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]; Said, Moustafa[2]; Mohaned Ali Akeila[3] and Sabry, M.Abdelrahem[1]

Dept. of Bacteriol, Mycol.& Immunol. Fac.of Vet. Med. Minufiya Univ.
Dept of Infectious Diseases, Fac.of Vet. Med(Moshtohor), Banha University
Dept. of Microbiology, Fac.of Vet. Med; Alex. University

Summary

In this study127 out of 262 faecal swabs collected from newlyborn diarrhoeic Frisian cattle calves harboured *Escherichia coli* with an incidence of (48.47%) non O_{157} and (10.30%) were O: $_{157}$. Serological testing showed that the most predominant serovars were O_1, O_8, O_{25}, O_{26}, O_{55}, O_{86}, O_{125}, O_{126}, O_{146}, O_{151} and two isolates were untypable as well as O_{157}:H_7 -ve .

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

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 which constitutes all the previous isolates but being cultured directly from faeces upon broth for 7 hrs, as well as the serotyped O157 isolates. And 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), similar results were obtained in the 4th group after 7hrs culture in broth from direct faecal samples. Also infant mouse assay was used to confirm the production of (Sta) toxine in the PCR positive isolates and 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 SLTII and eaeA genes in the O157 isolates revealed negative results. Which confirmed with VERO CELL ASSAY. From the present study we concluded that, *Escherichia coli* are persistently associated with newly born diarrhoeic calves especially the enterotoxigenic type (producing the heat stable toxine)which can be detected directly using PCR directly onto the samples.

Introduction:

Diarrhea of neonatal calves causes amajor 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 extraintestinal infections in which it can produce

Shiga-like toxins (Stx), heatlabile (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 Stx 1 and/or 2, causes the hemorrhagic colitis (HC) and the hemolytic-uraemic syndrome (HUS) in humans *(Nataro and Kaper 1998).* And another virulence associated factor expressed by STEC, is a protein called intimin, 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 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 *Escherichia coli* strains by amplifying genes encoding K99 and F41 fimbriae, heat-stable enterotoxin a, intimin, and Shiga toxins 1 and 2 *(Frank et al., 1998).*Therefore the present study was aimed to know :-

1- the prevalence of *E. coli* especially E.coli O157 from diarrhoeic calves, Isolation, identification .

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 effect of some acidifiers such as organic acid onto *E. coli* .

Material and Methods

Materials:

1.1. Faecal samples;

A total of 262 faecal samples were collected from Frisian cattle calves aged between 1 day and 30 days old and hade profuse watery diarrhea samples were collected early before the application of the antibiotic course .

1.2. Media and reagents prepared according to *Cruickshank et al., (1975) and Koneman et al., (1997).*

1.3. Biological reagents; Antiserum intended for the serological identification of *E.coli* O_{157} and other non O_{157} according to *(Sakazaki et al., 1992).* The antisera were intended for the (O) antigen for all isolates and for the (H) antigen for O_{157} the antisera were purchased from (Denker Seiken Co., LTD.Tokyo, Japan.).

1.3.1. 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}	------	--------	------

1.3.2. Diagnostic antisera for H antigen only used to detect the H7antigen of O_{157};

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}	-------	------

1.4. Reagents and chemicals used for polymerase chain reaction (PCR);

1. Oligonucleotide primers;

(a) The primers were selected to amplify E.coli heat stable toxin (STa) *gene (Ojeniy et al., 1994*) two primers were needed;

(I) upstream primer (STa1) with a sequence of 5'TCCGTGAAACAACATGACGG3'.

(II) Downstream primer STa2 of sequence of 5'ATAACATCCAGCACAGGCAG3'.These primers were synthesized and supplied by (Gentech. Co)

(b) The primers were selected to amplify *E.coli* shiga toxine2 (STX2) and attaching and effacing (eae) genes in entero haemorrhagic *E.coli* (EHEC) isolated from cattle faeces (Paton *et al.,* 1998) two primers are needed for STx2;

(I) upstream primer with sequence of; 5'GGCACTGTGAAACTGCTCC3'.

(II) Down stream primer with sequence of; 5'TCGCCAGTTATCTGACATTCTG3'

As well as two primers for attaching and effacing gene (eae);

(I)Upstream primer with sequence of 5'GACCCGGCACAAGCATAAGC3'.

(II) Down stream primer with sequence of 5'CCACCTGCAGCAACAAGAGG3'.

These primers were synthesized and supplied by (Gentech. Co.)

1.5. Laboratory animals;

2-4 days old suckling mice were used to detect enterotoxigenic activity of *E.coli* they were separated from their mothers immediately before use.

1.6. 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. And preserved at -20 this was added to Eagle's MEM medium to get a final concentration of 5%.

3- Trypsin solution (0, 25%w/v);

It was prepared by dissolving 2.5 g of Trypsin (Difco) in one liter of phosphate buffer saline.

5- Antibioitc solutions; Benzyl penicillin (2000unites /ml) and streptomycin (2000μg/ml) were stored in 5 ml aliquots at -20 oC was required for 500ml of cell cutler medium and added immediately befor use.

Polymyxine B (GIBCO Limited, UK) 0.1mg /ml solution of Polymyxine B was dissolved in phosphate buffer saline .it was used for releasing cell bound verotoxine from *E.coli* cells

6- Tryptic soy broth (Biolife);

It was used for growing the *E.coli* isolates for verotoxine and.

7- 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).*

2. Methods

2.1. Collection of samples;

262 rectal swabs and fecal samples were taken from diarrhoeic calves by means of sterile cotton swabs. The collected samples were transferred on ice bags to the laboratory of Bacteriology, Immunology and Mycology in the Faculty of Veterinary Medicine, Sadat city, Minufya University.

2.2. The samples were subjected to bacteriological examination and comlete identification according to *(Kudra et al., 1997) and Quinin et al., (2002). and*

2.3. Serotyping of *E.coli* were applied according to (Sakazaki *et al.,* 1992)

2.4. Utilization of Polymerase chain reaction for detection of the genes coding the heat stable enterotoxin (Sta), shiga like toxin2 (SLTII) and attaching and effacing (eaeA) genes of *E.coli* isolates.

2.4.1. 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 100μlof broth culture were centrifuged and the pellet was re suspend 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.

2.4. 2. Preparation of Oligonucleotide primers of Sta and SLT-II;
(Sambrook et al., 1989).

2.4.3 . Detection of PCR products (*Sambrook et al., 1989).*

3.1. Pathogenicity of E.coli supernatant culture media in Infant Mouse Assay (Phenotypic method) was carried out according to *Dean et al., (1972)*

3.2. 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* both isolated from diarrhoeic calves in relation to calve age.

Calf age	No. of examined animals	No of positive *E.coli* strains.			
		No. of non O:157		No. of non O:157	
		No of positive samples.	%	No of positive samples.	%
1-7 days.	68	62	91.17	4	5.8
7-15 days.	28	14	50	6	21.4
15-21days.	56	16	28.57	7	12.5
21-30 days	110	8	7.27	10	9
Total	**262**	**100**	**38.16**	**27**	**10.30%**

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

No. of E.coli isolates.	No of serotyped E.coli isolates.			
	No. of O:157		No. of non O:157	
127	No of samples.	%	No of samples.	%
	20	15.7	9	.07

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

Types of Ecoli serotypes.	Total No. of examined *E.coli.*
20 isolates were serotyped with the available antisrea.	One for each of; O_8,O_{25},O_{26},O_{55},O_{86},O_{125},O_{126},O_{146} ; 5 serotypes of O_1, 3 serotypes of O_{125} ; 3 seroeypes of $O_{151}$and 2 untypable .

(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.** *On photo 1*	**Samples show typical cultural and biochemical picture but not identified serologically.**	**Samples show negative cultural and biochemical picture and not identified serologically.**	**Samples taken representive to the past groups but the PCR technique made directly upon broth culture of faecal samples.**
Type of media upon which the isolates were grown before the technique	Nutrient agar slopes.	Nutrient agar slopes.	Nutrient agar slopes.	Nutrient broth Cultured by the filtrate of faecal samples.
Time of culture	24hrs	24hrs	24hrs	7hrs
No of tested samples	12 samples (10 being serologically detected + (2 untypable) and 9 (O157)	5 samples take numbers (1-5)	5 samples take numbers (6-10)	All the samples from the past groups cultured on broth and tested with pcr..
Results	-9 samples from 12 were positive.	-4 samples from 5 were positive.	-3 samples from5 were positive.	The samples showed typical results to their
Resultsfor O157 with shiga like toxin 2(slt2), attaching and effacing gene and	-ve	-ve	-ve	-ve

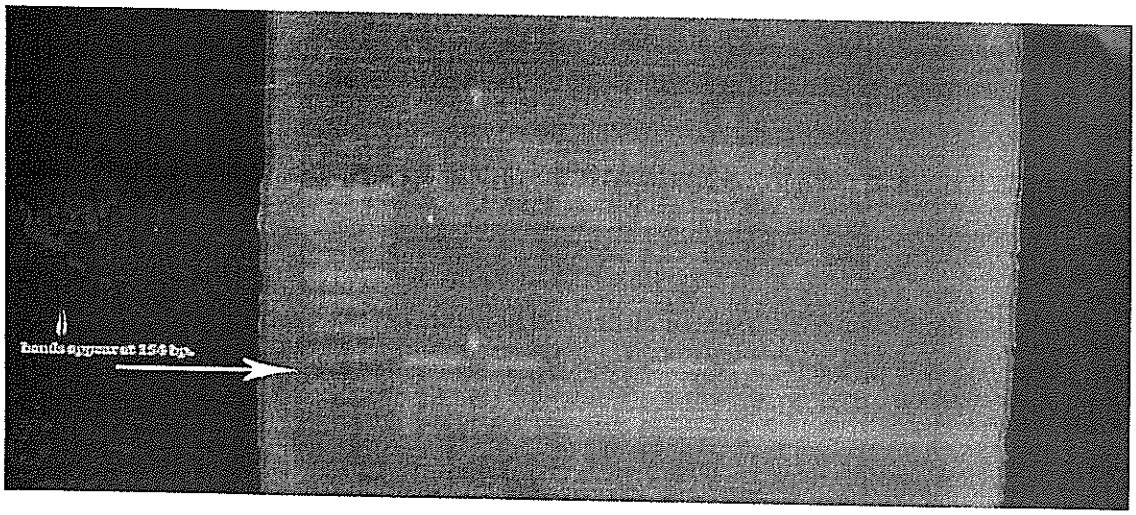

Photo (1-a) PCR photo (agarose gel) showing positive isolates to Sta gene(at 154 base pair) in the first group showing positive 4 isolates.

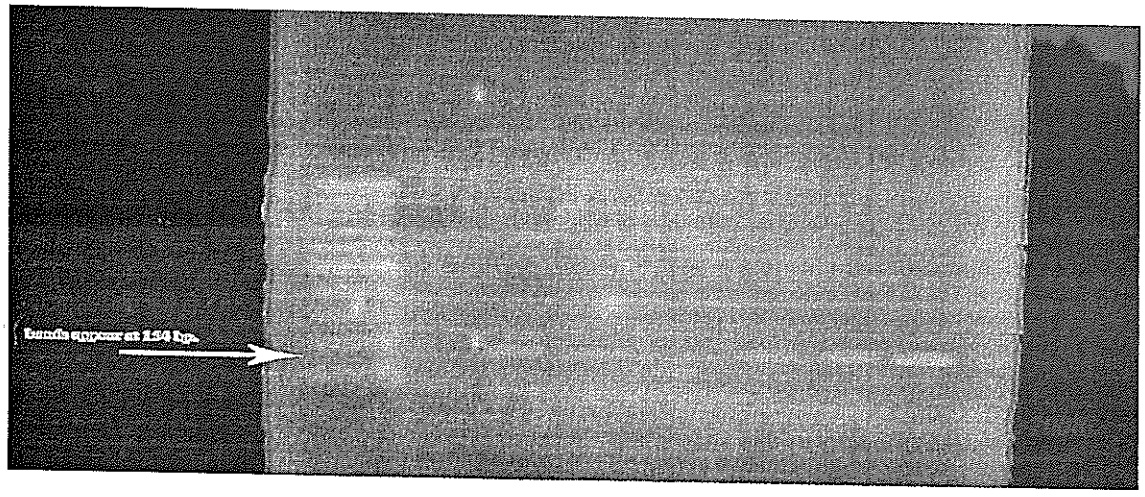

Photo (1-b) PCR photo (agarose gel) showing positive isolates to Sta gene(at 154 base pair) in the first group showing positive 5 isolates.

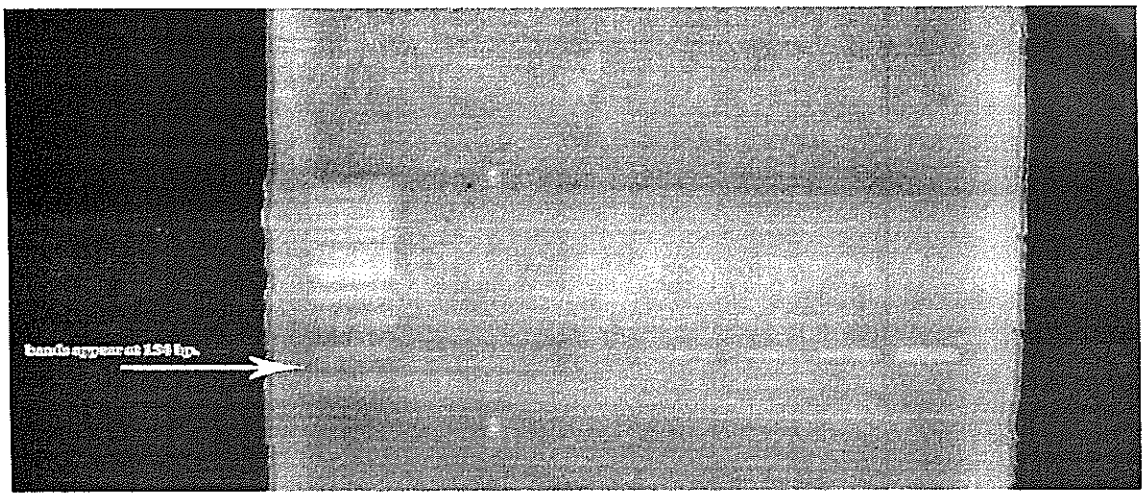

Photo (2)PCR photo (agarose gel) showing positive 4 isolates of the second group to Sta (at 154 base pairs).

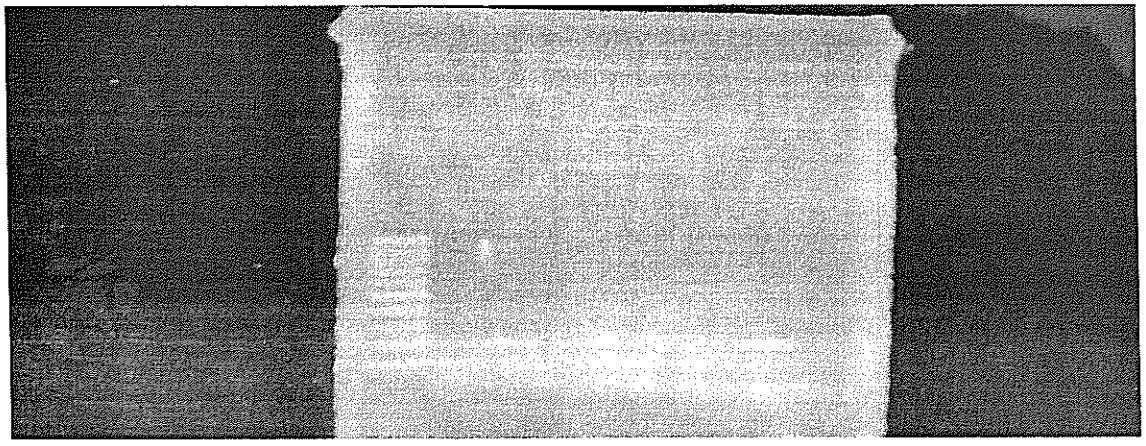

photo (3) PCR **photo** (agarose gel) showing positive 3 isolates of the third group.) to Sta (at 154 base pairs).

(Table 5): polymerase chain reaction (PCR) results for serologically identified isolates with the heat stable (Sta), shiga like toxin2 (SLTII) and attaching and effacing gene (eaeA).

Non O157			O157		
tested samples with Sta	No.of Positive samples	Percentage %	tested samples with Stashiga like toxin2 (SLTII) and attaching and effacing gene (eaeA).	No.of Positive samples	Percentage %
12	9	75%	9	0	0%

(Table 6): Relation between infant mouse assay and PCR.

E.coli samples no.	Results of infant mouse assay	Results of 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 haemolytic (enterotoxigenic and O: 157) E.coli isolates and PCR

E.coli serovars	No of tested isolates	Results of Haemolysis	Results of 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 is an important pathogen in bovine neonates, capable of causing Intestinal and extraintestinal infections (Gay and Besser 1994).

PCR is a genotypic technique which can be used extensively in 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 faecal swabs collected from newborn Frisian cattle calves showing clinical signs of diarrhoea were found to harbour *Escherichia coli* with an incidence of (48.47%) which were closely similar to those obtained with (Fecteau *et al.*, 1997) (51%). Also results obtained from table (2) showed that Out of the 127 were 100 (38.16%) of *E.coli* non O: 157 and that is closely similar to that revealed by (Kamel 2001) (37.7%), and 27 (10.30%) were O: 157 that's nearly similar to (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 confined to the (O) antigen only and that fulfilled with selecting (20) random samples with typical *E.coli* biochemical pattern to be serologically into the following serovars are 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 diarrhoea reported in literature but there located partial similarity in type of strain isolated from such cases (Farid *et al.*, 1976) who isolated *E.coli* serogroupes from faecal samples of buffalo and Frisian

calves in farms suffering from problems of enteritis in Egypt, And the similarly isolated serogroupes were O8, O26, O86, O125 and O126 from the whole isolated serogroupes. Ahmed (1977) who isolated *E.coli* serogroupes O26, O55,O86,O125, O126 from the whole isolated serogroupes from diarrhoeic buffalo calves, (Awad *et al.*, 1979) who isolated strains were grouped serologically into serogroupes, O26, O55,O86, O125, O126 and O128 were isolated from the whole isolated serogroupes. (Elsayed 1987) who isolated the following serogroupes from calf diarrhoea O55, O86, O125 from healthy cattle and O26, O125, O86 from diarrhoeic calves. (Franck *et al.*, 1998) who isolated O8 from Calves and pigs suffering from diarrhoea and STEC O157:H7, O157 NM, O5, and O8. (Hornitzky *et al.*, 2005) who isolated Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) cells from 191 fecal samples from cattle with gastrointestinal infections STEC serotypes were identified, including O5, O8, O26, O157. From the whole isolated subgroups.

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), 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 diarrhoea 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 increased intracellular cGMP levels, and 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 7hr or 24 hr culture growth) positive with PCR including the following O1, O126, O125, O25, O86, O8, O26 ,O151 and Untypable 2 those represents 75% and the same result confirmed with suckling mouse assay. And 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 62 of 88.7% .

Shiga toxin-producing *Escherichia coli* (STEC) are known for certain life-threatening infections like haemorrhagic colitis (HC), bloody diarrhoea, haemolytic uraemic syndrome (HUS) and thrombocytopenic purpura in humans. STEC occur in the faeces of domestic animals and are most frequently found in ruminants (Montenegro *et al.*, 1990). Although several of them can cause diarrhoea 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 a major source of infection for humans. (Wang *et al.,* 2002). VTEC was one of *E.coli* groups which is 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 presence of genes encoding Shiga toxin 1 and 2 (stx1 and stx2), H7 flagella (flicC), Enterohemolysin (hly) and intimin (eaeA) in *E.coli* isolates (n = 400). 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) so this 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) where the entire isolated O: 157 (9) isolates revealed negative results with the two genes as well as being confirmed with negative results on VERO cell assay and that seems to be in accordance with (Rogerie *et al.,* 2001) who isolated 105 *E.coli* isolates and 8 of them were negative for *stx*1 , *stx*2, and *eae* .

Haemolysin is a component that had been confirmed to be incorporated in virulence of *E.coli* (Emery *et al.,* 1992) and it was also confirmed that the introduction of the gene of Haemolysin will increase virulence (Emo¨dy *et al.,* 1980). Haemolysin is cytotoxic for leukocytes, and at sublethal levels, inhibits phagoctosis and chemotaxis. (Cavalieri and Synder 1982) Haemolysin show vitality for *E.coli* in the mechanism of acquiring and assimilating iron from its host (Cavalieri *et al.,* (1984). *Escherichia coli* haemolysin HylA belongs to a family of exoproteins, broadly disseminated among gram-negative pathogens, which has been named RTX (for repeats of glycin in toxins) (Welch 1995).Haemolysin produced by *E.coli* strains designated α-Haemolysin and β- haemolysin .The α-haemolysin is excreted as extracellular protein, where as beta–haemolysin 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 *E.coli* isolates non O157 showed positive results with haemolysin and most of them showed also positive results with heat stable enterotoxin this shows accordance in that haemolysin is associated with virulent strains and the higher the percentage of virulent strain (enterotoxigenic), the higher the percentage of haemolytic strains and this shows accordance with (Abd

EL-Wahed 2005) who noted that 66.07% of isolated *E.coli* were haemolytic and most of them were enterotoxigenic. (Ameen 2006) those reported that 69.35% of the isolated strains were beta haemolytic. (El-Ashker 2006) who isolated *E.coli* from diseased calves and 95% of them showed *α*-haemolysin.

Also from the results in table (7) which revealed that 3(33.3%) out of (9) *E.coli* O157 were positive for haemolysin and this result is closely related to what had been obtained with (Duby *et al.*, 2000) those found that 30.4% of E.coli isolated from diarrhoeic goats were haemolytic and reported that the virulence of haemolytic strains is multifactorial and this depends on their association with other virulence factors.

References

Abd-El-Wahed, M. A. (2005): Virulence determinant of enterotoxigenic *E.coli* and it's relation to adhesive antigen K99 associated with diarrhoea in newlyborn calves. M.V.Sc.Thesis, Fac.Vet.Med., Zagazig Univ.

Ahmed, S. A. (1977): Studies on colibacillosis among newly-born buffalo calvesin Egypt Ph.D.Thesis Cairo.Univ.Fac.Vet.Med

Ameen, N.R.B. (2006): A contribution on virulence markers of Escherichia coli isolated from newly born buffalo calves. M.V.Sc.Thesis. Fac.Vet.Med.,Alex.Univ.

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.

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

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.

Cavalieri, S. J. and Synder, I. S. (1982): *Escherichia coli* alpha haemolysin on human peripheral leukocyte function in vitro.Infect.Immun., 37:966-974.

Cavalieri, S. J.; Bohach, G. A. and Synder, I. S. (1984): *Escherichia coli* alpha haemolysin characteristics and probable role in pathogenicity.Microbiol.Rev., 48:326-343.

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.

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.

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 diarrhoea in children in Honolulu. J.Infect.Dis., 125(4):407-411.

Devenish, j.; Gyles, C. and Maree, J. (1998) : Binding of E.coli verotoxin to cell surface protein on wild type and globotrisoylceramide –defecient vero cells. Can.J.Microbiol., 44 (1) : 28-34.

Duby, S.; Sharda, R.; Chharbra, D.; Tiwari, S. and Pillai, A. G. R. (2000): Congo red colony pigmentation and haemolysin production by E.coli strains from diarrhoeic goats. Ind. J.Comp. Microbiol. Immun. Infect. Dis., 21(2):157-158.

El-Ashker, H. M. (2006): Correlation between antigenic structure of Escherichia coli and it's virulence Ph.D.Thesis, Fac .Vet. Med., Cairo Univ.

Elsayed, A. S. A. (1987): The rate of Enterobacteriaceae group in enteritis of buffalo calves. M.V.Sc. Thesis, Cairo Univ. Fac. Vet. Med.

Emery, D. A.; Nagarja, K. V.; Show, D. P.; Newman, J. A. and White, D. C. (1992): Virulence factors of *Escherichia coli* associated with colisepticeamia in chickens and turkeys. Avian Dis., 36(3):504-511.

Emo¨dy, T.; Safonova, P. N. V.; Kuch, B. and Golutva, N. K. (1980): alpha-haemolysin:an additive virulence factor in *Escherichia coli*. Acta Microbiol. Acad. Sci. Hung., 27:333–342.

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 serogroupes among live calves. Vet. Med., 23B (1) 38.

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 bactereamic neonatal calves Vet. Microbiol., 78:241-249.

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.

Gay, C. C. and Besser, T. E. (1994): *Escherichia coli* septicaemia in calves.InGyles, C.L. (Ed), *Escherichia coli* in Domestic coli in domestic animals and Humans, 1thed. CABInternational, Walligford, p.75.

Han, Y. and Linton, R. H. (2004) : Fate of *Escherichia coli* O_{157}:$H_7$and Listeria monocytogenes in strawberry juice and acidified media at different pH values and temperatures. Journal of Food Protection, 67 (11):2443–2449.

Hornitzky, M. A.; Mercieca, K.; Bettelheim, K. A.; and Djordjevic, S. P. (2005): Bovine faeces from animals with gastrointestinal nfections 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.

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.

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 diarrhoea in Slovakia BMC Vet Res., 2 : 10.

Koneman, E. W.; Alle, S. D.; Dauda, M. W.; sohrechenberger, P. C. and Winn, W. C. (1997): Colour Atlas and text book of diagnostic Microbiology .FifthEdition, Lippincott, Philadelphia, New york.

Kudra, L.T.; Hatfield, P. G. and Hovde, C. J. (1997): characterization of Escherichia coli O157:H7and other shiga- toxin producing *E.coli* serotypes isolated from sheep.J. Clin. Microbiol., 35,892-899.

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.

Montenegro, M. A.; Bulte, M.; Trumpf, T.; Aleksic, S.; Reuter, G.; Bulling, E. and Helmuth, R. (1990) : Detection and characterization of faecal verotoxin-producing *Escherichia coli* from healthy cattle. J. Clin. Microbiol., 28, 1417–1421.

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.

Nataro, P. J. and Kaper, J. B. (1998): Diarrheagenic *Escherichia coli*.Clin. Microbiol. Rev., 11:142-201.

Ojeniyi, B.; Ahrens, P. and Meyling, A. (1994):Detection of fimberial and toxin genes in *E.coli* and their prevalence in piglets with diarrhoea .The application of colony hybridization assay, polymerase chain reaction and phenotypic assays .J.Vet.Med.B.,41:49-59.

Paton , J. C. and Paton , A. W (1998): Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infection. Clin. Microbiol. Rev., 11:450- 479.

Quinin, P. J.; Markey, B. K.; Donlley, W. J. C. and Leonard, F. C. (2002): Vetrinery Microbiology and Micribial Disease Textbook MPGBooks LTD, Bodmin, CornWall, P.111.

Radostits, O. M.; Leslie, K. E. and Fetrow, J. (1994): Health management of dairy calves. Herd Health Food Animal Production Medicine. 2nd edn. Saunders, Philadelphia, 184-213.

Rogerie, f.; Marecat, M.; Gambadeb, S.; Duponda, F.; Beauboisb, P. and Langea, M. (2001): Characterization of Shiga toxin producing *E.coli* and O_{157} serotype *E. coli* isolated in France from healthy domestic cattle. Internat. J. of Food MicrobioL., 63:217-223.

Sakazaki, R. (1992): Serotyping of diarrheagenic *E.coli*. Media Circle, 34:117.

Sambrook, J.; Fritsh, E. F. and Maniatis, T. (1989): Molecular cloning .Alaboratory Manual, 2nd Ed., Cold spring harbor Laboratory cold spring New York.

Sears, C. L. and Kaper, J. B. (1996): Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol. Rev., 60:167–215.

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* O_{157}:H_7 Serotype, and Components of the Type 2 Shiga Toxin Family by Multiplex PCR. J. Clin. Microbiol., 40(10): 3613–3619.

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.

Wieler, L. H. and Bauerfeind, R. (2003): STEC as a veterinary problem. Diagnostics and prophylaxis in animals. With acute neonatal diarrhoea. Nord. Vet. Med., 35:346–352.

Zschock, M.; Hamann, H. P.; Kloppert, B. and Wolter, W. (2000): Shiga-toxin-producing *Escherichia coli* in faeces of healthy dairy cows, sheep and goats: prevalence and virulence properties. Lett. Appl. Microbiol., 31: 203–208.