Phylogenetic Analysis of *Escherichia coli* Isolates from Healthy and Diarrhoeic Calves in Mashhad, Iran

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Summary


*Escherichia coli* is a normal inhabitant of the gastrointestinal tract of vertebrates. Certain *Escherichia coli* strains have been associated with neonatal diarrhoea in ruminants. These strains can be assigned to one of the four main phylogenetic groups, A, B1, B2 and D. Several studies have shown the relationship between phylogeny and pathogenicity of *E. coli*, a great deal can be obtained by determining the phylogroup of unknown *E. coli* strains. In this study, we aimed to evaluate the influence of diarrhoea on the genetic composition of *E. coli* populations isolated from calves. A total of 80 *Escherichia coli* isolates were obtained from healthy and diarrhoeic calves. Phylogenetic grouping was done based on the Clermont triplex PCR method using primers targeted at three genetic markers, chuA, yjaA and TspE4.C2. According to our results, phylogenetic group A strains was the most prevalent in both healthy (37.5%) and diarrhoeic calves (55%). Group B1 contained 27.5% of isolates in healthy calves, followed by group B2 (17.5%), and group D (7.5%). Also, four isolates from healthy calves were not included in the major phylogenetic groups or subgroups. A total of 14% and 4% of isolates from diarrhoecic calves belonged to phylogroups B2 and D respectively. Although no isolate from diarrhoeic calves was found to belong to group B1, there was no significant difference between healthy and diarrhoeic calves for other phylogroups. There was not a dramatic shift in *E. coli* phylogroup/subgroup due to occurrence of diarrhoea in calves, except for phylogroup B1 which was higher in healthy calves. This can be due to the difference in secretions of digestive system in diarrhoeic calves which can prevent the conditions for instability of *Escherichia coli* isolates of phylogroup B1. The majority of isolates from both healthy and diarrhoeic calves belonged to non-pathogenic phylogentic group A and B1.

Key words: calves, diarrhoea, *Escherichia coli*, phylogenetic group
INTRODUCTION

*Escherichia coli* is a normal inhabitant of the gastrointestinal tract of vertebrates. Its colonisation in the mammalian intestinal tract from environmental sources occurs shortly after birth and persists as one of the important members of the normal flora of the intestine throughout life (Quinn et al., 2011). Certain *E. coli* strains have been associated with neonatal diarrhoea in ruminants which causes considerable economic losses in dairy industry all around the world (Shahrani et al., 2014). *E. coli* strains may be assigned to one of the main phylogenetic groups: A, B1, B2 and D, which classify into seven subgroups (A₀, A₁, B₁, B₂, B₂, D₁ and D₂), according to the combination of the two and three genetic markers (chuA, the outer-membrane hemin receptor gene, and yjaA, which encodes an uncharacterised protein) and a DNA fragment that has been recently identified as part of a putative lipase esterase gene, TspE4.C2 based on triplex PCR (Clermont et al., 2000; Escobar-Paramo et al., 2004; Tenaillon et al., 2010). This method, which assigns strains to their correct MLST-based phylogroup, is acceptably accurate (80–85%) and has been found satisfactory (Gordon et al., 2008). Phylogenetic groups are different in characteristics such as virulence factors, ecologic niches, life history, carbohydrate fermentation, antibiotic resistance, growth rate and size of the genome (Berghtrsson et al., 1998; Lecointre et al., 1998; Walk et al., 2007).

Previous studies have shown that strains from phylogroups B2 and D contained more virulence factors than strains from the phylogroups A and B1 (Johnson et al., 2001; Bashir et al., 2012). The diarrhoeagenic *E. coli* strains belong to groups A, B1 and D, the commensal strains belong to groups A and B1, whilst the extra-intestinal pathogenic strains usually belong to groups B2 and D (Ferjani et al., 2012).

Up to now, there have been very few studies on phylogenetic group determination based on the health status of calves in Iran. Therefore, the aim of this study was to compare the phylogenetic groups of *Escherichia coli* isolates from healthy and diarrhoeic calves by Clermont triplex PCR method.

MATERIALS AND METHODS

*E. coli* isolates

This study was performed from September 2012 till July 2013, on 80 isolates of *E. coli* from faeces of 40 healthy calves and 40 calves with clinical diarrhoea. The bacterial strains were isolated from faecal samples of Holstein calves aged <1 month, from 5 farms located in north-east of Iran. The isolates were identified as *E. coli* based on standard biochemical tests (Seifi et al., 2015). Isolated strains which exhibited a biochemical profile for *E. coli* were kept as stock in nutrient broth with 15% glycerol at −20 °C for further experiments.

DNA extraction

DNA template preparation was performed by the boiling method as followed. First, a few colonies were resuspended in 500 μL sterile distilled water. The cells were lysed by heating at 95 °C for 10 min. After heating, they were immediately put on ice for 5 min. The supernatant was then harvested by centrifugation at 11,000 rpm for 10 min.
Determination of *E. coli* phylogenetic groups

We determined four phylogenetic groups of *E. coli* (A, B1, B2 and D) by use of triplex PCR as described by Clermont *et al.* (2000). Briefly, the genomic DNA of bacterial strains was amplified by triplex PCR using primers targeted at three markers, chuA, yjaA and TspE4.C2. The primer pairs used for PCR amplification is shown in **Table 1**. Multiplex PCR reaction was performed in a 25 μL reaction mixture, containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂, pH 8.7), dNTP (200 μM), each primer (0.4 μM), Taq DNA polymerase (1U), and template DNA (2 μL). Negative controls (reaction lacking the template DNA) and a positive control (ECOR 62) were included in all performed amplifications. The PCR reaction was performed as follows: initial denaturation at 94 °C for 5 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a final extension step at 72 °C for 7 min (Clermont *et al.*, 2000). Reactions were placed in a thermal cycler (Biorad, Germany) without mineral oil. Amplification products were separated in 1.5% agarose gels containing ethidium bromide.

### Statistical analysis

Phylogenetic relationship between the groups and subgroups and health status of calves (diarrhoeic and healthy) was evaluated by SPSS 20 using the Chi-square and Fisher exact tests with significance set at P<0.05.

**RESULTS**

Subtype distribution of isolates is shown in **Table 2**. According to multiplex PCR-based phylotyping, group A contained the majority of the collected isolates from both healthy and diarrhoeic calves. A total of 15 isolates (37.5%) from healthy calves belonged to phylogenetic group A, followed by group B2 (11 isolates, 27.5%), B1 (7 isolates, 17.5%), and D (3 isolates, 7.5%). All of the strains of group A were found to belong to subgroup A₁. Four and seven isolates (10% and 17.5%) of group B2 belonged to subgroup B2₂ and B2₃, respectively. A total of 22 isolates (55%) from diarrhoeic calves belonged to group A, followed by group B2 (14 isolates, 35%), and group D (4 isolates, 10%). No strains were found to belong to group B1. All strains of group A were found to belong to subgroup A₁. Nine and five isolates (22.5% and 12.5%) of group B2 belonged to

**Table 1.** Oligonucleotid primers used for detection of phylogenetic groups

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Product size (bp)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChuA.1</td>
<td>GAC GAA CCA ACG GTC AGG AT</td>
<td>279 bp</td>
<td>chuA</td>
</tr>
<tr>
<td>ChuA.2</td>
<td>TGC CGC CAG TACCAA AGA CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YjaA.1</td>
<td>TGAAGTGTCAGGAGACGCTG</td>
<td>211 bp</td>
<td>yjaA</td>
</tr>
<tr>
<td>YjaA.2</td>
<td>ATG GAG AAT GCC GTTC TCT AAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TspE4.C2.1</td>
<td>GAG TAA TGT CGG GGC ATT CA</td>
<td>152 bp</td>
<td>TspE4.C2</td>
</tr>
<tr>
<td>TspE4.C2.2</td>
<td>CGC GCC AAC AAA GTA TTA CG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Phylogenetic analysis of E. coli isolates from healthy and diarrhoeic calves in Mashhad, Iran

Table 2. Phylogenetic analysis of 80 E. coli isolates

<table>
<thead>
<tr>
<th>Phylogroup/subgroup</th>
<th>Number (%) of isolates</th>
<th>from healthy calves</th>
<th>from diarrhoeic calves</th>
<th>from all calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0 (chuA-, yjaA-, TspE4.C2-)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A1 (chuA-, yjaA+, TspE4.C2-)</td>
<td>15 (37.5)</td>
<td>22 (55.0)</td>
<td>37 (46.25)</td>
<td></td>
</tr>
<tr>
<td>B1 (chuA-, yjaA-, TspE4.C2+)</td>
<td>7 (17.5)</td>
<td>–</td>
<td>7 (8.75)</td>
<td></td>
</tr>
<tr>
<td>B2 (chuA+, yjaA+, TspE4.C2+)</td>
<td>7 (17.5)</td>
<td>5 (12.5)</td>
<td>12 (15.0)</td>
<td></td>
</tr>
<tr>
<td>D1 (chuA+, yjaA-, TspE4.C2-)</td>
<td>1 (2.5)</td>
<td>4 (10.0)</td>
<td>5 (6.25)</td>
<td></td>
</tr>
<tr>
<td>D2 (chuA+, yjaA-, TspE4.C2+)</td>
<td>2 (5.0)</td>
<td>–</td>
<td>2 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Untypable (chuA-, yjaA+, TspE4.C2+)</td>
<td>4 (10.0)</td>
<td>–</td>
<td>4 (5.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>40</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

subgroup B2 and B2, respectively. All strains of group D were found to belong to subgroup D1. No strains from healthy and diarrhoeic calves were found to belong to subgroup A0. Four isolates were untypeable by this method (Fig. 1).

There was not any significant difference between healthy and diarrhoeic calves for E. coli phylogroups distribution (P=0.217). The binary comparison of phylogenetic group B1 showed that the frequency of group B1 in isolates from healthy calves was higher than that of isolates from diarrhoeic calves (P=0.012). All phylogenetic groups and subgroups were present in isolates of healthy and diarrhoeic calves, but no isolates were found to belong to subgroup A0. Subgroup D2 and phylogroup B1 were not found among isolates from diarrhoeic calves. This can be due to the difference in secretions of digestive system in diarrhoeic calves which can prevent the conditions for instability of Escherichia coli isolates. E. coli strains in gut normal microflora may therefore be the natural reservoir of pathogenic strains and they may be derived from commensal strains by the acquisition of chromosomal or extra-chromosomal virulence operons (Duriez

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DISCUSSION

Previous studies have shown that phylogenetic groups, subgroups and genetic markers are not randomly distributed in different hosts such as human and animals and that the frequency of phylogenetic groups in mammals is dependent on host food regimen, body volume and climate conditions (Gordon & Cowling, 2003; Mokracka et al., 2011). In this study, 80 Escherichia coli strains isolated from faecal samples of healthy and diarrhoeic calves, were evaluated. There was not any significant difference between healthy and diarrhoeic calves for E. coli phylogroups distribution (P=0.217), but the binary comparison of phylogenetic group B1 showed that the frequency of group B1 in isolates from healthy calves was higher than that of isolates from diarrhoeic calves (P=0.012). All phylogenetic groups and subgroups were present in isolates of healthy and diarrhoeic calves, but no isolates were found to belong to subgroup A0. Subgroup D2 and phylogroup B1 were not found among isolates from diarrhoeic calves. This can be due to the difference in secretions of digestive system in diarrhoeic calves which can prevent the conditions for instability of Escherichia coli isolates. E. coli strains in gut normal microflora may therefore be the natural reservoir of pathogenic strains and they may be derived from commensal strains by the acquisition of chromosomal or extra-chromosomal virulence operons (Duriez...
M. Barzan, M. Rad, G. R. Hashemi Tabar & M. Azizzadeh

et al., 2001). In our study the majority of isolates belonged to the non-pathogenic phylogenetic group A, but certain virulence factors may be mobilised on genetic elements and transferred to normally commensal strains via horizontal exchange (Picard & Gouillet, 1988). Our data showed that phylogroup A (A1) was the most prevalent among isolates from healthy and diarrhoeic calves. This result was similar to previous studies which showed that isolates of phylogroup A were the most prevalent E. coli isolates in animal intestinal tract (Johnson et al., 2003; Escobar-Paramo et al., 2006; Asai et al., 2011). Other studies revealed that phylogroup B1 was dominant among isolates from healthy cattle (Alizade et al., 2014). The analysis demonstrated that phylogroup B1 was more prevalent in isolates from diarrhoeic and septi- mic calves in Iran (Ghanbarpour et al., 2009; Bihannic et al., 2014; Staji et al., 2015). It is interesting to note that subgroup B23 was found among isolates from both healthy and diarrhoeic calves, whereas subgroup A0 was not found in all isolates. These results are in contrast with the study conducted by Carlos et al. (2010) – according to their results subgroup B23 was present only in the human sample and they suggested that B2 strains, especially subgroup B23, could be a good indicator of human faeces contamination. It was similar to previous study which showed that isolates of ruminants (sheep, goat and cattle) and dogs belonged to subgroup B23 (Derakhshandeh et al., 2014). It is difficult to explain but environmental and ecological conditions, geographic variation, host species and health status play an important role in E. coli phylogroup distribution.

In conclusion, our results showed that there was not a significant shift in E. coli phylogroup/subgroup due to occurrence

Fig. 1. Triplex PCR profiles specific for E. coli phylogenetic groups. Each combination of chuA and yjaA genes and DNA fragment TSPE4.C2 amplification allowed phylogenetic group determination of a strain. Lane 1: Marker 100 bp plus (Fermentas); lanes 2 and 4: negative controls; lane 3: positive control; lane 5: Group A (A1), lane 6: Group B1, lanes 7–8: Group B2; lanes 9–10: Group D and lanes 11–14: untypeable.
of diarrhoea. Its effect on the presence or absence of some phylo-groups was obvious. As different phylogroups have various features in many aspects such as ability to cause disease, these findings would be important to formulate prevention programmes and effective therapies for calves’ diseases. Further studies need to be done on the shift of E. coli phylogroups related to the health status of calves in different geographical regions of Iran.

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