Studies on the *Escherichia coli* virulence factors coding heat stable toxin, Verotoxin and gene for attaching and effacing associated with diarrhea in calves using PCR

By

Abdelaziz A. Mosaad[1], El-Sayed M. Ibrahim[2], Mohaned Ali Akeila[3] and Sabry M. Abdelrahem[1]

Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Minufiya University
Department of Animal Medicine, Faculty of Veterinary Medicine (Moshtohor), Benha University
Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University

Summery

In this study 127 out of 262 fecal swabs collected from newly born diarrheic Frisian calves harboured *Escherichia coli* with an incidence of 48.47% non O157 and 10.30% were O157. Serological testing showed that the most predominant serovars were O1, O8, O25, O26, O55, O86, O125, O126, O146, O151 and 2 isolates were untypable as well as O157:H7 negative.

All bacterial isolates showed growth after various treatments tested for biochemical and hemolytic activity and showed no changes. Studying the virulence factors revealed that 80% of *E. coli* isolates non O157 and 33.3% of isolates of O157 were hemolytic.

Using PCR to detect the presence of Sta gene in four groups (first group contains 12 serotyped isolates, second group contains 5 non serotyped isolates but showing typical biochemical activity like *E. coli*, third group contains 5 non serotyped isolates and showing different biochemical activity from *E. coli* and the fourth group constitutes all the previous isolates but being cultured directly from feces upon broth for 7 hrs, as well as the serotyped O157 isolates. The results revealed that 9 (75%) out of 12 serotyped isolates (in the 1st group), 4 (80%) out of 5 isolates (in the 2nd group), 3 (60%) out of 5 (in the 3rd group) showed similar results to the 4th group after 7 hrs growth in broth from direct fecal samples.

The infant mouse assay was used to confirm the production of (Sta) toxin in the PCR positive isolates. We found that all the isolates showed positive result with PCR were questionably positive with infant mouse assay.

Using Multiplex PCR to detect the presence of SLT2 and eaeA genes in the O157 isolates revealed negative results which confirmed by Vero cell assay.

From the present study we concluded that *Escherichia coli* are persistently associated with newly born diarrheic calves, especially the enterotoxigenic type (producing heat stable toxin) which can be detected directly using PCR from the fecal samples.

Introduction

Diarrhea of neonatal calves causes major economic losses directly through mortality and the need for treatment, and indirectly from poor growth after clinical disease. It has

been estimated that neonatal calf diarrhea accounts for approximately 75% of the mortality of dairy calves under 3-weeks of age (Radostits *et al.,* 1994)

Escherichia coli is an important pathogen in bovine neonates, capable of causing intestinal and extra-intestinal infections in which it can produce Shiga-like toxins (Stx), heat labile (LT) or heat-stable (ST) enterotoxins (Gay and Besser 1994).

Infection by enterotoxin producing *E. coli* (ETEC) results in diarrheic secretions due to the action of one or more enterotoxins and can lead to dehydration and death (Butler and Clarke 1994).

The Shiga-toxin produced by *E. coli* strains (STEC) is similar to the Shiga-toxin produced by Shigella dysenteriae type 1. E. coli producing Stx1 and/or 2 causes the hemorrhagic colitis (HC) and the hemolytic-uremic syndrome (HUS) in humans (Nataro and Kaper 1998). Another virulence associated factor expressed by STEC (protein called intimin) is responsible for the intimate attachment of STEC to intestinal epithelial cells causing effacement lesions of the intestinal mucosa.

PCR is specific and sensitive tool could be used for rapid diagnosis of the vast array of Escherichia coli serotypes causing problems in animals. Hence, it will be useful in the epidemiological studies as well as treatment and prevention of such infections and more useful for identification of *E. coli* strains which cause diarrhea in calves. Multiplex PCR was developed to identify enterotoxigenic, attaching and effacing, and Shiga toxin-producing E. coli strains by amplifying genes encoding K99 and F41 fimbriae, heat-stable enterotoxin, intimin, and Shiga toxins 1 and 2 (Frank *et al.,* 1998).

Therefore the present study was aimed to:

1. Investigation of the prevalence, isolation, and identification of *E. coli*, especially O157 in diarrheic calves.
2. Investigation of *E. coli* virulence factors such as (heat stable enterotoxin, Verotoxin and eaeA gene for attaching and effacing by PCR.
3. Studying the effectiveness of some acidifiers such as organic acid onto *E. coli*.

Materials and Methods

Materials:

1. Fecal samples: A total of 262 fecal samples were collected from Frisian calves aged between 1 and 30 days old and had profuse watery diarrhea. Samples were collected early before the application of the antibiotic course.
2. Media and reagents: Media and reagents were prepared according to Cruickshank et al., (1975) and Koneman *et al.,* (1997).
3. Biological reagents: Antisera were used for the serological identification of *E. coli* O157 and other non O157 according to (Sakazaki *et al.,* 1992). The antisera were used for the O antigen for all isolates and for the H antigen for O157. The antisera were purchased from (Denker Seiken Co., LTD.Tokyo, Japan).

Diagnostic O polyvalent and monovalent antisera include:

Polyvalent sera	Monovalent sera						
Polyvalent 1	O_1	O_{26}	O_{86a}	O_{111}	O_{119}	O_{127}	O_{128}
Polyvalent 2	O_{44}	O_{55}	O_{125}	O_{126}	O_{146}	O_{166}	------
Polyvalent 3	O_{18}	O_{114}	O_{142}	O_{151}	O_{157}	O_{158}	------
Polyvalent 4	O_6	O_{27}	O_{78}	O_{148}	O_{19}	O_{168}	------
Polyvalent 5	O_{20}	O_{25}	O_{63}	O_{153}	O_{176}	------	------
Polyvalent 6	O_8	O_{15}	O_{115}	O_{169}	-------	-------	------
Polyvalent 7	O_{28}	O_{112}	O_{124}	O_{136}	O_{144}	-------	------
Polyvalent 8	O_{29}	O_{143}	O_{152}	O_{164}	------	-------	------

Diagnostic antisera for H antigen only used to detect the H7 antigen of O157 and include:

H_2	H_4	H_5	H_6	H_7	H_9	H_{10}	H_{11}
H_{12}	H_{16}	H_{18}	H_{19}	H_{20}	H_{21}	H_{27}	H_{28}
H_{34}	H_{40}	H_{41}	H_{42}	H_{45}	H_{51}	-------	------

4. Reagents and chemicals used for polymerase chain reaction (PCR):
   1) Two primers were selected to amplify *E. coli* heat stable toxin (STa) gene (Ojeniy *et al.,* 1994).
      a. Upstream primer (STa1) with a sequence of
         5'-TCCGTGAAACAACATGACGG-3'
      b. Downstream primer (Sta 2) with sequence of
         5'-ATAACATCCAGCACAGGCAG-3'

      These primers were synthesized and supplied by (Gentech Co.).
   2) The primers were selected to amplify *E. coli* shiga toxin 2 (STx2) and attaching and effacing (eae) genes in enterohemorrhagic *E. coli* (EHEC) isolated from cattle feces (Paton *et al.,* 1998).
      a. Upstream primer with sequence of:
         5'-GGCACTGTGAAACTGCTCC-3'
      b. Down stream primer with sequence of:
         5'-TCGCCAGTTATCTGACATTCTG-3'
   3) Two primers were selected to amplify the attaching and effacing gene (eae) (Paton *et al.,* 1998).
      a. Upstream primer with sequence of:
         5'-GACCCGGCACAAGCATAAGC-3'
      b. Down stream primer with sequence of:
         5'-CCACCTGCAGCAACAAGAGG-3'
5. Laboratory animals: Two-four day-old suckling mice were used to detect the enterotoxigenic activity of *E. coli*. They were separated from their mothers immediately before use.
6. Media and reagents used for Vero cell assay:
   1) Minimum essential medium (MEM): Eagle's MEM with EARL'S salt solution was obtained from (Gibco Limited, UK). This medium was used for the cultivation of Vero cells.

2) Fetal calf serum (Gibco Limited, UK): Fetal calf serum was first inactivated by heating for 30 minutes at 56°C then preserved at -20°C. It was added to MEM medium to get a final concentration of 5%.
3) Trypsin solution (0.25%w/v): It was prepared by dissolving 2.5 gm of Trypsin (Difco) in one liter of phosphate buffer saline.
4) Antibiotic solutions: Benzyl penicillin (2000 unites/ml) and streptomycin (2000µg/ml) were stored in 5 ml aliquots at -20°C. It was required for 500 ml of cell culture medium and added immediately before use. Polymyxine B (GIBCO, UK) 0.1 mg/ml solution of polymyxine B was dissolved in phosphate buffer saline. It was used for releasing cell bound Verotoxin from *E. coli*.
5) Tryptic soy broth (Biolife): It was used for the growth of *E. coli* isolates for Verotoxin production.
6) Vero cells (African Green Monkey Kidney Cells): These cells were kindly supplied by (Veterinary serum and vaccine research Institute, Abbassia, Cairo). It was cultured and maintained as recommended by Devenish *et al.,* (1998).

Methods:

1. Collection of samples: 262 rectal swabs and fecal samples were taken from diarrheic calves by sterile cotton swabs. The collected samples were transferred to the laboratory of Bacteriology, Immunology and Mycology in the Faculty of Veterinary Medicine, Sadat city, Minufiya University in ice bags.
2. The samples were subjected to bacteriological examination and complete identification according to Kudra *et al.,* (1997) and Quinin *et al.,* (2002).
3. Serotyping of *E. coli* was applied according to Sakazaki *et al.,* (1992).
4. Utilization of Polymerase chain reaction for detection of the genes coding the heat stable enterotoxin (Sta), shiga like toxin 2 (SLt2) and attaching and effacing (eaeA) genes of *E. coli* isolates.
5. Obtaining bacterial template DNA: DNA template was prepared from *E. coli* cells isolates that were grown in LB broth for an overnight at 37 °C. 100µl of broth culture were centrifuged and the pellet was resuspended in distilled water. The genomic DNA was extracted by boiling of the suspension for 10 minutes and supernatant was used as a template for PCR. 10µl of the DNA solution were used as template for PCR for each reaction.
6. Preparation of Oligonucleotide primers of Sta and SLT-II (Sambrook et al., 1989).
7. Detection of PCR products was performed according to Sambrook *et al.,* (1989).
8. Pathogenicity of *E. coli* supernatant culture media in Infant Mouse Assay (Phenotypic method) was carried out according to Dean *et al.,* (1972).
9. Detection of *E. coli* producing shiga like toxin using Vero cell assay (phenotypic method) was carried according to Han and Linton (2004).

Results

Table 1: The incidence of *E. coli* isolated from diarrheic calves in relation to age

Calf age (days)	No of examined animals	No of positive *E. coli* strains			
		Non O157		O157	
		Positive samples	%	Positive samples	%
1-7	68	62	91.17	4	5.8
7-15	28	14	50	6	21.4
15-21	56	16	28.57	7	12.5
21-30	110	8	7.27	10	9
Total	**262**	**100**	**38.16**	**27**	**10.3**

Table 2: The total numbers of serotyped *E. coli* isolates

No of *E. coli* isolates	No of serotyped *E. coli* isolates			
	Non O157		O157	
	Positive samples	%	Positive samples	%
127	20	15.7	9	0.7

Table 3: Serotyping of 20 randomly selected *E. coli* using the available antisera

E. coli serotype	Number of isolates
O1	5
O8	1
O25	1
O26	1
O55	1
O86	1
O125	3
O126	1
O146	1
O151	3
Untypable	2
Total	20

Table 4: Polymerase chain reaction (PCR) for the detection of heat stable toxin (Sta gene) from the isolated *E. coli* and the original samples

Type of samples	Samples identified serologically	Samples show typical cultural and biochemical picture but not identified serologically	Samples show negative cultural and biochemical picture and not identified serologically	Samples taken from the previous groups but PCR is done directly on the both culture of the fecal samples
Type of media upon which the isolates were grown before the technique	Nutrient agar slope	Nutrient agar slope	Nutrient agar slope	The filtrated fecal samples were cultured on nutrient broth
Time of culture	24 hrs	24 hrs	24 hrs	7 hrs
Number of tested samples	12 samples (10 serologically detected + 2 untyped) and 9 O157	5 samples (1-5)	5 samples (6-10)	All the previous samples cultured on the broth and tested with PCR
Results	9 out of 12 were positive	4 out of 5 were positive	3 out of 5 were positive	Typical results as the original samples
Results for O157 with shiga like toxin 2, attaching and effacing gene	-ve	-ve	-ve	-ve

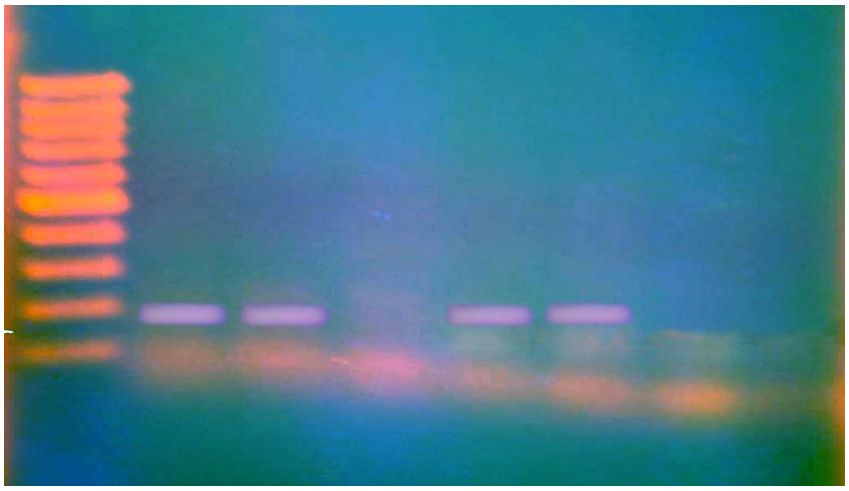

Photo 1-a: PCR photo (agarose gel) showing positive isolates to Sta gene (154 base pair) in the first group showing 4 positive isolates

Photo 1-a: PCR photo (agarose gel) showing positive isolates to Sta gene (144 base pair) in the first group showing 5 isolates positive

Photo 2: PCR photo (agarose gel) showing 4 positive isolates of the second group to Sta (at 154 base pairs)

Photo 3: PCR photo (agarose gel) showing 3 positive isolates of the third group to Sta (at 154 base pairs)

Table 5: Polymerase chain reaction (PCR) results for the serologically identified isolates with the heat stable (Sta), shiga like toxin 2 (SLT2) and attaching and effacing gene (eaeA)

Non O157			O157		
Tested samples with Sta	Positive samples	%	Tested samples with Sta, SLT2, and eaeA	Positive samples	%
12	9	75	9	0	0

Table 6: Correlation between infant mouse assay and PCR

Sample number	Infant mouse assay	PCR
O1	(0.075)+ve	+ve
O126	(0.071)+ve	+ve
O125	(0.074)+ve	+ve
O25	(0.07)+ve	+ve
O86	(0.07)+ve	+ve
O8	(0.08)+ve	+ve
O26	(0.07)+ve	+ve
O151	(0.07)+ve	+ve
O146	(0.060)-ve	-ve
O55	(0.065)-ve	-ve
Untypable 1	(0.065)-ve	-ve
Untypable 2	(0.074)+ve	+ve
1	(0.075)+ve	+ve
2	0.072)+ve	+ve
3	(0.07)+ve	+ve
4	(0.073)+ve	+ve
5	(0.062)-ve	-ve
6	(0.07)+ve	+ve
7	(0.073)+ve	+ve
8	(0.071)+ve	+ve
9	(0.06)-ve	-ve
10	(0.064)-ve	-ve

Table 7: Correlation between hemolytic activity of *E. coli* isolates (enterotoxigenic and O157) and PCR

E. coli serovars	No of tested isolates	Haemolysis	PCR
O:1	5	+ve	+ve for Sta
O:125	3	+ve	+ve for Sta
O:25	1	+ve	+ve for Sta
O:8	1	+ve	+ve for Sta
O:26	1	+ve	+ve for Sta
O:151	3	+ve	+ve for Sta
O:55	1	+ve	-ve for Sta
O:157	9	3	-ve for Sta
Untypable 2	1	+ve	+ve for Sta

Discussion

Escherichia coli are an important pathogen in bovine neonates. They are capable of causing intestinal and extra-intestinal infections (Gay and Besser 1994).

PCR is a genotypic technique which can be used extensively in the diagnosis of bacterial samples with higher specificity and sensitivity which can reach a level of 99%. This detection technique could be suitable for use in a clinical laboratory (Begum *et al.,* 1993).

Results obtained from Table 1 show that 127 out of 262 fecal swabs collected from newborn Frisian calves showing clinical signs of diarrhea were found to harbor *Escherichia coli* with an incidence of 48.47% which were closely similar to those obtained by Fecteau *et al.,* (1997) (51%). Also the results that obtained from Table 2 showed that out of 127 samples 100 (38.16%) were non O 157 *E. coli*. These results are closely similar to that obtained by Kamel (2001) (37.7%). Out of 127 samples 27 (10.30%) were O 157 *E. coli*. These results are nearly similar to that reported by Cobbold and Desmarchelier (2001) (9.4 %).

Serotyping of *E. coli* plays a major role in the history of pathogen and has a vital means by which pathogenic strains are differentiated (Natario and Kaper 1998).

Serotyping of *E. coli* in the present study was confined to the O antigen only and that fulfilled with selecting 20 random samples with typical *E. coli* biochemical pattern. The results showed that the examined samples are serologically belong to the following serovars O1, O8, O25, O26, O55, O86, O125, O126, O146, O151 and two isolates were untypable. Although not completely similar to those isolated from cases of diarrhea reported in literature but there are partial similarity in the type of the strains isolated from such cases (Farid *et al.,* 1976). This author isolated *E. coli*

serogroups from fecal samples of buffalo and Frisian calves in farms suffering from problems of enteritis in Egypt. He found that the most predominant serogroups were O8, O26, O86, O125 and O126. Ahmed (1977) isolated *E. coli* serogroups of O26, O55, O86, O125, and O126 from diarrheic buffalo calves. Awad *et al.*, (1979) isolated and serologically grouped *E. coli* into serogroups O26, O55, O86, O125, O126 and O128 from the whole isolated serogroups. Elsayed (1987) isolated the following serogroups O55, O86, and O125 from healthy cattle and O26, O125, O86 from diarrheic calves. Franck *et al.*, (1998) isolated O8, STEC O157:H7, O157 NM, O5, and O8 from calves and pigs suffering from diarrhea. Hornitzky *et al.*, (2005) isolated Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) from 191 fecal samples from cattle with gastrointestinal infections. From these samples, STEC serotypes were identified including O5, O8, O26, and O157.

The virulence of *E. coli* is multifactorial and got many components which characterize the virulent strain from the non virulent one. The most important virulence factor in *E. coli* is the ability to produce toxins either heat labile (LT) and heat stable (ST) enterotoxins or shiga like toxins (Stx)(Gay and Besser 1994).

E. coli strains that produce enterotoxins (enterotoxigenic) *E. coli* [ETEC] are among the most important causes of diarrhea in neonatal swine and cattle. It was reported that there located two types of heat stable toxin STa, STb (Khac *et al.*, 2006) and it was also demonstrated that calf ETEC produces heat-stable enterotoxin a (STa), which causes hyper secretion into the gut lumen (Butler and Clarke 1994). STa stimulates GC activity, leading to increase the intracellular cGMP levels. This activity leads ultimately to stimulation of chloride secretion and/or inhibition of sodium chloride absorption resulting in net intestinal fluid secretion (Sears *et al.*, 1996). Our study had been directed toward the detection of presence of heat stable toxin either with the genotypic methods (PCR) or the phenotypic method by the utilization of suckling mouse assay.

Results shown in Table 5 showed that 9 out of 12 *E. coli* isolates were (either with 7 hr or 24 hr culture growth) positive with PCR including O1, O126, O125, O25, O86, O8, O26 ,O151 and untypable 2 representing 75% of the examined samples. These results were confirmed with suckling mouse assay. This result is closely nearer to that obtained by Ameen (2006) who found that the number of *E. coli* isolates producing Sta was 55 out of 62 (88.7%).

Shiga toxin-producing *E. coli* (STEC) are known for certain life-threatening infections such as hemorrhagic colitis (HC), bloody diarrhea, hemolytic uremic syndrome (HUS) and thrombocytopenic purpura in humans. STEC occur in the feces of domestic animals and are most frequently found in ruminants (Montenegro *et al.*, 1990). Although several of them can cause diarrhea in calves (Mohammad *et al.*, 1985), but most are harboured by asymptomatic, healthy animals like cattle, sheep and goats (Zschock *et al.*, 2000).

STEC strains elaborate two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or Verotoxin (VT1 and VT2). STEC that have been associated with HC and HUS in humans are called enterohemorrhagic *E. coli* (EHEC) and are capable of producing shiga toxins. Shiga toxin-producing *E. coli* are found in the intestine of healthy cattle, which are major source of infection for humans (Wang *et al.,* 2002). VTEC was one of *E. coli* groups which characterized by the production of potent cytotoxins that inhibit protein synthesis within eukaryotic cells. These toxins are either termed verocytotoxin (VT), because of their activity on Vero cells, or Shiga toxins (Stx), because of their similarity with the toxin produced by *Shigella dysenteriae* (Wieler and Bauerfeind, 2003).

Multiplex polymerase chain reaction (PCR) was used to detect the presence of genes encoding Shiga toxin 1 and 2 (Stx1 and Stx2), H7 flagella (flicC), enterohemolysin (hly) and intimin (eaeA) in *E. coli* isolates. Shiga toxin-producing isolates were tested for production of Shiga toxins (Stx1 and Stx2 and enterohemolysin of the *E. coli* O157:H7/H- strains (Murinda *et al.,* 2004). Therefore, our study aimed to detect the presence of shiga toxin 2 and eaeA gene as virulence related genes with PCR and detection of the presence of shiga toxin 2 gene with the Vero cell assay. The results were showed in Table 5. All the isolated O157 (9 isolates) revealed negative results with PCR for the two genes. The results were confirmed with negative results with Vero cell assay. Our results here seem to be in accordance with the results obtained by Rogerie *et al.,* (2001) who isolated 105 *E. coli* isolates and 8 of them were negative for stx1, stx2, and eae.

Hemolysin is a component that had been confirmed to be incorporated in the virulence of *E. coli* (Emery *et al.,* 1992) and it was also confirmed that the introduction of the gene of hemolysin will increase virulence (Emo¨dy *et al.,* 1980). Hemolysin is cytotoxic for leukocytes and, at sub-lethal levels, inhibits phagoctosis and chemotaxis (Cavalieri and Synder 1982). Hemolysin shows vitality for *E. coli* in the mechanism of acquiring and assimilating iron from its host (Cavalieri *et al.,* (1984). *Escherichia coli* hemolysin (HylA) belongs to a family of exoproteins, broadly disseminated among gram-negative pathogens, which has been named RTX (for repeats of glycine in toxins) (Welch 1995). Hemolysin produced by *E. coli* strains are designated as α-Hemolysin and β- hemolysin. The α-hemolysin is excreted as extracellular protein where as β-hemolysin is cell associated. They are cytolytically active protein that causes lysis of erythrocytes (Emery *et al.,* 1992).

The results obtained in Table 7 revealed that 16 (80%) of non O157 *E. coli* isolates showed positive results with hemolysin and most of them were also positive with heat stable enterotoxin. These results indicated that hemolysin is associated with virulent strains and the higher the percentage of virulent strain (enterotoxigenic), the higher the percentage of hemolytic strains and this shows accordance with the results obtained by Abd EL-Wahed (2005) who noted that 66.07% of isolated *E. coli* were hemolytic and most of them were enterotoxigenic. Ameen (2006) reported that 69.35% of the isolated strains were beta hemolytic. El-Ashker (2006) found that 95% of the isolated *E. coli* from diseased calves showed α-hemolysin.

Also the results in Table 7 revealed that 3 (33.3%) out of (9) O157 *E. coli* were positive for hemolysin. This result is closely related to that had been obtained by (Duby *et al.*, 2000) who found that 30.4% of *E. coli* isolated from diarrheic goats were hemolytic. He reported that the virulence of hemolytic strains is multifactorial and this depends on their association with other virulence factors.

References

1. Abd-El-Wahed, M. A. (2005): Virulence determinant of enterotoxigenic *E. coli* and it's relation to adhesive antigen K99 associated with diarrhea in newly born calves. M.V.Sc. Thesis, Zagazig Univ., Fact. Vet. Med.
1. Ahmed, S. A. (1977): Studies on colibacillosis among newly-born buffalo calves in Egypt. Ph.D. Thesis, Cairo Univ. Fact. Vet. Med.
2. Ameen, N.B. (2006): A contribution on virulence markers of *E. coli* isolated from newly born buffalo calves. M.V. Sc. Thesis, Alex. Univ., Fact. Vet. Med.
3. Awad, F. I., Zien El Abidin, Y., EL-Mulla, A., and Ahmed, S. A. (1979): Studies on the identification and sensitivity of *E. coli* isolated from newly-born buffalo-calves. J. Egypt. Vet. Med. Assoc. 14 (4), 99.
4. Begum, D., Strockbine, N. A., Sowers, E. G., and Jackson, M. P. (1993): Evaluation of a technique for identification of Shiga-like toxin-producing *Escherichia coli* by using polymerase chain reaction and digoxigenin-labeled probes. J. Clin. Microbiol., 31: 3153-3156.
5. Butler, D. G. and Clarke, R. C. (1994): Diarrhea and dysentery in calves, *in* C. L. Gyles (ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, United Kingdom, p. 91-116.
6. Cavalieri, S. J. and Synder, I. S. (1982): *E. coli* alpha hemolysin on human peripheral leukocyte function in vitro. Infect. Immun., 37: 966-974.
7. Cavalieri, S. J., Bohach, G. A., and Synder, I. S. (1984): *E. coli* alpha hemolysin characteristics and probable role in pathogenicity. Microbiol. Rev., 48: 326-343.
8. Cobbold, R. and Desmarchelier, P. (2001): Characterization and clonal relationships of shiga-toxigenic *Escherichia coli* (STEC) isolated from Australian dairy cattle. Vet. Microbiol., 79: 323-335.
9. Cruickshank, R., Duguid, J. P., and swain, R. H. (1975): Medical microbiology.12th Ed., ChurchiL Livingstone, Edinburgh. Correlation between Enterohemolysin production and Verocytotoxicity of *E. coli.* Ind. J. Comp. Microbiol. Immun. Infect. Dis., 22 (1): 96-97.
10. Dean, A. G., Ching, Y., Williams, R. G., and Harden, L. B. (1972): Test for *Escherichia coli* enterotoxin using infant mice: Application in a study of diarrhea in children in Honolulu. J. Infect. Dis., 125 (4): 407-411.
11. Devenish, j., Gyles, C., and Maree, J. (1998): Binding of *E. coli* Verotoxin to cell surface protein on wild type and globotrisoylceramide-deficient vero cells. Can. J. Microbiol., 44 (1): 28-34.
12. Duby, S., Sharda, R., Chharbra, D., Tiwari, S., and Pillai, A. G. R. (2000): Congo red colony pigmentation and hemolysin production by *E. coli* strains from diarrheic goats. Ind. J. Comp. Microbiol. Immun. Infect. Dis., 21(2):157-158.

13. El-Ashker, H. M. (2006): Correlation between antigenic structure of *Escherichia coli* and its virulence Ph.D. Thesis, Fact .Vet. Med., Cairo Univ.
14. Elsayed, A. S. A. (1987): The rate of Enterobacteriaceae group in enteritis of buffalo calves. M.V.Sc. Thesis, Cairo Univ. Fact. Vet. Med.
15. Emery, D. A., Nagarja, K. V., Show, D. P., Newman, J. A., and White, D. C. (1992): Virulence factors of *Escherichia coli* associated with colisepticemia in chickens and turkeys. Avian Dis., 36(3):504-511.
16. Emo¨dy, T., Safonova, P. N. V., Kuch, B., and Golutva, N. K. (1980): Alpha-hemolysin: an additive virulence factor in *Escherichia coli*. Acta Microbiol. Acad. Sci. Hung., 27:333-342.
17. Farid, A., Ibrahim, M. S., and Refai, M. (1976): Studies on colibacillosis in calves in Egypt I-Mortality among buffalo and Frisian calves II-Incidence of *E. coli* serogroups among live calves. Vet. Med., 23B (1) 38.
18. Fecteau, G., Van Metre, D. C., Pare , J., Smith, B. P., Higgins, R., Holmberg, C. A., Jang , S., and Guterbock, W. M. (1997): Virulence factors in *Escherichia coli* isolated from blood of bacteremic neonatal calves Vet. Microbiol., 78: 241-249.
19. Franck, S. M., Bosworth, B. T., and Moon, H. W. (1998): Multiplex PCR for enterotoxigenic, Attaching and Effacing, and Shiga Toxin-Producing *Escherichia coli* Strains from calves. J. Clin. Microbiol., p. 1795-1797.
20. Gay, C. C. and Besser, T. E. (1994): *Escherichia coli* septicemia in calves. In Gyles, C.L. (Ed), *Escherichia coli* in Domestic coli in domestic animals and Humans, 1thed. CAB International, Walligford, p.75.
21. Han, Y. and Linton, R. H. (2004): Fate of *Escherichia coli* O157:H7 and *Listeria* monocytogens in strawberry juice and acidified media at different pH values and temperatures. Journal of Food Protection, 67 (11): 2443-2449.
22. Hornitzky, M. A., Mercieca, K., Bettelheim, K. A., and Djordjevic, S. P. (2005): Bovine feces from animals with gastrointestinal infections are a source of serologically diverse atypical enteropathogenic *Escherichia coli* and shiga toxin-producing *E. coli* strains that commonly Possess intimin. J. Appl. and Environ. Microbiol. P.3405-3412.
23. Kamel, S. (2001): Laboratory diagnosis of some bacterial agents causing pneumonia and/or enteritis in ovines, caprines and bovines. Journal of Food Microbiol., 63: 217-223.
24. Khac, H. V., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, E. J., Mora, A., Dahbi, G., López, C., González, E., and Blanco, J. (2006): Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with post weaning diarrhea in Slovakia BMC Vet Res., 2 : 10.
25. Koneman, E. W., Alle, S. D., Dauda, M. W., Sohrechenberger, P. C. and Winn, W. C. (1997): Color Atlas and text book of diagnostic Microbiology. Fifth Edition, Lippincott, Philadelphia, New York.
26. Kudra, L.T., Hatfield, P. G., and Hovde, C. J. (1997): Characterization of *Escherichia coli* O157:H7 and other shiga-toxin producing *E. coli* serotypes isolated from sheep. J. Clin. Microbiol., 35: 892-899.
27. Mohammed, A., Pieiris, J. S. M., Wijewants, E. A., Mahalingam, S., and Gunasekara, G. (1985): Role of verotoxigenic *Escherichia coli* in cattle and Buffalo calf diarrhea. FEMS. Microbiol. Lett., 26: 281-283.

28. Montenegro, M. A., Bulte, M., Trumpf, T., Aleksic, S., Reuter, G., Bulling, E., and Helmuth, R. (1990): Detection and characterization of fecal verotoxin-producing *E. coli* from healthy cattle. J. Clin. Microbiol., 28: 1417-1421.
29. Murinda, S. E., Nguyen, L. T., Landers, T. L., Draughon, F. A., Mathew, A. G., Hogan, J. S., Smith, K. L., Hancock, D. D., and Oliver, S. P. (2004): Comparison of *Escherichia coli* isolates from humans, food, and farm and companion animals for presence of Shiga toxin-producing *E. coli* virulence markers. Foodborne Pathog. Dis. 1(3):178-84.
30. Nataro, P. J. and Kaper, J. B. (1998): Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142-201.
31. Ojeniyi, B., Ahrens, P., and Meyling, A. (1994): Detection of fimberial and toxin genes in *E. coli* and their prevalence in piglets with diarrhea. The application of colony hybridization assay, polymerase chain reaction and phenotypic assays. J. Vet. Med. B., 41: 49-59.
32. Paton , J. C. and Paton , A. W (1998): Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infection. Clin. Microbiol. Rev., 11: 450- 479.
33. Quinin, P. J., Markey, B. K., Donlley, W. J. C., and Leonard, F. C. (2002): Veterinary Microbiology and Microbial Disease Textbook MPG Books LTD, Bodmin, CornWall, P.111.
34. Radostits, O. M., Leslie, K. E., and Fetrow, J. (1994): Health management of dairy calves. Herd Health Food Animal Production Medicine. 2nd Ed. Saunders, Philadelphia, 184-213.
35. Rogerie, F., Marecat, M., Gambadeb, S., Duponda, F., Beauboisb, P., and Langea, M. (2001): Characterization of Shiga toxin producing *E. coli* and O157 serotype *E. coli* isolated in France from healthy domestic cattle. Int. J. of Food Microbiol. 63: 217-223.
36. Sakazaki, R. (1992): Serotyping of diarrheagenic *E. coli*. Media Circle, 34: 117.
37. Sambrook, J., Fritsh, E. F., and Maniatis, T. (1989): Molecular cloning. Laboratory Manual, 2nd Ed. Cold spring harbor Laboratory cold spring New York.
38. Sears, C. L. and Kaper, J. B. (1996): Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol. Rev., 60: 167-215.
39. Wang, G., Clark, C. G., and Rodgers, F. G. (2002): Detection in *Escherichia coli* of the Genes Encoding the Major Virulence Factors, the Genes Defining the *E. coli* O157:H7 Serotype, and Components of the Type 2 Shiga Toxin Family by Multiplex PCR. J. Clin. Microbiol., 40(10): 3613-3619.
40. Welch, R. A., Bauer, M. E., Kent, A. D., Leeds, J. A., Moayeri, M, Regassa, L. B., and Swenson. D. L. (1995): Battling against host phagocytes: the wherefore of the RTX family of toxins. Infect. Agents Dis., 4: 254-272.
41. Wieler, L. H. and Bauerfeind, R. (2003): STEC as a veterinary problem. Diagnostics and prophylaxis in animals with acute neonatal diarrhea. Nord. Vet. Med., 35: 346-352.
42. Zschock, M., Hamann, H. P., Kloppert, B., and Wolter, W. (2000): Shiga-toxin-producing *Escherichia coli* in feces of healthy dairy cows, sheep and goats: prevalence and virulence properties. Lett. Appl. Microbiol., 31: 203-208.