Molecular Screening of Certain Virulence Encoding Genes Associated with E. coli Strains Isolated from Diarrheic Calves

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ABSTRACT

Calf diarrhea is main causes of mortality among neonatal calves in large-scale cattle operations. The disease syndrome usually associated with severe economic losses. Bacteria represent the common causes of diarrhea in calves. In this study, we are collected one hundred fecal samples from diarrheic mixed-sex neonatal calves aged 1-60 days located at El-Behera Governorate were examined the presence of E. coli by bacteriological tests and further screened for the presence of some virulence encoding genes. Results were showed that 95% were positive for E. coli. The isolates belonged to 9 different serogroups namely O1, O27, O126, O119, O158, O146, O25, O148, and O115. The confirmed isolates were further examining for the existence of some virulence encoding genes (stx1, stx2, eaeA, and hlyA). The results appeared that, stx1, stx2, eaeA, and hlyA were successfully amplified in 66.6 %, 41 %, 16.6 %, and 16.6 % of the examined isolates, respectively. In conclusion, the comprehensive understanding of the virulence encoding determinants and the subcellular mechanism of E. coli pathogenesis will help develop accurate preventive and curative measures to decrease E. coli induced calf diarrhea in large-scale cattle farms.

Keywords: E. coli, Virulence genes, Diarrhea

INTRODUCTION

Diarrhea is a main clinical sign connected with mortality in calves (Butler, and Clarke, 1994). Diarrhea in newly born calves has been considered as one of the more important health troubles affecting dairy herds worldwide (Lage et al., 1993). Many factors increased the occurrence of calf diarrhea including, failure of the passive colostral transfer to the calf and environmental factors (Butler and Clarke, 1994). Several enteropathogens considered main causes of calf diarrhea including E. coli, Salmonella, Clostridium, Cryptosporidium species, rotavirus (RV), bovine corona virus, and Eimeria spp (Cho, and Yoon, 2014). E. coli is gram-negative, facultative anaerobic bacterium of the family Enterobacteriaceae. Morphological character of E. coli is rod-shaped, flagellated, non-sporulating. There are six main categories of E. coli strains, including enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enterohaemorrhagic E. coli (EHEC), Shiga toxin-producing E. coli (STEC), enteroinvasive E. coli (EIEC), enteroaggregative E. coli (EAEC), and diffusively adherent E. coli (DAEC) (Xia et al., 2010). The importance of E. coli as a main cause of diarrhea in calves has been confessed for many years (Cho, and Yoon, 2014; Butler, and Clarke, 1994). E. coli is leading to several problems as diarrhea and
hemorrhagic colitis (Ellaithi, 2004; Mohamed, 2009 and Malik et al., 2013). Strains of E. coli that cause enteric disease (diarrhoeagenic) and causing infections in humans and in animals caused by acquired virulence factors (Stenutz et al., 2006). Enteropathogenic E. coli appeared in the first 30 days of calf's life and causing diarrhea trans their virulence factors: adherence and enterotoxins. Enterotoxigenic E. coli (ETEC) adherently enterocyte trans fimbriae and mediates diarrhea by secreting heat-stable enterotoxin (sta) (Levine, 1987; Nataro and Kaper, 1998). The enteropathogenic E. coli EPEC does not produce Shiga toxin (Shahran et al., 2014), causing attaching-effacing (A/E) lesions on intestinal cells by intimin. EHEC enterohemolysin had plasmid hemolysin (Beutin et al., 1989) is known to enhance their pathogenic potential. E. coli can be isolated from healthy calves (Osek 2001; Mainil 2000; Herrera-Luna et al., 2009) because EPEC and STEC are opportunistic pathogens. Also, there are many risk factors, e.g., numbers of animals in farm, farm size, therapeutic treatment, housing is very important in controlling E. coli infections (Lundborg et al., 2005; Gulliksen et al., 2009 and Windeyer et al., 2014). Some of E. coli strains produce Shiga-like toxins (stx1 and stx2) and form A/E lesions as STEC, VTEC and EHEC. The common various gene of diarrheagenic E. coli strains is horizontal gene transfer (HGT). Specifically, Shiga toxin-producing E. coli and other E. coli strains can acquire virulence genes via horizontal gene transfer causing the emergence of new pathotypes of E. coli (Müller et al., 2007) and menacing on public health. ETEC was isolated from calves suffering from diarrhea by many authors worldwide (Dereje, 2012; Masud et al., 2012). Disentanglement of pathogenic E. coli by serological and molecular techniques are depend on O-H antigens and detection virulence markers (Nataro and Kaper, 1998; Ghanbapour and Oswald, 2009; Bandyopadhyay et al., 2011; Nguyen et al., 2011; Shams et al., 2012). E. coli has virulence encoding gene including (stx (1 and 2), hlyA and eaeA). The production of Shiga toxins (stx) is the main virulence property associated with STEC pathogens (Paton et al., 1998). Stx(1 and 2), these are toxins produced by STEC (verotoxin –Shiga-like toxin) because these toxins are similar to the Shiga toxin which produced by Shigella dysenteriae and S. sonnei, and they interfere with synthesis of protein and causing apoptosis in target cells while effectiveness of stx1 toxicity in Vero cells are 10-fold powerful than stx2 (Melton-Celsa, 2014). Molecular identification of virulence factors assists the diagnosis reliance on the pathogenicity of every species whose differs successively to the virulence factors. Rate of change in virulence factors considered as monition about the endemic case of the pathogen with a subsequent commending for restriction from the affected location (Badouei et al., 2010). Polymerase Chain Reaction (PCR) is a common nucleic acid-based method for screening of the virulence factors of E. coli strains and other enteric pathogens (Osek et al., 1999). Therefore, in this study we are isolated strains of E coli from neonatal calves suffering from diarrhea in private farms located at El-Behera Governorate. In addition to using PCR in detection of some virulence encoding genes (stx1 and 2), hlyA, and eaeA).

**MATERIALS AND METHODS**

**Samples**

One hundred samples were collected from mixed-sex neonatal calves aged 1-60 days, suffering from diarrhea belonged to commercial private dairy farms (about 7 farms) located at El-Behera Government. The fecal samples were collected in sterile bags and immediately transferred to the lab in University of Sadat City for bacteriological isolation and identification.

**Isolation and identification of E. coli**

Transfer the fecal samples into MacConkey broth then sub culturing into specific medium on MacConkey agar plate (Oxoid) at 37°C for 24 hrs. The suspected typical colonies of E. coli that appeared as rose pink colonies and cultured on EMB media (Oxoid). The typical colonies characteristic metallic sheen appearance (fish eyes) were further identified based on the biochemical tests were performed according to (Cruickshank et al., 1975; Koneman et al., 1983): -

**a- Indole test:**

To 48 hours culture incubated at 37°C in 1% peptone water, 1 ml of ethyl ether was added. The tubes good shaking and allowed to stand until ether rise to the surface to each tube 0.5 ml of the Kovac's reagent was trickled down on
side of the tube, the formation of a red ring (surface layer) after 10 minutes was considered a positive reaction.

**b- Methyl Red Test:**
Five ml buffered glucose broth tube inoculating with pure culture, incubated at 37°C for 24 hours, to each tube, 5 drops of Methyl Red reagent were added the development of a red color was considered a positive test.

**c- Voges – Praskauer test:**
From 48 hours culture incubated at 37°C in 5 ml buffered glucose phosphate broth, 1 ml taken on a test tube and 0.6 ml of alcoholic solution of alpha–naphthol and 0.2 ml of 4% potassium hydroxide solution were added, the tubes standing for 24 hours, pink coloration of the mixture was a positive result.

**d- Citrate utilization test:**
Slants and butts of Simmon citrate agar tubes stabbing from pure cultures and incubated at 37°C for 48 hours, the development of blue color pinpointed utilization of citrate.

**Serotyping of E. coli**
The isolates were further characterized by specific media and serotyped based on O antigens. The E. coli isolates were analyzed for their somatic antigen (serogroup) Kok et al., (1996) where 26.3% (25/95 isolates) belonged to twelve O serogroups (O1, O27, O126, O119, O158, O146, O25, O148, and O115) and 52% (13/25 isolates) are non-identified serogroup (table 3).

**Virulence encoding gene of E. coli**
These provenly the presence of some virulence encoding genes using PCR. The results of PCR (Fig.1) performed on 12 samples showed that eaeA gene (890 bp) was successfully amplified in O126 and O158 while it was absent in O1, O25, O27, O115, O119, O146, and O148. The data also showed that stx1gene (614 bp) was successfully amplified in O27, O15, O119, O126, O146, and O158 while it was absent in O1 and O25. Concerning the stx2 gene (779 bp), results showed that the stx2 gene was successfully amplified in O1, O119, and O126 while it was absent in O25, O27, O115, O146, O148, and O158. Finally, our results demonstrated that, hlyA gene (165 bp) was successfully amplified in O25 and 148, while it was absent in O1, O27, O115, O119, O126, O146 and O158 (Table 4).
Table (1): Primer sequences of E. coli used for PCR identification system

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5′ → 3′)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>F-5′ ACACTGGATGATCCTCAGTGG '3</td>
<td>614</td>
<td>Dhanashree and Mallya (2008)</td>
</tr>
<tr>
<td></td>
<td>R-5′ CTAATCCCCCCTCATTATG '3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>F-5′ CATGACAACGACGACGAGTT '3</td>
<td>779</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-5′ CCTGCACTGAGCAGCAGCCTTGG '3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>F-5′ GTGGCGAATACTGGCGAGACT '3</td>
<td>890</td>
<td>Mazaheri et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>R-5′ TAAATCCACGCCCAGTGCGCAAAAA'3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hylA</td>
<td>F-5′ ACATGTGGTTTATTCTGGA '3</td>
<td>165</td>
<td>Fratamico et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>R-5′ CTTACGTGACCATACATAT '3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (2) Prevalence of E. coli in diarrheic calves

<table>
<thead>
<tr>
<th>Number of fecal samples</th>
<th>Negative samples</th>
<th>Percentage</th>
<th>Positive samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>5</td>
<td>5%</td>
<td>95</td>
<td>95%</td>
</tr>
</tbody>
</table>

Table (3) serotyping in E. Coli isolates

<table>
<thead>
<tr>
<th>Number of Serotyping samples</th>
<th>Non-identified serogroup</th>
<th>Percentage</th>
<th>Identified serogroup</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>13</td>
<td>52%</td>
<td>12</td>
<td>48%</td>
</tr>
</tbody>
</table>

Table (4): Occurrence of virulence genes of enteropathogenic E. coli isolated from the examined fecal samples of diarrheic calves.

<table>
<thead>
<tr>
<th>E. coli Serotype</th>
<th>No. of ex. isolates</th>
<th>stx1</th>
<th>stx2</th>
<th>eaeA</th>
<th>hylA</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>3</td>
<td>NO</td>
<td>%</td>
<td>NO</td>
<td>%</td>
</tr>
<tr>
<td>O25</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O27</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O115</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O119</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>O126</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>O146</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O148</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O158</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>


(Fig.1): Agarose gel electrophoresis of multiplex PCR of stx1 (614 bp), stx2 (779 bp), eaeA (890 bp) and hlyA (165 bp) virulence genes for characterization of Enteropathogenic E. coli. Lane M: 100 bp DNA ladder, Lane (C+): positive Control E. coli for stx1, stx2, eaeA and hlyA genes, Lane (C-): negative Control, Lane (1): O1, Lane (2): O1, Lane (3): O1, Lane (4): O25, Lane (5): O27, Lane (6): O27, Lane (7): O115, Lane (8): O119, Lane (9): O126, Lane (10): O146, Lane (11): O148, Lane (12): O158.
DISCUSSION

E. coli is a commensal organism causing diarrhea in newly born calves, especially calves which take little amount of colostrum after birth (Malik et al., 2013). A better understanding of the main causes of diarrhea in calves and virulence encoding determinants will be useful to establish accurate curative and preventive measures. Therefore, this work was performed to determine the most important E. coli strains isolated from diarrheic calves and the existence of some virulence encoding genes. In this study, 100 samples were collected from calves suffering from diarrhea aged (1-60 days) located at EL-Behera governorates were examined for E. coli. Also, examined for the presence of some virulence encoding genes (stx1, stx2, hlyA, and eaeA). Our results showed that 95% of the collected samples were positive E. coli. The result is agreed with the finding previously obtained by (Begum et al., 2014), where 88.5% of fecal samples were positive E. coli. While a lower percentage was obtained by (Oporto et al., 2008), where only 35.9% of the examined samples were positive for E. coli. Lower results were also obtained by (Luna et al., 2009), where (18.9%) of the tested sample were positive for E. coli. Furthermore, Haggag and Khaliel (2002) reported that 82% % of the tested sample were positive for E. coli. Many factors are affecting the incidence of E. coli in newly born calves including management practices and overcrowding and malnutrition (Abdulgayeid et al., 2015). Also, the variation in these results might be attributed to many factors including, sampling area, age of calves, and many other factors not investigated under the conditions of the current study. The confirmed isolated were further identified into serogroups based on O and H antigens, our results showed that 26.3% (25/95 isolates) belonged to twelve O serogroups (O1, O27, O126, O119, O158, O146, O25, O148, and O115) and 52% (13/25 isolates) belonged to a non-identified serogroup. These results are agreeing with the findings reported in many studies (Joon and Kaura, 1993; Hussain et al., 2003; Wani et al., 2004; Osman et al., 2012). The serogroups O1, O115, O119, and O146 recovered in our study have also been shown in diarrheic calves (Mohammed et al., 2019). The serogroups O1 showed in our study have also been recovered in diarrheic calves (Wani et al., 2004 and Bhat et al., 2017). The serogroups O25 and O119 showed in the current study have also been recovered in diarrheic calves (Osman et al., 2013). The serogroups O126 showed in this study have also been recovered in diarrheic calves (Bhat et al., 2017). The confirmed isolates were further tested for the existence of some virulence encoding genes. In this current study showed that, eaeA gene (890 bp) was successfully amplified in O126 and O158. stx1 gene (614 bp) was successfully amplified in O27, O15, O119, O126, O146, and O158. Stx2 gene (779 bp), was successfully amplified in O1, O119 and O126. Finally, hlyA gene (165 bp) was successfully amplified in O25 and 148. Our results showed that, stx1 gene is the most prevalent gene, this is agreed with the findings Giovanna et al. (2012). The lower prevalence of eaeA gene has been observed in other studies (Hornitzky et al., 2005; Fremaux et al., 2006). Previous reports showed the most pathogenic strains of E. coli which isolated from the feces of goat, sheep and cattle were not harbor eaeA gene (Kobayashi et al., 2001; Pradel et al., 2001); Blanco et al., 2004; Vu-Khac and Cornick, 2008). The results in this study are agreed with discovering of (Nasr-Eldin et al., 2018), by using molecular characterization presence in E. coli isolates toxin genes: heat-stable enterotoxin (sta), (stx1-stx2). Strains carrying eaeA with stx1 or stx2 variants are considered as STEC while strains carrying eaeA but not stx1 and stx2 variants are considered as potential EPEC (Ishii et al., 2007) as in O126 and O158. Shiga toxin Stx2 has highly cytotoxic effect on endothelial cells and is connected with dangerous infections as in O1 (Bertin et al., 2001; Caprioli et al., 2005). Stx2 gene was more prevalent than stx1 and that both were connected with eaeA gene in STEC strains (Wani et al., 2003). This finding, however, is in contrast to an earlier report that relates signs with the presence of stx1 with eaeA genes as in O158 (Sandhu et al., 1996); eaeA genes were detected in 16.6% of E. coli isolates, which is in agreement with the findings of (Ishii et al., 2007) where only 19.3% of E. coli isolates harbor eaeA encoding gene. Previous reports
showed that, horizontal gene transfer from other pathogroups causing diarrheogenic *E. coli* and STEC were acquire virulence genes leading to the evolvement of divergently pathogroups (Müller et al., 2007; Johura et al., 2016). The high prevalence of EHEC and the presence of STEC–ETEC hybrid indicating their importance in the etiopathogenesis of diarrhea in calves and reinforcing the role of these animals as a reservoir of potentially pathogenic *E. coli* to humans. STEC and EHEC in normal and diarrhoeic faeces of young cattle was performed by Epidemiological studies. EHEC are considered a subset of STEC, and many studies have accentuated the importance of cattle as a reservoir of both pathotypes in Brazil (Salvadori et al. 2003; Aidar-Ugrinovich et al., 2007; Pigatto et al., 2008) and worldwide (Gyles 2007; Foster and Smith 2009; Moxley and Smith 2010). Finally *E. coli* is one of the main common diseases of newly born calves (9–10 days of age) characterized by watery diarrhea and the affected calves die within 2–3 days. Calf diarrhea appeared higher in medium and large sized dairy farms than small dairy farms (Yeshiwas and Fentahun, 2017).

Finally, results in this study with others show that *E. coli* one of the most important causes of calves' diarrhea and PCR is considered as a reliable technique for the determination of virulence encoding genes of *E. coli*. In addition, regular screening of *E. coli* isolated from calves suffering from diarrhea for the presence of virulence encoding genes.

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