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Some bacteriological and molecular studies on *Clostridium perfringes* and *Escherichia coli* isolated from calves suffering from enteritis.

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Abstract

The present study aims to investigate the presence of the predominant types of *Clostridium perfringes* and *Escherichia coli* isolated from calves infected with enteritis in Kafr El-Sheikh Governorate. A total of 80 faecal samples were aseptically collected from diarrhoeic calves and were processed for the isolation and identification of *C. perfringens* and *E. coli*. The incidence of *C. perfringens* was 35%, the recorded isolates were subjected to biochemical tests, Nagler`s test and dermonecrotic reactions in albino guinea pigs which proved that the recorded 21 isolates were *C. perfringens* type A (n=16) and type D (n=5). Conventional polymerase chain reaction (PCR) confirmed the presence of alpha toxin gene in *C. perfringens* type A that gave specific amplicon at 402 bp, also alpha and epsilon toxin genes in *C. perfringens* type D which gave characteristic bands at 402 and 541 bp respectively. Rate of isolation of *E. coli* was 75%. Serogrouping of isolated strains of *E.coli* revealed 6 (O) serogroups which were O1, O44(n=2, each), O55, O86a, O125 and O128 (n=1, each). For detection of virulence genes upon 6 *E. coli* serogroups, O55 was carried two virulence genes (*stx1, eae A*) by PCR assay while the remain serogroups were negative. Antimicrobial susceptibilities of *C. perfringens* isolates to 10 antimicrobial agents showed sensitivity to Chloramphenicol (60%), Ciprofloxacin (40%), Nalidixic acid (30%), Vancomycin (20%), while the isolates showed 100% resistance against Erythromycin, Metronidazol and Penicillin–G. Antibiogram pattern upon *E. coli* serogroups were high sensitive to Norfloxacin (100%), Gentamicin (75%), Chloramphenicol and Ciprofloxacin (50%, each), while the most isolates showed the highest resistance against Amoxicillin–Clavulanic acid (100%), Cefotaxime and Spiramycin (50%, each). So it could be concluded that the main cause of enterotoxaemia in young calves is *Clostridium perfringens* Type A and its toxin (alpha toxin) which lead to sudden death in young calves also diarrhea in calves is commonly caused by enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) have also been identified as causes of diarrhea in calves. PCR has established a sensitive and reliable investigative tool for the rapid detection of *C. perfringens* and virulence genes in *E. coli*.

Keywords: *Bacteriological studies, toxins and virulence genes, C. perfringens, E. coli and calves enteritis.*

Introduction

Enteritis in young calves considered generally to be the main hazard to calf health. Diarrhea as a symptom of enteritis is one of the major health problem in many farms associated with newly born calves as considerable number could be lost **(Quigiey *et al*., 1995)** and **(Wells *et al.*, 1996).**

Calf diarrhea caused by bacterial infection has a bad effect on the dairy industry all over the world when calves are reared intensively. It involves significant economic loss for labor and capital, calf mortality, loss in calf value and veterinary costs **(Pereira *et al*., 2011)** and **(de Verdier *et al*., 2012).**

Diarrhea is a major problem in livestock production in Egypt and throughout the world **(Farid *et al.,* 2001)** and **(Ibrahim, 2007)**. Enteritis in newborn calves causes high morbidity and mortality, leading to significant economic losses in Egypt **(Novert and Hammad, 2001)** and **(Ashraf, 2007).**

Clostridium perfringens is a Gram-positive, anaerobic bacterium that causes a wide range of disease in animals. It is widespread in the environment (e.g., in soil and sewage) and is commonly present in the gastrointestinal tract of animals **(Prescott *et al*., 2016)** and **(Smith, 2014).**

Clostridium perfringens classified into five types (A-E) according to the production of four major toxins (alpha, beta, epsilon and iota) **(Silva *et al*., 2009)** and **(Silva and lobato, 2015)**. Type A isolates produce alpha toxin only; type B isolates produce alpha, beta and epsilon toxins; type C isolates produce alpha and beta toxins; type D isolates produce alpha and epsilon toxins; and type E isolates produce alpha and iota toxins **(Timbermont *et al*., 2009).** These specific toxins causing the clinical signs and a syndrome attributable to each type. Each toxin type is associated with specific enteric infections of various animal species **(Ashgan *et al*., 2013)** and **(Ohtani and Shimizu, 2016)**.

Detection of *C. perfringens* toxin types and subtypes is critical to ensure a good understanding of the epidemiology of *C. perfringens* infections and may be useful in the development of effective control of the disease **(Das *et al*., 2012).**

The treatment of *C. perfringens*-associated diseases primarily involves antibiotic therapy **(Ramsey and Tennant, 2010)** and several studies have evaluated the *in vitro* antimicrobial susceptibility of *C. perfringens* to commonly used drugs **(Salvarani *et al*., 2012).**

The most calves are affected with *E. coli* within the first 3 days of life. There are many types of *E. coli* some are normal flora; different types cause septicemia; others are invasive; Enterotoxigenic *E. coli* (ETEC) is the most common cause of newly born calf diarrhea **(Bispham *et al.,* 2001).***Escherichia coli* is an important pathogen in bovine neonates, capable of causing intestinal and extra intestinal infections **(Gay and Besser, 1994).**

Diarrhea due to *E. coli* is one of the most common diseases of young calves **(Uhde *et al*., 2008).** *E. coli* diarrhea in newborn calves (9–10 days of age) is usually characterized by watery white or yellowish diarrhea, rapid onset and time course, and high mortality. In affected calves, diarrhea typically begins within 36–72 hours of birth, and affected calves die within 2 –3 days. Some calves die several hours after appearing healthy and free of diarrhea. The scouring calf losses fluids, rapidly dehydrates, and suffers from electrolyte loss and acidosis. Infectious agents may cause initial damage to the intestine, but death from scours usually results from dehydration, acidosis, and loss of electrolytes. Identification of infectious agents that cause scours is essential for implementation of effective preventive and treatment measures **(Radostits *et al.,* 2007).**

PCR is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive. A PCR which detects genes according to **(Stone *et al.,* 1994)** and **(China *et al.,* 1996).**

Antimicrobial agents are considered popular to fight diarrhea in calves. Nevertheless, their wide spectrum of activity, the emergence of microbial tolerance of different antimicrobial agents has become a well-known phenomenon, which represents a major concern **(Hajipour *et al*., 2013).**

Aim of work: is to detect the causative agents causing diarrhea in calves especially *C. perfringens* and *E. coli* ,toxin typing of *C. perfringens*, grouping of isolated strains of *E. coli* and

detection of virulence genes of *E. coli* by PCR in addition to antimicrobial pattern upon isolated strains of *C. perfringens* and *E. coli.*

Materials and Methods

Collection of samples

A total of 80 fecal samples were collected from the rectum using sterile swabs from diarrheic calves1-6 months age showed signs of diarrhea, at different farms of Kafr EL-Sheikh Governorates. Samples were transferred directly to the laboratory, in an ice box and kept in retail package under complete aseptic condition without delay and subjected to bacteriological examination.

Isolation and identification of *C. perfringens* and *E. coli*:

It was performed for *C. perfringens* by cultivation of each sample on an enrichment cooked meat medium (CMM) (oxoid) then detection of the haemolytic activity of *C. perfringens* toxins onto 10% sheep blood agar medium with neomycin sulphate (200 µg /ml) (**Smith and Holdeman, 1968).** Biochemical identification of the recovered isolates were applied as described by **(Koneman *et al.,* 1992).** Detection of lecithinase activity of *C. perfringens* alpha toxin on lecithin of an enriched egg yolk agar medium (oxoid) was performed **(Murray *et al.*, 2003).** Typing of the recovered isolates using dermonecrotic reactions in albino guinea pigs was applied according to **Quinn *et al.*, 2002**., but it was performed for *E. coli* by inoculated in MacConkey broth (oxoid) then streaked onto MacConkey agar (oxoid) and incubated aerobically at 37°C. After an overnight incubation, lactose fermenting colonies were streaked onto Eosin Methylene Blue (EMB) agar (oxoid) then incubated aerobically at 37°C overnight. Morphological, cultural and biochemical examinations were carried out according to **Murray *et al.* (2003).** Serogrouping of the isolated *E. coli* (8) were performed at the serology unit in Animal Health Research Institute, Dokki, Giza by slide agglutination test using polyvalent and monovalent diagnostic *E. coli* antisera using Mast diagnostics Kit (Mast Group Ltd., Merseyside, UK) according to (**Quinn *et al.*, 1994).**

Detection of *C. perfringens* toxin genes and virulence genes of *E. coli* by PCR

DNA extraction from *C. perfringens* isolates: DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the isolates suspension was incubated with 10 µl of proteins K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions for *C. perfringens* and *E. coli*.

Target gene	Primers sequences (5-3)	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Alpha toxin	GTTGA-TAGCGCAGGACATGTTAAG	402bp	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Yoo *et al.*, (1997)**
	CATGTAGTCATCTGTTCCAGCATC							
Beta toxin	ACTATACA-GACAGATCATTCAACC	236 bp						
	TTAGGAG-CAGTTAGAACTACAGAC							
Epsilon toxin	ACTGCAACTAC-TACTCATACTGTG	541 bp						
	CTGGTGCCTTAATAGAAAGACTCC							
eaeA	ATGCTTAGTGCTCTGGTTTAGG	248 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min	**Bisi-Johnson *et al.*, (2011)**
	GCCTTCATCATTTCGCTTTC							
Stx1	ACACTGGAT-GATCTCAGTGG	614 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min	**Dipineto *et al.*, (2006)**
	CTGAATCCCCCTCCATTATG							
Stx2	CCATGACAACG-GACAGCAGTT	779 bp						
	CCTGTCAACTGAGCAGCACTTTG							

Antibacterial sensitivity pattern of the isolated *C. perfringes* and *E. coli*

Antimicrobial susceptibility tests were performed using Kirby Bauer's disc diffusion method. The following antibiotics discs used for *C. perfringes* were: Erythromycin (E, 15μg), Metronidazole (MTZ), Penicillin (P,10μg), Amoxicillin–Clavulanic acid (AMC, 30μg), Vancomycin (VA, 30μg), Moxifloxacin (MO, 5μg), Nalidixic acid (NA, 30μg) , Ciprocin (CIP, 5μg), Chloramphenicol (C, 30μg) and Tetracycline (TE, 10μg) (Oxoid, Basing Stoke, UK)., but for *E.coli* the antibiotic discs tested were the following: Norfloxacin (NOR, 10μg), Gentamicin (CN, 10μg), Chloramphenicol (C, 30 μg), Ciprocin (CIP, 5 μg), Amoxicillin–Clavulanic acid (AMC,30μg), Cefotaxime (CTX, 30μg), Spiramycin (SP, 100μg), Pefloxacin (PEF, 5μg), Nitrofurantoin (NIT, 300μg) and Spectinomycin (SH, 20μg) (Oxoid, Basing Stoke, UK). The zone diameter interpretative criteria of *C.perfringes* and *E.coli* were used to classify isolates as susceptible, intermediate or resistant according to the Clinical and Labora-

tory Standards Institute (CLSI) guidelines **(CLSI, 2016).**

Results

1-Incidence of *Clostridium perfringes* isolated from diarrheic calves.

Concerning to isolation and identification of samples selected from different parts in governorate as fecal sample of diarrheic calves results indicated that 28 samples out of 80 which represented (35%) were positive for *C. perfringens*.

2-Typing of *Clostridium perfringes* in Guniea pigs shown in Table(2).

Table (2). Typing of lecithinase +ve strains of *C. perfringenes* by dermonecrotic reaction in Guniea pigs.

Types of isolates	Number of isolates	Percentage
A	16	76.19
B	0	0
C	0	0
D	5	23.81

3- Multiplex PCR for *C. perfringens* toxins genes.

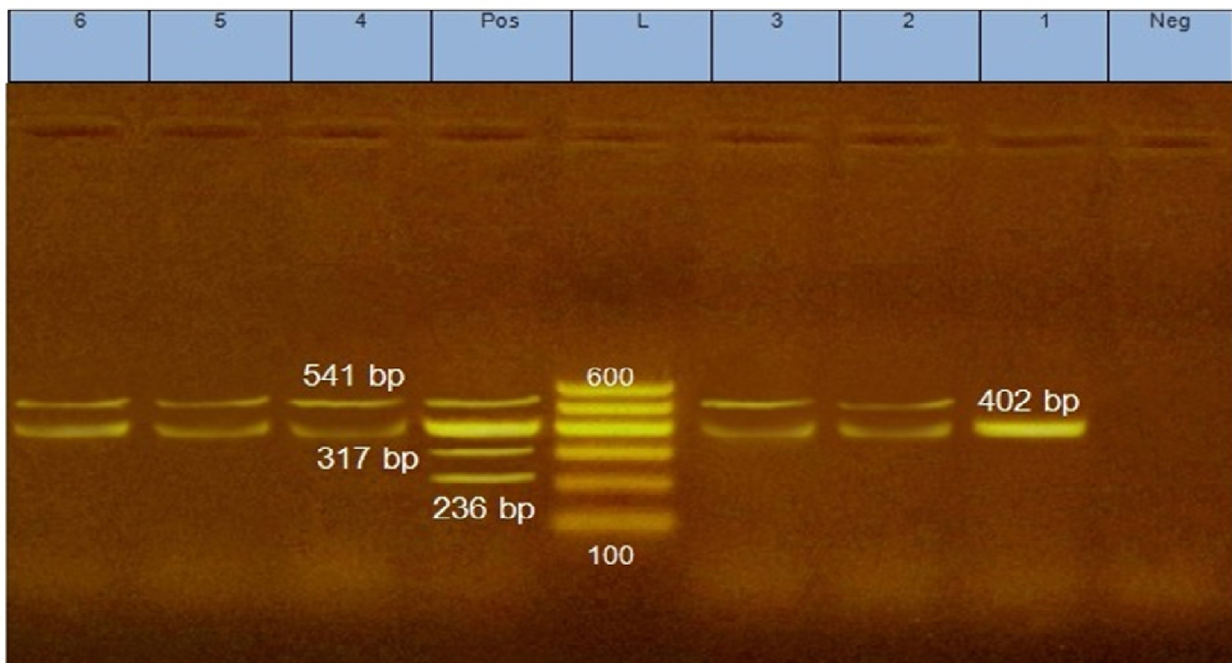

Fig. (1): Agarose gel electrophoresis of multiplex PCR for *C. perfringens* toxins genes.
Lane L: Molecular size marker (100-600 bp).
Lane Pos. and Neg.: Positive and negative controls.
Lane 1: Positive *C. perfringens* strain type A for alpha toxin gene at 402bp.
Lane 2, 3, 4, 5 & 6: Positive *C. perfringens* strains type D for both alpha and epsilon toxin genes at 402 and 541 bp, respectively

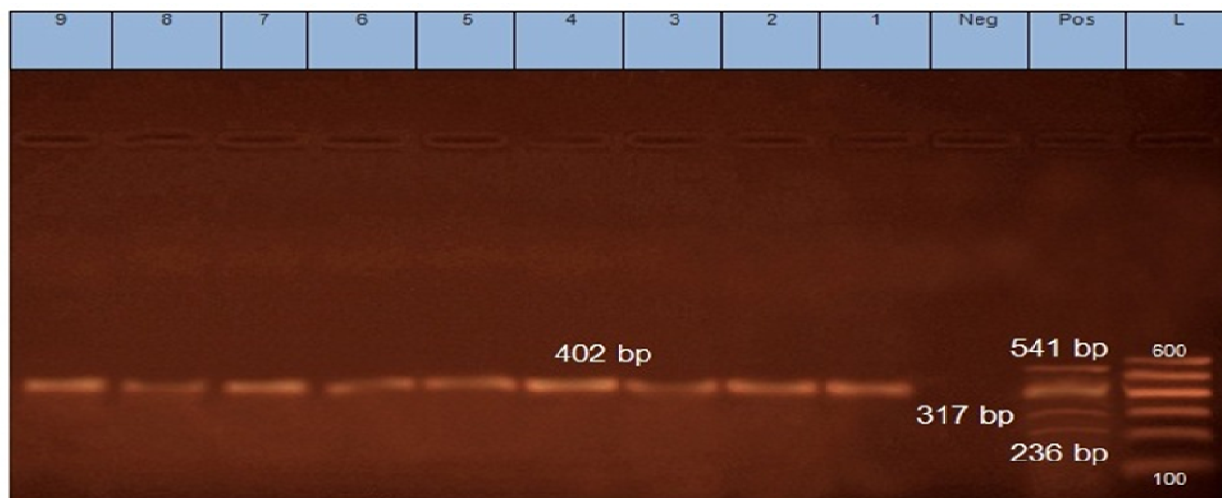

Fig. (2): Agarose gel electrophoresis of multiplex PCR for *C. perfringens* toxins genes.
Lane L: 100-600 bp molecular size marker.
Lane Pos. and Neg.: Positive and negative control.
Lane 1, 2, 3, 4, 5, 6, 7, 8 & 9: Positive *C. perfringens* strains for alpha toxin gene at 402 bp.

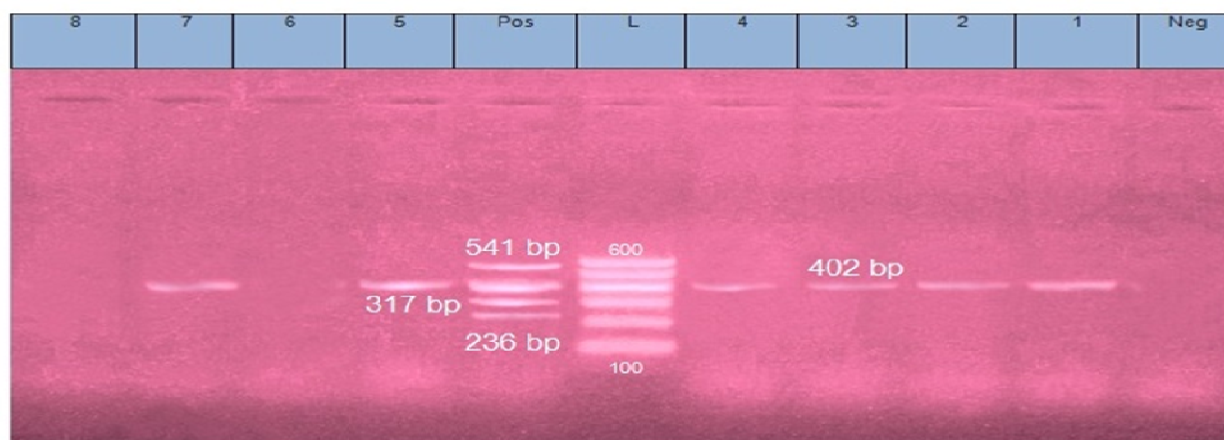

Fig. (3): Agarose gel electrophoresis of multiplex PCR for *C. perfringens* toxins genes.
Lane L: 100-600 bp molecular size marker.
Lane Pos. and Neg.: Positive and negative control.
Lane 1, 2, 3, 4, 5 & 7: Positive *C. perfringens* strains for alpha toxin gene at 402 bp.
Lane 6 & 8: Negative for *C. perfringens*.

4- Incidence of *Escherichia coli* isolated from diarrheic calves.

Our results showed that 60 out of 80 diarrheic calves samples which represented (75%) were positive for *Escherichia coli* by isolation and identification .

5- Serotyping and virulence genes of *E. coli* isolates recovered from diarrheic calves.

The serological examination of 8 randomly selected *E. coli* isolates resulted in detection of 6 different serogroups including O1 and O44 (two strains for each) and O55, O86a, O125 and O128 (one strain for each) and the isolates were screened for harboring (*stx1, stx2* and *eae A*) genes O55 only was noted for harboring *stx1* and *eae* genes as shown in Table (3).

Table (3). Serotyping and virulence genes of some randomly selected *E. coli* isolates:

Serogroups of *E. coli*	Total no. of strains	*stx*1	*stx*2	*eae*
O1	2	-	-	-
O44	2	-	-	-
O55	1	+	-	+
O86a	1	-	-	-
O125	1	-	-	-
O128	1	-	-	-

6- Detection of virulence genes in different serogroups of *E. coli* isolated from diarrheic calves by PCR:

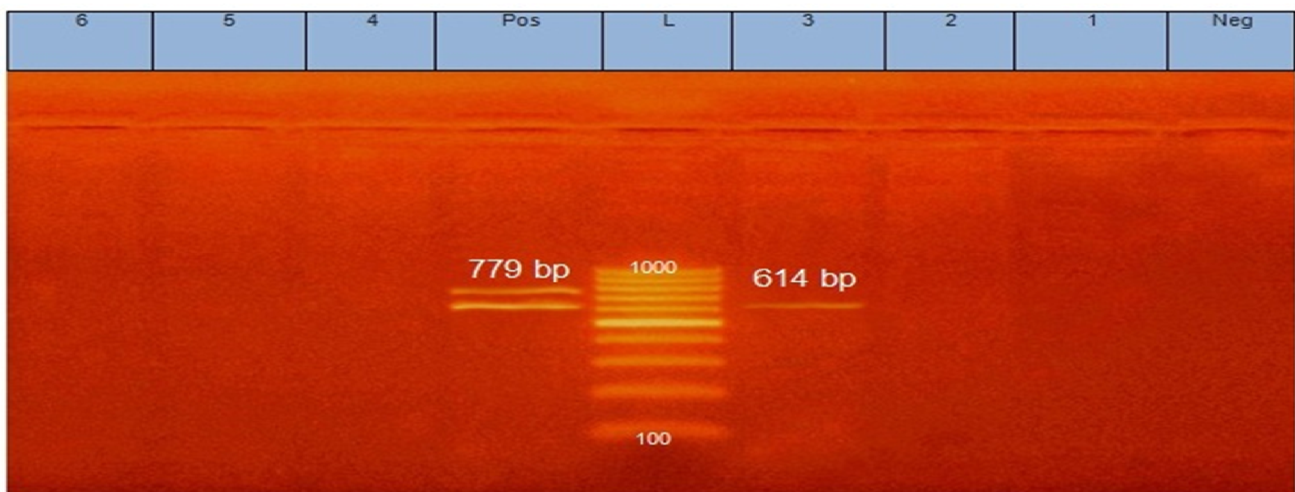

Fig. (4): Agarose gel electrophoresis of multiplex PCR for some virulence genes of *Escherichia coli*.
Lane L: 100-1000 bp molecular size marker.
Lane Pos.: Positive control *E. coli* strain positive for *stx1* and *stx2* gene at 779 and 614 bp, respectively.
Lane 3: Positive *E. coli* strain serotype O55 for *stxl* gene.

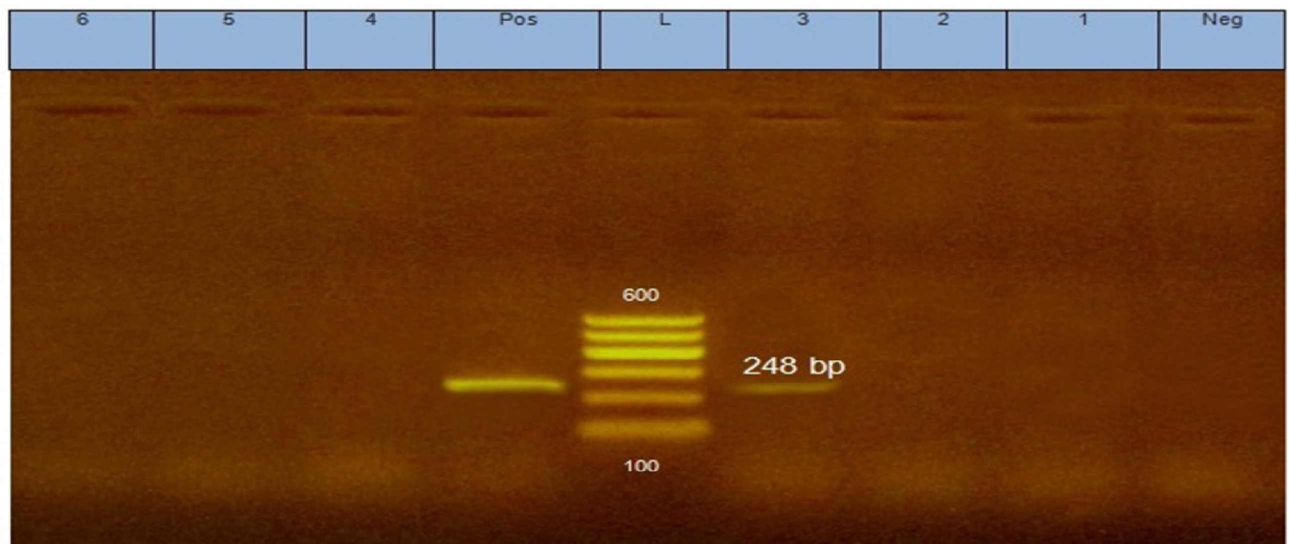

Fig. (5): Agarose gel electrophoresis for *eaeA* virulence gene of *Escherichia coli*.
Lane Pos.: Positive control.
Lane 3: Positive *E. coli* strain serotype O55 for *eaeA* gene at 248 bp.

7- Antimicrobial susceptibility of *C. perfringens*

Antimicrobial susceptibilities of *C. perfringens* isolates to 10 antimicrobial agents showed sensitivity to Chloramphenicol (60%), Ciprofloxacin (40%), Nalidixic acid (30%), Vancomycin (20%), while the isolates showed 100% resistance against Erythromycin, Metronidazol and Penicillin – G. as shown in table (4).

Table (4). Antimicrobial sensitivity test for isolated *C. perfringens* (n=10).

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Erythromycin (E)	0	0	0	0	10	100
Metronidazol (MTZ)	0	0	0	0	10	100
Penicillin – G (P)	0	0	0	0	10	100
Amoxicillin (AML)	1	0	0	0	9	90
Vancomycin (VA)	2	20	0	0	8	80
Moxifloxacin (MO)	1	10	2	20	7	70
Nalidixic acid (NA)	3	30	1	10	6	60
Ciprocin (CIP)	4	40	2	20	4	40
Tetracycline (TE)	0	0	6	60	4	40
Chloramphenicol (C)	6	60	3	30	1	10

8- Antimicrobial susceptibility of *E. coli* isolates

Antibiogram pattern of *E. coli* isolates were high sensitive to Norfloxacin (100%), Gentamicin (75%), Chloramphenicol and Ciprofloxacin (50%, each) ,while the most isolates showed the highest resistance against Amoxicillin–Clavulanic acid (100%), Cefotaxime and Spiramycin (50%, each).as shown in table (5).

Table (5). Antimicrobial susceptibility of *E. coli* isolates: (n=8).

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Amoxicillin–Clavulanic acid (AMC)	0	0	0	0	8	100
Cefotaxime (CTX)	2	25	2	25	4	50
Spiramycin (SP)	4	50	0	0	4	50
Nitrofurantoin (NIT)	5	62.5	0	0	3	37.5
Pefloxacin (PEF)	5	62.5	0	0	3	37.5
Spectinomycin(SH)	3	37.5	2	25	3	37.5
Chloramphenicol (C)	4	50	2	25	2	25
Ciprocin (CIP)	4	50	2	25	2	25
Gentamicin (CN)	6	75	1	12.5	1	12.5
Norfloxacin (NOR)	8	100	0	0	0	0

Discussion

C. perfringens is generally found in gastrointestinal tracts of man and animal and usually presents as mixed infection in which the primary pathogen has paved the way for the anaerobe by damaging the tissue and causing anaerobiosis **(Secasiu *et al.*, 1997)**

As evident from our results, the bacteriological examination and lecithin's activity of diarrheal faecal samples revealed that 28 out of 80 examined samples were positive for *C. perfringens* with a percentage of 35% of calves infected with enteritis. This results was lower than that results obtained by **Ammar *et al.* (2008)**in which they isolated *C. perfringens* from Kafrelsheikh, El-dakahlya and El-sharkia with a percentage of 48.6%, 71.43% and 51.85% respectively. And also our results was lower than that obtained by **Rahsan and Halil Ibrahim (2007)** who found that a total of 122 (81.33%) faecal samples collected from the diarrhoeic calves were positive for *C. perfringens* toxins. Our results was nearly similar to results that obtained by **Marina *et al.* (2008)** who isolated *C. perfringenes* from diarrheic calves with a percentage of 36.2%.

In this study typing of lecithinase positive strains of *C. perfringenes* by dermonecrotic reaction in Guniea pigs indicated that type A was the most predominant one and represented by 76.19%, and type D was 23.81% as shown in Table (3), these results was agreed with that obtained by **Nora *et al.* (2014)** who reported that *C. perfringenes* type A was the most predominant one and represented by 73.3%, 91.1%, 64.1% and 78.9% in Kafr El sheikh, El fayoum, Kalubyia and Beheira, respectively. Also, our results were similar to that obtained by **Ammar *et al.* (2008), Manteca *et al.* (2002) and Jonathan (2005)** but not agreed with that obtained by **Eman and Mona (2007)** who recovered *C. perfringens* type D (75%), type A (8.3%) and non-toxigenic ones (16.7%).

PCR is more accurate and faster than use of lab animals **(Daube *et al.*, 1994)** and may be used to differentiate *C. perfringens* into its five toxin types **Songer and Meer (1996)** and **Yamagishi *et al.*, (1997).**

In the present work, multiplex PCR was proved to be a reliable and sensitive protocol for genotyping of the untypable *C. perfringens* isolates recovered from diarrhoeic calves in which among 23 isolates, 16(69.56%) were typed as *C. perfringens* typeA and gave a characteristic band at 402 bp. This result agreed with **Aschfalk and Muller, (2002)** who examined fecal samples for occurrence of *C. perfringens* by PCR for the gene encoding, and all isolates were *C. perfringens* type A. also these results similar to that obtained by **Yoo *et al*, (1997)** who used a multiplex PCR to investigate the most prevalent type of the organism in calves showing diarrhea, enterotoxaemia, only *C. perfringens* type A was isolated from these calves. Also our results revealed that among 23 isolates, 5(21.74%) were typed as *C. perfringens* type D. This result was agreed with **Kumar *et al.*, (2014)** who reported genotyping of the 97 isolates of *C. perfringens* by a multiplex PCR from enterotoxaemia suspected flocks of sheep and observed 67.01% and 21.65% isolates as type A and D, respectively.

Our results revealed that *E. coli* was isolated from calves affected with enteritis with incidence of 75%, these results were agreed with (**Abubaker *et al*, 2015** and **El-Seedy *et al*, 2016)** who isolated *E. coli* from diarrhoeic calves with a percentage of 76% and 75.6%, respectively and the results were nearly similar to the results obtained by (**Yeshiwas and Fentahum, 2017)** who isolated *E.coli* from fecal samples collected directly from the rectum of diarrheic calves with a percentage of 70.6%. But our results were higher than that obtained by (**Manickam and Ponnusamy, 2017** and **Elham *et al.*, 2012)** who isolated *E. coli* from diarrhoeic calves with a percentage of 36.66% and 47.5% respectively.
Table (3) showed that different serogroups of

E. coli were recovered from diarrhoeic calves as O1, O44, O55, O86, O125 and O128. Similar *E. coli* serogroups had been isolated from diarrhoeic calves were reported by (**Rigobelo *et al*, 2006)** who isolated O55, O86a,O128 and O125 and (**Saridakis *et al*, 1997)** reported the isolation of *E. coli* serogroups O128, O125, O44 and O114 from diarrheic calves in Brazil. Our results disagreed with results obtained by (**Tan Duc *et al.*, 2011)** who reported that most prevalent serogroups of *E. coli* isolated from diarrhea in calves were O15, O20, O103 and O157

In Fig (4), the results of agarose gel electrophoresis of *stx1* gene for characterization of *E. coli* showed that, the *stx1* gene was detected in 1 isolate out of 6 tested isolates with a percentage of (16.6%). These findings were similar to that obtained by (**Jin *et al*, 2012)** who detected *stx1* gene with a percentage of 16.5% of the *E. coli* isolates. On the other hand, our results were lower than the results obtained by (**Oliveira *et al,* 2007)** who stated that out of 109 STEC isolates, 43 (39.5%) carried *stx*1gene also (**Shahrani *et al.*, 2014)** who detected *stx1* in EHEC with a percentage of 96.23%in addition to (**Ashraf *et al.*, 2017)** who detected *sxt1* in *E. coli* isolated from diarrheic buffalo calves with a percentage of 42.8%.

In figure (5), the results of agarose gel electrophoresis of (*eaeA*) gene for characterization of *E. coli* showed that, the (*eaeA*) gene was detected in 1 isolate out of 6 isolates tested with a percentage of (16.6%).These findings were similar to that obtained by (**Ashraf *et al.*, 2017)** who discovered (*eaeA*) gene in 2 *E. coli* isolates only (14.3%) and **Islam *et al*, 2008** who stated that *eae A* was found in 14.4% in tested *E. coli* isolates.. Also, These results were near that obtained (**Oliveira *et al.*, 2007)** who stated that there was no intimin. But, there were high records as, (**Borriello *et al.*, 2012)** who char-acterized the 120 *E. coli* isolates for the presence of the virulence factors and the (*eae A*) were positive in all isolates.

The results of antibiotic sensitivity tests of *C. perfringes* (Table, 4) revealed that most of *C. perfringes* isolates were resistant to Erythromycin, Metronidazol and Penicillin – G by 100%, but sensitive to Chloramphenico l60%, Ciprocin 40%, Nalidixic acid 30% and Vancomycin 20%, These results were nearly similar to that reported by (**Mafruza *et al.,* 2012** and **Sawsan and Ola, 2013)**. On the other hand, these results disagreed with the results reported by (**Oliveira *et al.*, 2016)** who found that all *C. perfringens* strains were susceptible to metronidazole, and penicillin while Two strains (4.9%) were resistant to erythromycin.

The results of antibiotic sensitivity tests upon examined strains of *E. coli* (Table 5) revealed that most of *E. coli* isolates were resistant to Amoxicillin–Clavulanic acid (AMC)100%, Cefotaxime (CTX) and Spiramycin (SP) by 50%. but sensitive to Norfloxacin (NOR) 100%, Gentamicin (CN) 75%, Chloramphenicol (C) and Ciprocin (CIP) by 50%. These results were similar to that reported by (**Kaura *et al*, 1988**, **Ahmed *et al*, 1986** and **Genovese *et al*, 2006)** who stated that calf isolates were resistant to amoxicillin. And these results were dissimilar with the findings of (**Joshi*et al*, 1986)** who reported that high percentage of *E. coli* isolates were sensitive to tetracycline and doxacilline.

Conclusion

It could be concluded that *C. perfringens* types A, D were recorded in calves infected with enteritis, moreover, type A was the most predominant one. Multiplex PCR has been proved to be a reliable, sensitive and specific protocol for detection of alpha and epsilon toxin genes, also (PCR) has been proved to be a reliable, sensitive in detection of *E. coli* virulence genes *(stx1, eae A).*

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