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

Reda Tarabees[1], Gamal Younis[2], Heba El-Khetaby[3*]

(1) Department of Bacteriology, Mycology and Immunology Faculty of Veterinary Medicine, University of Sadat City, Minufiya, Egypt.

(2) Department of Bacteriology, Mycology and Immunology Faculty of Veterinary Medicine, Mansuora University, Egypt

(3) Directorie of veterinary Medicine, Almeria, Alexandria, Egypt

**corresponding author: hepahamdy20@gmail.com* *Received: 2/8/2020 Accepted: 27/9/2020*

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 ofsome virulence encoding genes (*stx*1, *stx*2, *eae*A, and *hly*A). The results appeared that, *stx*1, *stx*2, *eae*A, *and hyl*A were successfully amplified in 66.6 %,41, 6 %,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 transtheir 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 (Shahrani *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 were 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; Ghanbarpour and Oswald, 2009; Bandyopadhyay *et al*., 2011; Nguyen *et al*., 2011; Shams *et al*., 2012). *E. coli* has virulence encoding gene including (*stx (*1and 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 dystenteriae* and *S. sonnei*, and they interfere with synthesis of protein and causing apoptosis in target cells while effectiveness of *stx*1 toxicity in Vero cells are 10-fold powerful than *stx*2 (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 (*stx*1and 2), *hly*A, and *eae*A).

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-Behira 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 was 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 coloration 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). A collection of 12 O-serotypings were available at the Central Lab, Ministry of Health. This strains consisted of (.O1, O27, O126, O119, O158, O146, O25, O148 and O115)

Genomic DNA extraction and PCR conditions

Extraction of bacterial DNA according to (Shah *et al.*, 2009), briefly DNA extraction was performed from pure strains of *E. coli* cultured on nutrient broth and incubated overnight using The QIAamp DNA Mini Kit Multiplex Polymerase chain reaction (PCR) for the detection of *E. coli* virulence encoding genes using specific primers (Table 1). The PCR reactions were completed in PCR tubes of 50 µl, containing 5 µl DNA template, 1 µl of each primer (0.5 µM), 25 µL of 2x multiplex master mix (Takara) and the final volume was adjusted to 50 µL with PCR water. The PCR conditions were started with initial denaturation 95 °C 3 m and followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1-10 m and a final at extension 72°C for 7 m.The reference strain *E. coli* O157:H7 Sakai represented (control positive for *stx*1, *stx*2, *eae*A and *hyl*A) and *E. coli* K12DH5α represented (a non-pathogenic negative control strain) were obtained from Food Analysis Center, Fac. Vet. Med., Benha Univ.) The amplified products were then resolved by electrophoresis in 2% agarose gel at 100 V for 60 min. Gels were stained with ethidium bromide solution and documentation was done using the Gel Doc system

RESULTS

Serotyping of E. coli

Our results showed that among the examined 100 samples collected from neonatal calves suffering from diarrhea, 95% (95/100) were positive for E. coli(Table 2). The isolates were further identified E. *coli* based on 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 *eae*A 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 *stx*1gene (614 bp) was successfully amplified in O27, O15, O119, O126, O146, and O158 while it was absent in O1 and O25. Concerning the *stx*2 gene (779 bp), results showed that the *stx*2 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, *hly*A 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

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
*stx*1	**F-5′** ACACTGGATGATCTCAGTGG **′3** **R-5′** CTGAATCCCCCTCCATTATG **′3**	614	**Dhanashree and Mallya (2008)**
*stx*2	**F-5′** CCATGACAACGGACAGCAGTT **′3** **R-5′** CCTGTCAACTGAGCAGCACTTTG **′3**	779	
*eae*A	**F-5′** GTGGCGAATACTGGCGAGACT **′3** **R-5′** TAAATCCACGCCCAGTCGCAAAAA′3	890	**Mazaheri *et al*. (2014)**
*hyl*A	**F-5′** ACGATGTGGTTTATTCTGGA **′3** **R-5′** CTTCACGTGACCATACATAT **′3**	165	**Fratamico *et al*. (1995)**

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

Number of fecal samples	Negative samples	Percentage	Positive samples	Percentage
100	5	5%	95	95%

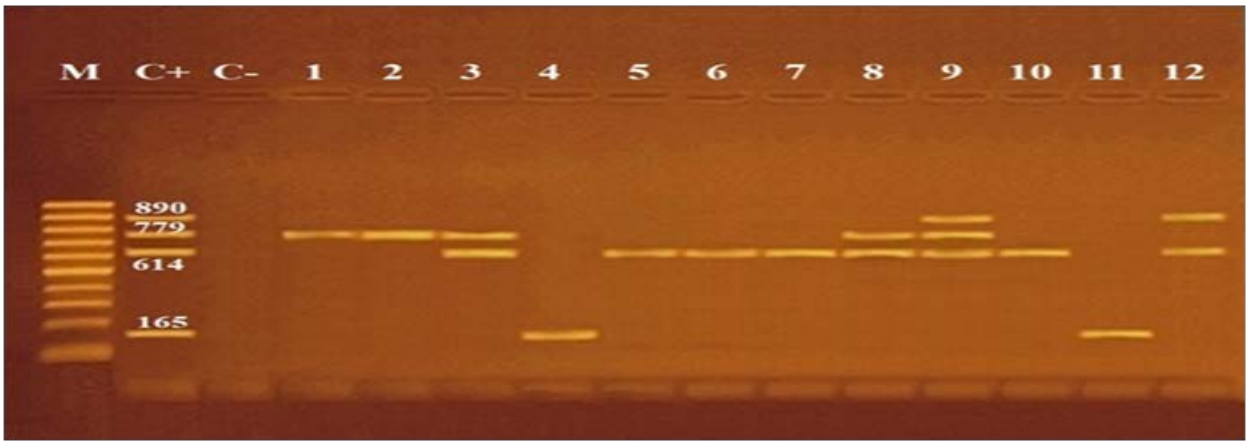
Table (3) serotyping in *E. Coli* isolates

Number of Serotyping samples	Non-identified serogroup	Percentage	Identified serogroup	Percentage
25	13	52%	12	48%

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

E.coli	**No. of ex.**	***stx*1**		***stx*2**		***eae*A**		***hyl*A**	
Serotype	**isolates**	**NO**	**%**	**NO**	**%**	**NO**	**%**	**NO**	**%**
O1	**3**	1	33.3	3	100	0	0	0	0
O25	**1**	0	0	0	0	0	0	1	100
O27	**2**	2	100	0	0	0	0	0	0
O115	**1**	1	100	0	0	0	0	0	0
O119	**1**	1	100	1	100	0	0	0	0
O126	**1**	1	100	1	100	1	100	0	0
O146	**1**	1	100	0	0	0	0	0	0
O148	**1**	0	0	0	0	0	0	1	100
O158	**1**	1	100	0	0	1	100	0	0

***stx*1:** Shiga- toxin 1 gene**, *stx*2:** Shiga- toxin 2 gene**, *eae*A:** intimin gene ***hyl*A:**haemolysingene.



(Fig.1): Agarose gel electrophoresis of multiplex PCR of *stx*1 (614 bp), *stx*2 (779 bp), *eae*A (890 bp) and *hly*A(165 bp) virulence genes for characterization of *Enteropathogenic E. coli* Lane M: 100 bp DNA ladder, Lane (C+): positive Control *E. coli* for *stx*1, *stx*2, *eae*A and *hly*A 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 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 (*stx*1, *stx*2,*hyl*A, and *eae*A). 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 furtherer 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 showed 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 (Cho and Yoon, 2014). The serogroups O158 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, *eae*A gene (890 bp) was successfully amplified in O126 and O158. *stx*1gene (614 bp) was successfully amplified in O27, O15, O119, O126, O146, and O158. *Stx*2 gene (779 bp), was successfully amplified in O1, O119 and O126. Finally, *hly*A gene (165 bp) was successfully amplified in O25 and 148. Our results showed that, *stx*1 gene is the most prevalent gene, this is agreed with the findings Giovanna *et al.* (2012). The lower prevalence of *eae*A 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 *eae*A 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 *eae*A with *stx*1 or *stx*2 variants are considered as STECwhile strains carrying *eae*A but not *stx*1 and *stx*2 variants are considered as potential EPEC (Ishii *et al.*, 2007) as in O126 and O158. Shiga toxin *Stx*2 has highly cytotoxic effect on endothelial cells and is connected with dangerous infections as in O1 (Bertin *et al.*, 2001; Caprioli *et al.*, 2005). *Stx*2 gene was more prevalent than stx1 and that both were connected with *eae*A 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 *stx*1 with *eae*A genes as in O158 (Sandhu *et al*.,1996), *eae*A 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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