# Detection of Alpha Toxin Gene of *Clostridium perfringens* Type-A Isolated from Diseased Rabbits and Chicken by Polymerase Chain Reaction

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## Abstract

***C.perfringens* strains which isolated from chickens (necrotic enteritis) and rabbits (enterotoxaemia) were typed using mice serum neutralization test (SNT) as biological assay *in vivo* and by PCR method as a molecular technique *in vitro*. It was found that all toxigenic *C.perfringens* isolates were belonged to serotype A and it was the dominant type, and the alpha toxin is considered to be a key virulence determinant in necrotic enteritis in chickens and enterotoxaemia in rabbits. Comparison of the PCR data with results obtained by SNT showed good correlation. PCR proved to be efficacious, accurate, sensitive, specific, faster and superior test than SNT. The results of PCR technique indicated that the amplified DNA products of *C.perfringens* isolates from rabbits and chicken were in the same molecular size.**

## Introduction

*C.perfringens* is the most widely occurring pathogen, it is commonly found in the gastrointestinal tract of birds and mammals as well as in the soil ( **Smith (1975) and Willis (1984)**. *C.perfringens* isolates are classified into five toxigenic types (A, B, C, D and E) on the basis of production of four major lethal toxins; alpha, beta, epsilon and iota **(McDonel, 1986)**, and each of these toxin types is responsible for a specific disease syndrome **(Hatheway, 1990)**. One of the most important toxin is alpha toxin which is produced by all types of *C.perfringens*, but type A is known to produce high level of alpha toxin which has been regarded as the major virulence factor for the pathogenesis of several enteric diseases including fatal enterotoxaemia in animals and necrotic enteritis (NE) in poultry **(, Songer (1996)**. *C.perfringens* type A alpha toxin associated with necrotic enteritis in chicken is characterized by severe necrosis of the mucosa of the small intestine leading to high mortality rates causing massive losses in broiler chickens **(AlSheikhly**

**and Truscott, 1977; Fukata et al., 1988; Ficken and Wages, 1997; Wages and Opengart, 2003 and Williams, 2005).**

In rabbits, enterotoxaemia caused by *C.perfringens* infection is characterized by high mortality rate, sudden death among kids at weaning age (3-4 weeks), causing severe economic losses in commercial rabbit farms and the alpha toxin is responsible for the development of this diarrheal disease **(Saad, 1994 and Diab et al., 2003).**

Toxigenic typing of *C.perfringens* isolates has been performed by seroneutralization assay in mice (intravenously) or Guinea pigs (dermonecrotic test) as a conventional method **(, Brooks et al. (1957), Sterne and Batty (1975), Smith (1979) and Walker (1990)**. But it is almost impossible to classify the isolates into toxigenic types by colony morphology and biochemical properties; also dependence on in vivo method for toxin detection has become limited in routine diagnosis of *C.perfringens* infection **(, Carter (1984).**

*In vitro*, alpha toxin was detected from *C.perfringens* isolates by ELISAs as described before **(by Lovland et al. (2003), McCourt et al. (2005) and Ahmad et al. (2006).**

A new typing method was developed by Daube et al. (1994) based on the amplification of toxin gene by PCR. This PCR enables reliable and specific detection of the toxin genes cpa, cpb and cpe of *C.perfringens*. The alpha toxin gene is located on the chromosome of all strains and is produced by all types of *C.perfringens* (A, B, C, D and E) and *C.perfringens* type A strains produce the chromosomal encoded alpha toxin.

The PCR assay was performed using specific primers, and the toxin genes were amplified with a single pair of primer (single PCR) or two pairs of primers (duplex PCR) or specific combination of many pairs of primers (multiplex PCR) for genotyping of *C.perfringens* isolates **(Uzal et al. (1996), Yoo et al. (1997), Meer and Songer (1997), Yamagishi et al. (1997), Engestrom et al. (2003), Nauerby et al. (2003) and Baums et al. (2004).**

So the aim of this work was to isolate and identify *C.perfringens* strains from diseased chickens and rabbits, typing of these isolates by seroneutralization test in mice and dermonecrotic test in Guinea pigs as a classical biological assay (*In vivo*), and using of PCR *in vitro* as a molecular technique for detection of alpha toxin gene of isolated *C.perfringens* strains, to detect if there is a difference between the avian (chicken) and mammalian (rabbit) strains.

## Material and methods

Samples:

A total of 30 samples were collected from each of intestinal tracts of chickens with necrotic enteritis, and another 30 intestinal samples were taken from rabbits with clinical symptoms of enterotoxaemia.

*C.perfringens* type A reference strain:

A reference type A of *C.perfringens* strains of rabbit and chicken was obtained from Anaerobic Department was used as positive control in the PCR assay.

*C.perfringens* types A, B, C, D and E antiserum:

*C.perfringens* types A, B, C, D and E antitoxin were obtained from Wellcome Research Laboratories, Beckenham, England for typing of *C.perfringens* isolates.

Toxigenicity test:

The content of each sample was mixed with equal volume of saline, centrifuged at 3000 rpm for 15 minutes, 0.3 ml of the supernatant was injected in three mice I/V, if the mice died within 24 hours indicate presence of toxin.

Isolation and identification of *C.perfringens* isolates:

The intestinal content of each sample was cultured on cooked meat media, streaked on neomycin sulfate blood agar plates, incubated anaerobically at 37°C for 24 hours (**according to Willis (1977)**. *C.perfringens* were identified by characteristic dual haemolysis, and the

typical colonies were picked up and inoculated onto toxin production media **(according to Roberts et al. (1970)** and streaked on egg yolk agar plate for detection of alpha toxin lecithinase activity **(MaClung and Toabe, 1947).**

Typing of isolated strains:

1. By using seroneutralization test (*in vivo*):

*C.perfringens* isolates were typed by SNT in mice I/V and in Guinea pigs (dermonecrotic test) with specific antisera of each *C.perfringens* type, as performed by **(Oakley and Warrack (1953), Smith and Holdman (1968) and Sterne and Batty (1975).**

2. By polymerase chain reaction (PCR) assay:

2.1.Specific forward and reverse primers encoding the conservative part of the alpha toxin gene of *C.perfringens* type A were used to amplify DNA fragment about 324 bp as described by **(Songer and Bueschel (1999)** The primers sequence were as follow
Forward primer 5\ GCT AAT ACT GCC GTT GA
Reverse primer 3\ CCT CTG ATA CAT GTA AG

2.2. Extraction of DNA: DNA was extracted from bacterial isolates according to **(Sambrook et al. (1989).**

2.3. PCR protocol was performed according to **(Kadra et al. (1999) and Nauerby et al. (2003)** as follows:
**Step 1:** denaturation at 95°C for 5 minutes for one cycle.
**Step 2**: 35 cycle as
Denatuartion at 94°C for 1 minut.
Annealing temperature at 55°C for 1 minute.
Extension at 72°C for 1 minute.
**Step 3**: one cycle for final extension at 72°C for 10 minutes.
The PCR products were stored at -20 °C until use.

2.4. Screening of PCR products: by using 1.5% agarose gel electrophoresis and then photographed according to **(Sambrook et al. (1989).**

## Results and Discussion

Different variant of diseases caused by alpha toxin of *C.perfringens* type A have been reported as important in commercial poultry especially in intensive broiler production Ficken and Wages (1997), in egg layers Dhillon et al. (2004) and in rabbit farms Diab et al. (2003). In recent years the incidence of *C.perfringens* associated diseases as necrotic enteritis has increased due to prevention of antimicrobial feed additives Heier et al. (2001) and Collier et al. (2003).

Diagnosis of these variant of *C.Perfringens* associated diseases require different approaches as P.M examination and detection of alpha toxin which is considered the major virulence factor in the pathogensis of necrotic enteritis in chickens (Fukata et al., 1988) and enterotoxaemia in rabbits.

From Table (1), it was shown that the incidence of the toxigenic isolates from rabbits was 66.6% and from chickens was 50%. On typing of these isolates by SNT it was found that all isolates were *C.perfringens* type A, these findings agree with the results obtained by Al-Sheikhly and Truscott (1977), Fukata et al. (1988), Hofshagen and Stenwig (1992), Ficken and Wages (1997), Heier et al. (2001), Justin et al. (2002), Sheedy et al. (2004), Rehman et al. (2006) and Zhang et al. (2006) who stated that all isolates from broiler were *C.perfringens* and it was defined as the strain producing alpha toxin.

Dependence on *in vivo* method of toxin detection has become a limitation to routine diagnosis of *C.perfringens* infection Carter (1984).

Toxigenic typing using SNT has some disadvantages as it requires a large number of experimental animals and using of monovalent antisera and may yield false negative or positive results Yoo et al. (1997) and this traditional method also lack of specificity and consuming time.

So PCR assay was developed by Daube et al. (1994) and Songer (1996) as a diagnostic assay for the detection of *C.perfringens* toxins which could offer a considerable advantages over the conventional method. So the using of PCR as recent technique was developed for detection of the alpha toxin gene of *C.perfringens*, and PCR enables the selective amplification of DNA sequence and to identify the gene

encoding alpha toxin in *C.perfringens* isolated strains from gastrointestinal tract of diseased rabbits and chickens.

Typing can be done by a single PCR reaction that uses primers pairs specific for the alpha toxin gene (pLc). The extracted DNA of *C.perfringens* isolates were tested with the forward and reverse primers of alpha toxin Fig.(1).

The results observed in Fig.(1) revealed positive amplification of about 324 bp fragments of alpha toxin gene from the extracted DNA of *C.perfringens* isolates of chickens and rabbits.

PCR results revealed that all isolates of *C.perfringens* present in the intestinal tract of diseased rabbits and chickens carried the cpa gene and all belonged to the toxin type A.

These results agree with the results reported by several authors as Cowen et al. (1987), Hofshagen and Stenwig (1992), Baba et al. (1997), Das et al. (1997), Yoo et al. (1997), Heier et al. (2001), Engestrom et al. (2003), Baums et al. (2004), Lovland et al. (2004), Van-Immerseel (2004) and Laila Mohamed et al. (2006) where they analyzed *C.perfringens* isolates by PCR and found that the alpha toxin gene of *C.perfringens* belonging to type A and genotyping of these isolates by PCR were type A.

Comparison of PCR data with results obtained by seroneutralization assay with mouse model showed a good correlation and the isolated strains were identified as *C.perfringens* type A producing alpha toxin which is the major factor inducing necrotic enteritis in chickens and enterotoxaemia in rabbits.

**Table (1)** Incidence of toxigenic isolates of *C.perfringens* from diseased rabbits and chickens

| Source of samples | Number of examined samples | Toxigenic isolates | % |
|---|---|---|---|
| Rabbit | 30 | 20 | 66.6 |
| Chickens | 30 | 15 | 50 |

From fig. (1) it was found that the size amplified DNA fragment of isolated *C.perfringens* either from chicken or from rabbits are the same.

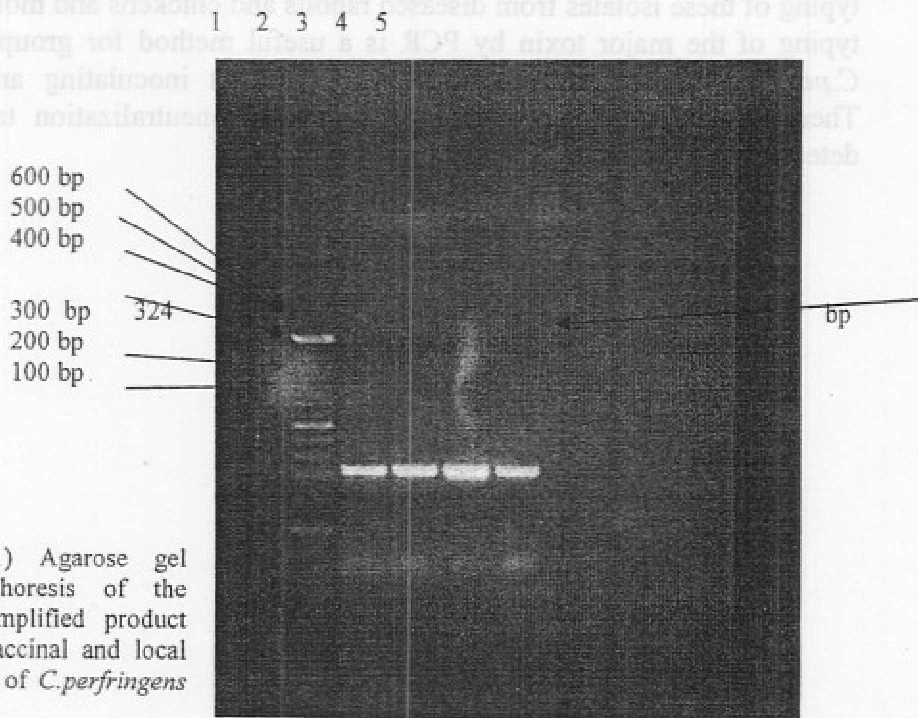


Fig. (1) Agarose gel electrophoresis of the PCR amplified product from vaccinal and local isolates of *C.perfringens* type A.

Lane (1) 100 bp molecular weight marker (Invitrogen).
Lane (2) Amplified 324 bp of vaccinal strain of chicken.
Lane (3) Amplified 324 bp of vaccinal strain of rabbit.
Lane (4) Amplified 324 bp of local isolates of chicken.
Lane (5) Amplified 324 bp of local isolates of rabbit.

These results agree with Titball (1993) and Titball et al. (1999) where they demonstrated the gene encoding alpha (cpa) is present in all strains of *C.perfringens* and also with that obtained by Tso and Siebel (1989) and Sheedy et al. (2004) as they stated that by analysis of alpha toxin of avian-derived strains demonstrated high homology to mammalian derived strains and the alpha toxin gene appeared to contain nearly a similar in coding sequence in the two different isolates.

In conclusion, this study indicated that PCR assay has several performance advantages, as it is easy, rapid, accurate, specific and sensitive for detection of the alpha toxin gene of *C.perfringens* and typing of these isolates from diseased rabbits and chickens and molecular typing of the major toxin by PCR is a useful method for grouping of *C.perfringens* isolates into toxin types without inoculating animals. Therefore, PCR could be used instead of seroneutralization test for detection and typing of *C.perfringens*.

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# الكشف عن جين الألفا توكسين لميكروب الكلوستريديم برفرنجنز نوع (أ) المعزولة من الأرانب والدجاج المصابة بإستخدام تفاعل البلمرة المتسلسل

علاء عبد الفتاح محمد علي المنيسي – سهام عبد الرشيد الزيدي
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تم إستخدام إختباري التعادل السمي في الفئران كإختبار تقليدي حيوي وإختبار تفاعل البلمرة التساسل كإختبار تقني جزيئي معملي لتصنيف عترات ميكروب الكلوستريديم برفرنجنز المعزولة من الدجاج المصاب بالإلتهاب المعوي التنكرزي والأرانب المصابة بالتسمم المعوي. وقد وجد أن كل المعزولات الضارية لميكروب الكلوستريديم برفرنجنز تنتمي للنوع "أ" وهو النوع السائد وأن السم ألفا يعتبر هو مفتاح الضراوة لمرض الإلتهاب المعوي التنكرزي في الدجاج والتسمم المعوي في الأرانب. وبمقارنة النتائج بإختبار السم التعادلي بالنتائج التي تم الحصول عليها بإختبار تفاعل البلمرة المتسلسل أظهرت تطابقاً جيداً. ولقد أثبت إختبار تفاعل البلمرة المتسلسل كفاءته من حيث الدقة والتخصص والحساسية والسرعة عن إختبار التعادل السمي في الفئران و خنازير غينيا. أيضاً أثبتت نتائج إختبار تفاعل البلمرة المتسلسل أن نواتج تكبير الحامض النووي الديوكسي ريبوزي (DNA) لمعزولات بكتيريا الكلوستريديم برفرنجنز من الأرانب والدجاج لها نفس الحجم الجزيئي.