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Typing of *Clostridium Perfringens* Isolates Recovered from Necrotic Enteritis in Turkeys in Egypt by Multiplex PCR

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Abstract*: Necrotic enteritis in turkey poults has emerged in Egypt in the last years as an economic problem causing great losses and great concern for the breeders. The present study was undertaken to study the incidence of the disease and types of the Clostridium perfringens involved in apparently healthy and diseased turkeys. A total of 122 intestinal turkey poults samples (39 intestinal tissue and its contents and 83 cloacal swabs) was examined, of which 56 (45.9%) were culture positive. Clostridium perfringens was confirmed by cultural and biochemical characters in 45 isolates from 75 diseased turkey poults and 11 isolates from 47 apparently healthy turkey poults. The 56C. Perfringens isolates were analyzed by multiplex PCR in order to determine the toxin genes for the molecular typing. A mixture of primers of alpha toxin (324bp), beta toxin (196bp), epsilon toxin (655bp), iota toxin (446bp) and enterotoxin (233bp) were used. In all tested isolates the CPA gene was detected, confirming the 56 isolates as Clostridium perfringens. In one of the isolates the CPA (324bp) and CPE (23bp) genes were detected indicating that it was C. perfringens type A with enterotoxin (0.82%). In another isolate the presence of 2 bands (324bp) & (196bp) confirmed the isolate as C. Perfringens type C (0.82%).A third isolate was confirmed as C. perfringens type E (0.82%) as it showed 2 for the CPA(324bp)a iA (446bp)genes Fig.(4).It was concluded that53 isolates had one band (324bp) for the CPA gene only, i.e. they contained alpha toxin and thus they were confirmed as C. perfringens type A (48.6%), of these 45 isolates were from 75 diseased turkey samples were type A without enterotoxin (60%), 8 isolates from 47 healthy turkey samples were Type A without enterotoxin (17%).From 47 apparently healthy turkey samples one isolate was Type A with enterotoxins, another isolate was Type C, and a third isolate was Type E .Types B and D were not identified.*

Keywords: *Clostridium perfringens (CP), Necrotic enteritis (NE), turkeys, multiplex PCR*

1. INTRODUCTION

Avian necrotic enteritis was first described in 1961 [1]and since then it has been reported to occur in almost all poultry-producing countries [2-11].Studies showed that the subclinical form of necrotic enteritis is a worldwide problem with an average of 80% of the flocks having had clostridium diagnosed [2]. A follow-up study in 2005 indicated an increased incidence of clostridial enteritis in all regions of the world. Recent European surveys confirmed the severity and the widespread of the problem. Necrotic enteritis is caused by toxins produced by *Clostridium perfringens*, which is often found in the intestinal tract of healthy birds, and when it grows in the intestinal tract, it can produce toxins. The disease may occur in the form of outbreaks in poultry, and especially in broiler and turkey flocks, causing acute clinical disease characterized by necrotic enteritis[13].

Clostridium perfringens is a Gram-positive, spore-forming and anaerobic bacterium responsible for a wide range of diseases in humans and animals[14-16]. *Clostridium perfringens* is commonly classified to toxigeno types based on the types of toxins they produce. The main toxins produced by strains of *CP* are α, β, ε and ι [13,17]. The conventional method of *Clostridium perfringens* typing is based on the detection and typing of the toxins with toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals. Moreover, it is time consuming. In recent years, molecular techniques such as polymerase chain reaction (PCR) are increasingly used to type *CP*[13,18-21].

The aim of this research was to determine the prevalence of necrotic enteritis in turkeys and identify the different types of *Clostridium perfringens* isolated from living and slaughtered turkeys by PCR molecular method.

2. MATERIALS AND METHODS

2.1. Sampling

A total number of 122 samples (39 intestinal samples and 83 faecal swabs samples) were collected from apparently healthy and diseased turkey poults. They were obtained from different slaughter houses and butchers' shops in Giza (27) and from Kafr Ghataty farm (95).

2.2. Material used for DNA Extraction, PCR, and Agarose Gel Electrophoresis

All chemicals and reagents were molecular biology grade. All reagents and buffers were prepared according to[22]**.**

a. TrisEdita buffer (TE):10mM Tris HCL and 1mM EDTA (pH was adjusted to 7.6)

b. PCR master mix: (5x Taq Master Mix, Jena bioscience).

c. Oligonucleotide primers:

Primers for the five toxin genes (alpha, beta, epsilon, iota and enterotoxin) of *C. perfringens* were selected from the published paper [23] as follows:

Nucleotide Sequence (5` To 3`)

cpa:	Forward	GCTAATGTTACTGCCGTTGA
	Reverse	CCTCTGATACATCGTGTAAG`
cpb:	Forward	GCGAATATGCTGAATCATCTA
	Reverse	GCAGGAACATTAGTATATCTTC`
etx:	Forward	GCGGTGATATCCATCTATTC
	Reverse	CCACTTACTTGTCCTACTAAC
iA:	Forward	ACTACTCTCAGACAAGACAG
	Reverse	CTTTCCTTCTATTACTATACG`
cpe:	Forward	GGAGATGGTTGGATATTAGG
	Reverse	GGACCAGCAGTTGTAGATA

2.3. Methods

2.3.1. Bacteriological Examination

Each sample was inoculated into a tube of sterile freshly prepared cooked meat medium as an enrichment medium, and then the tube was incubated an aerobically in anaerobic jar using anaerobic gas generating kits at 37°C for 24-48 hours. For isolation of *Clostridium perfringens*, a loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (150-200 µg /ml). The plate was incubated an aerobically at 37°C for 24-48 hours. The suspected colonies of *Clostridium perfringens* were picked up and examined for their morphological and biochemical characters [24]

2.3.2. PCR For Genotyping of Clostridium Perfringens

A total of 52 *Clostridium perfringens* isolates obtained from intestinal and cloacal swabs samples was subjected to genotyping by using multiplex PCR.

2.3.3. Extraction of Clostridium Perfringensdna [25]

Pure colonies of *Clostridium perfringens* that showed double zone of hemolysis on blood agar were grown over night in 5 ml brain heart infusion at 37°C under anaerobic condition. One ml of culture was centrifuged at 5000xg (rcf) for 15 minutes, the cell pellet was washed twice with 500 µl TE then resuspended in 200µl TE. The mixture was vortexed, boiled directly at 100°C for 10 minutes for cell lysis then cooled on refrigerator for 5 minutes. Finally the suspension was centrifuged at 13,000xg for 5 minutes. Five µl of supernatant was used as template DNA.

2.3.4. PCR amplification and cycling protocol [23]:

DNA samples were amplified in a total of 25µl of the following reaction mixture: 5µl of DNA as template, 0.34mM of each *cpe* oligo, 0.36mM of each *cpb* oligo, 0.44mM of each *etx* oligo, 0.5mM of

each *cpa* oligo, 0.52mM of each *iA* oligo, 1X of PCR master mix and completed to 25µl by DNase-RNase-free water. PCR cycling program was performed in the thermal cycler. A negative control PCR mixture with no template DNA was included.

2.3.5. PCR Cycling Protocol for the Five Toxin Genes Of Clostridium Perfringens using a Multiplex was Carried out According to Van Astenet Al.(2009) as Follows:

Initial denaturation at 94°C for 3 minutes, 35cycles of denaturation:94°C for 1 minute, annealing:55°C for 1 minute, extension: 72°C for 1 minute., final extension 72°C for 10 minute. After amplification, 10ul of amplified PCR products were mixed with 2ul of the 6x loading dye. Samples were electrophoresed on 1.5% agarose gel.

3. RESULTS

3.1. Isolation and Bacteriological Identification

As shown in Table 1, a total of 122 intestinal turkey poults samples (39 intestinal tissue and its contents and 83 cloacal swabs) was examined; 56 (45.9%) were culture positive. *Clostridium perfrigens* was confirmed by cultural and biochemical characters in 45 isolates from 75 diseased turkey poults and 11 isolates from 47 apparently healthy turkey poults.

Table1. *Results of microbiological examination*

Collection Date	Location	Sex	Age(Days)	Sample Status	Taken Medicine	Type of samples	No.of Samples	Positive	Negative
Jan-27	KafrGhatati's Farm	Male	110	apparently healthy	Yes	cloacal swabs	20	5	15
Dec-11	slaughter house and butcher’s shops	Male&Female	Different	apparently healthy	No	intestinal samples	7	2	5
Jun-15	Slaughter house and butcher’s shops	Male &female	Different	apparently healthy	No	intestinal samples	20	4	16
Jan-12	KafrGhatati's Farm	Male&Female	95	Diseased	Yes	cloacal swabs	11	7	4
May-12	KafrGhatati's Farm	Male&Female	63	Diseased	No	cloacal swabs	16	6	10
Jun-12	KafrGhatati's Farm	Male	135	Diseased	No	cloacal swabs	36	23	13
Jun-12	KafrGhatati's Farm	Male	135	Diseased	No	Intestinal samples	12	9	3

3.2. Molecular Typing of Clostridium Perfringens Isolates

The 56*Clostridium perfringens* isolates were analyzed by multiplex PCR in order to determine the toxin genes for the molecular typing. A mixture of primers of alpha toxin (324bp), beta toxin (196bp), epsilon toxin (655bp), iota toxin (446bp) and enterotoxin (233bp) were used. In all tested isolates the CPA gene was detected (Fig. 1, 2, 3), so the 56 isolates were confirmed as *Clostridium perfringens*.

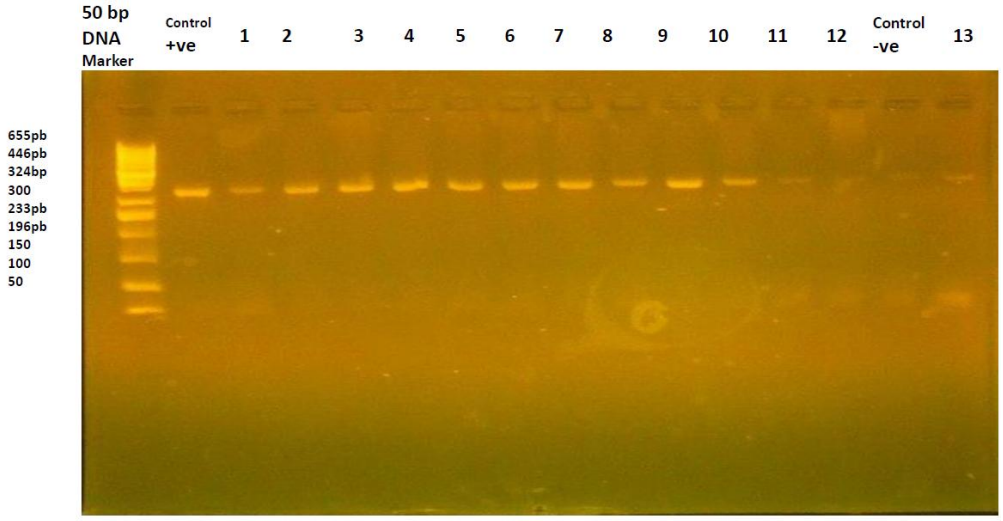

Figure1. ***Multiplex PCR gel amplification product*** *of alpha toxin (324bp), beta toxin (196bp), epsilon toxin (655bp), iota toxin (446bp) and enterotoxin (233bp)* ***Lane 1:*** *DNA Marker (50 bp),* ***Lane 2:*** *positive control,* ***Lane15?:*** *negative control and* ***Lane 3-14 and 16:*** *C. perfringens field isolates.*

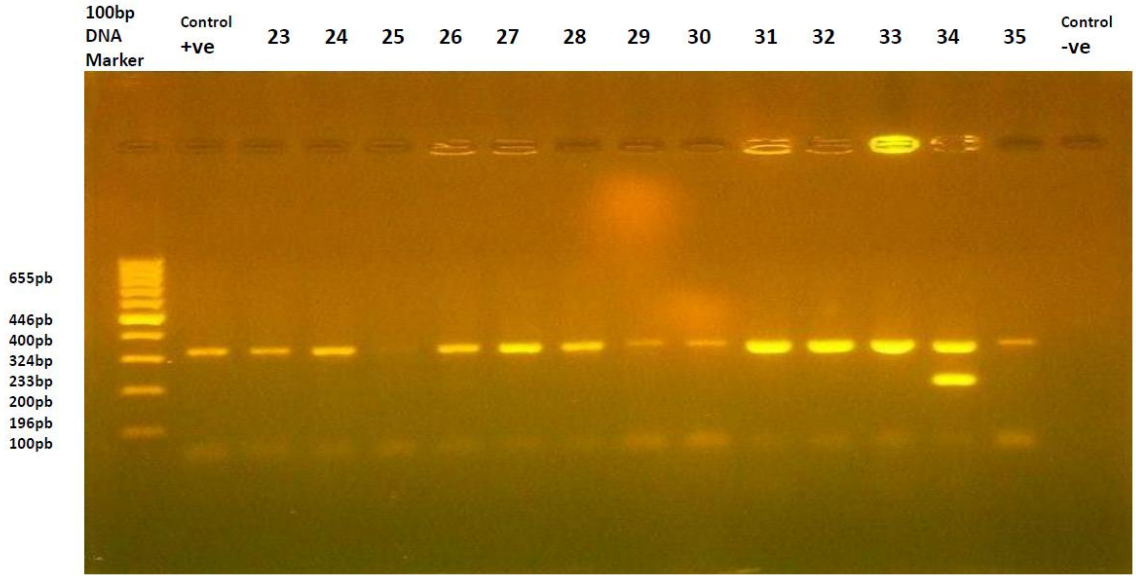

Figure 3. ***Multiplex PCR gel amplification product*** *of alpha toxin (324bp), beta toxin (196bp), epsilon toxin (655bp), iota toxin (446 bp) and enterotoxin (233bp)* ***Lane 1:*** *DNA Marker (100bp),* ***Lane 2:*** *positive control,****Lane16:*** *negative control and* ***Lane -15:*** *Clostridium perfringens field isolates.*

In one of the isolates the CPA(324bp) and CPE (233bp) genes were detected in the sample No. 34, confirmed the *Clostridium perfringens* type A with enterotoxin (0.82%). Fig.(3)**.** In another isolate (the sample NO. 37) the presence of 2 bands (324bp) & (196bp) confirmed the isolate as *Clostridium perfringens* type C (0.82%) . Fig.(4). The isolate no 39 was confirmed as *Clostridium perfringens* type E (0.82%) as it showed 2 for the CPA(324bp) and iA (446bp) genes Fig.(4).

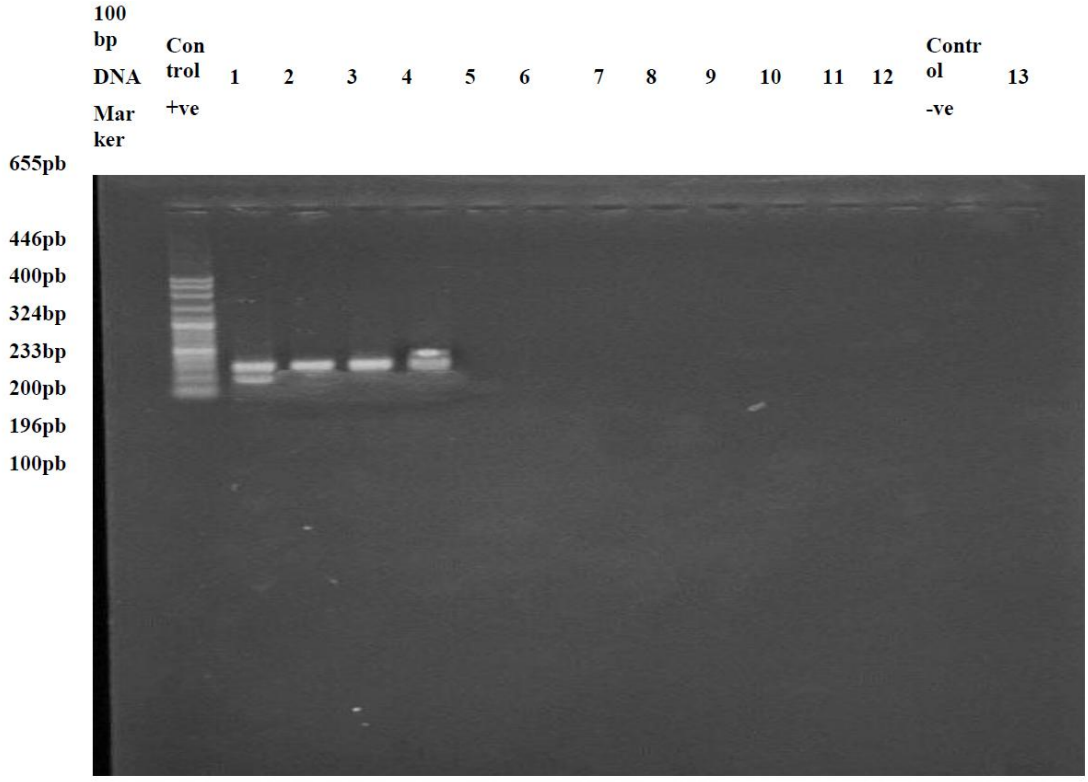

Figure4. ***Multiplex PCR gel amplification product*** *of alpha toxin (324bp), beta toxin (196bp), epsilon toxin (655 bp), iota toxin (446 bp) and enterotoxin (233bp)* ***Lane 1:*** *DNA Marker (100bp),* ***Lane 2-5:*** *C. perfringens field isolates.*

As shown in Table 2, it is clear that53 isolates had one band (324bp) for the CPA gene only, i.e. they contained alpha toxin and thus they were confirmed as *C. Perfringens* type A (48.6%), of these 45 isolates were from 75 diseased turkey samples were type A without enterotoxin (60%), 8 isolates from 47 apparently healthy turkey samples were Type A without enterotoxin (17%).

Table2. *Results of molecular typing of Clostridium perfringens isolates*

Clostridium perfringens	**No. Of Samples**	**Percentage**
Type A	53	43.5%
Type A with enterotoxins	1	0.8%
Type C	1	0.8%
Type E	1	0.8%
Negative	66	54.1%
Total	122	100%

Percentage was calculated according to the number of examined samples.

From 47apparentlyhealthy turkey samples one isolate was Type A with enterotoxins (2.1%), another isolate was Type C (2.1%), and a third isolate was Type E (2.1%) .Types B and D were not identified.

4. DISCUSSION

The interest in necrotic enteritis in poultry in Egypt dated back to 1977[26], when it was attempted to isolate anaerobic bacteria from the intestinal tract of normal and dead chicken sand to study the effect of alpha and beta toxins of *Clostridium perfringens* Type A and C introduced by different routes or given ration infected with *Clostridium perfringens* type C. Recently, the interest was directed to the application of molecular biology methods for typing the *Clostridium perfringens*[11]and to study the molecular diversity of Alpha Toxin produced by strains causing avian necrotic enteritis[27]**:**

In the present investigation the prevalence of *Clostridium perfringens* is Egyptian turkey poults was determined. The results show, that *Clostridium perfringens* is widespread in the investigated flocks. In turkeys, the prevalence of *Clostridium perfringens* is males was higher than in females, a result similar to that reported by [8]**:**, who attributed this difference to be due to difference in age. This is true in the present study, as infected males constituted 73.21%, while both males and females were 26.79%. There was no significance difference in the prevalence of *Clostridium perfringens* in birds that received medication or those not treated and there was also no seasonal variation. In the present study 94.64% of examined isolates were classified as *Clostridium perfringens* Type A, a result similar to that obtained by several authors world-wide [13.17.19-]*Clostridium perfringens* Types C and E were isolated each only once, while Types B and D were not detected. On the other hand, Type D was the second most detected toxovar, and especially prevalent in turkeys , while C was rarely isolated[8].It is interesting that C is increasingly encountered, particularly in Europe[8], however, this type was detected only once, besides another isolate belonging to type E, indicating the almost absolute predominance of Type A in Egypt.

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