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

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

S Torkan\textsuperscript{a}, MA Bahadoranian\textsuperscript{b}, F Khamesipour\textsuperscript{c*}, MU Anyanwu\textsuperscript{d}

ABSTRACT. This study was conducted to investigate the presence of some virulence and antimicrobial resistance genes in *E. coli* isolates from diarrhoeic 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 *eae* (1), 7 (50%) were positive for *aeae* (2), 7 (50%) were positive for *eae* (2), 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)*, *su (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, *Escherichia coli*, genes, virulence.

RESUMEN. El objetivo de este estudio 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 *eae* (2), siete (50%) fueron positivos para *eae* (2), y uno (7,1%) fue positivo para el aislamiento de *cnf* (1). De los 14 aislamientos probados para determinar la presencia de genes de resistencia antibacteriana, 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)*, *su (1)* y *cml (A)*, mientras que uno (7,1%) del aislado fue positivo para *ere*. Los resultados mostraron que cepas de *E. coli* enteroinvasiva (EIEC), *E. coli* shiga toxigénica (STEC) y *E. coli* necrotóxica (NTEC) que albergan varios genes que codifican para la resistencia antimicrobiana podrían estar involucradas 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, Tajbaksh 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 diarrhoeic 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), entero toxinogenic/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 (stx1 and stx2), cytotoxic necrotizing factor (cnf) encoded by cytotox necrotizing factor genes 1 and 2 (cnf1 and cnf2), 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 (IAUVTHI), 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.

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Target virulence gene</th>
<th>Primers Sequence</th>
<th>Amplicon size (base pair)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga-like toxin</td>
<td>Stx (1)</td>
<td>F: 5’- CCTGATTATGTTGCGGAAGG- 3’&lt;br&gt;R: 5’- CACCAGACATTGTAACCCTG- 3’</td>
<td>348</td>
<td>56</td>
<td>(Cebula et al 1995)</td>
</tr>
<tr>
<td></td>
<td>Stx (2)</td>
<td>F: 5’- ATCTATTACCACGGAGGTACG- 3’&lt;br&gt;R: 5’- GCGTCATCGTATACACAGGACC- 3’</td>
<td>584</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attaching and effacing factor</td>
<td>eae</td>
<td>F: 5’- TGGCGGCACACGGGCGCA- 3’&lt;br&gt;R: 5’- CGTCGCCGGACGAGATCT- 3’</td>
<td>629</td>
<td>56</td>
<td>(Heuvelink et al 1995)</td>
</tr>
<tr>
<td>Cytotoxic necroptizing factor</td>
<td>Cnf (1)</td>
<td>F: 5’- GGCGGAAGTCAGAAGAATTA- 3’&lt;br&gt;R: 5’- TGGCGTCACCTCTCAACCAG- 3’</td>
<td>1111</td>
<td>56</td>
<td>(Toro et al 2005)</td>
</tr>
<tr>
<td></td>
<td>Cnf (2)</td>
<td>F: 5’- TATCATAGGGCAGGAGAGCGACC- 3’&lt;br&gt;R: 5’- GTAAGTACATAATACCATTTTCCA- 3’</td>
<td>1240</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 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 following standard procedures.

ANTIMICROBIAL RESISTANCE AND VIRULENCE GENOTYPE OF THE E. coli ISOLATES

DNA of 14 isolates was extracted using bacteria DNA extraction kit following the manufacturer’s instructions. Using Ependorf Mastercycler, the presence of the following 5 virulence genes: Shiga-like toxin genes stx (1) and stx (2), attaching and effacing factor 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

OCURRENCE OF VIRULENCE GENES IN E. coli ISOLATES FROM DIARRHOEIC 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
TORKAN ET AL

Table 2. PCR primers used for detection of antimicrobial resistance genes.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Target resistance gene</th>
<th>Primers Sequence</th>
<th>Amplicon size (base pair)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Streptomycin        | aad (A1)                | F: 5´- TATCCAGCTAAAGCGCAACT- 3´  
|                     |                         | R: 5´- ATTTGCCGACTACCTTTGTC- 3´   | 447                       | 58  | (Puno-Sarmiento et al 2013) |
|                     | tet (A)                 | F: 5´- GTTTCACTCGAAGCGCTGCA- 3´  
|                     |                         | R: 5´- CTGTCGCCAGACTGTTGATGA- 3´  | 577                       | 57  |
| Tetracycline        | tet (B)                 | F: 5´- CCTCAGCTTGCTCAAGCCGTG- 3´  
|                     |                         | R: 5´- GCACCTTGCGTATGACTCTT- 3´    | 634                       | 56  | (Puno-Sarmiento et al 2013) |
| Trimethoprim        | dfr (A1)                | F: 5´- GGAGTGTCAAAAGGTGAACAGC- 3´  
|                     |                         | R: 5´- GAGCCGAAAGCTTTGGAATAAAC- 3´  | 367                       | 45  | (Torkan et al 2015) |
| Fluoroquinolone     | qnr                     | F: 5´- GGTATGGATATTATGTATGAAG- 3´  
|                     |                         | R: 5´- CTAATCAGCCGAGCTATTTA- 3´    | 670                       | 50  | (Li 2005) |
| Gentamicin          | aac (3)- (IV)           | F: 5´- CTTCAGGTGCAAAGTGTTG- 3´  
|                     |                         | R: 5´- TCAATCTCGTTCCTCCGCTCAT- 3´   | 286                       | 55  | (Van et al 2008) |
| Sulfonamide         | sul (1)                 | F: 5´- TGCGCTTGTGATTATCTCC- 3´  
|                     |                         | R: 5´- CGCAGATAAAATCCACACACATG- 3´   | 768                       | 47  | (Van et al 2008) |
| Cephalothin         | bla (SHV)               | F: 5´- TGGCCAGACCTGACAGCGAAA- 3´  
|                     |                         | R: 5´- TTTCCTGCTGAACTGGTCTTGGC- 3´   | 467                       | 47  | (Van et al 2008) |
| Ampicillin          | CITM                    | F: 5´- GCCGCGAACCTGACAGCGAAA- 3´  
|                     |                         | R: 5´- TTTCCTGCTGAACTGGTCTTGGC- 3´   | 419                       | 52  | (Van et al 2008) |
| Erythromycin        | ere                     | F: 5´- AGTGGGCTCAATGACGCGTTGAG- 3´  
|                     |                         | R: 5´- CGACTCTTCTCGGATGACAGGCG- 3´   | 547                       | 57  | (Van et al 2008) |
| Chloramphenicol     | cml (A)                 | F: 5´- CGGCACACCGTGTTTGTATATC- 3´  
|                     |                         | R: 5´- CACCTTGCGCTGCCCATATTAG- 3´    | 698                       | 58  | (Van et al 2008) |

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 2012), 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.
Koochakzadeh (2014) reported 36.64% pathogenic E. coli prevalence among 57 faecal isolates from 252 equidae/canidae. In Argentina, Bentacour et al. (2015) reported 18.9% faecal pathogenic E. coli prevalence among 79 faecal E. coli isolates from dogs with diarrhoea. 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 (Hammermueller 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/canidae. 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 (Hammermueller 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 (Hammermueller 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 *stx*1, 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, Koochkazadeh *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 harbouring 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). The 50% *eae* gene prevalence noted in this study is positive for the *eae* gene only. This nullifies possible involvement of EPEC/AEEC strains in diarrhoel disease in the sampled dogs (Bentancor *et al* 2007, Shahrani *et al* 2014). EPEC strains are defined as *eae*-harbouring diarrhoegenic *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 harbouring the plasmid-encoded bundle forming pili (*bfp*) gene, are regarded as typical EPEC (tEPEC) while *bfp* non-harbouring 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 harbour *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 diarrhoegenic *E. coli* pathotype isolated from dogs with diarrhoea in Iran. EHEC strains harbouring 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 diarrhoegenic 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, Koochkazadeh *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 interferes with the phagocytic 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 harboured _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 harboured, 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 harboured 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 _aac(3)-IV_ genes encode aminoglycoside adenyltransferases and acetyltransferases 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 _aac(3)-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 harboured by _E. coli_ colonising dogs in Iran. Other tetracycline-resistant genes which are thought to confer resistance through ribosomal protection and enzymatic inactivation (Ndé and Logue 2008, Torkan et al. 2015) may also be harboured 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 (Hammermueller 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 harbouring _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 sul1 (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 sul1 (1) gene is among the sulfonamide determinants encoding dihydropyurate synthase (DHPS) which is not inhibited by sulfonamide in E. coli (Emne 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 acetyltransferases, 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 diarrhoeagenic 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 cef (1) gene in E. coli isolates from companion animals in Iran.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the valuable contribution of Dr. Hassan Montaz and Dr. Elnaheh Tajbaksh.

REFERENCES


Costa D, P Poeta, Y Saenz, AC Coelho, S Matos, L Vinue, J Rodrigues, C Torres. 2008. Prevalence of antimicrobial resistance and resistance...
ANTIMICROBIAL RESISTANCE, DIARRHEA, DOGS, Escherichia coli, GENE, VIRULENCE

Hales BA, CA Hart, RM Batt RM, JR Saunders. 1992. The large plasmids found in enterohemorrhagic and enteropathogenic Escherichia coli constitute a related series of transfer-defective Inc F-IIA replications.