PCR based detection of Alpha toxin gene in *Clostridium perfringens* strains isolated from diseased broiler chickens

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A B S T R A C T

Necrotic enteritis is a highly prevalent global disease of poultry caused by *C. perfringens*, the disease causes severe economic losses in poultry industry due to bird losses and costs of treatment and preventive measures. In order to investigate the prevalence, the antimicrobial susceptibility as well as molecular characterization of Alpha toxin of *C. perfringens*, a total of 85 samples (intestine = 46 and liver =39) were collected aseptically from freshly dead chickens (with history of sever enteritis, 2-4 weeks old) from commercial broilers farms at Ismailia Governorate. Egypt. The collected samples were subjected to bacteriological examination where the total percent of the isolated *C. perfringens* strains was (57. 6%) (n=49). The antibiotic sensitivity test was carried out using disc diffusion method where the isolated strains were sensitive to Ciprofloxacin (100%), Amoxicillin clavulinc acid (100%) and penicillin (91,8%) and highly resistant to neomycin (100%), Streptomycin (100%) and Erythromcin (89,8%). PCR protocol was applied for amplification and detection of Alpha toxin gene in the isolated *C. perfringens* strains, where all the tested strains were carried Alpha toxin gene with specific amplicon size at 402 bp. Briefly, combination of phenotypic and genotypic analysis of *C. perfringens* is a valuable epidemiological tool for identification of isolates. PCR is a rapid and specific diagnostic tool used for genetic detection of alpha toxin of *C. perfringens*. Antimicrobial susceptibility testing is necessary to determine the drug of choice and monitoring resistance to different antibiotics.

Key words: necrotic enteritis, *C. perfringens,* Alpha toxin, PCR, Antibiotic sensitivity

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1. INTRODUCTION

Necrotic enteritis is an emerging worldwide disease of poultry. It is characterized by sever diarrhea, necrotic inflammation of the intestinal tract and necrotic foci in liver, kidney and cecum (Kohler, 2000). The disease resulting in severe economic losses that exceed 2 billion US dollar / year due to bird losses, treatment costs and cost of disease preventive measures (Lovland and Kaldhusdal, 2001). Necrotic enteritis has two clinical forms in chickens; the first form (acute form) is mainly characterized by sever intestinal necrosis and mainly accompanied with high mortalities. In addition, the other form of the disease (subclinical form) is mainly characterized by mucosal damage and mainly associated with lower mortality rate (Lovland and Kaldhusdal, 2001). The disease is mainly transmitted by ingestion of contaminated food and water. its main source is the contaminated environment and litter (Craven et al., 2003). *C. perfringens* is a Gram-positive, spore forming, anaerobic bacilli which normally inhabits the intestinal tracts of both human and animals. The microorganism is the principle cause of necrotic enteritis in poultry. (Immerseel et al., 2004; Labbe and Juneja, 2006; Timbermont et al., 2011). *C. perfringens* is classified according to the production of 4 major toxins (alpha, beta, epsilon and iota)

into 5 different toxino-types including A, B, C, D and E (Yoo et al., 1997). All toxino-types of *C. perfringens* produce Alpha toxin which has lecithinase activity and causes pathological modifications and necrosis in tissues. Alpha toxin is primarily responsible for necrotic enteritis in poultry (Keyburn et al., 2010). *C. perfringens* type A and with less extent type C have been shown to be the major causes of necrotic enteritis in chickens (Cooper and Songer, 2009). The polymerase chain reaction based detection of Alpha toxin is essential for the typical identification of alpha toxigenic *C. perfringens* strains (Baums, et al., 2004). In order to control and prevent the necrotic enteritis in chickens, antibiotics should be mixed the bird water and ration with consideration to the bacterial resistance to several antimicrobial agents such as to tetracycline and erythromycin (Silva, et al., 2009).

This work was planned to investigate the prevalence of *C. perfringens* in broiler chickens, monitoring the antimicrobial susceptibility of the isolated strains as well as molecular characterization of Alpha toxin in the isolated *C. perfringens* strains.

2. MATERIAL AND METHODS

2.1. Sampling:

A total of 85 samples (intestine = 46 and liver =39) were collected aseptically from freshly dead chickens (with history of sever enteritis, 2-4 weeks old) from commercial broilers farms at Ismailia Governorate, Egypt. Samples were collected after postmortem examination and only intestine and liver showing necrosis or necrotic foci were collected. Samples were taken to the laboratory in an ice box as soon as possible.

2.2. Isolation and identification of C. perfringens:

Sterile saline was added to the collected samples and heated at 80° C for 20 min in water bath. Then the processed Intestinal contents and liver pieces were inoculated into Robertson cooked meat medium (Oxoid) and sterile liquid paraffin was poured to make a layer over the medium. Inoculated medium was incubated anaerobically at 37° C for 24h. Then a loopful of broth culture was streaked onto neomycin sulphate sheep blood agar plates and egg yolk agar, then the streaked plates were incubated anaerobically at 37° C for 48h. using Gaspak anaerobic jar. Suspected pure colonies were identified according to morphological characters using Gram's stain, hemolysis on blood agar (double zone), lecithinase activity and biochemically using methods described by (Quinn et al., 2002).

2.3. Antimicrobial susceptibility testing by disc diffusion method:

The susceptibility to (8) types of antibiotics (Penicillin, Amoxicillin- Clavulanic acid, Tetracyclin, Neomycin, Ciprofloxacin, Streptomycin, Chloramphenicol and Erythromycin) was tested according to the procedures of (NCCLS, 2007) using disc diffusion technique and anaerobic cultivation in anaerobic jar. The susceptibility of the strains was determined according to the size of inhibition zone.

2.4. PCR detection of Alpha toxin gene of C. perfringens:

2.4.1. Extraction of DNA from C. perfringens strains (Sambrook and Russel, 2001):

Fresh bacterial colonies on blood agar plates were picked and suspended in Eppindorf tubes containing 1 ml of distilled water. Following 3 cycles of freeze-thawing, the tubes were boiled for 20 min and finally centrifuged at 10000 rpm for 5 min. The supernatants (containing DNA) were transferred to a fresh tube and kept frozen until used as templates in PCR.

2.4.2. Estimation of purity and concentration of DNA according to (Sambrook and Russel, 2001):

The concentration and purity of DNA that had been extracted was determined by estimating the optical density at wave lengths of 260 and 280 nm using the

spectrophotometer. The concentration was calculated as follows: OD260 = 50 ug /ml. - Purity of DNA = OD260 nm/ OD280 nm. The purity of DNA had a value of 1.8, where the optimal one ranged between 1.8-2.

2.4.3. DNA Amplification (polymerase chain reaction):

DNA samples were tested [in 50 µl. Reaction volume in a 0.2 ml. PCR tube, containing PCR buffer] (50 mM Kcl, 10 mM tris - Hcl, 1mM $Mgcl_{2)}$ each dNTPS (Deoxy nucleotide Triphosphate) 200 uM each (dATP, dGTP, dCTP and dTTP), [Two primer pairs each at 50 picomol / reaction] and 0.5 of taq DNA polymerase. Thermal cycling in a programmable thermal cycler (Coy vorporation, Grasslake, Michan, USA) was done. A negative control PCR reaction with no template also was included in this assay.

Table (1): list of primers used for PCR assay:

Primer	Primer Sequence	Annealing temperature	PCR product	Reference
Alpha F.	5′-GTT GAT AGC GCA GGA CAT GTT AAG-3′	55°C	402 bp	*(Yoo, et al, 1997)*
Alpha R.	5′-CAT GTA GTC ATC TGT TCC AGC ATC-3′			

PCR program: Initial denaturation (95ºC - 5 min.). 30 cycles (denaturation at 94 ºC - 1 min.; annealing at 55 ºC - 1 min.; extension at72 ºC - 1 min). Final extension at 72°C for 3 min.

2.4.4. Screening of PCR products:

Ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volts for 1 hour. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

3. RESULTS

3.1. Postmortem examination of freshly dead broiler chickens:

Birds showed severe dehydration, severe necrosis and dilatation of small intestine which appeared to be friable and necrotic foci in liver (in many cases).

3.2. Prevalence of C. perfringens in broilers chicken:

As shown in Table (2), Out of (46) collected intestine samples, the prevalence of *C. perfringens* was (65,9 %) (n= 31). On the other hand, out of (39) collected liver samples, the prevalence of *C. perfringens* was (46,1 %) (n = 18). In addition, the prevalence of *C. perfringens* isolated from broilers chicken in relation to the total number of collected samples was (57,6 %) (Total number of isolated strains = 49).

3.3. The interpretation of antibiotic sensitivity test:

As shown in Table (3), the interpretation of antibiotic sensitivity test revealed that, the isolated *C. perfringens* strains were highly sensitive to Ciprofloxacin (100%), Amoxicillin clavulinc acid (100%) and penicillin (91,8%), moderately sensitive to Chloramphenicol (100%) and Tetracyclin (59,2%) and highly resistant to neomycin (100%), Streptomycin (100%) and Erythromcin (89,8%).

3.4. PCR detection of Alpha toxin gene of C. perfringens:

In the present work, the isolated *C. perfringens* strains were subjected to PCR amplification and detection of Alpha toxin gene, all the tested strains were positive for Alpha toxin gene with specific amplicon

size (402 bp). Figure (1), illustrate the positive amplification of Alpha toxin in the isolated *C. perfringens* strains, where Lane (1) :100 bp DNA ladder, lane (2): control negative and lanes (3-6): showed positive isolated *C. perfringens* strains (402 bp).

Table (2): No. and percentage of *C. perfringens* isolated from broilers chicken

Organ samples	No. samples	No. of *C. perfringens* strains	% of *C. perfringens* strains
Intestine	46	31	65,9 %
Liver	39	18	46,1 %
Total	85	49	57,6 %

Table (3): Interpretation of antibiotic sensitivity test:

Antimicrobial agents	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Penicillin	-	-	4	8,2	45	91,8
Ciprofloxacin	-	-	-	-	49	100
Neomycin	49	100	-	-	-	-
Chloramphenicol	-	-	49	100	-	-
Erythromycin	44	89,8	5	10,2	-	-
Tetracycline	20	40,8	29	59,2	-	-
Sreptomycin	49	100	-	-	-	-
Amoxicillin- Clavulanic acid	-	-	-	-	49	100

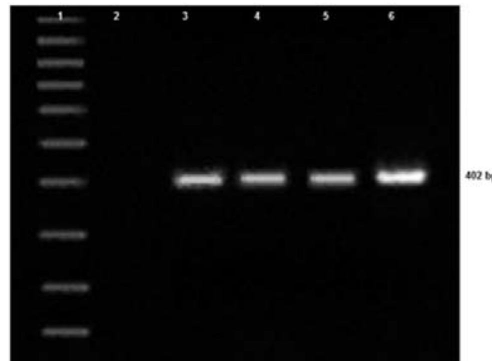

Fig. (1): Electrophoretic pattern of Alpha toxin PCR assay: Lane (1) :100 bp DNA ladder, lane (2): control negative and lanes (3-6): showed positive isolated *C. perfringens* strains (402 bp).

4. DISCUSSION

Necrotic enteritis is a highly prevalent worldwide disease of poultry caused by *C. perfringens*, resulting in severe economic losses. All types of *C. perfringens* produce Alpha toxin which is the main virulence factor incriminated in necrotic enteritis in chickens. In the present study, the prevalence of *C. perfringens* in affected chickens was (57. 6%) as shown in Table (2). High prevalence of *C. perfringens* in suspected cases with necrotic enteritis also recorded by (Malmarugan and Johnson, 2012; EI-Jakee et al., 2013). There are many predisposing factors that favor the occurrence of necrotic enteritis including stress; coccidiosis mucosal damage; poor sanitation; unbalanced nutrition and bad housing. All of these factors allow the heavy growth and massive toxin production of C. perfringens (Vissiennon et al., 1994; Craven, 2000; Jia et al., 2009). Regarding the results of antimicrobial susceptibility, as

shown in Table (3), the isolated *C. perfringens* strains were sensitive to Ciprofloxacin (100%), Amoxicillin clavulinc acid (100%) and penicillin (91,8%), moderately sensitive to Chloramphenicol (100%) and Tetracyclin (59,2%) and highly resistant to neomycin (100%), Streptomycin (100%) and Erythromcin (89,8%) These results nearly agreed with those obtained by (Gad et al., 2011; Llancol et al., 2012). Amoxicillin /clavulanic acid had proved strong bactericidal activity against *C. perfringens* strains which agrees with several studies performed in other countries (Gharaibeh et al., 2010). The resistance of *C. perfringens* to Macrolids group (erythromcin) is mainly due to the presence of the ermQ and ermB genes which are coded for enzymes production for the 23S rRNA dimethylation that resulting inhibition the antibiotic action on Bacteria (Berryman et al., 1994), while the resistance to tetracycline is mainly attributed to the presence of the tetP gene (Silva et al., 2009). Aminoglycoside (Streptomycin and neomycin) resistance is mainly occurred due to reduction of the antibiotic uptake (Davies and Wright, 1997). In this study PCR assay was used for amplification and detection of Alpha toxin gene, Fig. (1) illustrated the positive amplification Alpha toxin gene at 402 bp, all the tested isolated strains were positive (Alpha toxigenic strains); these results agreed with those obtained by (Effat et al., 2007). These findings confirm that the Alpha toxin is primarily responsible for necrotic enteritis in chickens. Polymerase Chain Reaction is mainly used in detection of virulence genes of *C. perfringens* due its high specificity and sensitivity (Baums, et al., 2004). All toxigenic types of *C. perfringens* able to produce Alpha toxin which has lecithinase activity and causes tissue necrosis especially in small intestine. Alpha toxin is mainly responsible for necrotic enteritis in chickens (Keyburn et al., 2010). In conclusion, the combination of phenotypic and genotypic analysis of *C. perfringens* is a valuable epidemiological tool for identification of isolates. PCR is a rapid and specific diagnostic tool used for genetic detection of alpha toxin of C. perfringens. Antimicrobial susceptibility testing is necessary to determine the drug of choice and for monitoring the resistance to different antibiotics.

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