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Clinical and sero-molecular characterization of *Escherichia coli* with an emphasis on hybrid strain in healthy and diarrheic neonatal calves in Egypt

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Abstract
The present study was carried out to characterize pathogenic *E. coli* in apparently healthy and diarrheic neonatal calves with special reference to the hybrid *E. coli* strains and evaluate their clinical and hematobiochemical consequences. One hundred and seventy calves (age 1-30 days) were divided into two groups: apparently healthy (n = 70) and diarrheic (n=100). Animals were subjected to thorough clinical, hematobiochemical and bacteriological examinations. Clinically, diarrheic calves showed various degree of diarrhea with the presence of cardinal signs of dehydration in moderate and severe cases. There was a significant increase ($p<0.05$) in the hemogram parameters with uremia and hyperkalemia in calves with severe diarrhea. The O-H serotyping of cultural and biochemically positive isolates identified 31 isolates belonging to 12 serotypes including O44:H18, O55:H7, O146:H21, O113:H4, O121:H7, O26:H11, O91:H21, O111:H2, O8, O127: H6, O86 and O128:H2. Molecular characterization of *E. coli* isolates on three toxin genes: heat-stable enterotoxin (*sta),* shiga toxin type 1 and 2 (*stx1* and *stx2)* revealed two well-known pathotypes (EPEC O44:H18, O55:H7, O146:H21, O113:H4, O121:H7 and EHEC O26:H11 O91:H21 O111:H2) with high frequency of enterohemorrhagic *E. coli* (EHEC). Molecular analysis also showed a number of *E. coli* isolates that carry *sta* and *stx1* or *sta* and *stx2* gene and belonged to O8, and O127:H6, O86 and O128:H2. These isolates were identified as hybrid *E. coli* strains (ETEC-STEC) and found in both apparently healthy and diarrheic calves. In conclusion, the present study identified high frequency of pathogenic *E. coli* in both apparently healthy and diarrheic calves. Serological and molecular analysis of *E. coli* isolates showed that high frequency of EHEC and presence of a new phenotype, STEC–ETEC hybrid, revealing their importance in the etiopathogenesis of diarrhea in calves and reinforcing the role of these animals as a reservoir of potentially pathogenic *E. coli* for humans.
Keywords: Calf, Diarrhea, *Escherichia coli*, Hybrid.

Introduction

Diarrhea in neonatal calves is the leading cause of calf's morbidity and mortality worldwide (Constable, 2004; Jor *et al*., 2010). It is a major cause of economic loss (Uhde *et al*., 2008; Bartels *et al*., 2010; Cho and Yoon, 2014) due to inefficient feed conversion, reduced live body weight, delay marketing, costly preventive and therapeutic program, deaths, loss of genetic material for herd improvement and decrease in the number of neonates for herd replacement and expansion (Uhde *et al*., 2008; Bartels *et al*., 2010; Cho and Yoon, 2014).

Diarrhea is a complex syndrome as it involves interaction between environmental, nutritional factors and infectious agents (Naylor, 2002). Several enteropathogens have been incriminated in calf diarrhea including *E. coli*, *Salmonella*, *Clostridium*, bovine coronavirus, bovine rotavirus group A, bovine viral diarrhea virus and *cryptosporidium* species (Kelling *et al*., 2002; Bhat *et al*., 2012; Singla *et al*., 2013; Cho, and Yoon, 2014).

Strain of *Escherichia coli* have long been recognized as a major enteropathogen in the first 28 days of calf's life. They mediate diarrhea in neonatal calves via their virulence factors: adherence and enterotoxins. Enterotoxigenic *E. coli* (ETEC) adheres to the enterocyte via fimbriae and mediates diarrhea by secreting heat-stable enterotoxin (*sta*) (Levine, 1987; Nataro and Kaper, 1998).

Unlike ETEC, Enteropathogenic *E. coli* (EPEC) strains do not produce toxins (DebRoy and Maddox, 2001) but produces an outer membrane protein, intimin, which mediates the intimate attachment of bacteria to the enterocyte, causing typical attaching and effacing (A/E) intestinal lesions. Shiga toxin-producing *E. coli* (STEC) may also be referred to as verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). They produce shiga-like toxins (*stx1* and *stx2*) and form A/E lesions.

EHEC are associated with cases of gastroenteritis in humans, which could progress to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). It is worthwhile to mention that horizontal gene transfer (HGT) is common among various strains of diarrheagenic *E. coli* strains. Specifically, STEC and

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other *E. coli* strains have the ability to acquire virulence genes via HGT leading to the emergence of new pathotypes of *E. coli* (Müller *et al*., 2007) which pose a potential public health threat.

Serological and molecular techniques are essential for detection and characterization of pathogenic *E. coli* and are based on O-H antigens and virulence markers, respectively (Nataro and Kaper, 1998; Ghanbarpour and Oswald, 2009; Bandyopadhyay *et al*., 2011; Nguyen *et al*., 2011; Shams *et al*., 2012). The clinical and hemato-biochemical alterations in *E. coli* diarrhea are complex in nature comprising serious imbalances of fluid, electrolyte and acid base status threatening the life of the calf (Singh *et al*., 2014). The evaluation of such alterations is important for determination of the proper medical intervention.

The present study aimed to characterize pathogenic *E. coli* with special reference to the hybrid strain in apparently healthy and diarrheic neonatal calves and evaluate their clinical and hematobiochemical consequences.

Materials and Methods

Animals and clinical examination

One hundred and seventy neonatal calves of both sex aged 1-30 days were included in the present study. Animals were belonged to commercial dairy farms at Assiut governorate- Egypt.

Neonatal calves were divided into two main groups: apparently healthy (n = 70) and diarrheic (n=100). Thorough clinical examination was conducted according to Radostits *et al*. (2007). The severity of diarrhea was assessed according to Naylor (2002). Special focus has been directed towards skin elasticity and mental state of the diarrheic neonatal calves through suckling affinity and calf demeanor. Animals were then subjected to laboratory and bacteriological examinations.

Fecal sampling

Fecal samples were collected directly from the rectum of apparently healthy and diarrheic calves using sterile swab tube, labelled, placed into a small Coleman cooler and transferred immediately to the laboratory for microbiological examination against *E. coli*.

Blood sampling and analysis

Blood samples were collected from all animals under investigation by means of jugular vein puncture. Whole blood samples were analyzed for complete blood picture (Total white and red cells counts, hemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration) using Vet hematology analyzer (Medonic CA620–Sweden). Serum samples were analyzed for blood serum levels of glucose, total protein, sodium, chloride, potassium, urea and creatinine spectrophotometriclly (Optizen 3220 UV, Mecasys Co. Ltd, Korea) using diagnostic test kits (Spectrum Diagnostics, Cairo, Egypt and Spinreact, Spain) and according to the recommendation of the expert panel of the International Federation of Clinical Chemistry (IFCC).

Cultural identification of E. coli

Fecal swabs from all examined animals were placed in 5 ml nutrient broth and incubated at 37°C for 24 h**.** A loopful of enrichment positive nutrient broth cultures was streaked on MacConkey agar medium (Oxoid, Thermo Scientific Uk) and incubated at 37°C for 24 h. Pink colony from MacConkey agar was streaked on Eosin methylene blue (EMB) agar medium (Oxoid, Thermo Scientific Uk) and incubated at 37°C for 24 h. Identification of suspected *E. coli* isolates was made based on Gram stain and their colonial characteristics on EMB agar (Oxoid, Thermo Scientific Uk) according to Cheesbrough (2004).

Biochemical identification of E. coli

Standard biochemical tests for detection of *E. coli* were performed on EMB positive isolates according to Kreig and Holt (1984) including indole production test, methyl - red test, Voges- Proskauer test, Simmon's citrate test, triple sugar iron (TSI) and urease test.

Serological characterization of E. coli isolates

The diagnostic antisera sets used for identification of *E. coli* were obtained from (Denka Seiken Co., Japan) and included two sets: Set 1(O- antisera) includes polyvalent antisera 1 (O1, O26, O86a, O111, O119, O127a and O128), polyvalent antisera 2 (O44, O55, O125, O126, O146 and O166), polyvalent antisera 3 (O18, O114, O142, O151, O157 and O158), polyvalent antisera 4 (O2, O6, O27, O78, O148, O159 and O168), polyvalent antisera 5 (O20, O25, O63, O153 and O167), polyvalent antisera 6 (O8, O15, O115 and O169), polyvalent antisera 7 (O28ac, O112ac, O124, O136 and O144) and polyvalent antisera 8 (O29, O143, O152 and O164). Set 2 (H- sera) includes H2, H4, H6, H7, H11, H18 and H21. Fifty random biochemically positive isolates were inoculated on TSA slants and subjected to serological examination according to Kok *et al*. (1996) using the aforementioned *E. coli* antisera sets.

Two separate drops of saline were put on a glass slide and a portion of the colony from the suspected culture was emulsified with the saline solution to give a smooth fairly dense suspension. To one suspension, control, one loopful of saline was added and mixed. To the other suspension one loopful of undiluted polyvalent "O" antiserum was added and tilted backward and forward for one minute. Agglutination was observed using indirect lighting over a dark background.

When a colony gave a strongly positive agglutination with one of the pools of polyvalent "O" antiserum, a further portion of it was inoculated onto a nutrient agar slant and incubated at 37°C for 24 hours to grow as a culture for testing with polyvalent "H" antiserum.

DNA extraction and amplification reaction

DNA extraction was performed according to Salvadori *et al*. (2003). Briefly, serologically positive isolates were grown on nutrient broth at 37ºC overnight. They were pelleted by centrifugation at 9184 *g* at 4ºC for 10 min (Hermle Labortechnik GmBH, Germany) and resuspended in 200 µL of sterile distilled water. Bacteria were lysed by boiling for 10 min; lysates were centrifuged at 9184 *g* at 4ºC for 10 min and 200 µL of the supernatant were utilized for polymerase chain reaction (PCR). PCR was performed according to Fagan *et al*. (1999) in a total volume of 25 µL composed of 12.5µL of EmeraldAmpGT PCR Master Mix (Takara Bio Europe, France), 1µM final concentration of both reverse and forward of *sta*, *stx1* and *stx2* primers (Alpha DNA, Canada) (Table 1), 6.5 µL molecular biology grade water (Takara Bio Europe, France) and 4 µL template of serotyped tested *E. coli*. Thermal cycler (Cyclogene Techno-US) was programmed as following: pre-denature at 95ºC for 5 min, denature at 95ºC for 1 min, annealing at 58ºC (*stx1* and *stx2*) and 57 ºC (*sta*) for 1 min, extension at 72ºC for 1 min, and final extension at 72ºC for 10 min. Total number of PCR cycles was 30 cycles for *stx1* and *stx2* and 35 cycles for *sta*. The reference strains for EHEC were *E. coli* O157:H7 Sakai (positive for *stx1*and *stx2)* and *E. coli* K12DH5α (a non-pathogenic negative control strain) that does not possess any virulence genes. PCR products were analyzed following standard protocol of agarose gel electrophoresis and visualized by gel documentation system.

Statistical analysis

Obtained data were statistically analyzed using Statistical software (Prism system). Data were presented as mean ± standard error of mean. The significance of variations between control and disease was determined using T-test.

Results

Clinical finding

Fifty nine percent of examined animals were affected by diarrhea of various degrees: mild, moderate and severe. Mild diarrheic neonatal calves (n = 45) passed thin scanty feces with frequent defecation. Calves in this group had a good demeanor, suckling affinity and barely detectable dehydration (skin folding test <1 sec). Moderate diarrheic neonatal calves (n = 40) passed thin to profuse watery feces. Animals in this group were commonly presented in sternal recumbency, weak suckling affinity with some degree of dehydration (skin folding test 6-8 sec). Severely diarrheic neonatal calves (n = 15) passed profuse watery feces and presented in lateral position with loss of consciousness. Mental state was exaggerated in few cases with convulsions. These animals showed severe degree of dehydration (skin folding test >8 sec), no suckling affinity, subnormal body temperature and weak pulse rate.

Isolation and bacteriological examination of E. coli

E. coli were successfully isolated on selective media, MaCconkey agar and EMB. *E. coli* gave pink colonies on MacConkey and black colonies with metallic sheen on EMB (Figure 1a,b). Based on results of selective media, 121 *E. coli* positive samples on EMB were obtained from 170 animals (80 diarrheic and 41 apparently healthy neonatal calves). The presumptive *E. coli* colonies were further subjected to Gram stain (Figure 1c) and biochemical tests.

Biochemical and serological identification of E. coli

Based on results of biochemical tests, out of 121 EMB positive fecal samples, 100 were biochemically identical as *E. coli* (70 diarrheic and 30 apparently healthy neonatal calves) (Table 2). Fifty random biochemically positive *E. coli* samples (35 from diarrheic and 15 from apparently healthy calves) were further subjected to serotyping. Serotyping of 50 random biochemically positive samples revealed that 31 isolates (21 from diarrheic and 10 from apparently healthy calves) were typed as pathogenic *E. coli* and belonged to 12 serotypes including O44:H18, O55:H7, O146:H21, O113:H4, O121:H7, O26:H11, O91:H21, O111:H2, O8, O127: H6, O86 and O128:H2. Data were presented in Table 3.

Molecular identification

A total of 31 positive serotyped *E. coli* were analyzed by PCR for detection of toxin genes, (*stx1*, *stx2*, and *sta*). Molecular characterization of serologically identified *E. coli* isolates revealed two well-known pathotypes of *E. coli*, (EPEC and EHEC that carry one or both *stx* genes) and one emerged (hybrid *E. coli*, STEC-ETEC, that carry one *stx* plus *sta* gene). Five isolates (2 apparently healthy and 3 diarrheic calves) were positive for *stx1* at 614 base pair (bp) belonged to O44:H18, O55:H7, and O146:H21. Three isolates from diseased animals were positive for *stx2* at 779 bp, belonged to O113:H4 and O121:H7. Fifteen isolates (4 healthy and 11 diseased) were positive for both *stx1* and *stx2* belonged to O26:H11, O91:H21 and O111:H2. Interestingly, 8 isolates were found to be STEC–ETEC hybrid strains. Three of the hybrid strain carried *sta* and *stx1* belonged to O8 and O127:H6 were isolated from both apparently healthy (n=1) and diarrheic calves (n=2). Five isolates of the hybrid strain carried *sta* and *stx2* belonged to O86 and O128:H2 were isolated from apparently healthy (n=3) and diarrheic calves (n=2). Data were presented in Tables 4 and 5 and Fig. 2 and 3.

Hematological and biochemical findings

Severely diarrheic neonatal calves showed significant increase ($p \leq 0.05, 0.01$) in the mean values of TWBCs, RBCs, Hb PCV, serum TP, urea, creatinine and K while significant decrease in serum Na in severe diarrhea. The mean value (±SEM) hematological and biochemical parameters in both apparently healthy and diarrheic neonatal calves were presented in Table 5 and 6.

Fig. 1. Cultural identification of *E. coli*. Inoculated MacConkey agar shows characteristic pink colonies (a), inoculated EMB agar shows characteristic green metallic sheen colonies (b) and Gram stain shows Gram negative coccobacilli (c).

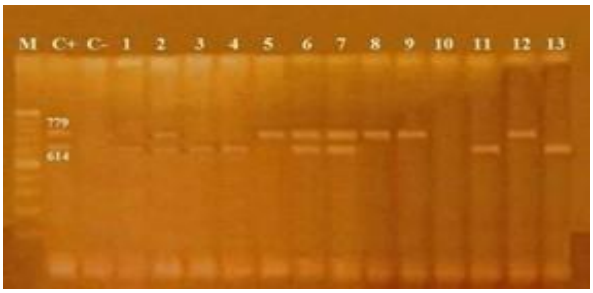

Fig. 2: Agarose gel electrophoresis of multiplex PCR of *stx1* (614bp) and *stx2* (779) genes for characterization of Enteropathogenic *E. coli*. 100 bp ladder as molecular size DNA marker (M), positive control (Sakai) for *stx1* and *stx2* genes (C+), negative control (*E. coli* K12DH5α) (C-), positive *E. coli* isolates (O8, O44, O55, O127 & O146) for *stx1* gene (lanes 1, 3, 4, 11 & 13, respectively), (O26, O91 & O111) for *stx1* and *stx2* genes (lanes 2, 6 & 7, respectively), (O86, O113, O121 & O128) for *stx2* gene (5, 8, 9 & 12, respectively), negative *E. coli* isolate (O124) for both *stx1* and *stx2* genes (Lane 10).

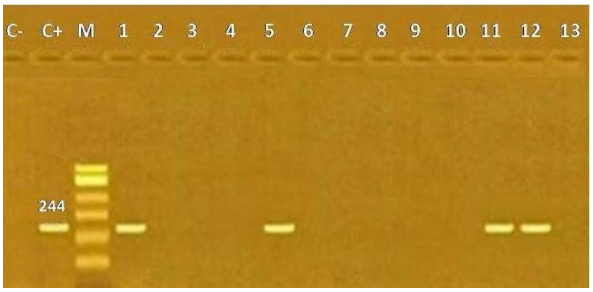

Fig.3: Agarose gel electrophoresis of uniplex PCR of *sta* (244bp) gene for characterization of enterotoxogenic *E. coli*. 100 bp ladder as molecular size DNA marker (M), control positive *E. coli* for *sta* gene (C+), control negative (C-), positive *E. coli* isolates (O8, O86, O127 and O128) for *sta* gene (lanes 1, 5, 11 and 12, respectively).

Discussion

Toxigenic *Escherichia coli* have been recognized as the most important cause of diarrhea in both human and animals in developing countries (Bouzari *et al*., 2007). Worldwide, *E. coli* diarrhea is still a persistent problem with significant economic and potential public health impact. Poor management and environmental factors enhance their interaction with their hosts causing dramatic illness and loss in neonatal animals. The present study was designed to screen for and characterize pathogenic *E. coli* with special reference to the hybrid strains in apparently healthy and diarrheic neonatal calves and evaluate their clinical and hematobiochemical changes. ETEC causes serious diarrhea in neonatal calves. Furthermore ETEC and shigatoxigenic *Escherichia coli* (STEC) have been incriminated in foodborne infections in humans. These two pathogroups are defined based on the pathogroup-associated virulence genes: *stx* encoding Shiga toxin for STEC and *sta* encoding heat-stable enterotoxin and/or *elt* encoding heat-labile for ETEC. The study looked into the toxin genes of serologically typed pathogenic *E. coli*. Along with the common pathogroups of *E. coli*, EPEC and EHEC, STEC/ETEC hybrid strains were detected. STEC and other diarrheogenic *E. coli* are able to acquire virulence genes via horizontal gene transfer from other pathogroups leading to the development of divergent pathogroups (Müller *et al*., 2007; Johura *et al*., 2016). Such divergent pathogroups are often assigned using different terminology such as "*hybrid*" "blended virulence profiles" and "virulence combination" (Bielaszewska *et al*., 2011; Mellmann *et al*., 2011; Johura *et al*., 2016). The coexisting STEC-and ETEC associated virulence genes in *E. coli* strains of human, animal, and environmental origins has been reported in in Germany, United States and Slovakia (Vu-Khac *et al*., 2007; Fratamico *et al*., 2008; Prager *et al*., 2011), some of which have been associated with human disease (Prager *et al*., 2011). Previous studies have identified STEC/ETEC hybrid strains from patients and animals in Finland (Martikainen *et al*., 2012) and from animal derived food in Burkina Faso (Nyholm *et al*., 2015a). Comparative genomics and characterization study of such strains has been conducted by Nyholm *et al*. (2015b) to determine their phylogenetic position among *E. coli* and to define the virulence genes they harbor.

Table 1. Sequence of primers used for PCR.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
stx1 (F)	5′ACACTGGATGATCTCAGTGG′3	614	Dhanashree and Mallya (2008)
stx1 (R)	5′CTGAATCCCCCTCCATTATG′3		
stx2 (F)	5′CCATGACAACGGACAGCAGTT′3	779	
stx2 (R)	5′CCTGTCAACTGAGCAGCACTTTG′3		
sta (F)	5′TCCGTGAAACAACATGACGG′3	244	Choi *et al*. (2001)
sta (R)	5′ATAACATCCAGCACAGGCAG′3		

stx1 = shiga toxin type 1; *stx2* = shiga toxin type 2, *sta* = Heat stable enterotoxin

Table 2. Eosin methylene blue agar medium and biochemical reactions of fecal isolates from diarrheic and apparently healthy neonatal calves.

Calves	Total samples	Culturing (n = 121 positive)	Biochemical tests (n = 100 positive)						
			No.	Test					
		EMB		IT	MR	VP	SC	UT	TSI
Diarrheic calves	100	80	70	+ve	+ve	-ve	-ve	-ve	acid butt slant with gas production
Apparently healthy calves	70	41	30	+ve	+ve	-ve	-ve	-ve	

(EMB): Eosin methylene blue; (+ve): positive; (-ve): negative; (acid): yellow color; (IT): indole production test; (MR): methyl red test; (VP): Voges-Proskauer test; (SC): Simmon's citrate test; (UT): urease test; (TSI): triple sugar iron.

Table 3. Typing of *E. coli* isolated from apparently healthy and diarrheic neonatal calves.

E. coli serotypes	No. of healthy calves	No. of diseased calves	Total No. of calves	Phenotype (Genotype)
O127:H6	1	1	2	STEC-ETEC *(sta/stx1)*
O8		1	1	STEC-ETEC *(sta/stx1)*
O86	1	-	1	STEC-ETEC *(sta/stx2)*
O128:H2	2	2	4	STEC-ETEC *(sta/stx2)*
O44:H18	-	1	1	EPEC (*stx1*)
O55:H7	1	2	3	EPEC (*stx1*)
O146:H21	1	-	1	EPEC (*stx1*)
O113:H4	-	2	2	EPEC (*stx2*)
O121:H7	-	1	1	EPEC (*stx2*)
O26:H11	1	4	5	EHEC (*stx1 & 2*)
O91:H21	1	2	3	EHEC (*stx1 & 2*)
O111:H2	2	5	7	EHEC (*stx1 & 2*)
Total No. of calves	10	21	31	

Table 4. Occurrence of virulence genes of shiga toxins (*stx1 and stx2*) and heat-stable toxin (*sta*) of *E. coli* strains isolated from clinically apparently healthy and diarrheic neonatal calves

Virulence factors	Serotype	Total isolates	Percentage (%)
stx1	O44:H18, O55:H7 and O146:H21	5	16.13
stx2	O113:H4 and O121:H7	3	9.68
stx1 and stx2	O26:H11, O91:H21 and O111:H2	15	48.39
sta and stx1	O8, O127:H6	3	9.68
sta and stx2	O86,O128:H2	5	16.13

Table 5. Mean value (±SE) of hematological parameters in apparently healthy and diarrheic neonatal calves.

Parameters	Healthy calves (n=10)	Mild diarrhea (n=45)	Moderate diarrhea (n=40)	Severe diarrhea (n=15)
TWBCs (x 10^9 /l)	7.97±0.5	9.33±0.22	11.28±0.89*	15.88±1.38**
RBCs (x 10^{12}/l)	7.80±0.65	10.03±0.22*	10.19±0.19**	12.64±2.2**
Hb (g/dl)	8.70±0.48	9.93±0.26	10.73±0.48	12.86±1.05*
PCV (%)	30.99±1.63	36.58±1.02	39.31±0.77	47.24±3.50*
MCV (fl)	31.70±0.60	34.39±0.7	35.01±3.01	36.38±1.17
MCH (pg)	14.10±0.70	13.46±0.4	13.02±0.52	12.32±0.30
MCHC (g/dl)	37.21±0.83	36.71±1.09	35.86±0.9	34.3±1.70**

(*): means significant changes from control at p≤0.05; (**): means significant changes from control at p≤0.001.

Table 6. Mean value (±SE) of blood Na, Cl, K^+, glucose, total protein, urea and creatinine concentration in apparently healthy and diarrheic neonatal calves.

Parameters	Healthy calves (n=10)	Mild diarrhea (n=45)	Moderate diarrhea (n=40)	Severe diarrhea (n=15)
Sodium (mmol/l)	145.30±1.57	142.70±0.88	136.70±1.89	133.10±4.09**
Chloride (mmol/l)	109.00±3.43	103.5±2.03	94.60±2.42	85.17±6.71*
Potassium (mmol/l)	4.52±0.37	4.36±0.18	4.50±0.18	7.08±0.54**
Glucose (mmol/l)	4.26±0.18	4.19±0.11	3.60±0.12	2.80±0.50*
Total protein (g/dl)	6.24±0.538	7.16±0.37	7.57±0.49	10.19±1.1*
Serum urea (mmol/l)	8.74±0.45	9.858±0.4	12.4±0.32	14.29±0.73**
Creatinine (mg/dl)	0.99±0.09	1.528±0.09	1.596±0.04	1.87±0.14*

(*): means significant changes from control at p≤0.05; (**): means significant changes from control at p≤0.001.

In ETEC, the genes that encode both heat-liable (LT) and heat-stable (ST) enterotoxins are found on plasmids, and studies have demonstrated that the enterotoxic activity of ETEC was transferred together with a self-transmissible plasmid. Other studies have demonstrated the experimental addition or subtraction of many features in a base model *E. coli* strain, enabling these modified strains to cause disease in their host (Kaper *et al*., 2004). The current study demonstrated the presence of STEC–ETEC hybrid among *E. coli* isolates and strongly suggested that this strain might contribute in the diarrhea in neonatal calves and pose a potential public health threat in Egypt.

Diseased calves showed various degrees of diarrhea based on their mental and hydration status (Naylor, 2002; Radostits *et al*., 2007). Mild diarrheic calves were able to stand without assistance and had good suckling affinity while moderately diarrheic ones were admitted in sternal recumbency, lethargic and weak suckling affinity. Severely diarrheic calves were presented in lateral recumbency with loss of consciousness and no suckling affinity.

The hydration status of diarrheic calves was assessed clinically according to skin elasticity test and appearance of eyeball. Mild diarrheic calves exhibited mild skin tenting and no significant changes in the eyeball while moderately diarrheic ones showed obvious skin tenting and mild to moderate sunken eyeball. On the other hand severely diarrheic neonatal calves expressed severe dehydration as indicated by severe decrease in skin elasticity and moderate to severe sunken eyeball. Heart rate was variable in diarrheic neonatal calves. Calves with severe diarrhea showed tachycardia (>150/min), week beats and slow jugular vein filling or bradycardia (< 90/min.) with cardiac arrhythmia. Such serious changes could be attributed to hypovlemia, hyperkalemia and metabolic acidosis.

Hematological and biochemical parameters showed significant increase in the mean values of TWBCs, RBCs, Hb and PCV ($p \leq 0.05$) in severe diarrhea. Such changes in the hemogram are usually associated with dehydration and *E. coli* infection. Relative hyperprotenamia was a constant finding in neonatal calf diarrhea and there was a direct relationship between the severity of dehydration and the concentration of total plasma protein. This finding could be attributed to body fluids losses. No change in the blood glucose level was

observed in mild cases of neonatal calf diarrhea. This could be attributed to the ability of calves to maintain their suckling affinity. On the other hand, there was significant hypoglycemia in moderate and severe cases of calf diarrhea. This could be attributed to weak or absence of normal suckling affinity, altered intestinal epithelial transport and developing endotoxic-septic shock (Naylor, 2002).

Uremia was a constant clinical finding especially in the late stage of neonatal calf diarrhea which is characterized by marked increase in serum urea and exerts its fatal effect in the pathogenesis of diarrhea. All succumbed diarrheic neonatal calves exhibited a high level in serum urea and creatinine. Serum urea and creatinine levels were significantly elevated in moderate and severe cases (Singh *et al*., 2014).

Hyponatremia was observed in calf diarrhea but the degree of hyponatremia was directly parallel with the severity of diarrhea. This could be attributed primarily to significant fecal losses of sodium ions in diarrheic neonatal calves.

Hyperkalemia was a characteristic finding and commonly noticed in severely diarrheic neonatal calves. It could be contributed to increased renal tubular reabsorption of potassium in response to acidosis in case of healthy renal tissue function but in case of compromised renal function (as indicated by increasing in the values of serum urea and creatinine), shift in potassium ions from intracellular fluid to extracellular fluid (K^+ - H^+ exchange) takes place in response to acidosis and hyperkalemia will developed (Naylor, 2002; Radostits *et al*., 2007; Singh *et al*., 2014).

Conclusion

The present study showed a high frequency of pathogenic *E. coli* in both apparently healthy and diarrheic calves. EHEC were the highest distributed *E. coli* strain among diarrheic and apparently healthy calves revealing its important participation in the etiopathogenesis of diarrhea in calves and reinforcing the role of these animals as a reservoir of potentially pathogenic *E. coli* for humans.

Additionally, the present study demonstrates, to the best of our knowledge, the emergence of a new phenotype, STEC–ETEC hybrid, that causes diarrhea in calves and pose a potential public health threat in Egypt. Further investigation on a large sample size is proposed to study genetic relatedness/phylogeny of this strain.

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Conflict of interest

The authors declare that there is no conflict of interest.

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