FULL PAPER *Bacteriology*

Molecular Characterization of Enterohemorrhagic *Escherichia coli* Isolates from Cattle

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ABSTRACT. A total of 21 (4.3%) enterohemorrhagic *E. coli* strains were isolated by biochemical tests and identification of the $eae^{+}stx1^{+}stx2^{+}$ genotype from 490 stool samples obtained from calves with diarrhea during 1-year period from a major farm in Tehran, Iran. All of the strains showed resistance to ampicillin, ciprofloxacin, trimethoprim, streptomycin, chloramphenicol and tetracycline, while 19% showed resistance to gentamicin. Out of 21 EHEC strains, 11 (53%) harbored class 1 integron. Two different amplification products, which were approximately 750 and 1,700 bp in size, were obtained from amplified variable regions (*in-F*/*in-R* primers) in 3 (14.3%) and 4 (19%) of the EHEC isolates, which corresponded to *dfrA7*(dihydrofolate reductase type I) and *dfrA1*/*aadA1*(dihydrofolate reductase/aminoglycoside adenyltransferase) resistance gene cassettes, respectively, and this was confirmed by sequencing. Genotyping analysis revealed a total of 16 pulsotypes that corresponded to 16 isolates with the similarity indices of 62% and 30% for the most and least similar isolates, respectively, 9 of which harbored class 1 integron. Analysis of pulsotypes showed an extensive diversity among the isolates harboring integron, which is indicative of a lack of any significant genetic relatedness among the isolates. No obvious relation could be deduced between integron content and special pulsotypes. The little data available on the genotyping patterns of EHEC isolates from cattle and their resistance gene contents emphasize the need to establish genotyping databases in order to monitor and source track the source of emergence and spread of new resistant and integron-carrying genotypes.

KEY WORDS: enterohemorrhagic *E. coli*, integrin, PFGE

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Enterohemorrhagic *Escherichia coli* (EHEC) has been globally recognized as an important food-borne pathogen. The principal virulence factor of EHEC is known as Shiga toxin or Verotoxin (VT), which was previously known as Shiga-like toxin (SLT). Human infections with Shiga-toxin-producing *Escherichia coli* (STEC) can be variable, from a simple or watery diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [35]. A variety of EHEC serotypes have been implicated in human foodborne diseases, among which serotype O157 is an important foodborne pathogen, and its first outbreak occurred in the United States in 1982; moreover, many other non-O157 serogroups have been reported as foodborne pathogens [26].

EHEC is transmitted by the fecal–oral route, and it can spread among animals via direct contact, water, feed, contaminated pastures or other environmental sources [15]. Ruminants, especially cattle and sheep, are the most important hosts for EHEC [13, 15], and EHEC can be transmitted to humans through the consumption of raw or undercooked meat of infected cattle or other food contaminated with animal feces [31]. EHEC multidrug-resistant (MDR) strains have been increasing recently, and they are one of the major health-care problems [24]. Emergence and spread of antimicrobial-resistant strains are the consequence of therapeutic and subtherapeutic usage of antibiotic in animals [1, 32, 34]. Spread of antimicrobial resistance can also occurr by horizontal transmission of antibiotic resistance genes among strains via different types of mobile DNA segments, such as plasmids, transposons and integrons [6, 33]. Integrons consist of two conserved sequences and a central variable region containing antimicrobial resistance gene cassettes [18]. The principle components of integron are the integrase gene (*int*I), the attachment site (*att*I) and the promoter (P_{ant}) [6]. Four classes of integrons have been reported up to now, and the class I integrons are more commonly recognized among clinical isolates [21, 30]. Several studies have shown the widespread distribution of integrons among enteric bacteria from different countries including Iran [12, 16, 19, 25, 26, 28].

EHEC infection outbreaks and sporadic cases have been increasing recently, and the largest outbreak of EHEC infection was reported in Japan in the summer of 1996 [23]. The pulsed field gel electrophoresis (PFGE) technique has also been used for identification of an undetected outbreak [4]. The clonal relatedness of human clinical EHEC isolates with those from cattle is a subject that needs to be studied to assess the significance of the animal-human manner of infection.

The aim of this study was to investigate the genetic diversity of different enterohemorrhagic *E. coli* isolates from cattle and to assess the distribution of class 1 integrons and their resistance gene cassettes among different EHEC genotypes.

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Table 1. Primers sequences used in this study

Primer	Sequence (5′-3′)	Amplicon size (bp)	Reference
eae	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	422	28
STEC/*stx1*	CAGTTAATGTGGTGGCGAAG CTGCTAATAGTTCTGCGCATC	894	5
STEC/*stx*$_2$	CTTCGGTATCCTATTCCCGG GGATGCATCTCTGGTCATTG	478	5
int	TGCGTGTAAATCATCGTCGT CAAGGTTCTGGACAGTTGC	900	2
in	GGCATCCAAGCAGCAAGC AAGCAGACTTGACCTGAT	Variable	9

MATERIAL AND METHODS

Specimens and culture process: Four hundred and ninety stool samples were collected from calves with diarrhea during a 1-year period from a major farm in Tehran, Iran. Each swab sample was inoculated to Amies transport media with charcoal (Merck KgaA, Darmstadt, Germany) and streaked on MacConkey agar (Merck KGaA) within 4 hr of collection. Up to 3 suspected colonies on MacConkey agar were selected and subjected to biochemical tests including gram staining, oxidase, indol, simmons citrate, urease and hydrogen sulfide as described previously [3]. The biochemically confirmed *E. coli* colonies were subjected to molecular analysis to search for the enterohemorrhagic pathotype.

Molecular diagnostic methods for EHEC and sequence analysis of integron contents: Genomic DNA was extracted from purified *E. coli* cultures using the boiling method. Accordingly, bacterial cells were pelleted by centrifugation and suspended in 50 μl of TE buffer and subsequently subjected to boiling for 10 min at 100°C. The lysate was centrifuged, and the supernatant was subjected to PCR assay using 3 different sets of primers targeting the *stx*1, *stx*2 and *eae* genes. Class 1 integron was detected among confirmed EHEC isolates using primers that specifically detect the conserved integrase gene (intF/int-R). The resistance gene cassette within the variable region of class 1 integron was further characterized using primers that spans between the 3′-CS and 5′-CS conserved regions of integron and amplifies the entire gene cassette integrated (in-F/in-R). The primer sequences used in this study are shown in Table 1. PCR was performed in a reaction mixture with a total volume of 25 μl containing 5 μl template DNA (20 *n*g), 2.5 μl 10X Taq polymerase buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl and 15 mM $MgCl_2$], 0.25 μl (100 pmol/μl) each of primers, 0.25 μl dNTPs (10 mM), 0.2 μl (5 U/μl) Taq DNA polymerase and 16.8 μl sterilized distilled water.

Amplification was done as follows: An initial denaturation step was performed at 94°C for 5 min followed by 30 cycles consisting of denaturation (94°C for 1 min), annealing (58°C for 1 min, separately adjusted for each set of primer pairs) and extension (72°C for 1 min), and this was followed by a final extension step at 72°C for 3 min.

Each PCR product from 3′-CS/5′-CS amplification was purified and directly sequenced (Macrogen Research, Seoul, Korea) and compared with the prototype strain *Salmonella enterica* serotype Typhimurium (accession number AB365868.1) as suggested previously [10].

Antimicrobial susceptibility testing: Antimicrobial susceptibility of isolates was examined using the disk diffusion method on Mueller–Hinton agar, as described by the CLSI guidelines [8].

Each isolate was tested for susceptibility to 7 antimicrobial agents: Ampicillin (AP) (10 mg), chloramphenicol (C) (10 mg) and streptomycin (ST) (10 mg) from Becton Dickinson and company (Sparks, MD, U.S.A.), and ciprofloxacin (CP) (1 mg), gentamicin (GM) (10 mg), trimethoprim (TR) (2/5 mg) and tetracycline (TE) (10 mg) from Difco Laboratories (Detroit, MI, U.S.A.).

GenBank accession numbers: One representative of each group of integrated gene cassettes with identical nucleotide sequences was deposited in the GenBank database. The assigned GenBank accession numbers are KC540778 and KC540779.

Pulsed-field gel electrophoresis: A PulseNet standardized protocol described for subtyping of *Escherichia* spp. was used for genotyping [29]. In brief, bacterial plate cultures were suspended in a cell suspension buffer (100 mmol l^{-1} Tris, 100 mmol l^{-1} EDTA, pH 8.0) and adjusted to absorbance values of 0.8 to 1.0 at a wavelength of 610 nm and used for preparation of agarose plugs with SeaKem Gold Agarose (Lonza, Rockland, ME, U.S.A.) and proteinase K. After a lysis step with 50 mmol l^{-1} Tris, 50 mmol l^{-1} EDTA (pH 8.0), 1% sarcosine, and 0.5 mg of proteinase K, followed by washing steps, 40 units of *Xba*I restriction enzyme (Roche Diagnostic Deutschland GmbH, Mannheim, Germany) was used for digestion of plug-embedded DNAs. A DNA molecular weight size marker was prepared by *Xba*I digestion of *Salmonella enterica* serotype Braenderup H9812 plugs. A CHEF Mapper XA System (Bio-Rad) was applied for electrophoresis with 200 V at 14°C for 18 hr with the pulsed time increasing from 2.16 sec to 54.17 sec. GelCompar II version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of PFGE-generated patterns using the Dice coefficient and UPGMA (unweighted pair group method with arithmetic averages) clustering. A dendrogram was constructed with an optimization value of 0.50% and a position tolerance of 1.0%.

RESULTS

A total of 21 (4.3%) enterohemorrhagic *E. coli* strains were identified in this study with the *eae*$^+$*stx1*$^+$*stx2*$^+$ genotype. Amplification bands of 422 bp, 894 bp and 478 bp were obtained for *eae*, *stx1* and *stx2*, respectively. The results of PCR analyses for each pair of primer sets are depicted in Fig. 1.

All of the strains showed resistance to Am, CP, TR, ST, C and TE, and 19% showed resistance to GM.

Distribution of class 1 integron: Out of 21 EHEC strains, 11 (53%) harbored an *integrase* gene specific for class 1 integron (*int*1) using primers *int1-F* and *int1-R* (5 conserved region) with a DNA fragment of 900 bp. Two different

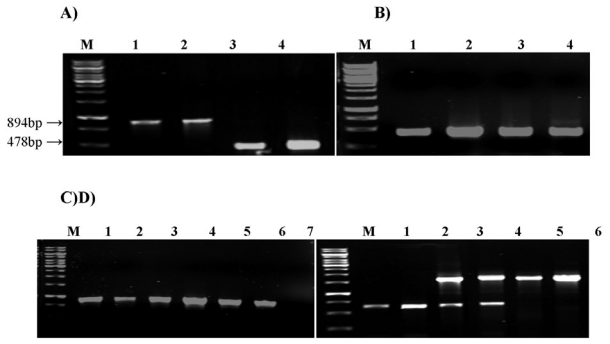


Fig. 1. A) PCR amplification of the *stx1* and *stx2* genes. Lanes 1 and 2, *stx1*; lanes 3 and 4, *stx2*. B) PCR amplification of the *eae* gene (lanes 1-4). C) PCR amplification of the class 1 integron integrase gene (*int*); lanes 1-6, isolates with class 1 integron; lane 7, negative control. D) PCR amplification of the internal variable region of class 1 integron. Lanes 1 and 2, 700 bp gene cassettes; lanes 3 and 4, 700/1,700 bp gene cassettes; lanes 5 and 6, 1,700 bp gene cassettes. The figure shows representative results for all the isolates tested.

amplification products, which were approximately 750 and 1,700 bp in size, were obtained from amplified variable regions (*in-F*/*in-R* primers) in 3 (14.3%) and 4 (19%) of the EHEC isolates. The amplified fragments corresponded to *dfrA7* (dihydrofolate reductase type I) and *dfrA1*/*aadA1* (dihydrofolate reductase/aminoglycoside adenyltransferase) resistance gene cassettes, respectively, which was confirmed by sequencing. Both of the amplification bands (750 and 1,700 bp) were simultaneously observed in 2 (9.5%) of the isolates indicative of the two gene cassettes. No product was produced from 2 int^+ isolates, suggesting an empty integron with no resistance gene cassette (Fig. 1).

Genotyping analysis: Genotyping patterns were compared using the Dice coefficient and UPGMA analysis, and the data revealed a total of 16 pulsotypes, which corresponded to 16 isolates, 9 of which were harboring class 1 integron. Analysis of pulsotypes showed extensive diversity among the isolates harboring integron, which is indicative of a lack of any significant genetic relatedness among the isolates. Similarity indices of 62% and 30% were obtained for the most and the least similar isolates, respectively (Fig. 2). Of the 21 isolates, 4 (19%) did not produce any restriction pattern, and their DNA repeatedly appeared as smears on the gel, even after addition of thiourea to the running buffer [22].

DISCUSSION

In pathogen detection systems before 2011, *E. coli* pathotypes, notably EPEC and EHEC isolates, had been identified by serotyping based on their O-group specificities, which was increasingly problematic due to the incapability of this method to clearly separate the pathotypes. Therefore, different molecular methods have been developed to more accurately assign the isolates to each pathotype based on their virulence-associated genes [5, 28]. Accordingly, the *eae*, *stx1* and *stx2* genes were used in this study for molecular identification of isolates.

All of the strains under study were resistant to all tested antimicrobial agents including Am, CP, TR, ST, C and TE. The most commonly used antibiotics in conventional dairies are penicillin and tetracyclines [11, 37]. This may correlate with the resistance of all isolates to these two groups of antibiotics. Our previously published data indicated the same resistance profile among enteropathogenic *E. coli* strains isolated from children with diarrhea [28]. A high level of resistance to different classes of antibiotics among enterohemorrhagic and enteropathogenic pathotypes of *E. coli* suggests the involvement of the same mechanisms in emergence of resistance in the two pathotypes; moreover, the circumstances of the intestinal environment in the acquisition of different mobile elements including integrons, transposons, etc, should not be ignored.

Distribution of class 1 integrons among members of the Enterobactericeae family is likely to be common around the world, and it has been shown to be associated with a variety of resistance gene cassettes, the encode for resistance to different antibiotics [14]. A total of 53% of our EHEC isolates harbored class 1 integron, and the most common integrated resistance gene cassettes belonged to the *dfr* and/or *aadA* family. The coexistence of these 2 families of resistance gene cassettes among human clinical isolates of *Shigella* spp., *Salmonella enterica* and *Escherichia coli* was previously reported by our laboratory [12, 28]. This emphasizes the wide distribution of these 2 gene cassette families among clinical and veterinary enteric bacteria, which provides evidence for horizontal gene transfer in the environment or intestinal tract of humans or animals; however, more studies are required to understand the exact mechanisms involved.

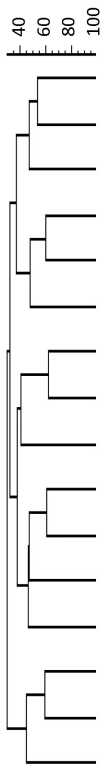
Guerra *et al*. reported the presence of class 1 integron in 41% of 105 EHEC isolates from humans with enteric disease (97 isolates) and cattle (8 isolates) that carried *dfrA15-aadA1*, *dfrA1-aadA1*, *aadA1* or no gene cassettes in their variable regions [17].

Cergole-Novella *et al*. reported the presence of class 1 integron in 22% of antimicrobial resistant Shiga-toxin-producing *E.coli* isolated from patients with diarrhea (21 isolates) and cattle (11 isolates) that harbored identical single gene cassettes (*aadA1*) in their variable regions [7].

Srinivasan *et al*. reported that the prevalence of class 1 integrons was 14% in enterohemorrhagic *E.coli* isolates from humans and animals and that the isolates uniformly contained the *aadA* gene [31].

These reports together with our findings suggest the worldwide distribution of the *aadA* and *dfrA* aminoglycoside resistance gene family among integrons, which further emphasizes the need to monitor their contents for fear of acquisition of resistance genes against newer antimicrobial agents.

Genotyping analysis revealed extensive heterogeneity among the isolates, which indicates a diverse population of EHEC in the intestinal tract of cattle. This finding is in contrast with genotyping studies on EHEC strains isolated from cattle and humans from Japan, which have shown a high degree of homogeneity among their isolates [36]. Changes in



Genotypes	Resistance patterns	Sizes of inserted gene cassettes (bp)	Gene Cassettes
eae+stx1+stx2+	*Amp / Cp/ Tmp/ Str/ Chl/ Tet/Gm*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	1700	*dfra1/aadA1*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet/Gm*	700-1700	*dfra7/dfra1/aadA1*
eae+stx1+stx2+	*Amp / CP/ Tmp/ Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp/ Str / Chl / Tet*	1700	*dfra1/aadA1*
eae+stx1+stx2+	*Amp / CP/ Tmp/ Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	700	*dfra7*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	700	*dfra7*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	1700	*dfra1/aada1*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet/Gm*	700-1700	*dfra7/dfra1/aada1*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	-	-
eae+stx1+stx2+	*Amp / CP/ Tmp/ Str / Chl / Tet*	700	*dfra7*
eae+stx1+stx2+	*Amp / CP/ Tmp / Str / Chl / Tet*	1700	*dfra1/aada1*

Fig. 2. UPGMA dendrogram showing banding patterns of *Xba*I-digested enterohemorrhagic *E. coli* isolates. The isolates are presented in comparison with their phenotypic resistance profiles and integron resistance gene contents.

the PFGE pattern of *Xba*I-digested DNA fragments of EHEC isolates have been shown in association with loss of Shiga toxin genes during maintenance or subcultivation, which is related to disappearance of a 70- or 80-kb fragment [27]. Nevertheless, little disparity has been reported in the PFGE pattern of EHEC isolates of different origins [20].

Isolates with identical integron contents showed different genotyping patterns, and no clear-cut relationship was obtained between the specific pulsotypes and their integron contents. This highlights the potential of integrons for integration within different EHEC isolates regardless of their genotypes. The antimicrobial resistance profiles were identical among the isolates, except for susceptibility to gentamicin. Diversity in the resistance profile of isolates was also not reflected in their genotype properties. Overall, these data suggest that the genotyping patterns of isolates are independent of their resistance properties and integron contents.

To our knowledge, little data exist on the genotyping patterns of EHEC isolates from cattle in relation to their resistance gene contents, which signifies the need for this to be worked on. This emphasizes the need for establishment of national programs in Iran and in the Middle East and exchange of genotyping databases in order to monitor and source track the source of emergence and spread of new resistant and integron-carrying genotypes within these areas.

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