Isolation and Virulence Genes Characterization of Diarrheagenic Escherichia coli from Calves

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ARTICLE HISTORY (17-349)
Received: October 23, 2017
Revised: March 20, 2018
Accepted: March 20, 2018
Published online: April 13, 2018

Key words: Diarrhea
Escherichia coli
Sensitivity
Virulence gene

ABSTRACT

Calf diarrhea due to Escherichia coli (E. coli) causes huge economic losses and possess an important veterinary health aspect. In current study, antibiotic profiling, molecular screening, and identification of stx-1, eaeA, escV and bfpA virulence genes of E. coli isolated from calves were carried out. Fecal samples from 28 diarrheagenic calves were taken, processed and cultured on MacConkey agar, followed by identification using biochemical tests. Antibiotic susceptibility of the isolates was checked by disc diffusion method. For molecular characterization, polymerase chain reaction (PCR) was performed by using specific primers. Out of 28 samples collected from diarrheagenic calves, 83% samples were found positive for E. coli on the basis of biochemical profiling. Antimicrobial susceptibility against various antibiotics exhibited that most of the E. coli isolates were multi-drug resistant. All the isolates showed 100% resistance to rifampicin, erythromycin and oxacillin. The E. coli isolates were not resistant to gentamycin. Analysis through PCR showed that 10% of the isolates were positive for stx-1 gene, however, bfpA, escV and eaeA genes were not detected. Thus, E. coli is one of the key bacteria causing calf diarrhea and it was found that among all the virulent genes the stx-1 plays a part in E. coli associated diarrheal infection in calves.

INTRODUCTION

Calf diarrhea is a common disease in young animals causing major economic and health losses in livestock production worldwide. Data reported by National Animal Health Monitoring System from United States in 2007 revealed that almost 57% mortality among young calves is due to diarrhea (Cho and Yoon, 2014). Both infectious and non-infectious factors i.e. enteric pathogens and environmental factors are crucially involved in the development of calf diarrhea. The multi-factorial nature of this disease hinders the effective control of the disease in animal production (Foster and Smith, 2009). Diarrhea in calves is the most significant disease among developed and under developed countries. The definitive hosts including man, birds and animals are most susceptible for enteric diseases caused by Eschericia coli (E. coli) (Andrade et al., 2012: Islam et al., 2015).

Based on virulence mechanisms, Diarrheagenic E. coli (DEC) is classified as enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enter-aggregative (EAEC), diffusely adherent (DAEC), and enteropathogenic E. coli (EPEC) (Hashish et al., 2016). Among all strains, EPEC and HEC affect the young calves between the ages of 2-8 weeks. EPEC is present in diarrheic and non-diarrheic calves producing characteristic lesions on intestinal epithelial cells brush borders and do not produce any kind of toxins (Abe et al., 2009). intimin is a virulence protein expressed in E. coli cell surface that helps in adhesion of E. coli to its corresponding receptor, the translocated intimin receptor (tir) on the host cells (Stevens et al., 2006) and the gene encoding for it are termed as eaeA gene (Andrade et al., 2012).

Presence and absence of EAF plasmid (adherence factor) classify EPEC into two types as atypical EPEC (aEPEC) and typical EPEC (tEPEC). The typical EPEC has Adherence Factor Plasmid (EAF plasmid) that encodes bfpA, Per regulators and virulence genes (Scalaetsky et al., 2010). Prevalence of aEPEC is more than tEPEC and is important in endemic disease and in
outbreaks (Ochoa and Contrera, 2011). The Shiga toxin producing E. coli (STEC) contains Shiga toxin 1 (stx1) or stx2 and also known as Vero-toxin producing E. coli (VTEC). EHEC and EPEC are more important in humans as compared to animals (Schroeder et al., 2012).

Resistant E. coli strains induce persistent and severe infection when compared with antibiotic susceptible ones (Lakshmidevi et al., 2014). It is also described that most prevalent resistances were observed against Ampicillin, Tetracycline, Streptomycin and Sulfonamides (Kolenda et al., 2015). Extensive use of antibiotics in developing countries is thought to be the main cause of resistance. This problem may also affect the isolates from adults with diarrhea. The current research was planned in order to detect presence of DEC and molecular characterization of virulence genes along with antibiotic sensitivity pattern study.

MATERIALS AND METHODS

Purification and Bio characterization: The present study was performed at Institute of Microbiology, University of Agriculture Faisalabad. Twenty-eight samples from the rectum of diarrheagenic calves under three months of age were collected and diluted with 150mM of normal saline followed by centrifugation at 4000 RPM for 5 minutes. Supernatant was inoculated on MacConkey’s agar medium plates and incubation was done at 37°C for 24 hrs. Isolates were identified for cultural and morphological features and preserved in lactose broth comprising 20% glycerol. Further confirmation of E. coli was done using the biochemical tests i.e. Indole, Methyl red, Voges-Proskauer and Triple Sugar Iron test (Brisse et al., 2006).

Antibiotic sensitivity profiling: Susceptibility of E. coli to commonly used antibiotics was done through standard disc diffusion method and zone of inhibitions were measured. Isolates were designated as susceptible, intermediate and resistant according to the Clinical and Laboratory Standards Institute (CLSI) standards (Bokhari et al., 2013). Antimicrobial agents tested were Penicillin (10µg), Cefuroxime (30µg), Tetracycline (30µg), Oxacillin (1µg), Gentamicin (10µg), Erythromycin (15µg), Amoxicillin (30µg) and Rifampicin (5µg) (Silva and Mendonca, 2012). E. coli (ATCC # 25922) strain was included as a quality control.

Molecular characterization of E. coli from diarrheagenic calves: DNA from E. coli was extracted with Phenol/Chloroform Method (Bergallo et al., 2006). E. coli was enriched in Brain Heart Infusion Broth for overnight incubation at 37°C followed by centrifugation at 8000 rpm. Pellet was used for DNA extraction. DNA concentration was measured using Thermo Scientific NanoDrop™ Spectrophotometer. All the positive isolates were characterized by using PCR for stx1, eaeA, bfpA and escV genes using specific set of primers (Table 1). Amplified PCR products were electrophoresed on agarose gel (John et al., 2008).

RESULTS

Prevalence of E. coli in diarrheagenic calves: Based on colony morphology on MacConkey’s agar medium followed by biochemical characterization, twenty-five (25) samples (83%) were found positive for the presence of E. coli collected from the diarrheagenic calves. Fig. 1 represents the percentage distribution of all samples collected from diarrheagenic calves.

Purification and microscopic examination of E. coli isolates from calves: After 24 hrs incubation on MacConkey’s agar medium, E. coli colonies appeared pink due to lactose fermentation on MacConkey’s agar medium as shown in Fig. 2. Under microscope, E. coli appeared as pink colored Gram-negative rods.

<table>
<thead>
<tr>
<th>Primer sequences used for amplification of eaeA, bfpA, Escv and Stx1 genes of E. coli</th>
<th>Amplicon size (bp)</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>eaeA1</td>
<td>AAACAGGTGAACACTTGGCC CTCTGCGAGTATTACCTCTGC</td>
<td>453</td>
<td>eaeA</td>
</tr>
<tr>
<td>eaeA2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bfpA</td>
<td>FAATGGTGTGCTTCCGCGTCTTGC</td>
<td>323</td>
<td>bfpA</td>
</tr>
<tr>
<td>bfpA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escv</td>
<td>TATTGCTGCCTTTATTATGCTG</td>
<td>543</td>
<td>Escv</td>
</tr>
<tr>
<td>KS 7</td>
<td>CCCCCATGCAAAAAACATTAAATAGC</td>
<td>282</td>
<td>Stx1</td>
</tr>
</tbody>
</table>
Bio characterization of E. coli isolated from calves: A total of 25 out of 28 E. coli isolates were positive for catalase, Indole and Methyl red tests. All these 25 isolates also showed fermentation of dextrose, lactose and/or sucrose in TSI medium.

Antimicrobial susceptibility of E. coli isolated from diarrheagenic calves: Antibiotic susceptibility pattern of E. coli to different antibiotics observed on Muller Hinton agar is shown in Fig. 3 (A & B). The percentage resistance of isolates against seven different antibiotics is shown in Table 2. The E. coli anti-biogram revealed 100% resistance against Oxacillin, Rifampicin and Erythromycin followed by Amoxicillin (60%). It showed less resistance to Cefotaxime (30%) and Tigecycline (33%). No resistance was observed against Gentamycin (0%). Fig. 4 represents the histogram showing results for antibiotic susceptibility trend of seven different antibiotics against E. coli isolates.

PCR amplification of E. coli isolated from calves: A total of 25 samples were selected for the amplification of eaeA, stx-1, bfpA and escV genes using the specific set of primers for each gene mentioned in Table 1. DNA concentration of all the 25 isolates positive for E. coli was analyzed by Nano drop spectrophotometer. The entire PCR product for each gene was subjected to gel electrophoresis. Among the 25 samples that were subjected to PCR for amplification, only two samples (10%) showed a band of 282 base pairs indicating the presence of stx-1 gene (Fig. 5). No bands were observed for the remaining three genes (eaeA, bfpA and escV) in any of the 25 samples.

DISCUSSION

Based on the virulence factors, DEC is classified into different groups named as EHEC, ETEC, DAEC, EIEC, EPEC and enteroaggregative E. coli (EAggEC).

Primary identification through culture characteristics and biochemical tests confirmed 25 (83%) positive isolates for E. coli. This high prevalence rate found was higher when compared to studies by Islam et al. (2015). A comparatively lesser percentage 25 to 49.8% was also reported by Malik et al. (2013) while relatively higher percentage (60%) was reported by Ansari et al. (2014). This difference in prevalence might be due to the selection of animals for sampling, health and immune status of calves, study area and the farm management practices.

Antibiotics are not advised for treatment of mild and acute diarrhea. Majority of the isolates showed drug resistance to seven antibiotics used in this study. A complete (100%) resistance was observed against Rifampicin and Oxacillin followed by Erythromycin (90%) and Ampicillin (60%). It was reported that E. coli is resistant to Ampicillin (41%) (Kelly, 2015). Average susceptibility for Tigecycline was also noticed and only 33% resistance was shown to it, while other report showed comparatively higher resistance to it (Badouei et al., 2014). No resistance was observed for Gentamycin. Resistance to Erythromycin (90%) was compatible with previous studies (85%). In diarrheic calves, complete resistance to Erythromycin (97.6%), was reported (Pourtaghi & Sodagari, 2016). Resistance to Rifampicin was also studied (Nazir and Hussain, 2007).
A 60% resistance was found in case of Amoxicillin, supported by a report in which almost same results were reported, i.e., 59.6% resistance (Kelly, 2015). E. coli was also found resistant to Rifampicin and Oxacillin, even though both are not used in veterinary practice. This resistance might be due to high genomic plasticity of E. coli as it can vary its virulence properties quite often. Moreover, it also carries mobile genes and plasmid DNA as it can infect both humans and animals. Transduction, insertion, conjugation, transformation are possible mechanisms for acquiring of mobile genes from the surrounding resistant strains thus facilitating resistance genes to get incorporate into the plasmids or host’s genome (Younis et al., 2009).

Table 2: Percentage resistance of E. coli isolates from calves to selected antibiotics

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Antibiotic</th>
<th>Disc conc.</th>
<th>No of E. coli isolates resistant</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amoxicillin</td>
<td>30µg</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>Rifampicin</td>
<td>5µg</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Gentamycin</td>
<td>10µg</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Cefotaxime</td>
<td>30µg</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Tigecycline</td>
<td>15µg</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>Erythromycin</td>
<td>15µg</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Oxacillin</td>
<td>1µg</td>
<td>25</td>
<td>100</td>
</tr>
</tbody>
</table>

Conclusions: Neonatal calf diarrhea caused by E. coli is an important disease of calves in early life. It has been found that stx-1 gene contribute to the virulence of E. coli infection in calves. Also, this study focuses on the demand for more epidemiological survey and detection of other pathogenic genes among diarrheic E. coli to find out the current scenario for pathogenic genes prevalence in diarrheic calves.

Authors contribution: MIA designed the experiment and write the paper. AP executed the experiments. FRA, AAB, SA, and SUR contributed in proof reading, formatting and writing.

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