Distribution Pattern of EcoR Phylogenetic Groups Among Shiga Toxin-Producing and Enteropathogenic Escherichia coli Isolated From Healthy Goats

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1. Background

Escherichia coli is distinguished as the key member of gut microbiota, which can be found in human and various warm-blooded animals (1). Phylogenetic analysis have indicated that E. coli can be categorized into four major phylogenetic groups (A, B1, B2, and D) which can be divided into seven phylogenetic subgroups A1, A2, B1, B2, D1, and D2 using a rapid polymerase chain reaction (PCR) based method for the detection of chuA (the outer-membrane hemin receptor gene), yjaA (the gene encodes an uncharacterized protein) and TSPE4.C2 (an anonymous DNA fragment) (2-5).

Recently, relationships have been demonstrated between E. coli phylogenetic characteristics and obtaining virulence genes (6, 7). Virulence factors may be found more commonly in strains of groups B2 and D, whereas majority of A and B1 strains are nonpathogenic and isolated from nondiarrheic fecal samples (8-10). Different phylo-groups have various features in aspects of their ecological niche, life history traits, ability to cause disease and phenotypic and genotypic characteristics (11, 12).

According to pathogenicity, E. coli can be divided into two groups including diarrheagenic and nondiarrheagenic strains. Diarrheagenic Escherichia coli (DEC) strains have been settled in at least six patho-types related to their pathogenicity and virulence factors comprising Shiga toxin-producing Escherichia coli (STEC), enterohaemagglutinative Escherichia coli (EAEC), enteropathogenic Escherichia coli (EPEC), enteroinvasive Escherichia coli (EIEC), enterotoxigenic Escherichia coli (ETEC), and diffusely adherent Escherichia coli (DAEC) (13, 14). There are different diseases in humans which are caused by STECs including hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), and diarrhea (1).

The strains possessing Shiga toxin genes (stx1, stx2, or both) are generally recognized as STEC. Some STEC strains containing locus of enterocyte effacement (LEE) are called enterohaemorrhagic Escherichia coli (EHEC) that produce attaching and effacing lesions. Within the LEE, eae is the most important gene encoding an outer mem-
brane protein, intimin (15). The EPEC strains harbor just LEE but not Shiga toxin genes (16). Epidemiologically, STEC is considered as one of the main food-borne risk factors in public health worldwide (17). Ruminants (cattle, sheep, and goats) have an important role as important of STEC strains (18, 19).

2. Objectives

The main origin of food-borne infections can be diverse related to common dietary habits in a nation’s culture. In Kerman Province (southeast of Iran), goats are slaughtered extensively to provide daily public meat consumption. Therefore, in this study, we investigated the distribution pattern of phylogenetic groups in STEC and EPEC isolates from 250 asymptomatic goats in Kerman City, Iran.

3. Materials and Methods

3.1. Sample Collection and Escherichia coli Strains

Two hundred and fifty fecal samples were obtained from healthy goats by moist sterile swabs in the local slaughterhouse in Kerman City, Iran, during 12 months. Amies medium (BBL, USA) was used to carry the samples to the laboratory through 6 hours. Then, the swabs were plated directly onto MacConkey agar (Merck, Germany) and incubated for 18 - 24 hours at 37°C. Two suspect lactose-positive colonies were selected for next steps. Validation of \textit{E. coli} strains was accomplished by usual biochemical tests (20). In this study, the standard strains EcoR62 and Sakai were used as positive-controls to identify phylo-groups and virulence genes, respectively. Finally, the confirmed \textit{E. coli} isolates were cultured in Luria-Bertani broth (Merck, Germany) for 18 hours without shaking and then sterile glycerol was added up to 25% and stored at -80°C.

3.2. Polymerase Chain Reaction (PCR) for EcoR Grouping and Virulence Genes (\textit{stx1}, \textit{stx2}, and \textit{eae})

First, one of two stored \textit{E. coli} isolates in LB broth were streaked on LB agar (Merck, Germany) and incubated at 37°C for 24 hours. DNA extraction was performed by Sodium hydroxide (NaOH) method; in brief, a well-defined single colony was selected from LB agar culture and suspended in NaOH (0.5 M), then 25 μL Tris (1 M; pH = 7.5) was added after 20 - 30 minutes and finally 450 μL distilled water was added to suspension. Afterward, samples were centrifuged at 13000 rpm for 1 minute, and the supernatants was transferred to a clean tube and stored at -18°C as templates for future analyses.

In order to obtain the STECs, multiplex-PCR was employed for detection of virulence genes \textit{stx1}, \textit{stx2}, and \textit{eae} as described previously by China et al. (21). Also, all isolates were subjected to multiplex PCR for determination of three phylogenetic markers \textit{chuA}, \textit{yjaA}, and DNA fragment TspE4.C2 described by Clermont et al. (2) (Table 1). Primers’ sequences were checked by basic local alignment search tool (BLAST).

For PCR, each prepared DNA (3 μL) was amplified in a total volume of 25 μL reaction mixture including: 0.3 μM of each primer, 0.2 mM of Deoxynucleoside Triphosphate (dNTP), 2 mM magnesium chloride (MgCl$_2$), 2.5 μL of 10 × PCR buffer, 1 unit Taq DNA polymerase (Cinna gen, Iran) and distilled water up to volume of reaction. The amplicons were electrophoresed on 1.5% agarose gel for 45 min at 85 V, stained using ethidium bromide and imaged with a GelDoc 1000 (Vilber Lourmat, France) image analysis station.

Table 1. Primers Used in This Study for Detection of Virulence Genes and Phylogenetic Markers

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’ to 3’)</th>
<th>Product Size, bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{eae}$^a$</td>
<td>AGGGCTTGTCACAGTTG CCATCGTCACCAGAGGA</td>
<td>570 (21)</td>
<td></td>
</tr>
<tr>
<td>\textit{stx1}$^a$</td>
<td>AGAGCGATGTTACGGTTTG TTGCCCCCAGAGTGGATG</td>
<td>388 (21)</td>
<td></td>
</tr>
<tr>
<td>\textit{stx2}$^a$</td>
<td>TGAGTTTTCTCTCGGTATC GTGTTTTCTGCTCTCA</td>
<td>807 (21)</td>
<td></td>
</tr>
<tr>
<td>\textit{chuA}$^b$</td>
<td>GACATCGTCTGACTCCTT GACGAACCACGTCAGGAT</td>
<td>279 (2)</td>
<td></td>
</tr>
<tr>
<td>\textit{yjaA}$^b$</td>
<td>TGCCGCCAGTACAAAGA CAGAAGCGAGGAGGAGTGA</td>
<td>211 (2)</td>
<td></td>
</tr>
<tr>
<td>TspE4.C2$^b$</td>
<td>GAGTAAACGCCGGGATTAC CGGCCCAACAAAGTATCAG</td>
<td>152 (2)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Cycling Conditions: 95°C for 3 min followed by 38 cycles at 95°C for 60 s, 53°C for 60 s, and 72°C for 60 s, a final extension step of 10 min at 72°C.

$^b$ Cycling Conditions: 94°C for 5 min, followed by 29 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension step of 7 min at 72°C.
4. Results

Two hundred and fifty \textit{E. coli} isolates were categorized into phylogenetic subgroups; phylo-group \textit{B1} was the most prevalent (57.6%), followed by phylo-groups \textit{A0} (20.4%), \textit{A1} (18.4%), \textit{D1} (2.8%), and \textit{B2} (0.8%). There were no isolate in \textit{B2} and \textit{D2} subgroups (Figure 1).

In total, 50 (20%) samples possessed at least one of the investigated virulence genes; \textit{stx1} (16.8%), \textit{stx2} (4.4%), and \textit{eae} (2.8%) (Figure 1). Thus, there are 41 (16.4%) STEC, 7 (2.8%) EPEC and 2 (0.8%) EHEC isolates in this research. The positive isolates for virulence genes were allocated into four phylogenetic subgroups \textit{A0} (16%), \textit{A1} (12%), \textit{B1} (68%), and \textit{D1} (4%) (Table 2).

Total number of forty three isolates possessed \textit{stx1}, \textit{stx2}, \textit{stx1/stx2}, and \textit{stx1/eae} profiles were distributed among \textit{B1} (32/43), \textit{A1} (5/43), \textit{A0} (5/43), and \textit{D1} (1/43) subgroups. Six isolates containing just \textit{eae} gene (EPEC) were assigned to three subgroups: \textit{A0} = 3 isolates, \textit{A1} = 1, \textit{B1} = 2 and \textit{D1} = 1 (Table 2).

![Figure 1. Multiplex Polymerase Chain Reaction Profiles of EcoR Phylo-Types (A) and \textit{stx1}, \textit{stx2}, and \textit{eae} Virulence Genes (B) Among \textit{Esherichia coli} Isolates From Fecal Samples](image)

| Table 2. Comparison of Phylo-Groups, Virulence Gene Profiles and Pathotypes of \textit{Esherichia coli} Isolates \textsuperscript{a,b} |
|-----------------|-----|-----|-----|-----|-----|-----|-----|
| Phylo-Subgroup  | \textit{A0} | \textit{A1} | \textit{B1} | \textit{B2} | \textit{D1} | Total |
| Virulence Gene Profile | | | | | | |
| \textit{stx1}    | 5 (16.6) | 3 (10) | 22 (73.3) | - | - | 30 (12) |
| \textit{stx2}    | - | - | 1 (100) | - | - | 1 (0.4) |
| \textit{eae}     | 3 (42.8) | 1 (14.2) | 2 (28.5) | - | 1 (14.2) | 7 (2.8) |
| \textit{stx1/stx2}| - | 1 (10) | 8 (80) | - | 1 (10) | 10 (4) |
| \textit{stx1/eae}| - | 1 (50) | 1 (50) | - | - | 2 (0.8) |
| Pathotypes       | | | | | | |
| STEC             | 5 (12.1) | 4 (9.2) | 31 (75.6) | - | 1 (2.4) | 41 (16.4) |
| EPEC             | 3 (42.8) | 1 (14.2) | 2 (28.5) | - | 1 (14.2) | 7 (2.8) |
| EHEC             | - | 1 (50) | 1 (50) | - | - | 2 (0.8) |
| Negative isolates| 38 (19) | 45 (22.5) | 110 (55) | 2 (1) | 5 (2.5) | 200 (80) |
| Total            | 46 (18.4) | 51 (20.4) | 144 (57.6) | 2 (0.8) | 7 (2.8) | 250 (100) |

\textsuperscript{a} Abbreviations: EPEC, enteropathogenic \textit{Esherichia coli}; EHEC, enterohemorrhagic \textit{Esherichia coli}; and STEC, shiga toxin-producing \textit{Esherichia coli}.

\textsuperscript{b} Values are presented as No (%).
5. Discussion

This is the first research on distribution of EcoR phylogenetic groups among E. coli isolates, considering STEC and EPEC isolates from healthy goats in southeast of Iran. The present study revealed that goats can be a potential reservoir of STEC in Kerman and showed that the majority of isolated STECs and non-STECs belonged to B1 phylogenetic group.

In current study, two hundred and fifty E. coli isolates from goat’s feces were mostly distributed into phylogroup B1, followed by groups A and A2. Carlos et al. (22) investigated the phylogenetic subgroups among 16 isolates from goats included: A0 (one isolate), A1 (n = 2) and B1 (n = 13). The groups B2 and D were rarely found among isolates of ruminants which this is probably related to gut characteristics and the diet of the hosts (22). The groups A and B1 are observed in nonhuman mammals more than humans (23). Also, group B1 in herbivorous and group A in carnivorous and omnivorous animals have been reported with more prevalence (24). Phylo-group B1 was found in most of hosts; therefore, it is not an important indicator of a special host’s feces. Overall, A and B1 groups probably are general while B2 and D are more special among the hosts and pathotypes of E. coli (22).

Our results showed that total number of STEC, EPEC and EHEC isolates were mostly belonged to groups B1 (68%) and A (28%) that are similar to results reported by Alizade et al. (25) in cattle and human at the same province. Bidet et al. (6) categorized their STEC isolates, like O157:H7, into group D and showed that cattle are the main reservoir. There are few studies about the phylogenetic affiliation of STEC and EPEC strains isolated from goats. According to previous studies, B2 and D strains are often more pathogenic than A and B1 strains (10, 26). However, based on previous studies it seems that commensal strains usually belong to groups A and B1 while the most of intestinal pathogenic isolates are associated to groups A, B1, and D (22).

Results of this study showed that the prevalence rates of STEC, EPEC, and EHEC strains were 16.4% (41 / 250), 2.8% (7 / 250), and 0.8% (2/250), respectively which these results are approximately in accordance with, Montaz et al. (27) reports from goat’s meat in Iran. The broad range of detection of STEC was reported in other investigations in Germany (56.1%), Spain (47.7%), Bangladesh (10.0%), and Nigeria (7.5%) (28-31). In the current study, the genotypic profile of stx proteins among the STEC isolates was stx1 and eae (12%) followed by stx1/stx2 (4%), eae (2.8%), stx1/eae (0.8%), and stx2 (0.4%) which is comparable with other studies carried out by other researchers; Oliveira et al. (32) reported the genetic profiles stx1, stx2, and stx1/stx2, with the prevalence rates of 24.1%, 34.7%, and 41.2%, respectively. These rates were 69.8% (stx1), 0.9% (stx2), and 22.5% (stx1 and stx2) in the study by Cortes et al. (29).

In most of comparative researches, EPEC has been isolated less than STEC. In a work, EPEC strains were found in 13% of healthy goats in the central region of Spain (33) whereas Krause et al. (34) did not detect the strains in Germany.

It is notable that in our report, only 2 isolates were EHEC (stx/eae-positive) and the most STEC isolates harboring stx2 were associated with stx1. In different studies on STECs isolated from sheep and goats, this pathotype mostly do not carry stx2 and eae (28, 35-38). Oliveira et al. (32) reported lacking of eae gene among the STECs from ruminants which is almost in agreement with our observation in goats. Since, there are other virulence genes which have been described in eae-negative STECs, such as soo (STEC-autoagglutinating adhesin) and astA (entero-aggregative heat-stable enterotoxin), we suggest checking these sequences in similar studies, too (39, 40).

There are several reasons probably related to the various ranges of prevalence of STEC and EPEC isolates. Among all of them, geographical variation, age, environmental conditions, seasonal difference, sampling method, detection technique, some farm management practices such as the administration of antibiotics, improper feeding, and use of contaminated food and water have recently emerged as the most important reasons (29, 41-43).

In conclusion, goats can be a potential reservoir of STEC strains in Kerman. The findings of our study show that B1 followed by A are the most prevalent phylogenetic groups among STEC and non-STEC isolates. Although the prevalence rate of EHEC isolates was low in our study, it is necessary to design the appropriate hygienic methods to reduce or eliminate the STEC and EHEC contaminations in slaughterhouses.

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Authors’ Contributions

Author Dr Maziar Jajarmi conducted literature search, provided summaries of previous research studies and performed sampling and laboratory tests. Author Dr Reza Ghanbarpour designed the study and wrote the protocol. Author Dr Hamid Sharifi conducted the statistical analysis. Author Dr Mehdi Golchin directed the accuracy of molecular tests and all authors contributed to and have approved the final manuscript.

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