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Full Length Research Paper

Multiplex PCR Analysis of *Clostridium perfringens* Isolates Associated with Necrotic Enteritis Outbreaks in Broiler Farms in Cairo

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During the winter of 2006, outbreaks of severe entritis affected many broiler farms in the AL-Fayoum governorate, south of Cairo, Egypt. To identify the causative agent(s), bacterial isolates (14) from the diseased chickens were characterized phenotypically and biochemically. All isolates exhibited characteristics of *Clostridium perfringens*. Subsequently, molecular typing of the bacterial isolates was performed by multiplex PCR using four sets of primers specific for the genes encoding the *C. perfringens* , , and toxins, respectively. A single amplicon, corresponding in size to the alpha ( ) toxin-encoding gene (approximately 402 bp), was amplified from all the bacterial isolates. It was therefore concluded that only *C. perfringens* type A was responsible for the disease outbreaks.

Key words: *Clostridium perfringens*- necrotic entritis – broiler chicken – multiplex PCR.

INTRODUCTION

Clostridia are commonly found in the environment, occur-ring in soil, sewage and water, as well as in the intestines of both man and animals. Members of the genus *Clostri-dium* are widely recognized as enteric pathogens for man, domestic animals and wildlife (Songer, 1996). *Clos-tridium perfringens*, a part of normal gut flora, is com-monly 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). Necrotic entritis, an important sporadic disease of broiler chicken, was first reported by Parish (1961) to be caused mainly by *C. perfringens* (Prukner et al., 1995). Mucosal damage-inducing factors such as coccidiosis (parasitism), high fiber litters, dietary changes, and poor hygienic and housing conditions are predisposing factors. These may produce a favourable growth environment for *C. perfringens*, resulting in its overgrowth and production of potent toxins that leads to necrotic entritis (Vissiennon et al., 1994). Moreover, the diminished use of growth-promoting antibiotics makes *C. perfringens*-induced nec-rotic entritis and subclinical infections important threats to poultry health (Gholamiandekhordi et al., 2006). *C. per-fringens* is a leading cause of food-borne poisoning in the USA (Rood, 1998). *C. perfringens* is a gram- positive spo-re-forming enteropathogenic anaerobe that produces a variety of fatal extracellular toxins, designated as alpha ( ),beta ( ), epsilon ( ), iota ( ) and theta ( ), as well as other minor toxins (Songer,1996). *C. perfringens* type A is 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 (Songer, 1996). Severe fatal diarrhoea, resulting from necrotic entritis, is due to the Clostridial exotoxins (Baba et al., 1992) . In the present study, the causative agent(s) of necrotic entritis disease outbreaks on chicken farms in Cairo, Egypt was investigated using phenotypic and molecular typing techniques.

MATERIAL AND METHODS

Bacterial isolation and phenotypic characterization

Infected chickens, which exhibited diarrhoea, reduced performance and depression, were killed and some of the intestinal contents and intestinal scrapings were taken and inoculated directly into cooked meat broth medium (Oxoid) and incubated anaerobically in an anaerobic Gas pack jar for 24 h at 37 ^{o}C. Loopfuls of growth were then streaked onto 5% sheep blood agar (Oxoid), supplemented with neomycin sulphate. The plates were incubated anaerobically for 24 h at 37^{o}C. The colonies were subjected to macroscopic examination,

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including morphotyping (shape, size and texture of the colonies on blood agar plates). In total, 14 bacterial isolations were made, all of which were also gram-stained and examined by light microscopy.

Biochemical characterization

The 14 bacterial isolates were subjected to biochemical identification patterns, including Catalase test, lecithinase activity on egg yolk salt agar (EYSA), haemolytic activity on sheep blood agar and sugar fermentation (glucose, maltose, lactose, inulin, dulicitol, man-nitol, inositol and salicin). The inoculated sugar media were incuba-ted anaerobically at 37^{o}C for 24 h and examined for acid and gas production (Cruickshank et al., 1975; Koneman et al**.,** 1988).

Mouse bioassays

Following culture of the *C. perfringens* field strains in cooked meat broth medium, as described above, the cells were harvested by centrifugation at 3 000 rpm for 15 min and the cell -free culture supernatants were recovered. White mice (25 - 40 g) were injected intraperitonealy with 0.3 ml of the culture supernatant and then observed over a period of three days for either death or disease symptoms. Reference strains of *C. perfringens* types A, B, C and D were included as positive controls, while the supernatant from non-inoculated cooked meat broth was also included. For *C. perfringens* type A, strains from both a rabbit and chicken was available. The reference strains were obtained from the Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt.

Preparation of cell lysates

Colonies from 24 h old cultures on blood agar plates were picked and suspended in 1.5 ml Eppindorf tubes containing 1 ml of distilled water. Following 3 cycles of freeze-thawing, the tubes were incubated at 95^{o}C for 20 min and finally centrifuged for 15 min at 5 000 rpm. The supernatants were collected and kept frozen until use as templates in the PCR. The DNA samples were analyzed spectrophotometrically at 260 and 280 nm to check the presence of DNA and its purity. Samples were also subjected to electrophoresis on a 1.5% (w/v) agarose gel in 1 × TBE buffer to ensure the presence of intact DNA. (Maniatis et al., 1982)

Oligonucleotide primers

Primers used in this study were designed according to Yoo et al. (1997), and were obtained from Metabion International AG (Germany). The sequence of the respective oligonucleotide primers were as follow:

C. perfringens Alpha (CPA) for amplification of the alpha ( ) toxin gene (402 bp):

Forward primer: 5'-GTTGATAGCGCAGGACATGTTAAG-3'; Reverse primer: 5'-CATGTAGTCATCTGTTCCAGCATC-3'. *C. perfringens* Beta (CPB) for amplification of the beta ( ) toxin gene (236 bp):

Forward primer: 5'-ACTATACAGACAGATCATTCAACC-3'; Reverse primer: 5'-TTAGGAGCAGTTAGAACTACAGAC-3'. *C. perfringens* Epsilon (CPE) for amplification of the epsilon ( ) toxin gene (541 bp):

Forward primer: 5'-ACTGCAACTACTACTCATACTGTG- 3'; Reverse primer: 5'- CTGGTGCCTTAATAGAAAGACTCC-3' *C. perfringens* Iota (CPI) for amplification of the iota ( ) toxin gene Forward primer: 5'-GCGATGAAAAGCCTACACCACTAC-3'; Reverse primer: 5'-GGTATATCCTCCACGCATATAGTC-3'.

Multiplex PCR

The PCR reaction mixture (25 µl) contained 5 l of bacterial lysate as template DNA, 2.5 l of 2 mM dNTP's, 2.5 l 10 × PCR buffer, 0.25 l of 5 U/ l *Taq* DNA polymerase (Vivantis, Malaysia), 1 l of each of the primers (10 pmol/ l) and 6.75 l distilled water. The PCR reaction mixtures were placed in a Biometra PCR thermal cycler. Following initial denaturation for 5 min at 94°C, the samples were subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. After the last cycle, a final extension for 10 min at 72°C was performed. The PCR reaction mixtures (10 l) were analyzed by electropho-resis on a 1% (w/v) agarose gel in the presence of 100-bp DNA ladder (Fermentas Life Science, EU). The agarose gel was supple-mented with ethidium bromide in order to visualize the DNA on an UV transilliminator.

RESULTS AND DISCUSSION

Clinical signs of diseased birds and post-mortem examination of dead ones

The diseased birds exhibited general signs in the form of depression, reluctance to move, pronounced apathy, ruffled feather and watery diarrhoea as well as high mortality rate. Moreover, the pathological lesions of dead birds revealed friable small intestine (jejunum and ilieum) distended with gas. The intestinal mucosas were covered by yellowish or green pseudo membrane.

Characterization of bacterial isolates

All bacterial isolates exhibited the characteristic features of *C. perfringens*. The colonial characters on blood agar showed dew drops smooth greyish convex colonies with a double zone of haemolysis. Microscopic characters revealed gram positive non motile rods.

Biochemical identification of the isolates showed catalase, lecithinase positive and a haemolytic activity on sheep blood agar showing double zone of haemolysis. Gas and acid from glucose, fructose, lactose sucrose and mannitol were seen, urease negative and gelatinase positive.

Results of pathogenicity test in white mice were observed during 3 days which ends with death. All mice injected with the bacterial culture filtrate died and those injecteds with control broth without bacteria were alive.

Multiplex PCR

Figure 1 and 2 shows amplification of *C. perfringens* type A chicken strain and type A rabbit strain and gave only one band of approximately 402 bp considering alpha tox-in gene. While type B gave three bands one of them is comparable to 402 bp, alpha toxin gene, one is below the first between 200 and 300 bp that is considered beta toxin gene 236 bp. Third band is higher than alpha toxin gene band that lies above 500 bp which is considered ep-silon toxin gene 541 bp. Type C gave two bands one for

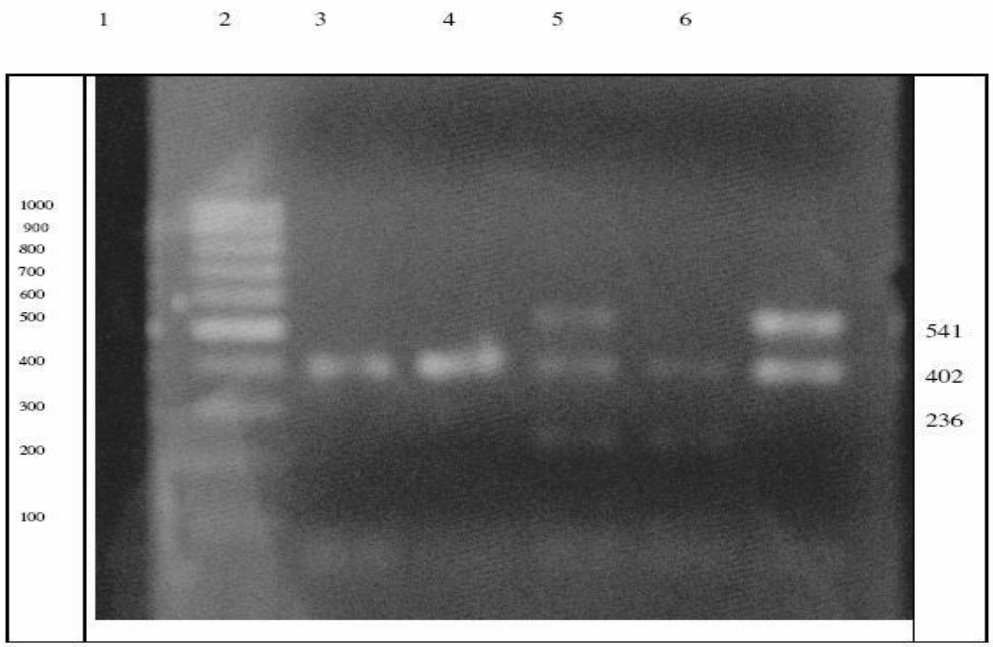

Figure 1. Multiplex PCR *Clostridium perfringens* reference strains. Lane 1, 100-bp DNA molecular marker; lane 2, *C. perfringens* type A (chicken); lane 3, *C. perfringens* type A (rabbit); Lane 4, *C. perfringens* type B; lane 5, *C. perfringens* type C; lane 6, *C. perfringens* type D.

alpha toxin gene and the other for beta toxin gene. Type D gave two bands one for alpha toxin gene and one abo-ve for epsilon toxin gene. There was no available refer-ence strain of *C. perfringens* type E which produces iota toxin. For that reason there was no band for iota toxin gene.

The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon and iota) and other toxins including enterotoxin (Hatheway, 1990). The patterns of production of the toxins are different, depending on the *C. perfringens* type. Therefore, the patterns have been used to type the bacterium into types A, B, C, D and E. The type could be different according to type of the animal's species.Molecular typ-ing allowed for an easier *in vitro* test. There was no avai-lable *C. perfringens* type E which does produce iota toxin beside alpha toxin. We used the primers for iota gene in case if one of the isolates could be type E.

We may recommend to use uniplex PCR (one set of primer in each run) firstly and then if it works multiplex PCR may be applied. The possibility of forming primer di-mers among primers may be greater in case of adding many sets of primers than presence of one set of primer.

In this study it was found that all the incriminated isolates were *C. perfringens* type A. All gave only one band very close to 400 bp as mentioned by Yoo et al. (1997) to be 402 bp. These findings agreed with the results reported by several authors which mentioned that *C. perfringens* type A was the most predominant type isolated from broiler chickens with necrotic entritis (Das et al., 1997).

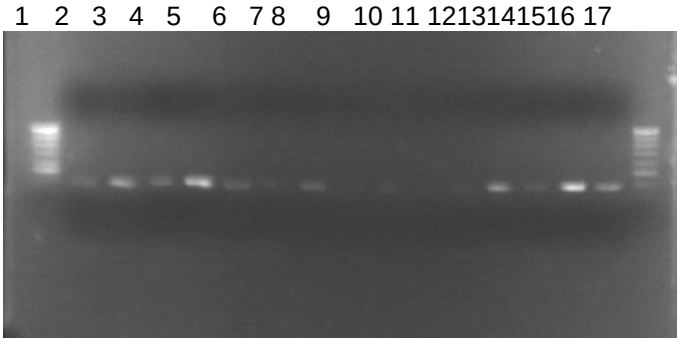

Figure 2. Multiplex PCR of DNA extracted from 14 field isolates of *C. perfringens* isolates in addition to a reference strain of *C. per-fringens* type A. Lanes 1 and 17, 100-bp DNA molecular marker; lanes 2 through 15, *C. perfringens* field isolates; lane 16, *C. perfrin-gens* type A reference strain. A single PCR-amplified DNA band, corresponding in size to the alpha ( ) toxin-encoding gene (approxi-
mately 402 bp), can be visualized on the agarose gel.

However, there was no *C. perfringens* type C or D isolated from broiler chickens demonstrated necrotic entritis and this disagreed with the results of Shane et al. (1985) and Heier et al. (2001) that isolated *C. perfringens* type D and type C from broiler chickens suffering from necrotic entritis.

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