OCCURRENCE AND CHARACTERISATION OF ENTEROHAEMORRHAGIC ISOLATES ESCHERICHIA COLI FROM DIARRHOEIC CALVES

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SUMMARY

The presence of major virulence factors of enterohaemorrhagic Escherichia coli (EHEC; stx1, stx2, eae, Ehly) were determined among isolates from 158 diarrhoeic calves by multiplex polymerase chain reaction (PCR). Strains positive for virulence factors were subjected to serotype specific PCR assays for O157:H7 and O111 antigens. Additionally, serogroups were determined by three monovalent antisera for O26, O111 and O157 somatic antigens and enterohaemolysin production were also shown phenotypically. Thirteen (8.2%) calves carried strains positive for one or more of the virulence factors tested, and eleven (6.9%) calves harboured the shiga toxin producing strains (stx1 or stx2). stx1 was detected in eight (5%) and stx2 in three (1.9%) calves. eae and Ehly were observed in the same frequency (6.3%) and were detected in parallel. Of the 13 virulence-positive strains, the predominant genotype was (stx1/eae/Ehly) at 53.8%. None of the EHEC in this study belonged to O157:H7 or O111 serotypes, but four strains (30.7%) belonged to the O26 serogroup. The results show the possible role of stx1/eae in calf diarrhoea and the particular importance of O26 EHEC. Calves can also act as a reservoir for EHEC and in the transmission of the disease to humans.

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) is a major cause of bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans. Domestic ruminants and especially cattle have been implicated as a principal reservoir of EHEC that causes human infection [8]. Pathogenic capacity of EHEC resides in a number of virulence factors including shiga toxins (stx1, stx2), intimin (eae) and enterohaemolysin. EHEC may produce two types of shiga toxins (stx1 and stx2), which are functionally and structurally related to shiga toxin of shigella dysenteriae type 1. Intimin encoded by the eae gene, located on the locus of enterocyte effacement, mediates the intimate attachment of bacteria to intestinal villi and induces attaching and effacing lesions [9]. It has been suggested that enterohaemolysin may complement or enhance the effects of shiga toxins [5]. Strains producing Ehly are not haemolytic on standard blood agar but produce a haemolytic zone on washed sheep blood agar supplemented with calcium. EHEC infections have been described in a wide range of both domestic and wild animal species, but their natural pathogenic role has been demonstrated only in young calves, weaning pigs and dogs [2]. Different serotypes of EHEC have been associated with diarrhoea in calves (mainly O5, O26 and O118). Recently, the possible roles of O26 and O111 EHEC have been described in association with calf diarrhoea [5]. Although E. coli O157 as an archetype of EHEC is associated with most of the outbreaks in humans worldwide, other serotypes particularly O26 and O111 have emerged as significant causes of human disease. The objectives of this study were to determine the distribution of major virulence factors of EHEC and the presence of important EHEC serotypes associated with human and calf disease, including O26, O111 and O157, in isolates from diarrhoeic calves in Iran.

MATERIAL AND METHODS

Specimen collection and E. coli strains

Faecal samples were obtained from 158 diarrhoeic calves from 10 geographically separate farms. Samples were collected using sterile swabs from 7- to 90-day-old calves with symptoms of diarrhoea or dysentery at the time of sampling. Specimens were sent to the laboratory in Amies transport medium and plated within 24 h of collection on MacConkey agar, Sorbitol MacConkey agar (SMAC) and O157chromagar (CHROMagar). Three to four suspected colonies including lactose fermenting colonies on MacConkey agar, sorbitol negative colonies on SMAC agar and mauve colonies on chromagar were randomly picked and sub-cultured.
Detection of virulence genes by multiplex PCR

All E. coli isolates were screened by multiplex PCR using four pairs of specific primers for stx1, stx2, eae and Ehly as described by Paton and Paton (Table 1) [7]. Total genomic DNA was extracted from overnight LB agar culture by the boiling method, as described by Zahraei Salehi et al. (2007) [10]. Positive controls and negative control (sterile water) were included in all PCRs.

PCR for rfbO157, rfbO111 and fliCH7

Two other PCR assays for the detection of O157, O111 and H7 antigens were carried out on virulence trait-positive strains (according to multiplex PCR results). O157 and O111 somatic antigen rfb genes were screened by specific primers in duplex PCR assay. PCR condition and thermal cycles were similar to virulence genes multiplex PCR [10]. PCR for fliCH7 was performed according to Gannon et al. (1997) as described previously [3]. Controls (positive and negative) were included in all PCR reactions.

Serogroup determination

Strains which were positive for virulence markers in the PCR assay were subjected to serogroup determination using commercially available O26, O111 and O157 monovalent antisera according to the manufacturer’s recommendations.

Enterohaemolysin activity of Ehly-positive strains

Production of enterohaemolysin was assessed in Ehly-positive strains phenotypically using washed sheep blood agar supplemented with 10 mM CaCl₂ (WSBA-Ca) as described previously [1]. Negative controls (Ehly negative strain) were used.

RESULTS

A total number of 375 isolates were confirmed as E. coli in biochemical tests and were subjected to multiplex PCR for virulence markers. Similar patterns of virulence markers were observed among isolates associated with each PCR positive sample; therefore, one strain per animal was applied for data analysis (n=13). Among 158 diarrhoeic calves, strains from 13 calves (8.2%) were positive in virulence markers multiplex PCR and 11 calves (6.9%) harboured shiga toxin producing strains. The most frequent shiga toxin was type 1 (stx1) and was present in 5% of samples (n=8); stx2 was detected only in 1.9% of specimens (n=3). eae and Ehly were found in the same frequency (6.3%) and were detected in parallel (n=10). Among the 13 virulence marker-positive strains, the predominant genotype was (stx1/eae/Ehly) with a frequency of 53.8% (n=7; Table 1) and individual virulence genes stx1, stx2, eae and Ehly were detected at frequencies of 61.5%, 23%, 76.9% and 76.9%, respectively (Table 2). In the serotype specific PCR assays, none of the isolates were found to be positive for O157:H7 and O111. In phenotypic serogroup determination using three monovalent antisera, four virulence marker positive strains belonged to O26 serogroup (30.7%) and were in agreement with the serotype specific PCR. None of these isolates reacted with O111 and O157 antisera. Seventy-five percent of O26 EHEC (n=3) showed prevalent genotype (stx1/eae/ Ehly) and 25% (n=1) only contained stx2. All Ehly-positive strains phenotypically produced enterohaemolysin on WSBA-Ca.

Table 2: Frequency of virulence gene genotypes of EHEC

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>Frequency (%)</th>
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<tr>
<td>stx1/eae/Ehly</td>
<td>7/13</td>
<td>53.8</td>
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<tr>
<td>eae/Ehly</td>
<td>2/13</td>
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<td>stx2</td>
<td>2/13</td>
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<td>7.7</td>
</tr>
<tr>
<td>stx2/eae/Ehly</td>
<td>1/13</td>
<td>7.7</td>
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</table>

Table 3: Frequency of individual virulence genes of EHEC

<table>
<thead>
<tr>
<th>Virulence marker</th>
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<th>stx2</th>
<th>eeae</th>
<th>Ehly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8/13</td>
<td>3/13</td>
<td>10/13</td>
<td>10/13</td>
</tr>
<tr>
<td>Frequency (%)</td>
<td>61.5%</td>
<td>23%</td>
<td>76.9%</td>
<td>76.9%</td>
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</tbody>
</table>
This study investigated the presence of major virulence factors of EHEC among 375 isolates from 158 diarrheic calves by efficient multiplex PCR, detecting genes of major virulence factors of EHEC (stx1, stx2, eae, Ehly) simultaneously. The findings showed that 6.9% of samples were positive for shiga toxins (stx1 or stx2). stx1 was present in 5% and stx2 in 1.9% of samples. stx+/eae+ strains were identified in 5% and stx−/eae+ strains in 1.3% of animals tested. In this study, primers for eae gene were able to target a conserved region of the intimin gene (eae) between EHEC and EPEC (enteropathogenic E. coli) [7]. Therefore, stx−/eae+ strains can be considered as EPEC, but coexistence of Ehly gene in eae+ strains suggests that these might be the former EHEC which lost the genes for shiga toxins during infection or sub-culture. Inability of primers to target stx variants could be another possible explanation for this observation [8]. Our results support other findings which reported higher frequency of stx1 in calves. In contrast, some studies have detected stx2 as a dominant shiga toxin among EHEC from calves [1, 10] Zahraei Salehi et al. (2007) examined 29 isolates from diarrheic calves in Iran and identified 55% of strains as stx2+ and 13.7% stx1+ [10]. In the present study, 6.3% of samples contained eae+ strains; interestingly, all of the eae+ strains also carried Ehly, and most of them included stx1. Another noteworthy observation in the present study was the fact that all E. coli strains associated with each EHEC positive sample were positive for similar patterns of virulence genes. In other words, if only one colony from each sample were positive for similar patterns of virulence genes, e.g. eae and stx was observed. The findings indicate that routine use of WSBACa is highly beneficial as a simple, low-cost screening medium for presumptive detection of EHEC in combination with other diagnostic methods. Evidence of enterohaemolysin production could fill at least a part of the gap in diagnosis of highly virulent EHEC in a serotype nonspecific manner.

REFERENCES
