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RESEARCH ARTICLE

Epidemiological Study of Diarrheagenic *Escherichia coli* Virulence Genes in Newborn Calves

Emad A Hashish[1]*, Hend M El Damaty[2], Yasmine H Tartor[3] and Ahmed M Abdelaal[4]

[1]Department of Clinical Pathology; [2]Department of Animal Medicine, Infectious Diseases; [3]Department of Bacteriology, Mycology and Immunology; [4]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
*Corresponding author: eagad@vet.zu.edu.eg

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A B S T R A C T

Diarrheagenic *Escherichia coli* (DEC) are considered one of the major etiological agents of neonatal calf diarrhea (NCD). One hundred calves (2-21 days-old) suffering from diarrhea in Sharkia Governorate, Egypt were used in this study. Six *E. coli* virulence genes were characterized by multiplex PCR including; heat-stable enterotoxins (STa; 33.3%), heat-labile enterotoxin (LT; 30%), two Shiga toxins: (Stx1; 86.67%), (Stx2; 26.67%) produced from Shiga toxin producing *E. coli* (STEC) and two enterotoxigenic *E. coli* (ETEC) adhesions (F5; 13.3%) and (F41; 16.67%). Each isolate was found to carry one or more virulence genes. Clinicobiochemical examination showed significant ($P<0.01$) increase in serum total protein, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN), and potassium levels. Meanwhile; serum albumin, A/G ratio, sodium and chloride levels were significantly ($P<0.01$) decreased. Multiplex PCR was a useful quick diagnostic tool for characterization and identification of *E. coli* virulence genes. Shiga toxin genes increase the risk for zoonotic STEC infection. Mixed *E. coli* infection is strongly associated with the reported cases of diarrhea due to the presence of several virulence genes in some isolates.

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INTRODUCTION

Escherichia (*E.*) *coli* is the most important bacterial cause of diarrhea in young animals. Several pathogenic and nonpathogenic factors are predisposed to calf diarrhea (Bartels *et al*., 2010; Izzo *et al*., 2011). Diarrheagenic *E. coli* (DEC) are recognized as the major cause of neonatal calf diarrhea (NCD) with severe lethal outcome and major damage to the livestock industry worldwide. Consequently, high mortality rate in calves under 3-weeks-old and up to 3-months-old has been reported (Windeyer *et al*., 2014). Besides, significant loss of other neonatal animal species such as lambs, suckling piglets and foals due to diarrhea has been reported (Cho and Yoon, 2014).

Single primary pathogen or co-infection can predispose to the development of diarrhea. Other factors such as nutritional factors, hygiene conditions and environmental factors could contribute to diarrhea (Fernandez *et al*., 2009). Based on the molecular, and pathological criteria, the DEC are classified into several pathotypes such as: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) enterinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Diffusely-adherent *E. coli* (DAEC) and vero- or Shiga-like toxin producing *E. coli* (VTEC or STEC) (Nagy and Fekete, 2005).

Virulence factors from several pathogenic *E. coli* strains might predispose to calves diarrhea. ETEC are common pathotype associated with infectious diarrhea in calves (Foster and Smith, 2009). They colonize the small intestine by their fimbriae without inducing significant morphological changes and predispose to severe watery diarrhea in newborn calves. Two main virulence factors are included, the fimbriae (pili) and the enterotoxins (Franck *et al*., 1998). There are two types of fimbriae (F5 and F41) and two types of secreted enterotoxins: STa and LT accounts for nearly most cases of ETEC infection in newborn calves. Enterotoxins reduce the intestinal absorption and increase the fluid and electrolyte secretion of small intestinal epithelial cells predisposing to diarrhea (Nagy and Fekete, 2005). Shiga toxin producing *E. coli* (STEC) are associated with dysentery in calves. They

secrete two different Shiga toxins: Stx1 and Stx2. Similarly; zoonotic *E. coli* O157:H7 it secretes Shiga toxins and termed EHEC. They are associated with hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Moxley, 2004). Other reports previously isolate *E. coli* O157:H7 strains from fecal samples of calves (Dastmalchi and Ayremlou, 2012).

The socioeconomic and zoonotic significance increase the concern regarding the importance of the DEC virulence factors. Consequently, the aim of the present work was to provide a critical analysis of the DEC virulence genes among NCD. Molecular characterization was done through multiplex PCR as a quick and reliable tool for screening of DEC (Bauer *et al*., 2007). Clinico-biochemical assays were utilized to access the general health condition through liver and kidney function tests in combination with the molecular diagnosis.

MATERIALS AND METHODS

Animals & clinical examination: One-year study for molecular characterization of the DEC in neonatal calves was carried out during the period of October 2013 to September 2014. Twenty calves showed no symptoms of diarrhea were used as negative control. The selected diarrheic calves (n=100) based on the signs of diarrhea on physical examination. Calves resided on several farms (each had >50 animals), in Sharkia Governorate, Egypt. Neonatal calves (2-21 days-old) showed symptoms of watery diarrhea at the time of sampling. Each calf underwent a clinical examination (Radostits, 2000), including general behavior, appearance, suckling reflex and degree of dehydration (skin tent test). Body temperature, pulse rate, respiratory rate, capillary refill time and case history were recorded. This study has been carried out in accordance with the principles and guidelines of Animal Care and Use with the help of veterinarians of the selected farms.

Fecal sampling and *E. coli* isolation: Fecal samples were collected using sterile rectal swabs, and directly placed into sterile plastic tubes containing Stuart medium. Samples were plated onto MacConkey's agar plates and incubated overnight at 37°C. Lactose positive colonies were randomly selected from each sample and confirmed to be *E. coli* by standard biochemical tests. Colonies were sub-cultured on Eosin methylene blue agar for characteristic metallic sheen of *E. coli*. Positive isolates were stored at -80℃ in tryptic soy broth containing 20% glycerol until use.

Blood sampling: Blood samples were collected from jugular vein. Blood (2.5 mL) were allowed to flow smoothly into vacutainer tubes containing no anticoagulant and left to clot. Serum was separated by centrifugation at 3000 rpm for 15 minutes and collected using sterile Pasteur pipettes. The collected serum was transferred to sterile labeled Eppendorf tubes for biochemical analysis. All calves were allowed to rest during and after the blood samples collection.

DNA extraction and Multiplex PCR: Multiplex PCR was used to identify STa, LT, Stx1, Stx2, F5 and F41 virulence genes (Franck *et al*., 1998). Bacterial cell boiling used for DNA extraction (Gholoobi *et al*., 2014). Collected DNA used for PCR reaction. Each reaction (40 µl) contains 5 µl of DNA template, 25 µl Max PCR Master Mix, 1 µl of each primer (20 pmol) (Table 1) and 9 µl of nuclease free water. The amplification includes, initial denaturation at 94°C for 5 minutes. Annealing temperature was 58°C for (Stx1 and Stx2), 57°C for (STa and LT) and 50°C for (F5 and F41) and extension at 70°C for 10 minutes. PCR products were separated by electrophoresis on 1.5% agarose gel. A 100 bp DNA Ladder was used. Gel was visualized by a gel documentation system.

Clinicobiochemical assays: Serum total protein, albumin (Fernandez *et al*., 1966), alanine aminotransferase (ALT), aspartate aminotransferase (AST) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Greco, 1950), blood urea nitrogen (Tabacco *et al*., 1979), creatinine (Heinegard and Tiderstrom, 1973), sodium, potassium and chloride levels were measured (Kulpmann, 1992). Serum globulin level was determined by subtracting the albumin from the total proteins. Albumin globulin ratio (A/G ratio) was estimated using albumin and globulin values.

Statistical analysis: Statistical Analysis System software package were used to analyze the data by using SAS for Windows, version 8 (SAS Institute, Cary, NC). Independent t-test has been utilized. Significant differences between means were represented by mean±SE at a level of ($P<0.01$).

RESULTS

Clinical examination of the diarrheic calves: Moderate to severe profuse watery diarrhea was observed in all infected calves. Nine of the infected calves appeared dull, depressed and recumbent with rapid respiration. All infected calves showed absence of suckling reflex, sunken eye with a space between eyeball and glob more than 0.5cm. Engorged eye capillary and congested conjunctiva mucous membrane, droopy ears, rough coat was reported in all infected calves. Corneal opacity was observed in one calf only. Six calves showed pyrexia with rectal temperature (39.5-41°C), meanwhile three calves had subnormal temperature (36-37.5°C) when compared with the control calves. Lateral recumbency and complete loss of consciousness has been reported. Severe case of diarrhea which ended with death was reported. Physical examination showed no clinical signs of disease in the control calves during the study period. One hundred neonatal calves with of diarrhea were used in this study. Five calves were at 2 days-old where four calves were at 4-5 days-old and their dams were not vaccinated during pregnancy. These calves were found to be raised in unhygienic conditions.

Identification of virulence genes of *E.-coli* among diarrheic calves by PCR: *E. coli* were isolated from thirty isolates (30%) and further characterized by multiplex PCR. Six DEC virulence genes (LT, STa, Stx1, Stx2, F5 and F41) were identified out of the thirty positive *E. coli* isolates. Multiplex PCR for the DEC virulence genes was summarized in Fig. 1. Amplicon of the

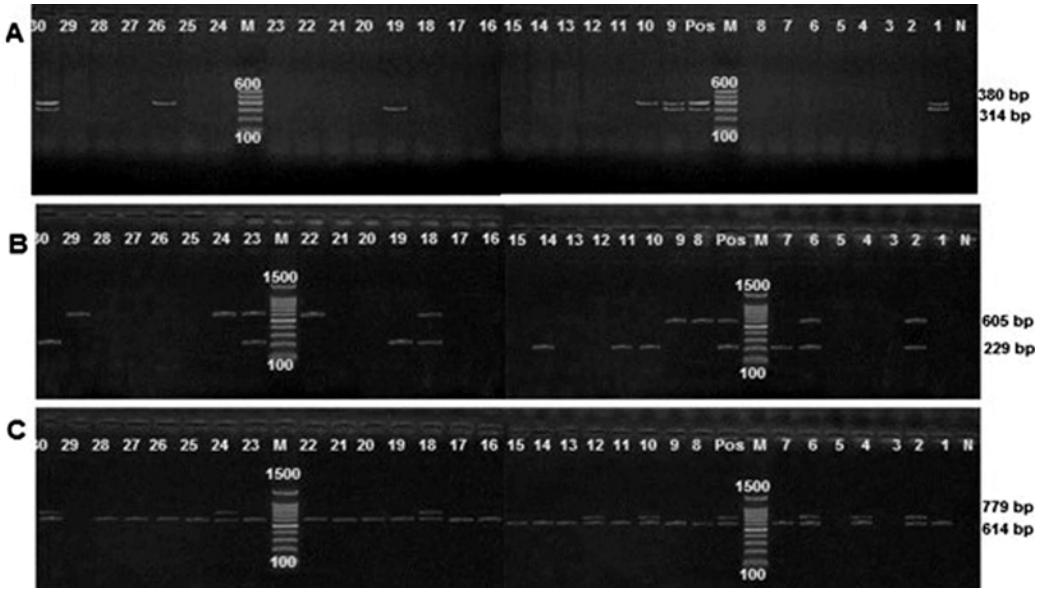

Fig. 1: Multiplex PCR for the DEC virulence genes where; N= negative control, Pos= positive control and M= DNA ladder. A) Lanes (1-30) showing the size of the fimbrial genes; F5 (314 bp) and F41 (380 bp). B) Lanes (1-30) showing the size of the enterotoxins; LT (605 bp) and STa (229 bp). C) Lanes (1-30) showing the size of the shiga toxins; Stx1 (614 bp) and Stx2 (779 bp).

Table 1: Primers used in multiplex PCR targeting virulence genes of thirty *E. coli* isolates from calves with diarrhea in Egypt. Three multiplex PCR reactions combine six genes as following; F5&F41, STa & LT and Stx1 & Stx2.

Target gene	Primers sequences 5 -3	Amplicon size	Reference
F5 (F)	TATTATCTTAGGTGGTATGG	314 bp	(Franck *et al.*, 1998)
F5 (R)	GGTATCCTTTAGCAGCAGTATTTC		
F41 (F)	GCATCAGCGGCAGTATCT	380 bp	(Franck *et al.*, 1998)
F41 (R)	GTCCCTAGCTCAGTATTATCACCT		
STa (F)	GAAACAACATGACGGGAGGT	229 bp	(Lee *et al.*, 2008)
STa (R)	GCACAGGCAGGATTACAACA		
LT (F)	GGTTTCTGCGTTAGGTGGAA	605 bp	(Lee *et al.*, 2008)
LT (R)	GGGACTTCGACCTGAAATGT		
Stx1 (F)	ACACTGGATGATCTCAGTGG	614 bp	(Dipineto *et al.*, 2006)
Stx1(R)	CTGAATCCCCCTCCATTATG		
Stx2 (F)	CCATGACAACGGACAGCAGTT	779 bp	(Dipineto *et al.*, 2006)
Stx2 (R)	CCTGTCAACTGAGCAGCACTTTG		

predicted size was confirmed, LT (605 bp), STa (229 bp), Stx1 (614 bp), Stx2 (779 bp), F5 (314 bp) and F41 (380 bp). Distribution of the virulence genes among the thirty *E. coli* isolates were summarized in Table 2. Enterotoxin-producing *E. coli* (ETEC) has been identified. The identified enterotoxins were LT gene (9 of 30, 30%) and STa gene (10 of 30, 33.30%). Four isolates (2, 6, 18 and 23) out of the 30 *E. coli* isolates shared both LT and STa genes (13.3%). Shiga toxin-producing *Escherichia coli* (STEC) were detected. The identified shiga toxin genes were Stx1 (26 of 30, 86.67%), Stx2 (8 of 30, 26.67%). Stx1 virulence gene was the predominant virulence gene. Eight isolates (2, 4, 6, 10, 12, 18, 24 and 30) out of the 30 *E. coli* isolates shared both Stx1 and Stx2 genes (26.67%). The ETEC fimbrial virulence genes have been identified. The adhesins genes identified were F41 (5 of 30, 16.67%) and F5 (4 of 30, 13.3%). Three isolates (1, 9 and 30) out of the 30 *E. coli* isolates shared both F41 and F5 genes (10%). The isolated DEC virulence genes are summarized in Fig. 1 and Table 2 and showed the prevalence of Stx1 gene among other virulence genes.

Clinicobiochemical parameters: Significant decrease ($P<0.01$) in the albumin level and the A/G ration with significant increase ($P<0.01$) in the globulin and total protein levels were detected among calves with diarrhea (Table 3). Alteration in the activities of hepatic marker enzymes such as serum alanine aminotransferase (ALT) and serum alkaline phosphatase (ALP) has been reported. A Significant increase ($P<0.01$) in the ALT and ALP levels with non-significant change in the serum AST activity were reported (Table 3). Alteration in the renal function tests and electrolyte has been detected. Serum urea and creatinine levels showed significant increases ($P<0.01$) in diarrheagenic calves. Significant decrease in the levels of sodium and chloride levels with significant increase in serum potassium levels ($P<0.01$) has been detected. Disturbance in serum electrolyte levels was reported in diarrheagenic calves. Significant decrease ($P<0.01$) in the serum sodium and chloride levels with significant increase ($P<0.01$) in the serum potassium levels in diarrheagenic calves was observed (Table 3).

DISCUSSION

This study focusing on the molecular characterization of the DEC virulence genes with clinicobiochemical assays in calves suffering from diarrhea. Interaction between several management and environmental factors

Table 2: Distribution of virulence genes among the thirty *E. coli* isolates from calves with diarrhea in Egypt

Virulence gene	F41	F5	LT	STa	Stx1	Stx2
Isolate 1	+	+	-	-	+	-
Isolate 2	-	-	+	+	+	+
Isolate 3	-	-	-	-	-	-
Isolate 4	-	-	-	-	+	+
Isolate 5	-	-	-	-	-	-
Isolate 6	-	-	+	+	+	+
Isolate 7	-	-	-	+	+	-
Isolate 8	-	-	+	-	+	-
Isolate 9	+	+	+	-	+	-
Isolate 10	+	-	-	+	+	+
Isolate 11	-	-	-	+	+	-
Isolate 12	-	-	-	-	+	+
Isolate 13	-	-	-	-	+	-
Isolate 14	-	-	-	+	+	-
Isolate 15	-	-	-	-	+	-
Isolate 16	-	-	-	-	+	-
Isolate 17	-	-	-	-	+	-
Isolate 18	-	-	+	+	+	+
Isolate 19	-	+	-	+	+	-
Isolate 20	-	-	-	-	+	-
Isolate 21	-	-	-	-	+	-
Isolate 22	-	-	+	-	+	-
Isolate 23	-	-	+	+	+	-
Isolate 24	-	-	+	-	+	+
Isolate 25	-	-	-	-	+	-
Isolate 26	+	-	-	-	+	-
Isolate 27	-	-	-	-	+	-
Isolate 28	-	-	-	-	+	-
Isolate 29	-	-	+	-	-	-
Isolate 30	+	+	-	+	+	+
Total	5 (16.67%)	4 (13.3%)	9 (30%)	10 (33.3%)	26 (86.67%)	8 (26.67%)

Table 3: Serum biochemical parameters in the negative control calves and *E. coli* Diarrheic calves

Parameters	Unit	Control calves	*E. coli* Diarrheic calves
Total protein	g/dl	67.34±1.53	82.53±2.00*
Albumin	g/dl	40.06±1.89	30.50±2.25*
Globulin	g/dl	26.74±2.00	52.03±2.44*
A/G	Ratio	1.50±0.42	0.59±0.33*
ALT	U/L	19.95±0.45	23.80±0.25*
AST	U/L	35.70±1.00	45.50±1.75*
ALP	U/L	16.94±0.30	18.50±2.25
BUN	mmol/L	12.83±1.25	27.55±2.00*
Creatinine	mmol/L	109.06±4.89	232.60±6.72*
Sodium	mEq/L	133.55±2.00	120.95±1.35*
Potassium	mEq/L	5.30±0.75	6.25±0.22*
Chloride	mEq/L	98.75±2.25	79.50±1.00*

Values (mean±SE) bearing asterisk in arrow differ significantly (P<0.01) as compared to control.

could end up with diarrhea. Infectious diarrhea in calves is commonly associated with DEC. Therefore, screening of the DEC virulence genes is roughly needed. Persistence of the problem of NCD might associated with the poor environmental hygiene, failure to clearly understand the disease ecology, and biased epidemiological data (Younis *et al*., 2009). Although medications, and herd management have been implemented to minimize the economic loss, the NCD economic impact is still significant (Cho and Yoon, 2014). Several approaches should be considered for future control such as vaccination of the pregnant dams, and fluid therapy (Younis *et al*., 2009).

Multiplex PCR of fecal samples of calves with diarrhea identified six virulence genes associated with DEC. Four calves were at 2 days-old and 5 calves were at 4-5 days-old. The susceptibility of NCD was reported to be from the first 3-4 days of life or more than 1 week-old (Nagy and Fekete, 2005). ETEC are considered the major pathogen-causing diarrhea in new born calves (Younis *et al*., 2009). In the current work, multiplex PCR was performed to investigate the virulence genes encoding for F5, F41 fimbriae and enterotoxins LT, Sta, Stx1 and Stx2.

Interestingly, in this study, we found that the F5 and F41-positive strains are not usually associated or encoded with enterotoxin genes (STa and LT) when analyzed by PCR. The enterotoxins prevalence was reported as follow: LT (30%), STa (33.3%). However, more than 86.67% of the positive isolates contained genes for Shiga toxin which would suggests the emergence of a new phenotype causing diarrhea in calves in Egypt. In our study, the prevalence of ETEC fimbrial gene was F5 (13.3%) and F41 (16.67%). The prevalence of F5 was previously reported 10.36% (Younis *et al*., 2009). Lower frequency of ETEC fimbrial virulence genes (5.3%) was recorded (Shams *et al*., 2012). The differences in the prevalence might be due to the difference in number of the samples, variations in region, managemental and hygienic measurements. The ETEC fimbrial genes identified by PCR were simple and rapid. It is previously reported that ETEC fimbrial antigen characterization by PCR does not require high purity of sample (Franck *et al*., 1998). Shiga toxin virulence genes were the most prevalent in all *E. coli* isolates. In this study, out of the thirty *E. coli* isolates 26 were positive for Stx1 and 8 was positive for Stx2 (26.67%). Stx1 virulence gene was the predominant virulence gene (86.67%). High frequency of *E. coli* isolates carrying the Stx1 gene was observed (Wani *et al*., 2007). Higher prevalence of stx2 gene has been reported (Irino *et al*., 2005). In other study, the stx1 gene was not detected, while the stx2 gene was found to be 93.1% (Karmali *et al*., 2003). It is known that the Stx2 toxins resulted in HUS in human more frequently than Stx1. Although Stx1 gene was the predominant gene in our study, still the zoonotic health hazard might be associated with the contaminated environment in this area.

Clinicobiochemical assays were performed through measuring the changes in the liver and kidney functions of the selected calves. Alteration in the proteinogram with unusual hyperproteinemia and hyperglobulinemia was found in association with diarrhea. It is expected that hyperproteinemia might be due to inadequate water intake or excessive water loss. The increase in the serum globulins level might be due to the increase in the immunoglobulins which indirectly reflect the serum total protein levels. Previous hypoalbuminemia is associated with diarrhea in human and animal (Chunlertrith *et al*., 1992). This is consistent with the results of the present study with a decrease in the serum albumin level. Consequently a decrease in the A/G ratio will be noticed. Liver enzymes such as ALT and AST have been used as an indicator of liver function. Our results showed an increase in both enzymes. An increase in both enzymes indicates liver function damage during the neonatal calf diarrhea (Grodzki *et al*., 1991). Creatinine and blood urea nitrogen (BUN) are considered important markers of renal function (Gowda *et al.*, 2010). An increase in the levels of creatinine and BUN was detected. This indicates an alteration in the kidney function. It is well known that the most important complications of diarrhea are the dehydration, acidosis and electrolyte abnormalities. In our study, the electrolyte imbalance was represented by hyponatremia with hypochloremia. In addition,

hyperkalemia was clearly noticed in our study. Similarly, neonatal calves with acute diarrhea develop hyperkalemia with acid-base and electrolyte disturbances (Weldon *et al*., 1992). Overall, the biochemical assays revels that there was an alteration in the liver and kidney function in association with the calf diarrhea.

Conclusions: This study characterizes the virulence gene factors associated with calf diarrhea including the zoonotic STEC. It indicates that DEC plays a significant role in NCD. Clinicobichemical assays indicated that the liver and kidney function of calves have been affected. Consequently the health condition and productivity will be reduced. Multiplex PCR was found to be quick, specific and convenient for large-scale screening and accurate for the identification of DEC virulence genes. The prevalence of ETEC fimbrial genes, LT and STa enterotoxin was found to be mixed with Shiga toxin genes. Although, this study reveals a potential zoonotic hazard of STEC infection, we still lack in-depth studies to determine other STEC reservoirs. Further studies are still needed to investigate several animal species and determine the distribution of the infection between different animals. It is necessary to raise the awareness and hazard of STEC pathogen to prevent the transmission of this zoonotic pathogen within the surrounding environment.

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Author's contribution: EH and HE designed the experiment; HE and AA collected the samples needed for the experiment; EH, HE, YT and AA carried out the experimental procedures; HE and YT isolated the bacteria; EH, YT and HE carried out the PCR; EH and AA carried out the biochemical analysis. EH prepared the manuscript.

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