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# Characterization of *Clostridium perfringens* Isolated from Poultry

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**Abstract:** Clostridium perfringens organisms have an economic concern in poultry production. The goal of this study was to characterize C. perfringens isolated from poultry farms. Intestinal and liver samples were collected from apparently healthy and diarrheic chickens (n=120), ducks (n=90) and turkeys (n=90) aged 1-10 days old. C. perfringens was isolated from 33.3, 33.3 & 42.2% of apparently healthy chickens, ducks and turkeys respectively and 75, 66.7 & 62.2% from diarrheic chickens, ducks and turkeys respectively. Toxigenic isolates were typed using dermonecrotic test and PCR. Out of the 157 isolates 107 (68.2%) produced toxins. Toxigenic C. perfringens isolates collected from chickens were type A (53.8%) and type D (15.4%). While turkeys isolates were belonged to type A (38.3%), type D (19.1%) and type C (10.6%).The majority of toxigenic ducks isolates were type A (48.9%) followed by type C (17.8%). Diversity of 16 C. perfringens isolates were investigated using random amplified polymorphic DNA (RAPD). Also a trial was done to describe the *C. perfringens* toxins by SDS-PAGE. The results illustrated the diversity of C. perfringens isolates and the prevalence of these pathogens in poultry production sites.

**Key words:** Sheep blood agar · Egg yolk agar · Cooked meat media · DNA fingerprint · Nagler's test

## INTRODUCTION

*Clostridium perfringens* is the most important clostridial pathogen of poultry. It is one of the most frequently isolated bacterial pathogens in food-borne disease outbreaks in humans, after some other pathogens such as *Campylobacter* and *Salmonellae* [1]. Necrotic enteritis is accompanied with severe fatal diarrhea due to the production of toxins produced by clostridial microorganisms which destruct the epithelial lining of the intestinal mucosa followed by the invasion of clostridia especially the exotoxins into the blood stream [2, 3]. *C. perfringens* is capable of producing different histotoxic and enteric diseases in both humans and animals [4]. In immunocompromised farmer, *C. perfringens* type A is considered as a potential pathogen of sepsis in a clinical case of meningoencephalitis with subdural empyema [5]. Several typing systems, based on either phenotypic or genotypic characteristics of *C. perfringens*, have been applied. Currently, the most commonly used typing method in epidemiological studies of *C. perfringens* is pulsed-field gel electrophoresis (PFGE), RAPD-PCR and SDS. PCR genotyping assays have been also developed [6]. The objective of this study was to characterize *C. perfringens* isolated from poultry farms.

## MATERIALS AND METHODS

**Samples:** A total of 300 samples (liver n = 160 and intestine n = 140) were collected form apparently healthy (chickens n = 60, ducks n = 45 and turkeys n = 45) and diarrheic (chickens n = 60, ducks n = 45 and turkeys n = 45) birds with necrotic enteritis. Livers and intestines of each bird were collected and investigated for *C. perfringens* infection.

**Isolation and Identification *C. perfringens*:** According to Willis [7], the collected samples were inoculated into tubes of freshly prepared boiled and cooled cooked meat medium (Oxoid) and incubated anaerobically for 24 hours at 37°C. A loopful of inoculated fluid medium was streaked onto neomycin sulphate sheep blood agar plates

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Table 1: Oligonucleotide primers used for amplification of toxin genes of *C perfringens* isolates

| Primer | Specificity | Sequence | Annealing temperature | Size of the amplified product (bp) |
|---|---|---|---|---|
| Alpha | Reverse | 5'-CATGTAGTCATCTGTTCCAGCATC-3' | 59.6°C | 402 |
| | Forward | 5'-GTTGATAGCGCAGGACATGTTAAG-3' | | |
| Beta | Reverse | 5'-TTAGGAGCAGTTAGAACTACAGAC-3' | 56°C | 236 |
| | Forward | 5'-ACTATACAGACAGATCATTCAACC-3' | | |
| Epsilon | Reverse | 5'-CTGGTGCCTTAATAGAAAGACTCC-3' | 55°C | 541 |
| | Forward | 5'-ACTGCAACTACTACTCATACTGTG-3' | | |

[8]. The streaked plates were incubated anaerobically for 24 hours at 37°C using a Gaspak anaerobic jar [9]. Suspected *C. perfringens* colonies were cultured onto 2 plates of sheep blood agar and egg yolk agar. One plate was incubated aerobically and the other plate was incubated anaerobically. The colonies that grew only in anaerobic condition and lecithinase producer and showed double zone of haemolysis on blood agar were picked up and purified for identification tests [8, 10].

**Determination of Toxigenic *C. perfringens* Isolates:** Toxigenic isolates were characterized by Nagler's test [11] and dermonecrotic test in Guinea pigs [12]. Also, PCR was used to type toxigenic isolates using primers (Table 1) were designed according to Yoo *et al*. [13] and were obtained from Metabion International AG (Germany).

**Random Amplified Polymorphic DNA (RAPD) PCR:** DNA from the 16 *C. perfringens* isolates was extracted by hexadecyl trimethyl ammonium bromide (CTAB) according to Sambrook *et al.* [14]. RAPD PCR was applied according to Renders *et al.* [15], using 15 pmol primer "TGA GCA TAG ACC TCA". The reaction mixture performed in 25µl consisted of 2µl (20 ng) of extracted DNA template from bacterial cultures, 2.5µl 10X PCR buffer, 2.5µl $MgCl_2$ (25mM), 2.5µl dNTPs (5mM), 3µl (10 pmol primer, AmpliTaq DNA polymerase (5 units/µl) and the volume of the reaction mixture was completed to 25 µl using DDW. PCR Protocol was applied in the following steps, step 1-denaturation at 95°C for l0 minute, step 2-denaturation at 94°C for 1 minute, step 3- annealing temperature for each primer pair was adjusted for 1 minute, step 4-extension at 72°C for l minute. The PCR products were stored in the thermal cycler at 4°C until they were collected. Screening of PCR products was done by using gel electrophoresis 2% with ethidium bromide and visualized under short wave UV transilluminator. The gel was photographed in order to obtain a permanent record using a Polaroid Camera (Kodak, USA).

**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):** Six toxigenic *C. perfringens* isolates were characterized by SDS-PAGE in comparison with 2 *C. perfringens* type B (control positive). All reagents and solutions were prepared according to Johnson *et al.* [16] and Chart [17] and *C. perfringens* toxins were prepared according to Bullen [18].

## RESULTS

**Prevalence of *C. perfringens* in the Examined Poultry:** As shown in Table 2, *C. perfringens* was isolated from 20 out of 60 and 45 of 60 samples collected from apparently healthy and diseased chicken samples with incidence of 33.3% and 75%, respectively. It was isolated from 15 out of 45 and 30 out of 45 samples obtained from apparently healthy and diseased duck samples with incidence of 33.3 % and 66.7%, respectively. Moreover, the diseased turkeys voided *C. perfringens* in 28 out of 45 samples (62.2%) while the percentage was 42.2% among the apparently healthy turkey samples. In this study, *C. perfringens* was isolated from liver and intestinal samples of diseased chickens (50% and 100%, respectively) and diseased ducks (50% and 80%, respectively) as well as from diseased turkeys (40% and 80%, respectively) as shown in Table 2.

**Determination of Toxigenic *C. perfringens* Isolates:** It is clear that 107 out of 157 *C. perfringens* isolates were toxin producing isolates (68.2%). Table 3 illustrates that, the majority of chicken isolates belonged to toxin type A (53.8%) followed by type D (15.4%) and the majority of turkey isolates belonged to toxin type A with an incidence of 38.3% followed by type D (19.1%) and type C (10.6%). The majority of duck isolates belonged to type A (48.9%) followed by type C (17.8%). The toxigenic isolates were confirmed by PCR as shown in Fig. 1.

**Result of RAPD- PCR:** Sixteen *C. perfringens* isolates recovered from chickens (n = 5), ducks (n = 6) and turkeys (n = 5) were characterized using short sequence primer as shown in Figures 2-4. The RAPD-PCR profile analysis of chicken isolates showed that the used primer identified 4-8 bands varied from 199-1553 bp as shown in Fig. 2. The RAPD-PCR profile analysis of duck isolates showed

Table 2: Incidence of *Clostridium perfringens* among the examined samples

| | | Chickens (1-10 days old) | | | Ducks (1-10 days old) | | | Turkeys (1-10 days old) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Positive samples | | | Positive samples | | | Positive samples | |
| Source of the isolates | | No. of examined samples | No. | % | No. of examined samples | No. | % | No. of examined samples | No. | % |
| Apparently healthy birds | Intestine | 30 | 15 | 50 | 25 | 10 | 40 | 25 | 12 | 48 |
| | Liver | 30 | 5 | 16.7 | 20 | 5 | 25 | 20 | 7 | 35 |
| | Total | 60 | 20 | 33.3 | 45 | 15 | 33.3 | 45 | 19 | 42.2 |
| Diseased birds | Intestine | 30 | 30 | 100 | 25 | 20 | 80 | 25 | 20 | 80 |
| | Liver | 30 | 15 | 50 | 20 | 10 | 50 | 20 | 8 | 40 |
| | Total | 60 | 45 | 75 | 45 | 30 | 66.7 | 45 | 28 | 62.2 |
| Total | 120 | 65 | 54.2 | 90 | 45 | 50 | 90 | 47 | 52.2 | |

No. = Positive number of samples

% = was calculated according to the number of examined samples.

Table 3: Incidence of toxigenic and non toxigenic *C. perfringens* isolates

| | | Toxigenic isolates type | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | | C | | D | | Total | | Non toxigenic isolates | |
| Source of isolates | No of isolates | No | % | No | % | No | % | No | % | No | % |
| Chickens | 65 | 35 | 53.8 | -- | 0 | 10 | 15.4 | 45 | 69.2 | 20 | 30.8 |
| Ducks | 45 | 22 | 48.9 | 8 | 17.8 | -- | 0 | 30 | 66.7 | 15 | 33.3 |
| Turkeys | 47 | 18 | 38.3 | 5 | 10.6 | 9 | 19.1 | 32 | 68.1 | 15 | 31.9 |
| Total | 157 | 75 | 47.8 | 13 | 8.3 | 19 | 12.1 | 107 | 68.2 | 50 | 31.8 |

No. = Number

% = the percentages was calculated according to the total number of examined isolates

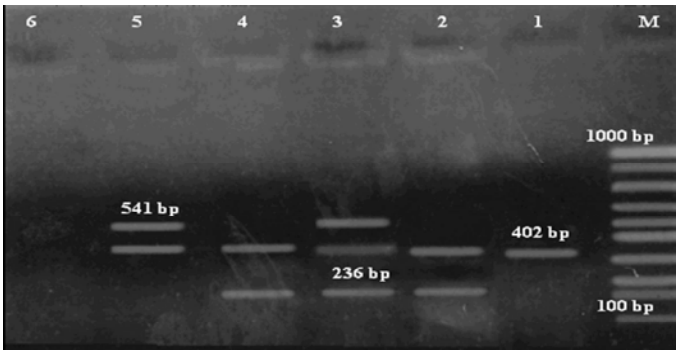


Fig. 1: Agarose gel electrophoresis of PCR products of *C. perfringens* isolates

Lane M: 100 bp DNA marker (Fermentas, Germany), Lane 1: *C. perfringens* type A, Lanes 2 & 4: *C. perfringens* type C, Lane 3: *C. perfringens* type B (positive control), Lane 5: *C. perfringens* type D & Lane 6: negative control.

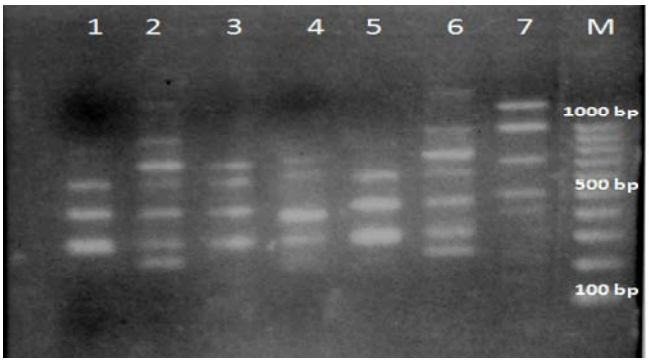


Fig. 2: DNA finger print of *C. perfringens* of chicken's origin using RAPD-PCR.

Lanes 1and 5: *C. perfringens* type B (positive control), Lanes 2 and 6: *C. perfringens* type A, Lanes 3 and 4: *C. perfringens* type C, Lane 7: *C. perfringens* type D and Lane M: 100 bp DNA marker (Fermentas, Germany)

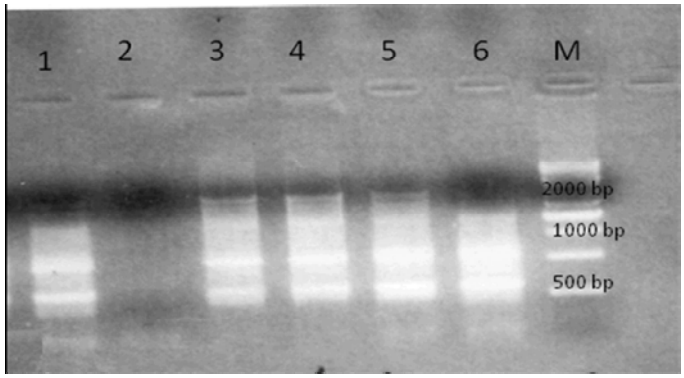


Fig. 3: DNA finger print of *C. perfringens* of duck's origin using RAPD-PCR.
Lanes 1, 3 and 4: *C. perfringens* type A, Lane 2: non toxin producing *C. perfringens* isolate, Lanes 5 and 6: *C. perfringens* type C, & Lane M: 100 bp DNA marker (Fermentas, Germany)

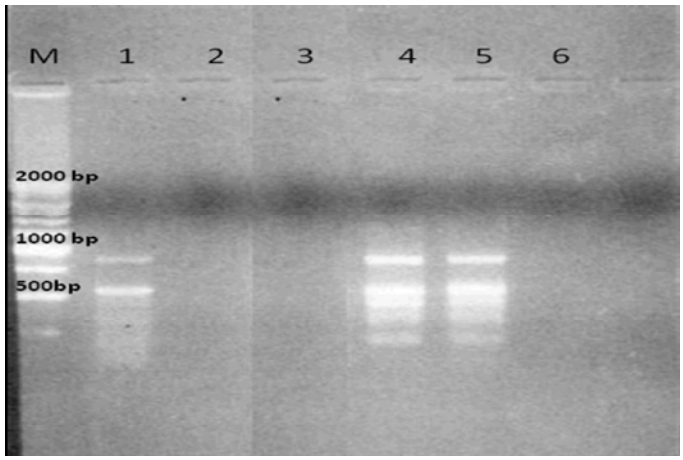


Fig. 4: DNA finger print of *C. perfringens* of turkey's origin using RAPD-PCR.
Lane M: 100 bp DNA markers (Fermentas, Germany), Lane 1: *C. perfringens* type A, Lanes 2 and 3: non toxin producing *C. perfringens* isolates, Lane 4: *C. perfringens* type C, Lane 5: *C. perfringens* type D and Lane 6: negative control

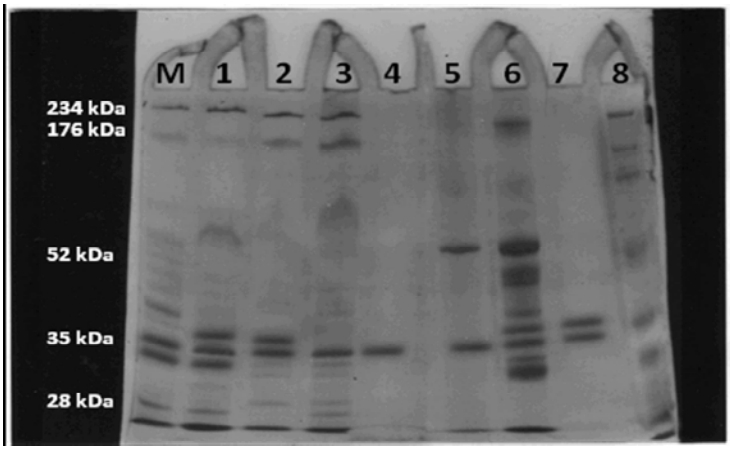


Fig. 5: SDS profile analysis of *C. perfringens* toxins.
Lane M: Promega protein marker from Sigma (28-234 kDa), Lanes 1 and 3: *C. perfringens* type B toxin (positive control), Lane 2: *C. perfringens* type C toxin (turkey isolate), Lane 4: *C. perfringens* type A toxin (chicken isolate), Lane 5: *C. perfringens* type A toxin (duck isolate), Lane 6: *C. perfringens* type D toxin (turkey isolate), Lane 7: *C. perfringens* type A toxin (turkey isolate) & Lane 8: *C. perfringens* type C toxin (duck isolate)

that the used primer identified 7-8 bands varied from 199-1725 bp as shown in Fig. 3, while, the non toxigenic isolate had one band at 263 bp. The RAPD-PCR profile analysis of turkey isolates showed that the used primer identified 5-7 bands varied from 260-865 bp as shown in Fig. 4, while the non toxigenic isolates had no amplified fragment.

**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of *C. perfringens* Isolates:** Characterization of 6 toxigenic *C. perfringens* isolates by SDS-PAGE technique are presented in Figure 5 in comparison with 2 *C. perfringens* type B (control positive). All isolates had band at 30-33.3 kDa. *C. perfringens* type A had one or 2 bands at 30-53.6 kDa. *C. perfringens* types B, C and D had a complex protein profiles with 7-10 bands. They had bands at 220.7-180.5, 40-52.2 and 20.8-28.1 kDa. *C. perfringens* types B and C had bands at 35.4-37.6 and 118-144.8 kDa. The most important results of this study indicate that, although the overall toxin profiles of *C. perfringens* isolates showed some similarity, there were marked differences in the intensity of individual bands between isolates.

## DISCUSSION

*C. perfringens* in poultry constitutes a risk for transmission to humans through the food chain. Colonization of poultry by clostridia is a very early event in the animals' life and can be transmitted within the broiler chicken operation. The percentage of *C. perfringens* positive samples from the hatcheries ranged from 13% to 23% with an overall incidence of 20% [19]. As shown in Table 2, *C. perfringens* was isolated from apparently healthy (33.3%) and diseased (75%) chicken samples. It was also isolated from apparently healthy (33.3 %) and diseased (66.7%) duck samples. Moreover, the diseased turkeys voided C. *perfringens* in 28 out of 45 samples (62.2%) while the percentage was 42.2% among the apparently healthy turkey samples. *C. perfringens* is a ubiquitous bacterium present in poultry houses and surroundings [20]. It is often found in the intestinal tract of healthy birds but it can cause outbreaks of disease in many species of poultry and especially in broiler and turkey flocks [21]. Toxins of *C. perfringens* isolates were detected using inoculation of guinea pig to detect necrosis and confirmed by PCR (Table 3 and Fig. 1). PCR was established to replace animal testing and to reduce cost and time [22]. Table 3 illustrates that 107 out of 157 *C. perfringens* isolates were toxin producing isolates (68.2%). It is clear that the majority of toxigenic isolates were type A with incidence of 53.8, 48.9 and 38.3 among chicken, duck and turkey isolates respectively. *C. perfringens* type A is the most common cause of food poisonings in the industrialized world [23]. Xiao *et al*. [24] identified 59 out of 98 food-borne isolates as *C. perfringens* type A. Type C was detected from ducks (17.8%) and turkeys (10.6%) while type D was detected from chickens (15.4%) and turkeys (19.1%). The majority of isolates from poultry belong to toxin type A but a few belong to type C [25].

RAPD-PCR can be used to generate a molecular fingerprint of *C. perfringens*. This technique can be compared to determine the relationship among sixteen *C. perfringens* isolates recovered from chickens (n = 5), ducks (n = 6) and turkeys (n = 5) using short sequence primer of the organisms (Figs. 2-4). RAPD-PCR profile analysis of chicken isolates showed that the used primer identified 4-8 bands varied from 199-1553 bp as shown in Figure 2. The used primer identified 7-8 bands among duck isolates varied from 199-1725 bp, while the non toxigenic isolate had one band at 263 bp (Fig. 3). The RAPD-PCR profile analysis of turkey isolates showed 5-7 bands varied from 260-865 bp, while the non toxigenic isolates had no amplified fragment as shown in Figure 4. Genetic characterization has revealed that healthy birds may carry two genotypes of type A organisms [20] to as many as five [26]. It is clear that, there are certain variations when using the specified random primer. SDS was a successful aid to classify various bacterial species and to identify protective antigens [27]. A trial was made in this study to characterize 6 toxigenic *C. perfringens* isolates by SDS-PAGE technique (Fig. 5) in comparison with 2 *C. perfringens* type B (control positive). All isolates had band at 30-33.3 kDa. *C. perfringens* types B and C had a band at 33to 35 kDa. *C. perfringens* type B and type C isolates, which produce beta-toxin (CPB), cause fatal diseases originating in the intestines of humans or livestock and CPB is a 35-kDa protein that forms pores in the membrane of susceptible cell lines, which leads to swelling and cell lyses [28]. Gao and McClane [29] concluded that *C. perfringens* enterotoxin is approximately 35 kDa polypeptide and causes the symptoms associated with several common gastrointestinal diseases. *C. perfringens* type A had one or 2 bands at 30 & 53.6 kDa. *C. perfringens* alpha-toxin is a 43-kDa protein and it is the major virulence factor in the pathogenesis of the isolates [30]. *C. perfringens* types B, C and D had a complex protein profiles with 7-10 bands. They had bands at 220.7-180.5, 40-52.2and 20.8-28.1 kDa.

*C. perfringens* types B and C had bands at 35.4-37.6 and 118-144.8 kDa. On the cellular and molecular level, *C. perfringens* enterotoxin inserts itself into the plasma membrane of enterocytes and forms a complex of ~90 kDa with claudin -3 and claudin -4 receptors [4]. In the present investigation *C. perfringens* type C produced band at 119-131 kDa. *C. perfringens* type C NCIB 10662 produced various gelatinolytic enzymes with molecular masses ranging from approximately 120 to approximately 80 kDa and 120 kDa gelatinolytic enzyme was present in the largest quantity in the culture supernatant [31]. SDS-PAGE analysis of *C. perfringens* showed three of the labeled bands of about 35 kDa, which is the expected size of monomeric toxin and of about 191 and 228 kDa, which are the expected sizes of hexameric and heptameric toxins, respectively [32].

## CONCLUSION

In conclusion the results indicated that, although the overall toxin profiles of *C. perfringens* isolates were showed some similarity, there were marked differences in the intensity of individual bands between isolate and there was greater diversity in C. perfringens populations.

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