ANTIMICROBIAL RESISTANCE GENOTYPES OF *E. coli* ISOLATES FROM DIARRHOEIC DOGS

Out of 14 isolates tested for the presence of antimicrobial resistance genes, 9 (64.3%) were positive for CITM gene, 6 (42.9%) were positive for *aad*(A1) and *bla*(SHV), 5 (35.7%) were positive for *tet*(A), *dfr*(A1) and *cat*(1), 4 (28.6%) were positive for *aac* (3)-IV, 3 (21.4%) were positive for both *tet*(B), *sul*(1) and *cml*(A), while 1 (7.1%) of the isolate was positive for *ere* (figure 3).

DISCUSSION

In this study, the presence of some virulence and antimicrobial resistance genes in *E. coli* isolates from dogs with diarrhoea in Iran was investigated. The presence of virulence and antimicrobial resistance genes in *E. coli*

strains harbored by companion animals is of public health concern because humans are in close contact with these animals (Puno-Sarmiento *et al* 2013). The presence of pathogenic *E. coli* strains in diarrhoeic companion animals is of greater importance because of high possibility of zoonotic transmission following widespread environmental contamination with these organisms (Geser *et al* 2011, Nguyen and Speradio 2012). In this study, isolation of 33 (47.1%) *E. coli* strains from 70 diarrhoeic dogs suggested the involvement of *E. coli* in a sizeable percentage of canine diarrhoea in Iran. The fact that virulence genes were detected in the isolates investigated indicates that they were pathogenic *E. coli* strains (Bentancor *et al* 2007, Shahrani *et al* 2014). Although the serotypes of the isolates in this study were not determined, they could belong to the serogroups capable of causing zoonotic infections (Morato *et al* 2009, de Almeida *et al* 2012, Tramuta *et al* 2014). The 47.1% pathogenic *E. coli* prevalence in this study is higher when compared with 44.4, 25 and 37.1% faecal pathogenic *E. coli* prevalence among 45, 68 and 70 dogs with diarrhoea reported in Canada (Hammemermulaer *et al* 1995), Brazil (Puno-Sarmiento *et al* 2013) and Egypt (Ali and Metwaly 2015), respectively. In Iran, Zahraei Salehi *et al*. (Zahraei Salehi *et al* 2011) reported 10% faecal pathogenic *E. coli* prevalence among 100 apparently healthy/diarrhoeic dogs
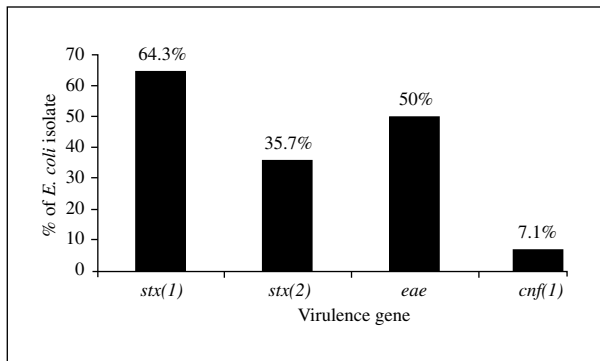
Figure 1. Frequency of occurrence of tested virulence genes in 14 *E. coli* isolates from dogs with diarrhoea.

Frecuencia de ocurrencia de genes probados de virulencia en 14 aislados de *E. coli* de perros con diarrea.
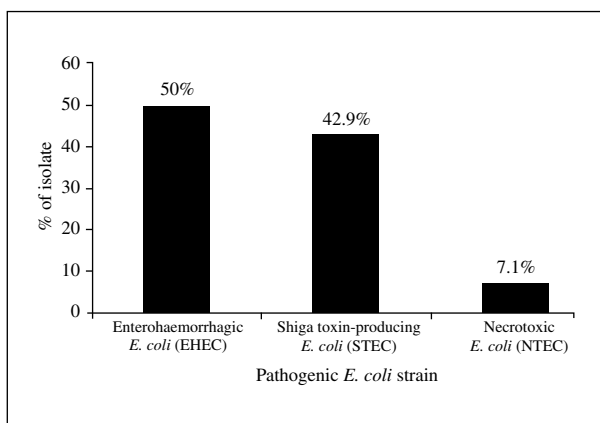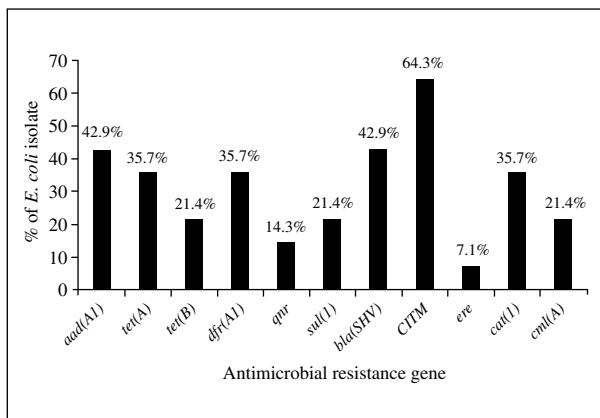

Figure 2. Distribution of pathogenic *E. coli* strains (pathotypes) among 14 strains isolated from dogs with diarrhoea.

Distribución de cepas patogénicas de *E. coli* (patotipos) entre 14 cepas aisladas de perros con diarrea.

Figure 3. Frequency of antimicrobial resistance genes in 14 pathogenic *E. coli* isolates from dogs with diarrhoea.

Frecuencia de genes de resistencia a los antimicrobianos en 14 aislados patogénicos de *E. coli* en perros con diarrea.

while Koochakzadeh *et al* (2014) reported 36.64% pathogenic *E. coli* prevalence among 79 faecal *E. coli* isolates from 252 equidae/canidae. In Argentina, Bentacour *et al*

(2007) reported 15.5% faecal pathogenic *E. coli* prevalence among 450 dogs. These findings are also lower than the result (47.1%) of the present study. The 47.1% pathogenic *E. coli* prevalence in this study is however lower when compared with 66.6% pathogenic *E. coli* prevalence among 51 dogs with diarrhoea reported in Egypt (Yunis *et al* 2015). Variations in prevalence of pathogenic *E. coli* strains in these studies may be due to differences in the level of contamination of dogs' environment, food and drinking water, age, immune status, stage of infection and number of samples analysed (Shaheen *et al* 2011, Yunis *et al* 2015). The focus of this study however was not on predisposing factors for faecal *E. coli* shedding but on isolation of *E. coli* from dogs with diarrhoea.

In this study, the presence of 3 important virulence genes *stx*, *eae* and *cnf* often haboured by pathogenic *E. coli* were investigated in 14 isolates which were categorised into pathotypes based on the virulence genes detected. It is noteworthy that 13 (92.8%) of the 14 isolates examined haboured *stx* gene. The *stx* genes encode shiga-like toxin (stx) also called verocytotoxin/verotoxin, a putative virulent factor involved in the pathogenicity of STEC also known as verocytotoxin-producing *E. coli* (VTEC) and EHEC strains (Paton and Paton 1998, Goldwater *et al* 2012, Nguyen and Speradio 2012, Shahrani *et al* 2014). The stx inhibits protein synthesis and allows invasion of the intestinal mucosa similar to what is observed in human shigellosis (Nguyen and Speradio 2012). The 92.8% *stx* gene prevalence in this study is higher when compared with 40 and 44.4% *stx* gene prevalence among 92 (from 25 diarrhoeic dogs) and 20 (from 45 diarrhoeic dogs) faecal *E. coli* isolates reported in Brazil (Paula and Marin 2008, Paula and Marin 2009) and Canada (Hammermueler *et al* 1995), respectively. In Iran, Zahraei *et al* (2011) reported 4% *stx* gene prevalence among 10 pathogenic *E. coli* isolates from 100 apparently healthy/diarrhoeic dogs while Koochakzadeh *et al* (2014) reported 18.9% *stx* gene prevalence among 79 pathogenic *E. coli* isolates from a population of 252 canidae/equidae. Their findings are also lower when compared with the results (92.8%) of the present study. Detection of *stx* (1) in 64.3% of the isolates as against *eae* (50%), *stx*(2) (35.7%) and *cnf*(1) (7.1%) in this study, suggested that *stx* (1) may be the dominant virulence gene harbored by *E. coli* strains isolated from dogs with diarrhoea in Iran. The 63.4% *stx*(1) gene prevalence recorded in this study is higher when compared with 8.9 and 7.6% *stx*(1) gene prevalence among 20 and 92 *E. coli* isolates from dogs with diarrhoea reported in Canada (Hammermueler *et al* 1995) and Brazil (Paula and Marin 2008, Paula and Marin 2009), respectively. It is also higher than 12.3% *stx*(1) gene prevalence among 57 faecal *E. coli* isolates from healthy dogs reported in Canada (Hammermueler *et al* 1995), and 18.9% prevalence among 79 faecal *E. coli* isolates from canidae/equidae reported in Iran (Koochakzadeh *et al* 2014). On the other hand, 35.7% *stx*(2) gene prevalence in this study is higher than 1.1, 22.2 and 5.4% *stx*(2)

gene prevalence among faecal *E. coli* isolates from dogs reported in Argentina (Bentancor *et al* 2007), Canada (Hammermueler *et al* 1995) and Brazil (Paula and Marin 2008, Paula and Marin 2009), respectively. But it is lower when compared with 60% *stx*(2) gene prevalence among 34 *E. coli* isolates from dogs with diarrhoea reported in Egypt (Yunis *et al* 2015). Thus, the result of this study suggested that stx especially the stx1, may be associated with majority of canine diarrhoea in Iran in which *E. coli* is isolated. This finding corroborates previous reports in Iran (Zahraei *et al* 2011, Koochakzadeh *et al* 2014). The differences in the prevalence of *stx* genes in the aforementioned studies indicate variation in the rate of contamination and infection by *E. coli* strains habouring these genes in the study areas.

In the present study, detection of *stx* and *eae* in 7 (50%) of the investigated isolates enabled their placement in the EHEC group (Bentancor *et al* 2007, Aslani *et al* 2008, Goldwater and Bettelheim 2012, Nguyen and Speradio 2012, Shahrani *et al* 2014, Ali and Metwaly 2015). The *eae* gene encodes intimin which enables adhesion of the *E. coli* isolates to the intestinal epithelial cells resulting in the classical histopathological attaching and effacing (A/E) lesions (Nataro and Kaper 2003, Goldwater and Bettelheim 2012, Shahrani *et al* 2014). Interestingly, none of the isolates in this study was positive for the *eae* gene only. This nullifies possible involvement of EPEC/ AEEC strains in diarrhoeal disease in the sampled dogs (Bentancor *et al* 2007, Shahrani *et al* 2014). EPEC strains are defined as *eae*-harbouring diarrhoeagenic *E. coli* that possess the ability to form A/E lesions on intestinal cells and that do not possess shiga-like toxin encoding genes (Moxley and Smith 2010, Shahrani *et al* 2014). EPEC strains habouring the plasmid-encoded bundle forming pilli (*bfp*) gene, are regarded as typical EPEC (tEPEC) while *bfp* non-habouring strains are atypical EPEC (aEPEC) (Moxley and Smith 2010, Ali and Metwaly 2015). Since this study did not detect EPEC strains, the presence of *bfp* gene in the isolates was not investigated. Nonetheless, the EHEC pathotypes in this study may habour *bfp* gene and this needs to be further verified. On the contrary, Zahraei Salehi *et al*. (Zahraei Salehi *et al* 2011) reported that 6 (6%) isolates among 10 pathogenic *E. coli* isolates from dogs without diarrhoea in Iran were EPEC strains. The 50% *eae* gene (combined with *stx* gene) prevalence noted in this study is higher when compared with 13, 17.6 and 20% *eae* gene prevalence among 19, 12 and 34 *E. coli* isolates from 146, 68 and 51 dogs with diarrhoea reported in Canada (Nakazato *et al* 2004), Brazil (Puno-Sarmiento *et al* 2013) and Egypt (Ali and Metwaly 2015), respectively. It is also higher than 8 and 10.5% *eae* gene prevalence among 36 and 86 *E. coli* isolates from dogs without diarrhoea reported in Canada (Nakazato *et al* 2004) and Brazil (Puno-Sarmiento *et al* 2013), respectively. This finding further suggests higher rate of environmental contamination and dog

infection with pathogenic *E. coli* strains in Iran than the other study areas.

In this study, the prevalence (50%) of EHEC pathotype is higher compared against 1 (7.1%) of the isolates which haboured *cnf* (1) only and was regarded as NTEC (De Rycke *et al* 1999; Landraud *et al* 2000, Salvadoris *et al* 2003, Shahrani *et al* 2014), and 6 (42.9%) which haboured *stx* only and were grouped as STEC (Aslani *et al* 2008, Shahrani *et al* 2014). This result suggested that EHEC strains may be the predominant diarrhoeagenic *E. coli* pathotype isolated from dogs with diarrhoea in Iran. EHEC strains habouring highly conserved plasmid families encoding for multiple virulence have been described (Wood *et al* 1986, Hales *et al* 1992, Nataro and Kaper 2003). EHEC are diarrhoeagenic strains incriminated in different types of diarrhoea in humans (Aslani *et al* 2008, Amisano *et al* 2011, Goldwater and Bettelheim 2012, Nguyen and Speradio 2012). Thus, isolation of EHEC from dogs with diarrhoea in this study, portends public health risk particularly to individuals that could have direct or indirect contact with these dogs (Nguyen and Speradio 2012). The 50% EHEC prevalence recorded in the present study is higher than 0.22% EHEC prevalence among 70 pathogenic *E. coli* isolates from 450 dogs reported in Argentina (Bentancor *et al* 2007). However, lack of EHEC detection in previous studies (Zahraei *et al* 2011, Koochakzadeh *et al* 2014) in Iran is attributed to the fact that the authors classified isolates which haboured both *stx* and *eae* genes as STEC strains. The stx is a major virulent factor involved in pathogenicity of the EHEC and STEC/ VTEC pathotypes (Paton and Paton 1998, Nguyen and Speradio 2012, Shahrani *et al* 2014). STEC strains have been associated with diarrhoea in dogs (Paton and Paton 1998, Paula and Marin 2008, Zahraei *et al* 2011). The 42.9% STEC prevalence observed in the present study is higher when compared with 13% STEC prevalence among 92 *E. coli* isolates from 25 dogs with diarrhoea reported in Brazil (Paula and Marin 2008, Paula and Marin 2009). It is also higher than 6% STEC among 10 pathogenic *E. coli* isolates from 100 healthy/diarrhoeic dogs reported in Iran (Zahraei *et al* 2011). In Turkey, Sancak *et al* (2004) reported a lower STEC prevalence of 24.6 and 28% among 57 and 82 dogs with acute and chronic diarrhoea, respectively. Thus, higher prevalence of STEC in this study suggested that the environment and/or food and drinking water of dogs in the present study could have been contaminated with STEC strains more than in the other study areas (Nguyen and Speradio 2012). The health status of the dogs and duration of infection (Sancak *et al* 2004) might also have affected the reported prevalence in the various studies. The finding of high STEC (42.9%) and EHEC (50%) prevalence in this study, portends serious threat to public health since STEC and EHEC strains causes highly fatal and untreatable infections such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) which causes renal failure in humans especially in children (Bentancor *et al* 2007,

Amisano *et al* 2011, Goldwater *et al* 2012, Nguyen and Speradio 2012, Shahrani *et al* 2014). Although the EHEC isolates in this study were not serotyped, their zoonotic significance cannot be ruled out since both EHEC O157 and non-O157 EHEC strains are known causes of HC and HUS (Goldwater and Bettelheim 2012).

The 7.1% NTEC strains observed in this study suggested that it may be the least predominant *E. coli* pathotype isolated from dogs with diarrhoea in Iran. Pathogenicity of NTEC strains is based on elaboration of cnfs as well as other virulent factors (Kavitha *et al* 2010, Shahrani *et al* 2014). The *cnf*(1) gene encodes cnf1, a toxin which interfere with the pahagocytic activities of polymorphonuclear cells thereby facilitating blood stream invasion by *E. coli* with subsequent apoptosis of intestinal epithelial cells (Emödy *et al* 2003, Kavitha *et al* 2010, Koochakzadeh *et al* 2014, Shahrani *et al* 2014). None of the isolate investigated in this study haboured *cnf*(2) gene which encodes cnf2 (Kavitha *et al* 2010). This suggested that all the NTEC strains obtained in this study belonged to the NTEC-1 pathotype (Kavitha *et al* 2010). Based on the type of *cnf* gene haboured, NTEC strains are grouped into two distinct homogenous categories NTEC-1 and NTEC-2, each of them being genetically linked to several other specific virulence markers (Kavitha *et al* 2010). The 7.1% *cnf* (1) gene prevalence in this study is lower when compared with 16.4% *cnf*(1) gene prevalence among 55 faecal *E. coli* isolates from healthy dogs reported by Siqueria *et al* (2009) in Brazil. Variation in NTEC-1 strain prevalence in these studies could also be due to differences in level of environmental, food and/or drinking water contamination by NTEC-1 strains in the study areas. Therefore, the environment of dogs in the present study could have been contaminated more with the organisms which resulted in higher infection and isolation rate.

Resistance to antimicrobial agents is encoded by chromosomal and plasmid genes haboured by bacterial organisms (Tenover 2006). These genes may be inherent or acquired via vertical or horizontal transfer (transformation, conjugation and transduction) mechanisms (Tenover 2006). Phenotypic resistance is determined by the genotype (Morrison and Rubin 2015). The *aad*(A1) and *aac3*-(IV) genes encode aminoglycoside adenyltransferases and acetyltranferaseses which mediate resistance to streptomycin and gentamicin, respectively (Szczepanowski *et al* 2009). These genes were detected in this study indicating that the isolates are aminoglycoside-resistant strains. Detection of *aad*(A1) gene in 6 (42.9%) of the investigated isolates as against 4 (28.6%) for *aac3*-(IV) gene, suggested acquisition of streptomycin resistance gene more than gentamicin resistance gene. The high acquisition of *aad*(A1) gene may be a result of selection pressure due to frequent use of streptomycin which is often combined with penicillin to elicit broad-spectrum action, in treating bacterial infections in companion animals. In this study, the presence of tetracycline resistance genes *tet*(A) and *tet*(B), showed that the isolates possessed multiple tetracycline determinants. The *tet*(A) and *tet*(B) genes are among several tetracycline determinants in *E. coli* which encode energy-dependent membrane-associated efflux proteins (Roberts 2005). Detection of *tet*(A) in 5 (35.7%) of the examined isolates as against 3 (21.4%) for *tet*(B) suggested that *tet*(A) may be the predominant tetracycline resistance gene haboured by pathogenic *E. coli* colonising dogs in Iran. Other tetracycline-resistant genes which are thought to confer resistance through ribosomal protection and enzymatic inactivation (Nde and Logue 2008, Torkan *et al* 2015) may also be haboured by the tetracycline-resistant gene-positive isolates in this study. However, the presence of these other genes was not verified in this study.

The emergence of β-lactam-resistant bacteria in companion animals and their transfer to humans pose serious risk to public health (Hammermueler *et al* 1995, De Rycke *et al* 1999). In this study, the presence of two determinants (CITM gene cluster and *bla*(SHV) gene) for β-lactam resistance in the isolates was investigated. Detection of CITM gene cluster in 9 (64.2%) of examined isolates suggested that among all the resistance genes tested, it is the most predominant. The high prevalence of CITM gene cluster may be a result of selection due to frequent exposure to β-lactams especially ampicillin. Beta-lactams are widely used in veterinary medicine for treating infections caused by *E. coli* in companion animals (Li *et al* 2007). In *E. coli*, the CITM gene cluster encodes AmpC β-lactamase which hydrolyses β-lactams (Van *et al* 2008). Detection of *bla*(SHV) in 6 (42.9%) of the examined isolates, suggested high prevalence of this SHV β-lactamase-encoding gene (Feria *et al* 2002, Ojdana *et al* 2014). The *bla*(SHV) gene encodes β-lactamase which mediates resistance to cephalothin, a first-generation cephalosporin. However, some variants of *bla*(SHV) encode extended-spectrum β-lactamase which hydrolyses third-generation cephalosporins (extended-spectrum β-lactams) (Bradford 2001, Bush and Jacoby 2010, Ojdana *et al* 2014), these variants have been reported in faecal *E. coli* isolates from dogs (Rocha-Gracia *et al* 2015, Schmidt *et al* 2015). Therefore, the 42.9% *bla*(SHV) detection rate in this study suggested that many dogs with diarrhoea in Iran may harbor extended-spectrum β-lactam (ESBL)-resistant *E. coli*. This finding is a cause for concern because extended-spectrum β-lactams are critical for treatment of bacterial infections in humans and animals (Bradford 2001) and *E. coli* isolates habouring *bla*(SHV) have been reported to exhibit multidrug resistance (Branger *et al* 2005, Bush and Jacoby 2010, Geser *et al* 2011). Thus, the presence of *bla*(SHV) gene in the examined isolates in this study, pose serious threat to public health as well as that of the examined dogs since compromise in antibacterial therapy may result following zoonotic transmission of the organisms (Warren *et al* 2001). In America, *bla*(SHV) was also detected in *E. coli* isolates from companion animals but with a lower 17% prevalence (Shaheen *et al* 2011).

The detection rate (14.3%) of fluoroquinolone determinant *qnr* gene in this study is surprising because fluoroquinolones are not known to be used in canine medicine in Iran. Nevertheless, the isolates could have acquired the gene from bacterial organisms from other sources. The presence of *qnr* gene in isolates in this study poses threat to public health. This is because *qnr*-plasmids are often associated with integrons and they carry multiple resistance determinants, thus providing resistance to several classes of antimicrobials including β-lactam and aminoglycoside (Kang *et al* 2005, Li 2005). In this study, the trimethoprim determinant *dfr* (A1) gene was haboured by 5 (35.7%) of the examined isolates. This rate of trimethoprim resistance gene acquisition is high, and may be due to selection resulting from frequent use of sulfonamide/trimethoprim combination (due to its broad-spectrum activity) in small animal medicine (Antunes *et al* 2005, Torkan *et al* 2015). This reason may also explain the 28.6% prevalence of *sul*(1) gene in the examined isolates. The *dfr*(A1) gene is one of the variants of *dfr* gene.; in *E. coli* it encodes dihydrofolate reductase (DHFR), thus countering the inhibitory effect of trimethoprim (Szczepanowski *et al* 2009). The *sul*(1) gene is among the sulfonamide determinants encoding dihydropteroate synthase (DHPS) which is not inhibited by sulfonamide in *E. coli* (Enne *et al* 2001). Detection of erythromycin determinant *ere* gene in 1 (7.1%) of the examined isolates, suggested that the gene was acquired at a low rate by the isolates. The low *ere* gene prevalence in this study may be related to the fact that erythromycin is not used for treatment of infections caused by Gram-negative organisms. Therefore, there may not have been selection pressure to necessitate acquisition of *ere* gene which encodes erythromycin methylases, the mediators of resistance to macrolides (Landraud *et al* 2000, Gaynor and Mankin 2003). In the current study, detection of *cat*(1) gene in 5 (35.7%) and *cml*(A) gene in 3 (21.4%) of the examined isolates, suggested that the isolates haboured different chloramphenicol determinants. The prevalence of these genes suggests that *cat*(1) gene may be the predominant chloramphenicol determinant haboured by *E. coli* isolates from dogs with diarrhoea in Iran. The high prevalence of these genes may be due to use selection pressure which resulted in acquisition of the genes at a high rate. In *E. coli*, the *cat*(1) gene is a variant of *cat* genes encoding chloramphenicol acetyltranferases, the major mediators of chloramphenicol resistance (Schwarz *et al* 2004, Torkan *et al* 2015) while the *cml*(A) is among the genes encoding chloramphenicol efflux proteins (exporters) (Schwarz *et al* 2004).

It is concluded that *E. coli* isolates from dogs with diarrhoea presented to IAUTH, Iran haboured various virulence and antimicrobial resistance genes. The isolates belonged to the EHEC, STEC and NTEC pathotypes with the EHEC strain being the most prevalent. The CITM gene cluster is the predominant antimicrobial resistance determinant haboured by the examined isolates. The

bla(SHV) gene which confers resistance to β-lactams including extended-spectrum β-lactams was detected in some of the examined isolates. Thus, antibacterial-resistant diarrhoeageenic *E. coli* strains are possible offenders in diarrhoeal diseases of dogs reared in Iran. This poses serious threat to public health following zoonotic transmission. However, further molecular studies to detect other virulent and antimicrobial resistance genes in the isolates obtained in this study is recommended. This study is the first report on detection of *cnf*(1) gene in *E. coli* isolates from companion animals in Iran.

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