Serological and Molecular Typing of *Clostriduim Perfringens* and Its Toxins Recovered from Weaned Rabbit's Flocks in Egypt

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Abstract: This study was carried out for serological and molecular typing of ***Clostridium perfringens (C. perfringens)*** and its toxins recovered from apparently healthy, diseased and dead weaned rabbits, as well as their feed and water. Identified 42 ***C. perfringens*** organisms representing 35 from rabbits and 7 from feed and water were subjected for determination of toxigenicity by intravenous inoculation (I/V) in Swiss mice. Toxigenic ***C. perfringens*** organisms were typed serologically using Nagler's test, dermonecrotic reaction in albino Guinea pigs and toxin antitoxin serum neutralization test (SNT) in Swiss mice. For confirmation of serological results, detection of alpha gene of ***C. perfringens*** was done using conventional polymerase chain reaction (PCR), followed by using of multiplex PCR to detect the toxin's types. Distribution of different ***C. perfringens*** types of surveyed rabbit's farms at different Egyptian governorate was carried out. In addition, the *in-vitro* antibiotic sensitivity of different ***C. perfringens*** types against different antimicrobial agents was performed. The results showed that 34 (97.14%) of ***C. perfringens*** recovered from rabbits were toxigenic, while 1.0 (2.86%) was none. Four out of 6 (66.66%) ***C. perfringens*** isolates from feed was toxigenic and 2 (33.33%) isolates was non-toxigenic. Only one (100%) ***C. perfringens*** isolate from water was toxigenic. Identical proving results were obtained using serological typing tests. Single and mixed types of toxigenic ***C. perfringens*** constituted 17 out of 35 (48.57%) for each, whereas only one type was none (2.86%). Single ***C. perfringens*** was representing 8 (22.85%) type A, 3 (8.57%) type B, 4 (11.43%) type D and 2 (5.71%) type E, whereas mixed types were 11 (31.42%) types (A and D), 2 (5.71%) types (A and E) and 4 (11.42%) types (B and D). Four ***C. perfringens*** isolates from feed was 3 type A (50%) and 1.0 type D (16.6%). The only ***C. perfringens*** isolate from water was type A (100%). Conventional PCR proved detection of alpha gene of ***C. perfringens,*** whereas multiplex PCR proved that ***C. perfringens*** type (A) was positive for alpha toxin at 324 base pair (bp), type (B) was positive for alpha toxin at (324 bp), beta toxin at (196 bp) and epsilon toxin at (655 bp), type (D) was positive for alpha toxin at (324 bp) and epsilon toxin at (655 bp) while type (E) was positive for alpha toxin at (324 bp) and iota toxin at (446 bp). The result of distribution of different ***C. perfringens*** types at different Egyptian governorate was recorded. *In vitro* antibiotic sensitivity test of single or mixed types of identified ***C. perfringens*** revealed sensitivity to amoxicillin/ clavulanic acid and ampicillin and resistance to colistine, erythromycin and lincomycine.
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1. Introduction

Rabbits are animals of an economic importance. The rabbit meat has many advantages as it is nearly white, fine grained, palatable, flavored, high in good quality protein content, low fat and caloric contents, contains a higher percent of minerals than other meats, nearly of the same nutritive value as beef meat, good meat-to-bone ratio and it is acceptable to the general consumer in most countries of the world (Reddy *et al.*, 1977; Lukefahr *et al.*, 1989).Weaning is considered as a stressful period for rabbits caused by abrupt changes of diet and environment. An immature immune system together with a transient decrease of nutrient digestibility places the animal in adverse conditions where digestive pathologies might take place (de Blas *et al.*, 2012).

Enteritis in rabbits is considered to be a major cause of disease, mortality and economic loss in domestic rabbits, particularly in younger ones (Peeters *et al.*, 1984).

There are many infectious agents known to play a role in inducing enteritis in rabbits, including intestinal and hepatic ***Eimeria***, and infections with bacteria such as ***Escherichia coli***, ***Clostridium*** species (spp.) and ***Bacillus piliformis***. Dietary and management factors are also recognized to have an effect on the incidence of enteric disease (Carman *et al.*, 1948; Patton *et al.*, 1978; Percy *et al.*, 1993).

Clostridium perfringens (C. perfringens) is the most widely occurring pathogenic bacterium and is certainly the most important cause of ***Clostridial*** enteric disease in domestic rabbits (Smith and Williams, 1984). Some types of ***C. perfringens*** (mainly type A) are consistently recovered both from the intestinal tracts of animals and from the environment, while others (types B, C, D, and E) are less common in the intestinal tracts of animals (Carter and Chengappa,

1991) and can occasionally be found in the environment in areas where disease produced by these organisms is enzootic (Niilo, 1980). ***Clostridial*** toxins are main pathogenic virulence factor of ***C. perfringens*** that have been associated with a wide range of diseases in both humans and domestic animals (Jin Zeng *et al.*, 2011).

Typing of ***C. perfringens*** into types A, B, C, D and E is based on production of four major toxins namely alpha, beta, epsilon and iota as determined by *in vivo* protection tests performed by intradermal injection of Guinea pigs or intravenous inoculation of mice (McDonel, 1980; Walker, 1990; Songer, 1996). Strains of ***C. perfringens*** type A produces alpha toxin which is the principle lethal toxin producing enterotoxaemia in rabbits, type B produces alpha, beta and epsilon toxins, type C induces alpha and beta toxins, type D produces alpha and epsilon toxins, while type E induces alpha and iota toxins (Kunstyr *et al.*, 1975; Molloy, 1978)

Routine diagnostic bacteriological cultivation of intestinal samples for ***C. perfringens*** followed by polymerase chain reaction (PCR) based genotyping colonies, also multiplex real-time PCR for detection of toxins were previously done by Albini *et al.*, (2008).

Antimicrobial therapy continues to be important in reducing losses due to ***Clostridial*** infections. Susceptibility, resistance and antibiotic profile of different antimicrobial agents against ***C. perfringens*** strains isolated during surveillance studies of ***Clostridial*** enteritis in rabbits were investigated by Agnoletti *et al.*, (2007); Marien *et al.*, (2008); Richez *et al.*, (2008); Saggiorato *et al.*, (2008).

So, the purpose of this study was typing of ***C. perfringens*** and its toxins serologically and molecularly, and also testing the sensitivity of different ***C. perfringens*** type's *in-vitro* to different antimicrobial agents.

2. Materials and Methods

Clostridial strains:

Morphologically and biochemically identified 42 field isolates of C. perfringens that isolated from apparently healthy, diseased and dead weaned rabbits as well as their feed and water were kindly obtained from Poultry and Rabbits Diseases Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

Determination of toxigenic strains of Clostridium spp. by intravenous (I/V) inoculation in Swiss mice:

This test was carried out according to the method of Mariano et al., (2007). From recovered toxigenic isolates of C. perfringens, about 5ml of 24 hrs cooked meat cultures of toxigenic strains of C. perfringens was added to 50 ml of toxin production medium for types A, B and C and incubated for 5-6 hrs. Another 5ml from the same 24 hrs cooked meat cultures was added to 50 ml of toxin production medium for types D and E, incubated for 48 hrs., then trypsinized to a final concentration of 0.1% and then incubated at 37°C for an hr. The cultures were centrifuged at 3000 rpm for 20 min. and 0.1 ml from the clear supernatant fluid was I/V inoculated in the tail vain of each of Swiss mouse. Mice were kept under observation for 48 hr. If the mice died during 48 hrs observation period, it will consider as highly toxigenic C. perfringens.

Nagler's test by half antitoxin plate:

This test was carried out according to the method of Smith and Holdman, (1968). It was done by spreading C. perfringens types A, B, C, D and E antitoxin separately on half of egg yolk agar plate and allowed to dry in incubator for half an hr. The suspected colonies were streaked across the plate starting from the half of plate without antitoxin and ending to side containing antitoxin. The plate was incubated anaerobically at 37ºC for 24 hrs. The release of alpha toxin that produced by all types of C. perfringens on lecithin was inhibited by the alpha antitoxin. In positive cases; opalescence should be clear on the side of the plate without antitoxin.

Typing of C. perfringens toxins by dermonecrotic test in albino Guinea pigs:

Preparation of the toxins was done according to Bullen, (1952). Application and interpretation of the test in albino Guinea pigs were adopted after Oakley and Warrack, (1953) and Quinn et al., (2002).

Toxin antitoxin serum neutralization test (SNT) in Swiss mice:

It was done as the method described by Smith and Holdman, (1968).

All the experiments on animals such as inoculation in Swiss mice, dermonecrotic reaction in albino Guinea pigs and toxin antitoxin SNT in Swiss mice was carried out according to the National regulations on animal welfare and Institutional Animal Ethical Committee (IAEC).

Genotyping of C. perfringens using polymerase chain reaction (PCR):

Identified pure colonies of C. perfringens were grown over night in 5 ml brain heart infusion broth supplemented with 1% sodium thioglycolate at 37°C under anaerobic condition. The process of DNA extraction was done as Sheedy et al., (2004). The concentration of DNA in µg /ml was measured at 260 and 280 nanometer (nm) by ultra-violet spectrophotometer and then the ratio of 260/280 was calculated. Pure DNA should have ratio of >1:8 that contamination with protein resulted in a significantly lower value. The DNA solution was kept at -20° C

until used. DNA samples were amplified according to the method of Tong and Labbe, (2003). Primers used for conventional and multiplex PCR was showed in Tables (1 and 2). DNA samples were amplified according to the method of August ynowicz et al., (2000). Cycling program of PCR was performed in the thermal cycler as in Tables (3 and 4). Specific amplicons were observed under ultraviolet trans illumination, compared with the marker and photographed by a digital camera.

Table (1): Primer of alpha toxin gene of C. perfringens used in conventional PCR

Primer name and direction	Nucleotide sequence
cpa: Forward Reverse	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG`

Cpa: C. perfringens alpha toxin

Table (2): Primers for the four toxins genes of C. perfringens used in multiplex PCR

Primer name and direction	Nucleotide sequence
cpa: Forward Reverse	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG`
cpb: Forward Reverse	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC`
etx: Forward Reverse	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC
iA: Forward Reverse	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG`

Cpa: C. perfringens alpha toxin, Cpb: C. perfringens beta toxin,
etx: C. perfringens epsilon toxin, iA: C. perfringens iota toxin

Table (3): PCR cycling protocol for alpha gene of C. perfringens using conventional PCR

Amplified DNA	Initial denaturation	Actual cycles	Final extension	Amplified product size (bp)
C. perfringens (alpha) toxin gene	94° C for 5 min.	35 cycles of : Denaturation: 94° C for 1 min. Annealing: 53° C for 1 min. Extension: 72° C for 1 min.	72° C for 5 min.	cpa: 324

Table (4): PCR cycling protocol for four toxins genes of C. perfringens using multiplex PCR

Amplified DNA	Initial denaturation	Actual cycles	Final extension	Amplified product size (bp)
C.perfringens toxin genes (alpha, beta, epsilon and iota)	94° C for 3 min.	30 cycles of : Denaturation: 94° C for 1 min. Annealing: 55° C for 1 min. Extension: 72° C for 1 min.	72° C for 5 min.	cpa: 324 cpb: 196 etx: 655 iA: 446

In vitro antibiotic sensitivity test:

The in-vitro antibiotic sensitivity test of single or mixed types of C. perfringens using disc diffusion technique was applied to according National Committee for Clinical Laboratory Standards (NCCLS), (1998).

3. Results and Discussion

Enteric diseases in weaned rabbits constitutes a great problem resulted in high morbidity and mortality as well as growth depression. ***C. perfringens*** is a widely occurring pathogenic bacterium in enteric diseases of domestic rabbits and its pathogenicity comes from the production of potent exotoxins (***Romero* et al., *2011).***

Since ***C. perfringens*** represents an intestinal commensal organism (Petit *et al.,* 1999), so we should differentiate between toxigenic and non toxigenic ones. Table (5) revealed the results of the prevalence of toxigenic and non- toxigenic types of ***C. perfringens*** recovered from surveyed rabbits and from feed and water at different Egyptian governorates. A total of 35 ***C. perfringens*** isolated from rabbits that previously identified morphologically and biochemically were subjected for determination of toxigenicity by I/V inoculation in Swiss mice. The results revealed that 34

out of 35 (97.14%) of ***C. perfringens*** were toxigenic, while 1.0 (2.86%) was none. The obtained results nearly agree with that proved by Abdel-Rahman *et al.*, (2006) who recorded that the incidence of toxigenic ***C. perfringens*** was 81.82% while, the non toxigenic ones was 18.18%. On the other hand, Mostafa, (1992) recovered 54.1% toxigenic ***C. perfringens*** and 45.9% non toxigenic ones. Moreover, Heba, (2010) found 67.3% and 32.7% toxigenic and non toxigenic ***C. perfringens***, respectively. The differences in the recovery percentages may be attributed to the difference in samples types or the status of the host.

Four out of 6 (66.66%) ***C. perfringens*** isolates from feed was toxigenic and 2 (33.33%) isolates was non-toxigenic. The only isolated ***C. perfringens*** isolate (100%) from water was toxigenic. This result accord with that recorded by Heba, (2010) who isolated ***C. perfringens*** from feed and water samples obtained from surveyed rabbit farms.

Recovered ***C. perfringens*** isolates from rabbits, feed and water were typed serologically using Nagler's test, dermonecrotic test in albino Guinea pigs and toxin antitoxin SNT in Swiss mice.

The results revealed that 34 out of 35 ***C. perfringens*** isolates from rabbits were positive for Nagler's reaction which appeared as opalescence on the plate's side without antitoxins indicating the toxigenicity of these isolates, whereas one isolate showed clear zone representing the non toxigenicity of that isolate (Fig. 1). The toxigenic 34 ***C. perfringens*** strains exhibited clear zone versus to side of the plate with antitoxin indicating that these strains produce alpha toxin which is secreted by all ***C. perfringens*** types. That result cleared a preliminary serological typing of the isolated ***C. perfringens***. Many investigators used Nagler's reaction as a serological tool for testing the toxigenicity and typing of ***C. perfringens*** only (Smith, 1955; Hartwigk and Ghenitir, 1969; Mostafa, 1992; Heba, 2010).

Dermonecrotic reaction in albino Guinea pigs was used for differentiation of different ***C. perfringens*** types. The results demonstrated that 17 out of 35 isolates of ***C. perfringens*** were single type (48.57%) which were as follow; 8 type (A) (22.85%) as they showed irregular areas of yellowish to green necrosis and the lesions tend to spread downward (Fig. 2A), 3 type (B) (8.57%) as they induced purplish yellow hemorrhagic necrosis (Fig. 2B), 4 type (D) (11.43%) as they showed circular white necrosis which were fully developed within in 24 hrs surrounded by small areas of purplish hemorrhagic necrosis (Fig. 2C) and 2 type (E) (5.71%) as they revealed irregular purplish hemorrhagic necrosis (Fig. 2D). In addition, 17 isolates were mixed types representing 11 types (A and D) (31.42%), 2 types (A and E) (5.71%) and 4 types (B and D) (11.42%). Finally, one isolate proved to be non toxigenic as it did not induce any reaction in Guinea pigs' skins. Similarly, Singh and Malik, (1968) revealed that out of 18 ***C. perfringens*** strains, 4 were type A, 2 were type C, 5 were type E and 4 were non toxigenic, also Mostafa, (1992) used dermonecrotic reaction for typing of ***C. perfringens*** and found types A, B, D and E, respectively at percentages of 15.65, 4.45, 8.89 and 71.11%, while mixed types (A and D) was isolated in percentage of 5%. Furthermore, Heba, (2010) isolated ***C. perfringens*** types A and D with percentages 47.3 and 20.0%, respectively using the same test.

As well, 4 toxigenic ***C. perfringens*** feed isolates were 3 type (A) (50%) and 1.0 type (D) (16.6%), while the toxigenic water strain was type A (100%) (Table, 6).

Single 17 toxigenic ***C. perfringens*** were examined serologically using toxin antitoxin SNT in Swiss mice. The results showed that all mice inoculated with each toxin and its corresponding antitoxin as well as negative control ones were still a life after 3 days observation period, while positive control mice were died within 24 hrs. The results of ***C. perfringens*** typing which obtained by SNT in Swiss mice were identical and confirmatory to the results of dermonecrotic reaction in albino Guinea pigs as reported by Rosskopf *et al.,* (2004) who applied toxin neutralization test in mice to confirm ***C. perfringens*** types B and D.

The PCR techniques in this study were used for as a recent, rapid and an accurate diagnostic tool for detection and typing of ***C. perfringens*** (Uzal *et al.*, 1997). Four ***C. perfringens*** types A, B, D and E were subjected for molecular confirmation of ***C. perfringens*** and its common toxin (alpha) using conventional PCR. The results illustrated that all tested types were positive for Cp alpha toxin at (324 bp) (Fig., 3A).

Multiplex PCR is a protocol for genotyping of ***C. perfringens*** as a reliable and specific test for detection of ***C. perfringens*** toxin genes alpha (Cpa), beta (Cpb), epsilon (etx), iota (iA), enterotoxin (Cpe) and beta-2 toxin (Baums *et al.*, 2004), so we used that technique as a final step for molecular typing of ***C. perfringens*** and its toxins. The results of multiplex PCR demonstrated that ***C. perfringens*** type (A) was positive for Cp alpha toxin at (324 bp), ***C. perfringens*** type (B) was positive for Cp alpha toxin at (324 bp), beta toxin at (196 bp) and epsilon toxin at (655 bp), ***C. perfringens*** type (D) was positive for Cp alpha toxin at (324 bp) and epsilon toxin at (655 bp) and ***C. perfringens*** type (E) was positive for Cp alpha toxin at (324 bp) and iota toxin at (446 bp) (Fig. 3B). Parallel results were found by Songer *et al.*, (1993); Songer and Ralph, (1996); Augustynowicz *et al.*, (2000) and Piatti *et al.*, (2004) who developed a PCR assay for detecting of ***C. perfringens*** alpha toxin gene which gave a characteristic band at 324 bp, however, many authors as Fach and Guillou, (1993); Augustynowicz *et al.*,

(2002); Eman *et al.*, *(*2006) and Heba, (2010) recorded that ***C. perfringens*** type A contained alpha toxin gene which gave a characteristic band at 1167 bp. That difference between our results and the others attributed to usage of different olignucleotide primers. The results of molecular typing of different ***C. perfringens*** types and its toxins accurately cleared the toxin type which is disagree with that reported by Wang, (1985) and Percy *et al.*, (1993) who mentioned the absence of information on the toxin type. This disagreement may be related to the advances in application of PCR techniques as a recent, rapid, reliable and an accurate method for typing of ***C. perfringens*** types and its toxins.

Most ***C. perfringens*** strains carrying the enterotoxin gene (Cpe) are classified as type A isolates. The Cpe gene can be present on either the chromosome or on a large plasmid (Collie and McClane, 1998). Discovered by Gibert *et al.*, (1997), toxin b2 is encoded from the Cpb2 gene, carried on plasmid. The Cpb2 gene can be found in all ***C. perfringens*** toxin types (Bueschel *et al.*, 2003).

Table (7) summarized the distribution of different types of isolated ***C. perfringens*** among surveyed rabbit's farms at different Egyptian governorates. The results indicated that, the total number of single type, mixed types as well as, non toxigenic type of ***C. perfringens*** was 35 representing 12 (Giza), 6 (El-Qaliubiya), 3 (El-Sharkia), 3 (Cairo), 3 (Port-Said), 3 (El-Fayoum), 3 (El-Menoufia) and 1 (Beni Suef). Reviewing available literatures, there was no recorded data about the distribution of different ***C. perfringens*** types at different Egyptian governorates.

The *in-vitro* sensitivity of the most prevalent toxigenic types of ***C. perfringens*** that recovered from surveyed rabbit's farms at different governorates of Egypt to different antibiotics was tabulated in Table (8). All types of ***C. perfringens*** were highly sensitive for amoxicillin/clavulanic acid and ampicillin. Strains of ***C. perfringens*** types (E, A and E), (A and E) and (B and D) showed sensitivity to nalidixic acid. Also, all types of ***C. perfringens*** revealed intermediate sensitivity to enrofloxacin, doxycline and penicillin, whereas resistance to colistine, erythromycin and lincomycine. These results are nearly similar to these recorded by Secasiu and Pastarnac, (1993) who tested the *in-vitro* the sensitivity of 78 strains of ***C. perfringens*** isolated from rabbits to some antimicrobial drugs and found that, they were sensitive to penicillin and ampicillin, Abdel-Rahman *et al.*, (2006) who observed that ***C. perfringens*** isolated from diarrheic rabbits was sensitive to ampicillin and resistant to gentamycin, Agnoletti *et al.*, (2010) who detected that rabbit's ***C. perfringens*** isolates were sensitive to tylosin and oxytetracyclin and Heba, (2010) who demonstrated that ***Clostridial*** isolates from diarrheic rabbits were highly sensitive to penicillin and tylosin. On the other hand, our results disagree with Mostafa, (1992) who reported sensitivity of ***Clostridial*** spp. to colistin sulphate and sulphaquinoxaline/ trimethoprim and resistance to penicillin and tetracycline, Abdel-Rahman *et al.*, (2006) who found that ***C. perfringens*** isolates were resistance to gentamicin, Heba, (2010) who recorded on resistance of ***Clostridial*** strains to nalidixic acid, gentamycin, ampicillin, oxytetracycline and doxycycline and Catalán *et al.*, (2010) who observed that strain H28 ***C. perfringens*** showed resistance to penicillin, ampicillin and tetracycline. This disagreement with other Egyptian investigators may be due to the hazardous usage of different antimicrobial agents, the difference of surveyed governorates and the time of surveillance.

Table (5): Prevalence of toxigenic and non toxigenic types of *C. perfringens* recovered from surveyed rabbits farms as well as from feed and water samples at different Egyptian governorates

No. of recovered *C. perfringens*		Toxigenic *C. perfringens*		Non-toxigenic *C. perfringens*	
		No.	%	No.	%
examined rabbits	35	34	97.14	1	2.86
feed samples	6	4	66.66	2	33.33
water samples	1	1	100	-	0

Table (6): Prevalence of *C. perfringens* isolated from feed and water samples in examined rabbit's farms

Types of samples	Recovered *C. perfringens*	Non- toxigenic *C. perfringens*		Toxigenic *C. perfringens*		Types of toxigenic *C. perfringens*			
	No.	No.	%	No.	%	A		D	
						No.	%	No.	%
Feed	6	2	33.33	4	66.66	3	50	1	16.6
Water	1	-	0	1	100	1	100	-	-

Table (7): Distribution of different types of *C. perfringens* among surveyed rabbit's farms at different Egyptian governorates

Governorates	No. of farms	non-toxigenic *C. perfringens* isolates		toxigenic *C. perfringens* isolates		*C. perfringens* types						
		No.	%	No.	%	A	B	D	E	A and D	A and E	B and D
Port-said	2	-	0	3	8.57	-	-	1	-	2	-	-
Giza	4	-	0	12	34.28	3	-	2	1	3	1	2
Cairo	2	-	0	3	8.57	1	-	1	-	1	-	-
Beni Suef	2	-	0	1	2.86	-	1	-	-	-	-	-
El-Fayoum	3	-	0	3	8.57	1	-	-	1	1	-	-
El-Qaliubiya	4	1	2.86	6	17.14	1	-	-	-	2	1	2
El-Sharkia	1	-	0	3	8.57	1	1	-	-	1	-	-
El-Menoufia	1	-	0	3	8.57	1	1	-	-	1	-	-
Total	19	1	2.86	34	97.14	8	3	4	2	11	2	4

%= Percentages were calculated according to the total No. of typed *C. perfringens* (35).

Table (8): *In-vitro* antibiotic sensitivity test of different *C. perfringens* types recovered from surveyed rabbit's farms at different Egyptian governorates.

Active principle	C. perfringens types																				
	A			B			D			E			A and E			A and D			B and D		
	R	IS	S	R	IS	S	R	IS	S	R	IS	S	R	IS	S	R	IS	S	R	IS	S
Amoxicillin / Clavulanic acid (2:1)			+			+			+			+			+			+			+
Cloxacillin		+			+		+			+			+			+			+		
Erythromycin	+			+			+			+			+			+			+		
Gentamicin	+				+			+		+			+				+			+	
Oxytetracycline			+		+		+				+		+				+			+	
Penicillin G		+			+			+			+			+			+			+	
Sulphquinoxaline / Trimethoprim		+		+			+				+		+			+			+		
Kanamycine		+		+			+			+			+			+			+		
Enrofloxacin		+			+			+			+			+			+			+	
Doxycycline		+			+			+			+			+			+			+	
Ampicillin			+			+			+			+			+			+			+
Colistine	+			+			+			+			+			+			+		
Lincomycine	+			+			+			+			+			+			+		
Tylosine			+		+			+				+			+			+			+
Tetracycline		+			+		+			+				+			+			+	
Chlorotetracycline		+		+			+			+				+		+				+	
Nalidixic Acid			+	+					+			+			+			+			+
Metronidazole		+		+			+			+			+				+			+	

R: Resistance IS: Intermediated sensitivity S: Sensitivity

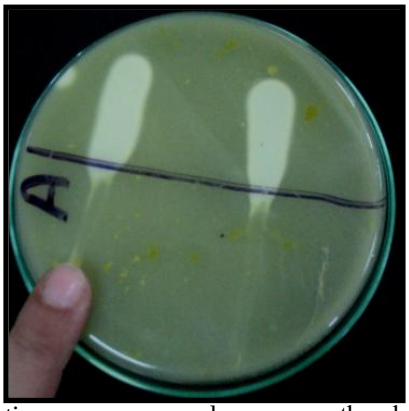

Fig. (1): Positive result of Nagler's reaction appears as opalescence on the plate's side without antitoxin and as clear zone on the another side of the plate with antitoxin

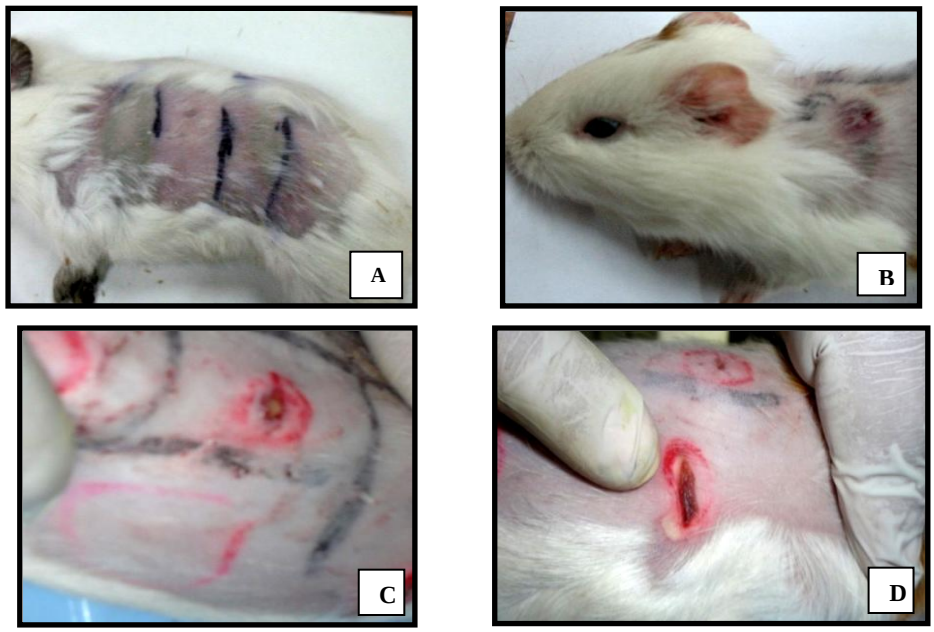

Fig. (2): Results of dermonecrotic reaction in Guinea pigs as the follow; **A:** ***C. perfringens*** type (A) shows irregular areas of yellowish to green necrosis and the lesions tend to spread downward, **B:** ***C. perfringens*** type (B) induces purplish yellow hemorrhagic necrosis, **C:** ***C. perfringens*** type (D) shows circular white necrosis which are fully developed within in 24 hrs surrounded by small areas of purplish hemorrhagic necrosis and **D:** ***C. perfringens*** type (E) reveals irregular purplish hemorrhagic necrosis.

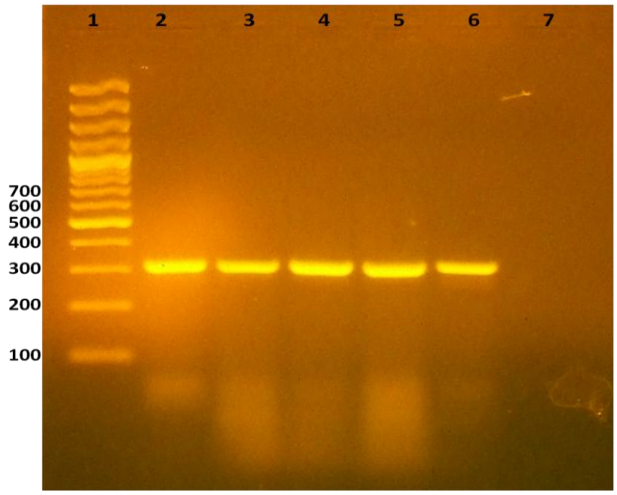

Fig. (3 A): Agarose gel photodocumentation of conventional PCR on genetic material extracted from ***C. perfringens*** types A, B, D and E. ***lane 1,*** molecular weight marker (1kb); ***lane 2-5,*** the samples are positive for Cp alpha toxin (324bp); ***lane 6,*** positive control (alpha) and ***lane 7,*** negative control.

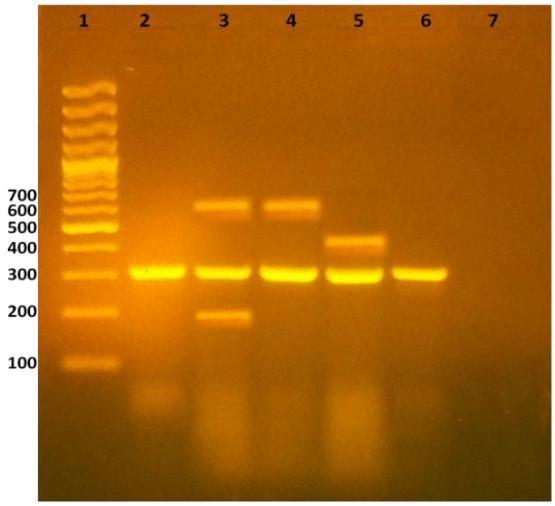

Fig. (3 B): Agarose gel photodocumentation of multiplex PCR on genetic material extracted from ***C. perfringens*** types A, B, D and E. ***lane 1,*** molecular weight marker (1kb); ***lane 2,***CpA sample is positive alpha toxin (324bp); ***lane 3,*** Cp B sample positive is for alpha toxin (324bp), beta toxin (196bp) and epsilon toxin (655bp); ***lane 4,*** Cp D sample is positive for alpha toxin (324bp) and epsilon toxin (655bp); ***lane 5,*** Cp E sample is positive for alpha toxin (324bp) and iota toxin (446bp); ***lane 6,*** is positive control (alpha toxin) and ***lane 7,*** is negative control.

Conclusion

In conclusion, the detection of toxingenicity of the isolated ***C. perfringens*** circulating strains in the Egyptian field is very important as some of them are non toxigenic. Also, accurate, rapid and reliable serological and molecular methods of diagnosis must be applied. It is very important to through light on the role of feed and water as an epidemiological source of infection for early weaned rabbits. Latterly, it is the must to carry out *in vit*ro sensitivity test of rabbits ***Clostridial*** strains to different antimicrobial agents as there are resistance to the common antibiotics used for prevention and control f ***Clostridial*** enteritis in rabbits.

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