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Necrotic enteritis caused by *Clostridium perfringens* in broiler chickens

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Abstract

Necrotic enteritis caused by *C. perfringens* is a complex and multi-factorial disease associated with high mortality rate and severe economic losses. Bacteriological examination for detection of *C. perfringens* was carried out on 50 Necrotic enteritis (NE) diseased broiler in Behira province .The results showed that *C. perfringens* was recovered as 20 isolates with percent of 40% of examined samples .Typing of *C. perfringens* isolates was done by dermonecrotic reaction test using intradermal injection in albino Guinea pigs, revealed that 16 isolates were toxigenic (80%) where all of them were of type A and 4 isolates (20%) were non toxigenic .Typing was confirmed by PCR technique. Antimicrobial susceptibility test for *C. perfringens* isolates showed that all tested isolates were sensitive to ciprofloxacin and Amoxycillin while most of them were resistance to tetracyclin , trimethoprim-sulfamethoxazole and enrofloxacin.

Keywords: *Clostridium perfringens; Necrotic enteritis, Broiler*

Introduction

Necrotic enteritis causing economic losses for the poultry industry considered over $6 billion, by lowering growth performance and increase cost of veterinary treatments **(Wade and Keyburn 2015).** It is the most common clostridial enteric disease in poultry, which typically found in broiler chickens **(Cooper *et al.,* 2013)**. It caused by the overgrowth of pathogenic *C. perfringens* which induces intestinal mucosal necrosis **(Prescott *et al.,* 2016).** It characterized by necrosis and inflammation of the GIT with a significant decrease in growth performance and, in clinical cases, a great increase in mortality rate. The total cost of NE outbreaks to be over $2 billion annually **(Vander Sluis, 2000).** *C. perfringens* is the main causative agent of NE; its source is ultimately the chickens themselves **(Cooper and Songer 2009).** *C. perfringens* is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium **(Baba *et al.,* 1997)** widespread in nature and commonly found in the intestines of humans and animals ***(Uzal et al., 2014)*** have five toxinogenic types (A, B, C, D, E), NE is caused by type A isolates **(Songer and Meer 1996)** and rarely caused by type C **(Engstrom *et al.,* 2003).** Clinical form of NE may cause high levels of mortality; the subclinical form of the disease is more significant. Diseased birds which remain untreated because the disease is undetected cause great economic losses for the poultry production **(Wade and Keyburn 2015).** Subclinical NE occurs without a substantial increase in mortality, but with clear signs of intestinal affection, there is damage of the intestinal mucosa, decrease performance effect digestion and absorption, resulting in weight loss, high feed conversion with lower production **(Cooper *et al.,* 2013)**. Necrotic enteritis is associated with severe fatal diarrhea due to the production of toxins by clos-

tridial microorganisms which damage the epithelial lining of the intestinal mucosa followed by the invasion of clostridia producing exotoxins into the blood stream **(Cowen *et al.,* 1987 and Baba *et al.,* 1992)**. During necropsy of affected bird typical necrotic lesions can be observed in the intestinal tract **(Kaldhusdal *et al.,* 2001 and Van Immerseel *et al.,* 2004),** range from friable walls and thin to clear extensive necrotic lesions **(Cooper and Songer 2010).**

Rapid techniques such as molecular characterization and toxinotyping were used for the detection of *C. perfringens* from suspected necrotic enteritis cases **(Thomas *et al.,* 2014)**.
The objective of this study was to characterize and identify C. *perfringens* causing necrotic enteritis from broiler farms.

Materials and Methods

1-Samples collection:

Intestinal samples from 50 chickens of 25 to 32 days of age were collected aseptically from flocks experiencing NE in broiler farms (marked depression, decreased appetite, ruffled feathers, enteritis and diarrhea). Each sample was kept in a sterile plastic bag in an ice box and brought to the laboratory without delay.

2-Bacteriological examination:

Samples from intestine were inoculated under a septic condition into broth of freshly prepared boiled then rapidly cooled cooked meat medium (CMM) (Oxoid) and incubated for 24 hours at 37°C anaerobically in a Gaspak anaerobic jar **(Willis, 1977)**: A loopful of inoculated medium was streaked onto neomycin sulphate (200ug/ml) sheep blood agar plates then re-incubated anaerobically for 24 h at 37°C **(Cruickshank, *et al.,* 1975).** Suspected *C. perfringens* colonies were tested on egg yolk agar medium for lecithinase activity. Typical colonies (lecithinase producer and showed double zone of haemolysis on blood agar medium) were picked up, sub-cultured and purified for further biochemical identification tests **(Koneman *et al.,* 1983).**

3- Antimicrobial susceptibility testing:

Ten *C. perfringens* isolates were tested by a disc diffusion method according to **Cruickshank *et al.,* (1975)**, isolates were examined in vitro against eight antibiotics, which included Penicillin (10 µg), amoxycillin (10 µg), ampicillin (10 µg), ciprofloxacin (5 µg), enrofloxacine (5 µg), Tetracycline (30 µg), Chloramphenicol 30 µg), and trimethoprim-sulfamethoxazole (1.25 + 23.75 µg) (Oxoid, UK).
The results were interpretteted according to **(NCCLS 2002)**.

4-Typing *C. perfringens* isolates by dermonecrotic test and neutralization in Albino Guinea pigs

according to (**Smith and Holdeman, 1968**) showed that all isolates were type A (which appeared as an irregular area of yellowish necrosis tended to spread downward) as reported by (**Sterne and Batty, 1975).** Followed confirmatory test for 6 isolates by polymerase chain reaction as follow:

5-DNA extraction:

Was performed for 6 isolates using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification: Primers were utilized in a 50 µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 2 µl of each primer of 10 pmol concentrations, 5 µl of DNA template and the mixture was completed by sterile DW to 50 µl. The reaction was performed in an Applied biosystem 2720 thermal cycler, programmed to: Primary denaturation 1 cycle of 94°C (5 min), followed by 35 cycles of 94°C (30 sec), 55°C (40 sec) and 72°C (45 sec) and a final cycle of 72°C (10 min) to allow the final DNA extension.

Analysis of the products:
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gel pilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and direction

Target gene	Direction	Primers sequences	Fragment length bp	
α/cpa	F	$^{5-}$GTTGATAGCGCAGGACATGTTAAG^{-3}	402	**According to Yoo *et al.*, 1997**
	R	^{5}-CATGTAGTCATCTGTTCCAGCATC^{-3}		
β/cpb	F	$^{5-}$ACTATACAGACAGATCATTCAACC^{-3}	236	
	R	$^{5-}$TTAGGAGCAGTTAGAACTACAGAC^{-3}		
ε/etx	F	$^{5-}$ACTGCAACTACTACTCATACTGTG-3	541	
	R	$^{5-}$CTGGTGCCTTAATAGAAAGACTCC^{-3}		
ί/iap	F	$^{5-}$GCGATGAAAAGCCTACACCACTAC^{-3}	317	
	R	$^{5-}$GGTATATCCTCCACGCATATAGTC^{-3}		

Results
Out of (50) collected intestine samples; the prevalence of *C. perfringens* was (40 %) (n=20) from the examined samples, table (2).
Table (3) showed that typing of (20) *C. perfringens* isolates by dermonecrotic test in Guinea Pigs revealed that 16 (80 %) of isolates were toxigenic and all of them were of type A and 4 isolates (20 %) were non toxigenic.
Conformation of tested isolates by PCR proved that they harbored only the *cpa* gene encoding the α-toxin production and none of these strains harbored the *cpb, etx, iap.*

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

No. of samples examined	+ Ve *C. perfringens*		- Ve *C. perfringens*	
	No.	%	No.	%
50	20	40	30	60

% calculated according to No of examined samples.

Table (3). Typing of *C. perfringens* isolates by dermonecrotic test in Guinea Pigs

No. of *C. perfringens* isolates	Types of toxigenic isolates		Non toxigenic isolates	
	Type A			
	No.	%	No.	%
20	16	80	4	20

% calculated according to No of *C. perfringens* isolates.

Table (4). Antimicrobial susceptibility test of *C. perfringens* toxigenic isolates (N=16):

Antimicrobial agents / Potency	Sensitive	Intermediate	Resistant
	No. %	No. %	No. %
Penicillin 10 µg/mg	14 (87.5)	2 (12.5)	-
Ciprofloxacin 5 µg/mg	16 (100)	- -	-
Chloramphenicol 30 µg/mg	-	16 (100)	- -
Tetracycline 30 µg/mg	-	1 (6.3)	15 (93.7)
Amoxycillin 10 µg/mg	16 (100)	- -	- -
trimethoprim-sulfamethoxazole25 µg/mg	- -	2 (12.5)	14 (87.5)
Enrofloxacine 5 µg/mg	- -	3 (18.8)	13 (81.2)
Ampicillin 10 µg/mg	14 (87.5)	2 (12.5)	- -

% calculated according to the No of tested isolates (16)

Analysis of the PCR Products:

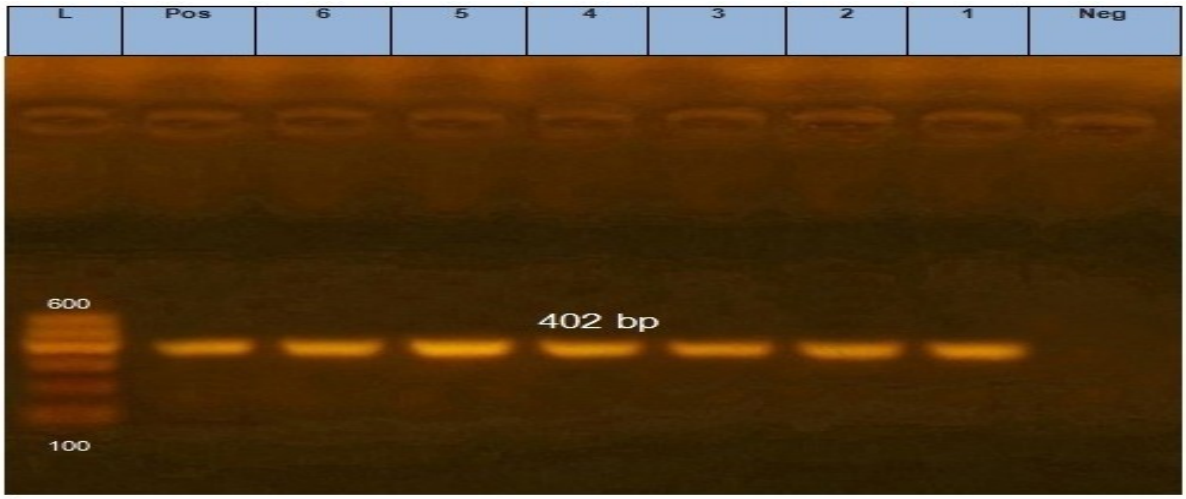

Photo. (1): Electrophoretic pattern of alpha toxin PCR assay: Lane (L):100 bp DNA ladder, lane (Neg): control negative, lane (Pos): control positive and lanes (1-6): showed positive isolated *C. perfringens* strains (402 bp).

Discussion

C. perfringens type A was mainly isolated from poultry chicken; produces a α-toxin, a phospholipase C that hydrolyzes phospholipids result in production of inflammatory mediators and acute death **(Titball., 1993)**. One of the most important toxins is α toxin which considered as the major virulence factor responsible for producing lesions in necrotic enteritis **(Gholamiandekhordi *et al.,* 2006** and **Van Immerseel *et al.,* 2008).**

Al-Sheikhly and Truscott, (1977) reported induction of NE by intro-duodenal infusions of both large volumes of *C. perfringens* broth culture and crude toxins which resulted in typical lesions of NE into chickens. During the experimental challenge the inoculated group develop signs of NE result after oral inoculation of *C. perfringens* type A concluded that α -toxin was the main toxin produced by *C. perfringens*, and then it was considered the most virulent factor in the pathogenesis of NE.

Fukata *et al.,* (1988) found that 21 out of 56 germ-free chickens, inoculated with *C. perfringens* either purified α -toxin or a supernatant of broth cultures died after inoculation, whereas no bird died after receiving a culture supernatant neutralized by anti alpha-toxin serum. Later on, **Hofshagen and Stenwig (1992)** found a significantly higher amount of α -toxin in isolates from birds with NE compared with isolates from birds without NE. In other study, **Cooper and Songer (2009)** suggested that immunization with α -toxin gave substantial protection against NE and **Rehman *et al.,* (2009)** concluded that α -toxin can damage the intestinal mucosal barrier. Contradicting these studies, in vitro study **(Gholamiandekhordi *et al.,* 2006)** demonstrated no difference in alpha toxin production between *C. perfringens* isolated from healthy flocks and those isolated from NE outbreaks flock, suggested that alpha-toxin of *C. perfringens* was not a major factor in producing NE in chickens came from a study using an a toxin negative mutant of *C. perfringens* generated from a virulent strain isolated from NE infected birds.

The *alpha* toxin is produced by all the types of *C. perfringens*. The toxin is a necrotizing toxin which is believed to be a virulence marker **(Murray *et al.,* 2003 and Ata *et al.,* 2013)**. It is the main virulence factor incriminated in necrotic enteritis in chickens. Necrotic enteritis (NE) caused by *Clostridium perfringens* type A is a persistent problem affecting 1.3 to 37.3% of rapidly growing broiler chickens, re-

sulting in direct and indirect economic losses **(Ficken and Wages, 1997).** In the present study, the prevalence of *C. perfringens* in affected chickens was (40%) which near to isolation rate recorded by **Ghada *et al.,* (2017)** as 49.7% when compared to low isolation rate reported by **Kalender and Ertas (2005) and Miah *et al.,* (2011)** who reported lower prevalence rate of necrotic enteritis 5 and 8% from intestine of broiler chickens, respectively and all isolates were of type A which generally agree with several studies found that most predominant type in chickens is type A (**Awad *et al.,* 1977, Latinovic, 1983, Demir *et al.,* 1989** and **Holfshagen *et al.,* 1992**). Variation found due to different methodologies used for isolation, classifying the microorganism as well as poultry farms management used. Photo (1) illustrated the positive amplification Alpha toxin gene at 402 bp, all the tested isolated strains were positive (Alpha toxigenic strains); these results were agreed with those obtained by **Effat *et al.,* (2007)** and **Algammal and Elfeil (2015)**, also these findings were agreed with results recorded by **(Keyburn *et al.,* 2010).** As shown in the results of table (4) the tested isolates of *C. perfringens* strains were sensitive to Ciprofloxacin (100%), Amoxicillin (100%) Ampicillin (87.5%) and penicillin (87.5%), moderately to Chloramphenicol (100%) and resistant to Tetracyclin (93.7%) , trimethoprim-sulfamethoxazole (87.5%) and enrofloxacine (81.2%). **Hamza *et al.,* (2017)** found the resistance to streptomycin, lincomycin, and trimethoprim-sulfamethoxazole was 100%, 100%, and 94% respectively, while appropriate percentages for ciprofloxacin, cefotaxime, and rifampicin were 41, 34, and 31, respectively. On the other hand, *C. perfringens* isolates showed high sensitivity to amoxicillin (94%) and ampicillin (97%). These results were in agreement with those of **Osman and Elhariri (2013)** who mentioned that *C. perfringens* isolates showed high resistance to streptomycin (100%), lincomycin (100%), and trimethoprim -sulfametho-xazole (98%). Furthermore, results reported by **(Silva *et al.,* 2014 and Abd El–Hamid *et al.,* 2015)** showed intermediate sensitivity of *C. perfringens* to cefotaxime, ciprofloxacin, and low sensitivity to lincomycin. **Abd-El Gwad and Abd El-Kader (2001)** demonstrated that *C. perfringens* isolates were highly sensitive to ampicillin, ciprofloxacin, and amoxicillin, this is consistent with the present results suggesting that ampicillin and amoxicillin may be the drugs of choice for *C. perfringens* infection **(Agunos *et al.,* 2012).** Studies have shown that amoxicillin is effective against necrotic enteritis and its use is suggested for prevention of *C. perfringens* infection **(Lanckriet *et al.,* 2010). Llanco *et al.,* (2012).** Found that all *C. perfringens* strains were susceptible to amoxicillin, amoxicillin-clavulanic acid, cefoxitin, chloramphenicol, enrofloxacin, metronidazole and penicillin-streptomycin and the resistant to tetracycline was observed as 32% of the tested strains. **Algammal and Elfeil (2015)** isolated *C. perfringens* strains which 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%). **Tsiouris *et al.,* (2010)** reported that crude supernatant from *C. perfringens* type A cultures induced necrotic lesion in broilers. Moreover, the development of NE lesions prevented partially by antibodies against C. *perfringens* toxin - α. The intestinal necrosis characteristic of NE is caused by the potent α -toxin produced by *C. perfringens* **(Al -Sheikhly and Truscott, 1977).**

Conclusion

C. perfringens type A was the predominant isolates from broiler farm during these study with characteristic sensitivity for amoxycillin and ciprofloxacin and resistant against tetracyclin, enrofloxacin and trimethoprim-sulfamethoxazole. Further studies needed for virulence genes of type A *C.perfringens* to control NE in broilers.

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