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Isolation of *Clostridium perfringens* from broiler and genotyping by multiplex PCR

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Abstract

This study was carried out to isolate ***Clostridium perfringens*** from broiler chickens and to detect the gene encoding the alpha toxin produced by all types of ***C. perfringens*** by polymerase chain reaction (PCR).

C. perfringens was isolated as 90 (64.3%) isolates out of 140 Intestinal and liver samples collected from both apparently healthy and diseased chickens aged about 22-40 days *C. perfringens* was isolated with a percentage of (57.1& 71.4%) of apparently healthy chickens and diseased chickens respectively . PCR analysis was conducted for molecular typing of twenty representative isolates it revealed that ***C. perfringens*** type **A** with alpha toxin 0f 324bp fragment.

Key words: *Clostridium perfringens, broilers, multiplex PCR*

Introduction

Clostridium perfringens is a Gram positive, spore forming and anaerobic bacteria that causes serious infections in humans and animals by toxins. *C. perfringens* is divided into five types, A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota.

All *C. perfringens* types produce α toxin. In addition, type B strains produce β and £ toxins, type C produces β toxin, type D produces £ toxin and type E produces Í toxin **(Hatheway 1990)**. Different types cause different diseases in animals and humans, thus determining the presence of these toxins is important when diagnosing a disease or investigating the epidemiology of *C. perfringens*.

Smyth and Martin (2010) recorded that necrotic enteritis is a serious disease of chickens and turkeys. *Clostridium perfringens* induced necrotic enteritis (NE) and subclinical disease have become important threats to poultry health and is one of the main causes of losses in broiler flocks due to high mortalities and reduction in growth rate, mortality can reach 1% per day with a total mortality of 10-40% **(McDevit *et al.* 2006).** *C.perfringens* is a member of normal intestinal flora that reproduces at high rates and produces toxins **(Kalender and Ertas 2005).** *C. perfringens* type A, which causes infection in chickens, has been reported to cause food poisoning in humans **(Gholamiandekhordi *et al*., 2006).**

NE occurs in broilers aged between two and six weeks **(Songer 1996; Cooper and Songer. 2010).** it also demonstrated that *C. perfringens* strains from a mammalian species and from normal chickens, can cause necrotic enteritis in chicken.

The incidence of *Clostridium perfringens*-associated with necrotic enteritis in poultry has increased in countries that stopped using antibiotic as growth promoters. It is also reported that coccidiosis plays a role in the occurrence of necrotic enteritis **(Al-Sheikhly and Saieg 1980; Baba *et al.,* 1997)** due to mucosal dam-

age also high fiber litters, dietary changes, and poor hygienic and housing conditions these are predisposing factors that may produce a favourable growth environment for the induction of *C. perfringens* and its overgrowth and production of potent toxins that leads to necrotic enteritis
(Vissiennon *et al.,* 1994; Janneke *et al.,* 2013).
Besides the economic importance of *C. perfringens* in poultry, it also constitutes a risk to the public health through the food chain **(Van Immerseel *et al.,* 2004; Hughes, *et al*., 2008)** poultry meat, have frequently been reported as the most common food vehicles **(Cooper *et al.,* 2010 ; Nowell *et al.*,2010).** Avian necrotic enteritis costs the world poultry industry an estimated $2 billion annually, largely due to the costs of antimicrobial prophylaxis and inefficient feed conversion (**Cooper *et al.*,2009; Van Immerseel *et al.,* 2009)**
Routine typing procedure consumes a lot of antisera and experimental animals. Additionally it is labor. In order to avoid high cost and the use of experimental animals, several researchers have been working on a PCR method for detection of genes encoding *C. perfringens* toxins also it is always possible to isolate *C.perfringens* from intestinal contents as it is normal habitant of the intestine therefore the detection of toxin produced by the bacteria is a convenient method so PCR can be used to determine the presence of toxin genes and for typing *C. perfringens* **(Meer and Songer. 1997; Yoo, *et al.,* 1997; Engström, *et al.,* 2003; Baums *et al*., 2004)**
The present work was planned to isolate *C. perfringens* from broiler chickens and to detect the type of it by detection of different toxin gene in the isolates by multiplex PCR.

Materials and Methods

Samples. A total of 140 samples were collected from slaughtered apparently healthy broiler chickens and diseased chicken aged about 22-40 days, samples obtained from animal production Farm Faculty of Agriculture, Assiut University and other private farms. Samples were Intestinal contents , Intestinal scrapings and livers. All samples were sent to the lab.as soon as possible for *Cl. perfringens* isolation.
Isolation and Identification. A portion of the collected samples were inoculated into tubes of freshly prepared boiled and cooled cooked meat medium (Oxoid) and incubated anaerobically in an anaerobic Gas Pak jar using Gas generating Kit ,B 36, Oxoid for 24 -48 hours at 37°C. A loopful of inoculated broth medium was streaked onto TSC agar (Tryptose Sulphite Cycloserine agar, Oxoid CM 857; Oxoid) and the plates were further incubated at 37°C for 48 hours anaerobically, in a Gas Pak system
In order to confirm *Cl. perfringens*, the suspect black colonies from each positive TSC agar plate were purified and gram-stained, examined by light microscopy, and also identified biochemically by using catalase test, lecithinase activity on egg yolk salt agar (EYSA), and litmus milk test according to **(Willis, 1977; Koneman *et al.,* 1992)**

DNA extraction.
Template DNA for multiplex PCR assay was prepared by rapid boiling procedures according to **(Sheedy *et al.,* 2004).** Briefly, all isolates were incubated in cooked meat broth (Oxoid CM0081) for 24 h. at 37°C in anaerobic conditions (top of tubes were covered by sterile liquid paraffin). One milliliter of each enrichment culture was separately transferred to a microcentrifuge tube and was centrifuged for 5 min at 12000 g. The pellets were resuspended in 200µl sterile distilled water and boiled for 20 min. in a boiling water bath. The suspensions were then centrifuged for 5 min at 12000 g and the supernatant was aspirated to a clean tube to be used as template DNA.

Multiplex PCR Assay to Determine the Toxin Genotype of Isolates.
The multiplex PCR assay of **Meer and Songer (1997)** was used to detect the presence of genes encoding alpha-toxin (*cpa*), beta-toxin

(*cpb*), epsilon-toxin (*etx*) and iota-toxin (*iap*). The names of target genes, nucleotide sequences of primers and amplicon sizes are shown in (Table 1) PCR was performed in a total reaction volume of 25µl, which contained 5µl of DNA as template,0.36 mM of each *cpb* primer, 0.44 mM of each *etx* primer, 0.5 mM of each *cpa* primer and 0.52 mM of each *iA* primer, 1X of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). The amplification cycles were carried out in a PT- 100 Thermocycler (MJ Research, USA). Amplification conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min was followed. Amplification products were electrophorezed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 50 bp DNA ladder was run simultaneously as a marker, both *Cl. perfringens* type C,D were used as positive control previously identified by PCR and toxin neutralization test in mice.

Table 1. Primers used in the study, their nucleotide sequences, their targeted genes and their products sizes.

Primer name	Primer sequences (5' – 3')	Target gene	PCR product size (bp)
cpa F *cpa R*	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	alpha toxin gene	324 bp
cpb *cpb R*	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	beta toxine gene	196 bp
etx F *etx R*	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	epsilon toxin gene	655 bp
iA F *iA R*	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	iota toxin gene	446 bp

Results

Prevalence of *C. perfringens* in the examined Poultry.

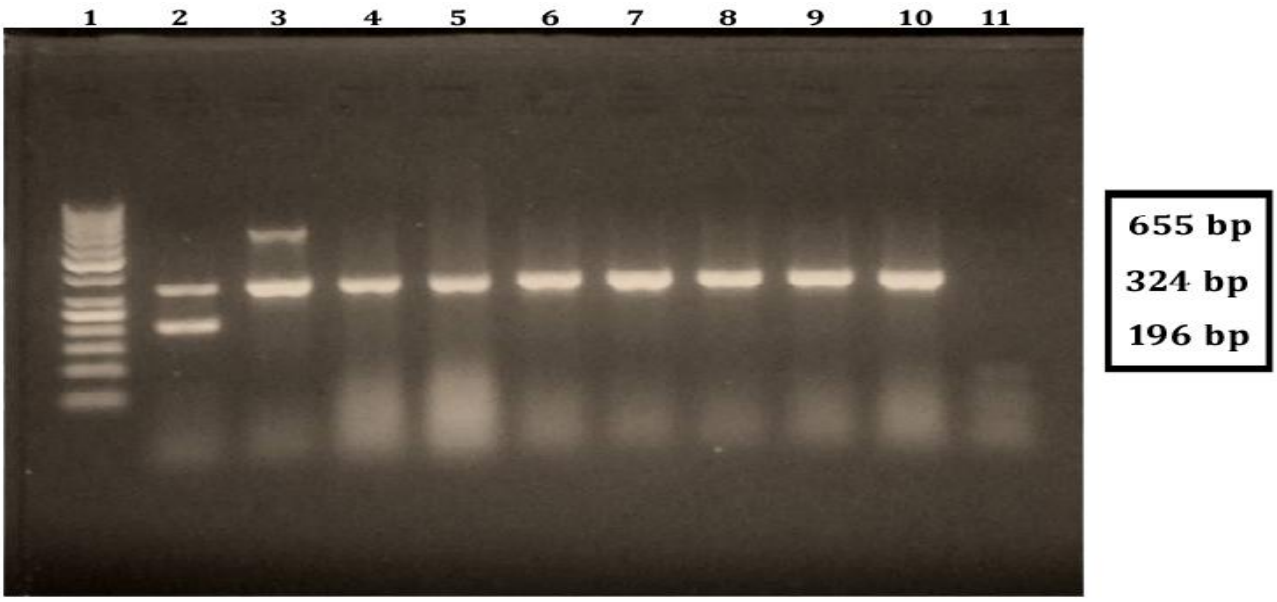
As shown in Table(2), ***C. perfringens*** was isolated from 90 out of 140 samples collected from apparently healthy and diseased chicken samples with incidence of 64.3% In this study, ***C. perfringens*** was isolated from liver and intestinal samples of examined chickens.

Table (2). Incidence of *C. perfringens* in examined samples.

Source of the isolates	Number of examined samples	Number of positive samples	Percentage %
Apparently healthy birds	Intestine 70 Liver -	40 -	57.1 -
	Total 70	40	57.1
Diseased, dead birds	Intestine 35 Liver 35	35 15	100 42.8
	Total 70	50	71.4
Total	140	90	64.3

On *perfringens* agar (TSC) the bacterial colonies were black with opaque halo, Microscopic characters revealed gram positive non motile rods. Biochemical identification of the isolates showed catalase, lecithinase, positive, all the isolates produce stormy fermentation and coagulation in litmus milk

Multiplex PCR. PCR analysis of twenty representative *Clostridium perfringens* isolates revealed that all the examined isolates were genotyped as ***C. perfringens*** type **A** where the only amplified gene was that of the alpha toxin as shown in **Figure 1**



FIGUR 1

Agarose gel electrophoresis of PCR product of some *C.perfringens*

Lane 1: 50 bp ladder DNA marker (50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900,1000bp)
Lane 2: C. perfringens type C as positive control (324 and 196 bp)
Lane 3: C. perfringens type D as positive control (324 and 655 bp)
Lanes 4-10: representative isolates identified to be C. perfringens type A (324 bp)
Lane 11: negative control

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. It is often found in the intestinal tract of healthy birds but it can cause outbreaks of disease in many species of poultry especially in broiler and turkey flocks **(Craven, *et al.*, 2001; Engström, *et al.*, 2003)** and also with (**McDevitt *et al.*, 2006 and Craven, *et al.*, 2001**) who analysed intestinal contents of broiler chickens and found that approximately 75% to 95% of the birds were have been colonized by ***C. perfringens*** with only a small proportion of these ever showing signs of the disease, this come in agreement with our results since we could isolate ***C. perfringens*** from apparently healthy (57.1%) and diseased, dead (71.4%) chicken samples.

In the present study, 90 ***C. perfringens*** strains were isolated from 140 samples of broilers; the isolates were reconfirmed on the basis of morphological and cultural characteristics. The obtained colonies were black on TSC agar at 37° C for 48 hrs. Microscopically all isolates were Gram-positive spore-forming bacilli, biochemical identification of the isolates showed both catalase and lecithinase positive **(Koneman *et al.,* 1992; El-Jakee *et al.,* 2010).**

In recent years many authers have focused on the molecular typing of ***C. perfringens*** by detection of toxin genes using multiplex PCR **(Engström, *et al.,* 2003; Heikinheimo, A. and Korkeala, H. 2005)**

PCR has been widely used in identifying the toxin genes of ***C. perfringens*** because of its high sensitivity, this test was established to replace animal testing and to reduce cost and time as reported by **(Baums, *et al*., 2004; Das *et al.,* 2008).** In the present study, the PCR protocol published by **(Meer and Songer 1997**) was chosen in order to determine the presence of major toxin genes *(cpa, cpb, etx and iA*) from ***C. perfringens*** isolates obtained from broiler chickens.

All the twenty isolates were ***C. perfringens*** type A with the gene coding for alpha toxin. These results come in accordance with **(Yoo *et al.,* 1997; Engström *et al.,* 2003; Kalender and Ertas 2005; Sheedy *et al.,* 2004; Effat *et al.,* 2007; Fayez *et al.,* 2013)** Who mentioned that ***C.perfringens*** type A was the most predominant type isolated from broiler chickens .

Conclusion

Clostridium perfringens Type A (α-toxin producer) was found to be wide spread in the poultry farms and is common in the intestinal tract of healthy birds. Isolation of *C. perfringens* type A in chickens slaughtered for human consumption has a crucial impact on public health. Cross contamination with *C. perfringens* occurring during slaughter and meat processing could be an important threat to public health. To combat the disease as well as to minimize the losses due to this bacterial infection in poultry, and reduce human exposure to this important microorganism proper investigation, detailed epidemiological studies are needed to give a certain idea on chicken enterotoxemia also further molecular analysis of the pathogen and the role of *cpa* gene in association with the disease is required to be understood for undertaking the development of control measures, especially for the formulation of cost effecting vaccine.

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