Implementation of a rapid procedure for distinguishing enterotoxigenic Clostridium perfringens

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Abstract: The objective of the present study is to develop an easy method for detection of toxigenic *C. perfringens* isolates .4 *C. perfringens* isolates (type A, B, C & D) were collected from chickens and reconfirmed on the basis of conventional tests and multiplex PCR. Antisera were prepared from *C. perfringens* types A, B, C & D separately in different groups of rabbits. The titres of the prepared hyperimmune sera were estimated by ELISA & staphylococci protein A (SpA) agglutination test. An attempt was carried out to detect *C. perfringens* toxins in infected fecal samples. The fecal samples were infected by 20 &40 µg /ml *C. perfringens* toxins (A, B, C & D) and examined by double sandwich ELISA & SpA agglutination methods. In addition the sensitivity of PCR for detection of *C. perfringens* types were compared with conventional culture technique among fecal samples contaminated with *C. perfringens* types and the results were discussed.
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Keywords: *C. perfringens,* ELISA, SpA agglutination, PCR.

1. Introduction

Clostridia are commonly found in the environment, occurring in soil, sewage, and waters, as well as in the intestines of both man and animals. Members of the genus *Clostridium* are widely recognized as enteric pathogens for man, domestic animals and wildlife (Songer, 1996). *Clostridium perfringens*, a part of normal gut flora, is commonly involved in diseases in most domestic animals and some wildlife, including horses, poultry, birds, rabbits, sheep, goats, cattle, mink, ostrich, dogs and cats (Nillo ,1993). Smyth and Martin (2010) recorded that necrotic enteritis is a serious disease of chickens and turkeys caused by *Clostridium perfringens* and demonstrated that *C. perfringens* strains from a mammalian species and from normal chickens, can cause necrotic enteritis in chickens. *Clostridium perfringens* is an important cause of both histotoxic and enteric diseases (Fernandez-Miyakawa *et al.,* 2008). *C. perfringens* type D is the etiological agent of enterotoxaemia (pulpy kidney disease) of several animal species (Blood *et al.,* 1983). According to current knowledge, the disease is caused by epsilon toxin, a major exotoxin produced by this microorganism (Uzal and Kelly, 1996).

Substantial interest and effort have been expended in establishing new methods as well as improving classical methods for the detection and isolation of *C. perfringens*. The objective of the present study is to develop an easy method for typing of *C. perfringens* isolates in the veterinary routine diagnostic laboratory.

2. Materials and Methods

Diagnostic antisera:

Diagnostic *C. perfringens* antisera type A, B, C and D were obtained from Welcome, Diagnostics Dartford, England from Microbiology department, Anaerobic Unit, Abbassia. They were used as control positive among the used serological test.

Identification of *C. perfringens* isolates:

Four isolates of *C.perfringens* type A, B, C and D were collected from chickens. Each isolate was inoculated into freshly prepared Robertson's cooked meat medium (Smith and Holdman, 1968), and incubated anaerobically at 37°C for 24 hrs. Then a loopful from this culture was streaked onto neomycin sulphate sheep blood agar (Carter and Cole, 1990). The streaked inoculated plates were incubated anaerobically at 37°C for 24-48 hrs. The catalase negative colonies were picked up and examined for their morphological, cultural and biochemical characters according to Koneman *et al.* (1992). Biotyping of *C. perfringens* isolates by dermonecrotic reaction in Albino Guinea pigs was carried out according to Stern and Batty (1975).

Multiplex PCR:

Template DNAs for PCR were prepared according to Osek and Winiarczyk (2001). Specific oligonucleotide primers for the toxin genes (alpha (α), beta (β) and epsilon (ε) of *C. perfringens* toxins

were selected on the base of published sequences (Effat *et al*., 2007) as shown in Table (1). The PCR product using 1.5% agarose gel electrophoresis and DNA molecular weight marker of 100 base pair ladder (Bioron GmbH) (Jena Bioscience - Germany) was analyzed according to Sambrook *et al*. (1989)

Table (1): Oligonucleotide primers for the toxin genes α, β and ε of *C. perfringens*

Primer Designation	5'----------3' sequence	Amplified product size (bp)
CP ALPHA toxin F	GTTGATAGCGCAGGACATGTTAAG	402 bp
CP ALPHA toxin R	CATGTAGTCATCTGTTCCAGCATC	
CP BETA toxin F	ACTATACAGACAGATCATTCAACC	236 bp
CP BETA toxin R	TTAGGAGCAGTTAGAACTACAGAC	
CP EPSILON toxin F	ACTGCAACTACTACTCATACTGTG	541 bp
CP EPSILON toxin R	CTGGTGCCTTAATAGAAAGACTCC	

Preparation of hyperimmune sera against *C. perfringens* isolates:

Sixteen healthy rabbits of New Zealand breed, each of 1.5-2 kg body weight were obtained from the farm of "Animal Health Research Institute, Dokki, Giza". They were rest under strict good hygienic conditions during the whole period of experiment and provided with a balanced diet. They proved to be free from clostridial infections. Before beginning of the experiment, 3 ml blood sample from the ear vein of each rabbit was taken and the serum was separated and kept as non-infected control negative sample and kept at -20°C. The rabbits were classified into 4 groups, each group contains 4 rabbits. The first group was injected with *C. perfringens* type A, the second group was injected with *C. perfringens* type B, the third group was injected with *C. perfringens* type C and the fourth group was injected with *C. perfringens* type D. Each animal of each group was inoculated with 18 hrs culture of *C. perfringens* grow in medium containing 2% (w/v) polypeptone (Daigo, Osaka, Japan), 1% (w/v) glucose, 0.5% NaCl (pH 7.2) and heated at 100°C for 60 min. The immunization was carried out by injection of 0.5, 1.0, 1.0, 1.0, 1.5, 2.0, 2.0, 3.0, 3.0 and 3.0 ml of the suspension, respectively into ear vein of a rabbit at 3 day intervals (Yamagishi *et al.*, 1971). The sera of inoculated rabbits were collected and kept at 20°C till use.

Preparation of toxin:

C.perfringens types A, B, C and D were cultivated in cooked meat broth anaerobically at 37°C for 24 hrs. Each one of *C. perfringens* type A, B, C and D was inoculated into toxin production medium (Roberts *et al.,* 1970) and incubated for 5 hrs except type D incubated for 48 hrs. Centrifugation for type A, B, C and D and the supernatant was taken. Trypsin was added to supernatant of type D and incubated for 1 hour. Dialysis was used for each toxin to remove small molecular contaminants according to Judson *et al.* (1987). The dialyzed toxin inside the dialysis bags were covered with polyethylene glycogen and left at 4°C to avoid protein denaturation until the solutions were concentrated to 5 ml (volume) in each bag. The bags were washed with distilled water and the solutions were collected in tubes and then deposited in a lyophilizer to concentrate the volume to 0.5 ml in each sample. The concentrated toxins were stored at -20°C till use. The protein content of 4 toxins of *C. perfringens* type A, B, C and D were measured using the modified Lowry's assay according to Lowry *et al.* (1951).

Antibody assay (titration):

Titration of the prepared hyperimmune sera was estimated by ELISA and SpA agglutination test.

Enzyme linked immunosorbent assay (ELISA):

Indirect ELISA was used to detect the antibodies against toxins of *C. perfringens* type A, B, C and D according to Harlow and Lane (1988). The sera were considered to be positive when the absorbency values were as or more than the cut-off value (the cut-off = double fold of the mean negative sera) according to Timmerck (1994).

SpA agglutination test (Subramanayam *et al.*, 2000):

A Cowan 1 strain of *S. aureus* (It was obtained in freeze lyophilized dried ampoules from the Namru 3 in Egypt) was spread over on brain heart infusion agar medium (Oxoid). Incubation was carried out at 37°C for 48 hrs, bacterial growth was scooped by a bent glass rod with PBS pH 7.2 into test

tubes. The cells were centrifuged at 2000 rpm for 15 minutes at room temperature. The cells were treated overnight with formalin to make a final concentration of 2% to inactivate the cells. Then the *S. aureus* cells were treated at 80°C for 5 minutes in a water bath. The cells were cooled immediately by immersing in an ice bath. Then cells were washed five minutes with PBS, pH 7.2 and 10% of *S. aureus* suspension was prepared in PBS. 2 folds serial dilution of antitoxins were added in U-shaped microtiter plate (25 µl/well). Amount of 25 µl of the prepared 10% SpA suspension was then added to each well and incubated at 37°C for 1 hr. with periodical shaking. 25 µl *of C. perfringens* toxin prepared in PBS were added to SpA mixture then incubated at 4°C. Agglutination was read within 2 hrs.

Detection of *C. perfringens* in fecal samples:

The prevalence of *C. perfringens* in feces was determined by selective culture, PCR, SpA and ELISA to permit validation of the ELISA, SpA and PCR.

Artificially contaminated fecal rabbit samples of enterotoxin for *C. perfringens* type A, B, C and D separately were prepared by adding 0.1 ml of toxin at concentration of 20 µg and 40 µg separately to 1 g of feces and 9 ml of water and centrifugation for 5 min at 500 xg. The double sandwich ELISA was performed according El Idrissi and Ward (1992) in 96-well disposable flat-bottomed plates to detect *C. perfringens* toxins in contaminated feces. As well as, SpA agglutination test (Subramanayam *et al.*, 2000) used for detection of *C. perfringens* toxins in the contaminated feces.

Sensitivity of the multiplex PCR

The sensitivity of the multiplex PCR assay for detection of *C. perfringens* in feces was conducted according to Kanakaraj *et al.* (1998). Artificially contaminated fecal rabbit samples of *C. perfringens* A, B, C and D were prepared by adding 0.1 ml of a 12 hrs BHI broth culture to 1 g of feces and 9 ml of water then centrifuged for 5 min at 500 xg. One ml of the supernatant was serially diluted in sterile water. From 0.8 ml aliquots of each dilution DNA template was extracted (Stahl *et al.*, 1988) and counts were performed by plating 100 µl aliquots on TSC agar.

The sensitivity of the PCR assay was calculated as the number of colony forming units (CFU) of *C. perfringens* in the greatest dilution which was PCR positive.

3. Results and Discussion

C. perfringens type A (α-toxin producer) is common in the intestinal tract of chicks, soil, dust-contaminated feed and litre (Kalender and Ertafi, 2005). The α-toxin (α) the principal lethal toxin of *C. perfringens* is a multifunctional phospholipase produced by nearly all isolates. The toxin is haemolytic, necrotizing and potently lethal. Detection of *C. perfringens* toxin types is critical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the development of effective preventive measures.

In the present study, 4 *C. perfringens* isolates (type A, B, C & D) were collected from chickens and reconfirmed on the basis of morphological, cultural and biochemical characteristics. All isolates were Gram-positive spore-forming bacilli as shown in Photo (1). Colonies of *C. perfringens* are up to 5 mm in diameter, circular, flat, greyish and surrounded by zone of double haemolysis after cultured anaerobically on blood agar at 37 °C for 48 hrs (Photo.2). The biochemical behaviour of the isolates complies with these of *Clostridium* typical reactions as described by (Quinn *et al.*, 2002). Toxins of *C. perfringens* isolates were detected using inoculation of guinea pig to detect necrosis as shown in photo. (3)

The collected strains were investigated for production of toxins by PCR. This test was established to replace animal testing and to reduce cost and time. Rapid detection of enterotoxigenic *Clostridium perfringens* in meat samples was accomplished by Yang et al. (2010) with an immunomagnetic separation polymerase chain reaction (IMS-PCR). Photo (4) shows amplification of alpha toxin gene (402 bp) from all isolates & amplification of beta toxin gene (236 bp) from *C. perfringens* type B & C, while epsilon toxin gene (541 bp) was recorded in *C. perfringens* type D. The α-toxin is present in all types of *C. perfringens* and lies within the chromosome of the bacterial DNA (Albini *et al.,* 2008). Molecular typing allowed for an easier in vitro test (Effat *et al*., 2007).

The development of a useful serological test is dependent upon the identification of defined antigens and the examination of the antibody responses to these antigens. Therefore, antisera were prepared from *C. perfringens* types A, B, C & D separately. Serum samples were collected from rabbits after inoculations with the isolates and the antibody titre against homologous isolate was estimated by ELISA & SpA agglutination test as shown in Tables (3&4). Specificity and avidity of antibody binding to target antigen is critical for the success of antibody-based pathogen detection methods (Bhunia, 1997).

The optical density (OD) of ELISA among the prepared hyperimmune sera was estimated as shown in Table (2) after application of checkerboard titration. It is clear that, positive ELISA OD 0.522, 0.687, 0.504 and 0.534 were recorded among the

prepared hyperimmune sera of *C.perfringens* type A, B, C, and D diluted to 1:800, 1:1600, 1:800 and 1:1600. Using an enzyme-linked immunosorbent assay (ELISA) for measuring levels of specific antibodies against alpha-toxin Heier *et al.*(2001) found a variation in level of maternal antibodies against α-toxin in broilers. The variation in antibody levels between broiler flocks of different origin indicated that some parent flocks has raised an antibody response to naturally-occurring alpha-toxin. If broiler chickens are able to mount a similar response, this response may have value for protection against the disease, as well as diagnostic value to reveal subclinical disease in broiler flocks (Lovland *et al.,* 2003).

In the present study, SpA agglutination test was used for estimation of *C. perfringens* antibodies among the prepared hyperimmune sera. Coagglutination is similar to latex agglutination technique for detecting antigen protein A, a uniformly distributed cell wall component of *S. aureus* is able to bind to the Fc region of most IgG isotype antibodies leaving Fab region free to interact with antigens present in the applied specimens. The visible agglutination of *S. aureus* particles indicates the antigen-antibody reaction. Data present in Table (3) illustrated that positive coagglutination antibody titers could be detected at 1/4 up to 1/64. 1/16 was used as a CoA reagent for various tests in 12 studies and the results obtained are in agreement with Rahman *et al.* (1989) in *Salmonella* enterotoxin system and with Batra *et al.* (1989) in *Brucella* antigens.

Cross reactivity in vitro was tested to figure out the possible diagnosis of *C. perfringens* using concentrations of 20 µg /ml toxins (types A, B, C and D). In this study cross reactivity between *C. perfringens* types was tested as shown in Figures (1-5) using ELISA. These cross reactivity may be due to use of crude toxins which contain α-toxins among all types. α-toxin, a necrotizing toxin commonly produced by all five types of *C. perfringens*, is believed to be a major factor responsible for the organism tissue pathology and has been suggested to be a key virulence determinant and predominant product of *C. perfringens* type A (Scott *et al.,* 2004). Also Effat *et al.* (2007) revealed that α-toxin gene is found in all types of *Clostridium perfringens*

An attempt was carried out to detect *C. perfringens* in infected feces using ELISA, coagglutination test & PCR assay. Fecal samples were infected by 20 &40 µg /ml toxins (A, B, C & D). The infected samples were examined by double sandwich ELISA methods as shown in Tables (4-7). It is clear that 20 µg /ml is a concentration of choice. The ELISA method of Vaikosen and Ikgatua (2005) was able to detect as low as 0.1 µg /ml of enterotoxin corrected optical density (OD) at 405nm, the value of 0.4 OD units was used to estimate the enterotoxin levels of the isolates, and mean value of 0.66 OD units for *C. perfringens* type D, while the mean value of 0.94 OD units for *C. perfringens* type C were obtained. The detection of *C. perfringens* in feces of horses by ELISA could be diagnostically beneficial in a clinical setting (Waggett *et al.,* 2010). Using specific antibodies for beta and epsilon toxins of *C. perfringens*, two double sandwich ELISAs were developed for detection of these toxins in buffers, culture supernatants and intestinal contents by El Idrissi and Ward (1992). In both assays absorbance readings were directly related to the Log_{10} of the toxin concentration over 3-4 points between 31 and 250 ng/ml for beta toxin and between 8 and 125 ng/ml for epsilon toxin. The reason for this difference may be due to we used crude toxins as antigens while El Idrissi & Ward (1992) used a purified activated epsilon toxin, which may have increased both the specificity and sensitivity of the test.

As shown in Tables (8-11) the infected fecal samples were analyzed by SpA agglutination test. The epsilon toxin was detected in five samples by coagglutination and mouse neutralization tests by Subramanayam *et al*. (2000), these samples were made by serial two-fold dilutions form 1/2 to 1/32. Previously Dobosch (1983) describe staphylococcal coagglutination procedure for assaying *C. perfringens* enterotoxin; its sensitivity and specificity were studied. *C. difficile* A and B toxins and *C. perfringens* type A enterotoxin was studied by Giulazian *et al*. (2008) employing the immunological test systems in the coagglutination test using the plates.

The sensitivity of PCR for detection of *C. perfringens* types were compared with conventional culture technique. As shown in Table (12) and photos.(5-8) *C. perfringens* could be isolated onto medium only from 10^3 dilution, meanwhile *C. perfringens* toxins could be detected from all dilutions of different *C. perfringens* types by PCR assay. PCR provides a simple and rapid assay for detection of *C. perfringens* under condition where the current levels of sensitivity and specificity are acceptable; it should be immediately useful in epidemiologic and diagnostic studies.

In conclusion, the *C. perfringens* toxin can be detected instantaneously (within 4 minutes) on a slide by SpA agglutination test. Moreover, this test requires minimum number and amounts of reagents which could be presented and used in the form of portable diagnostic kit for use in the farm premises. The results demonstrate the suitability and reliability of multiplex PCR for the routine diagnostic

laboratory. The procedure will improve the diagnosis of food-borne intoxications and will help to discover further epidemiological and aetiological aspects of these diseases.

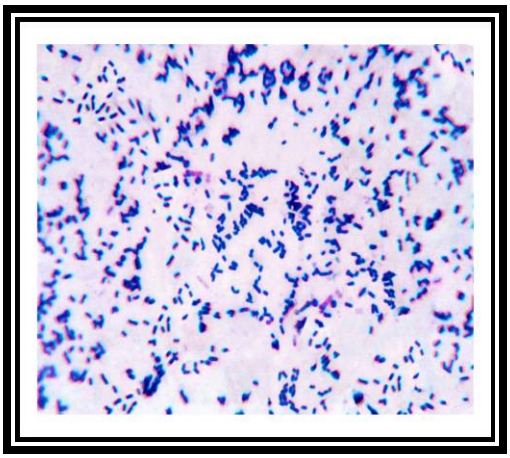

Photo (1): *C. perfringens* showed Gram-positive bacilli

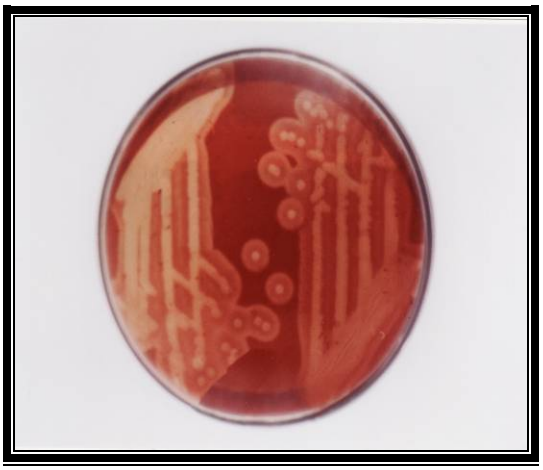

Photo (2): Double zone of hemolysis showed by *C. perfringens* grown on sheep blood agar.

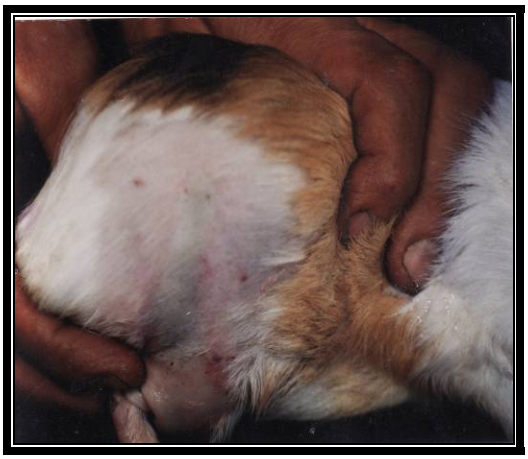

Photo (3): The dermonecrotic reaction of *C. perfringens* toxin type A

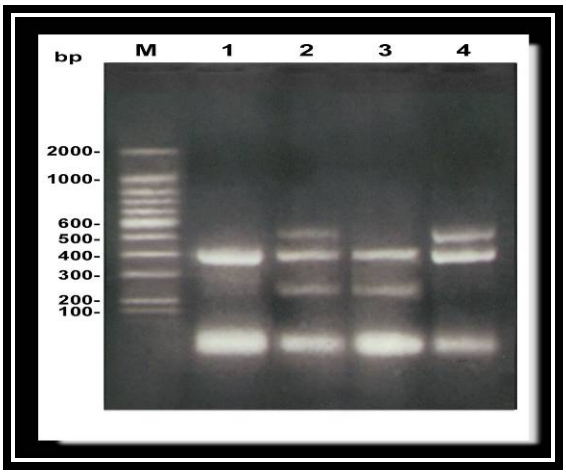

Photo (4): Amplification of *cpa* (402 bp), *cpβ* (236 bp) & *etx* (541 bp) genes from *C .perfringens* isolates using Multiplex PCR. Lane M:100 bp adder, Lane1: *C.perfringens* type A, Lane2*: C. perfringens* type B, Lane 3: *C. perfringens* type C, Lane 4: *C. perfringens type* D.

Table (2): Results of ELISA absorbance values among the prepared hyperimmune sera using *C. perfringens* toxin types A, B, C and D (20ug/ml).

Dilution of antisera	*C. perfringens* toxin types			
	A	B	C	D
1/50	0.851	1.521	0.980	1.022
1/100	0.811	1.103	0.850	0.972
1/200	0.742	0.950	0.801	0.842
1/400	0.651	0.870	0.622	0.685
1/800	0.522	0.742	0.504	0.601
1/1600	0.434	0.687	0.475	0.534

+ve OD≥ 0.5

Table (3) Results of SpA among the prepared hyperimmune sera using *C. perfringens* toxin types A, B, C and D (20 ug/ml).

Dilution of antisera	*C. perfringens* toxin types			
	A	B	C	D
1/4	+	+	+	+
1/8	+	+	+	+
1/16	+	+	+	+
1/32	+	+	+	+
1/64	+	+	+	+

Table (4): Results of ELISA among fecal sample infected with 40µg/ml and 20 µg/ml of *C. perfringens* toxin type A.

***C. perfringens* toxin type A**								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/800	0.792	0.530	0.623	0.369	0.560	0.301	0.523	0.286
1/1600	0.498	0.430	0.422	0.275	0.356	0.199	0.301	0.136

Table (5): Results of ELISA among fecal sample infected with 40 µg/ml and 20 µg/ml of *C. perfringens* toxin type B.

***C. perfringens* toxin type B**								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/800	0.560	0.329	0.880	0.721	0.602	0.301	0.591	0.276
1/1600	0.305	0.225	0.476	0.435	0.376	0.170	0.322	0.122

Table (6): Results of ELISA among fecal sample infected with 40 µg/ml and 20 µg/ml of *C. perfringens* toxin type C.

***C. perfringens* toxin type C**								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/800	0.512	0.310	0.690	0.331	0.700	0.495	0.430	0.272
1/1600	0.318	0.154	0.350	0.207	0.465	0.309	0.301	0.120

Table (7): Results of ELISA among fecal sample infected with 40µg/ml and 20 µg/ml of *C. perfringens* toxin type D.

C. perfringens toxin type D								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/800	0.523	0.312	0.602	0.414	0.530	0.295	0.834	0.552
1/1600	0.307	0.187	0.381	0.255	0.337	0.115	0.442	0.399

Table (8): Results of SpA test among fecal sample infected with *C. perfringens* toxin type A.

C. perfringens toxin type A								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/32	+	+	+	-	-	-	-	-
1/64	+	-	+	-	-	-	-	-

Table (9): Results of SpA test among fecal sample infected with *C. perfringens* toxin type B.

C. perfringens toxin type B								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/32	-	-	+	+	+	-	-	-
1/64	-	-	+	-	-	-	-	-

Table (10): Results of SpA test among fecal sample infected with *C .perfringens* toxin type C.

C. perfringens Toxin type C								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/32	-	-	+	+	+	+	-	-
1/64	-	-	-	-	-	-	-	-

Table (11): Results of SpA test among fecal sample infected with *C. perfringens* toxin type D.

C. perfringens Toxin type D								
Dilution of antisera	A		B		C		D	
	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml	40 µg/ml	20 µg/ml
1/32	-	-	+	-	-	-	+	+
1/64	-	-	-	-	-	-	-	-

Table (12): Sensitivity of the PCR assay for detection of *C. perfringens* toxins* in feces

different dilution of Feces infected with *C. perfringens* type A	Colony count on TSC agar	PCR
10^3	1-2 colonies	+ve
10^4	–	+ve
10^5	–	+ve
10^6	–	+ve

* *C. perfringens* toxin types A, B, C or D
TSC agar: trypticase soy agar.

Photo (5): Amplification of *Cpa* (402 bp) from feces infected with *C. perfringens* type A by multiplex PCR. Lane M: 100 bp ladder, Lanes1, 2, 3&4 *C. perfringens* type A (10^3, $10^{4,}$ 10^5 & 10^6 dilutions respectively).

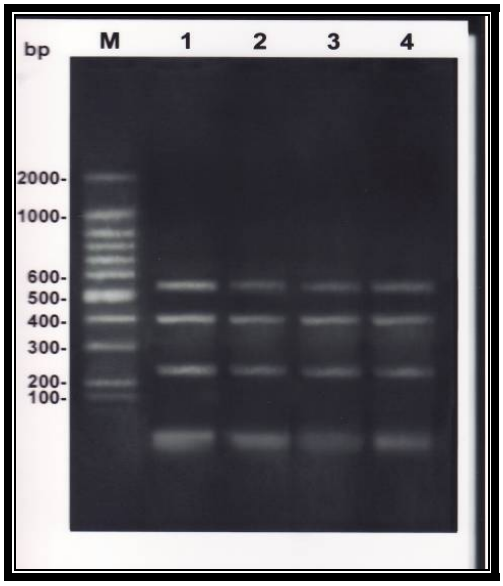

Photo (6): Amplification of *Cpa* (402 bp), *cpβ* (236 bp) and *etx* (541bp) genes from feces infected with *C. perfringens* type B by multiplex PCR. Lane M: 100 bp ladder, Lanes1, 2, 3&4 *C. perfringens* type B (10^3, $10^{4,}$ 10^5 & 10^6 dilutions respectively)

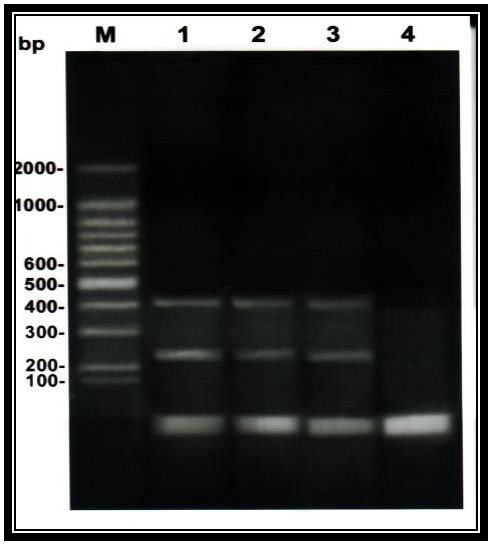

Photo (7): Amplification of *cpa* (402 bp) and *cpβ* (236 bp) genes from feces infected with *C. perfringens* type C by multiplex PCR. Lane M: 100 bp ladder, Lanes1, 2 and 3 *C.perfringens* type C (10^3, 10^4 & 105 dilutions respectively).

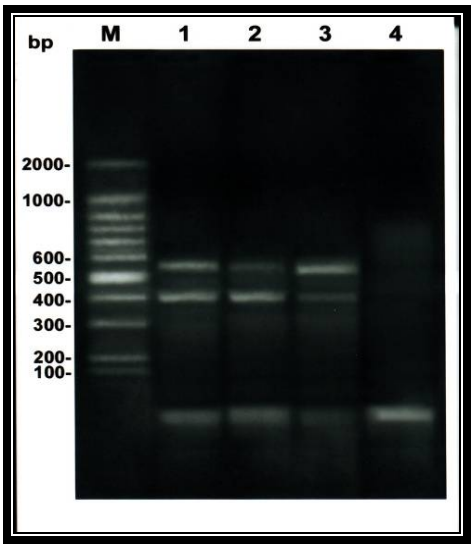

Photo (8): Amplification of *cpa* (402 bp) and etx (541 bp) genes from feces infected with *C. perfringens* type D by multiplex PCR. Lane M: 100 bp ladder, Lanes 1, 2 and 3 *C. perfringens* type D (10^3, 10^4 & 10^5 dilutions respectively).

References

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