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Research Article
Multiplex PCR and Detection of *netB* Gene of *Clostridium perfringens* from Broilers with Necrotic Enteritis

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Abstract
Necrotic Enteritis (NE) is the most common and financially devastating bacterial disease in modern broiler flocks. A previously unknown pore forming toxin, which called new toxin Perfringens necrotic enteritis B-like toxin (NetB toxin) and the encoding gene, *netB*, which is produced by some Australian strains of *Clostridium perfringens* has recently been reported. This toxin was reported to be critical for the development of necrotic enteritis in chickens. To investigate the occurrence of the toxin gene (*netB*) in non-Australian *C. perfringens* strains, intestinal samples of fifty diseased broilers chickens and fifty apparently healthy broilers in Egypt were examined. The *netB* gene was found in some isolates while alpha toxin, which encoded by cpα gene was detected in all isolates from chickens. In contrast, none of the isolates carried the enterotoxin gene. The present study indicates the role of *netB* in the induction of necrotic enteritis in correlation with α-toxin while, no significant effect of enterotoxin of *C. perfringens* in the occurrence of this disease in broiler chickens.

Key words: Multiplex PCR, *Clostridium perfringens*, *netB* gene, enterotoxin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Clostridium perfringens (CP) is the most important clostridia pathogens causing necrotic enteritis in poultry[1]. Extensive economic loss result from high mortality especially in 2-5 week-old chicks[2], reduced bird performance and carcass condemnation at slaughter[3]. The classification of *C. perfringens* isolates has traditionally been based on seroneutralization using expensive antiserum in mice and guinea pigs that take long time, which then have been replaced by molecular techniques as Multiplex PCR assay for cpα, cpβ εtx, ιAp, cpε and cpβ2 gene encoding the α, β, ε, ι, entero and β2-toxin, respectively[4]. The Molecular characterization and toxinotyping are considered as a rapid tools for detection of *C. perfringens* from suspected necrotic enteritis cases[5]. *Clostridium perfringens* has been classified into five toxigenic types (A, B, C, D and E) according to its ability to produce the major lethal toxins[2]. One of the most important toxins was α toxin which considered as the major virulence factor responsible for producing lesions in necrotic enteritis[6] through inducing mucosal damage in the intestinal tract of chickens[7]. A *netB* was firstly discovered in an Australian strain of *C. perfringens* type A[8], this toxin was shown to be critical for the pathogenesis of necrotic enteritis caused by *C. perfringens* strains in broilers through causing damage to host cell[9]. Moreover, necrotic enteritis in broiler chickens is associated with *netB* positive *Clostridium perfringens* type A strains[10]. Here we report an investigation of the occurrence of *C. perfringens* toxins and in particular, we examine *netB* occurrence with respect to the disease NE in broilers chickens.

MATERIALS AND METHODS

Samples: A total of 100 intestinal samples (50 from apparently healthy and 50 diseased suffering from brownish diarrhea, ruffled feather and weight loss with bloody intestinal content and necrosis in intestinal mucosa at postmortem examination) of 1-6 weeks hopper broilers.

Isolation and identification of *Clostridium perfringens*: Collected samples were introduced into tubes of freshly prepared cooled cooked meat medium and incubated anaerobically using a Gas pak anaerobic jar for 24 h at 37°C, subculture on Neomycin sulphate sheep blood agar plates for isolation of *C. perfringens*.

Biochemical and virulence activities: Suspected colonies were tested for oxidase, catalase, stormy fermentation, sugar fermentation reactions (glucose lactose, maltose and sucrose) and lecithinase activity.

Table 1: Oligonucleotide primers for detection of toxins, alpha (α), beta (β) and epsilon (ε)[13,14], *netB* gene[9] and enterotoxin(cpe)[15]

Toxin	Gene	Sequence 5'-3'	(bp)
Alpha (α)	cpα	GCTAATGTTACTGCCGTTGA	
		CCTCTGATACATCGTGTAAG	324
Beta (β)	cpβ	GCGAATATGCTGAATCATCTA	
		GCAGGAACATTAGTATATCTTC	196
Epsilon (ε)	εtx	GCGGTGATATCCATCTATTC	
		CCACTTACTTGTCCTACTAAC	655
netB toxin	*netB*	GCTGGTGCTGGAATAAATGC	
		TCGCCATTGAGTAGTTTCCC	383
Enterotoxin	cpe	GGA GAT GGT TGG ATA TTA GG	
		GGA CCA GCA GTT GTA GAT A	233

Determination of toxigenic *Clostridium perfringens* isolates:

- Through dermonecrotic test in guinea pigs[11,12]
- Molecular typing of toxigenic strains by application of multiplex PCR for detection of toxins, alpha (α), beta (β) and epsilon (ε)[13,14], uniplex for detection of *netB* gene[9] and enterotoxin (cpe)[15]

DNA extraction: *Clostridium perfringens* genomic DNA of the isolates were extracted[16] using DNA Purification Kit QIAamp® DNA Mini Kit (Cat. No. 51304-Qiagen) according to the instructions of the manufacturer. DNA concentration was determined spectrophotometrically at 260/230 nm.

PCR amplification: Oligonucleotide Primers for detection of toxins alpha (α), beta (β) and epsilon (ε)[13,14], *netB* gene[9] and enterotoxin[15]. As illustrated in Table 1. The PCR reaction was performed in a Thermal Cycler (Bio-Rad, S-1000 USA) in a total reaction volume of 50 μL containing 25 μL Dream Green PCR Reddy Mix (Dream *Taq* Green PCR Master Mix (2X) Fermentas Company, cat., No.K1080, USA.), 5 μL target DNA, 2 μL of each primers (containing 10 p mole μL^{-1}) and the mixture was completed by sterile DW to 50 μL. Amplification for detection the toxin (α, β and ε) was obtained with 35 cycles following an initial denaturation step at 95°C for 10 min. Each cycle comprised denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec and synthesis at 72°C for 90 sec, for detection of *netB* gene denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72°C for 1 min) steps were performed for 35 cycles. The final extension step occurred at 72°C for 10 min. Amplification for the detection of enterotoxin was 5 min initial step at 94°C followed by 30 cycles at 94°C for 1 min, 55°C for 2 min and 72°C for 3 min and a final extension step at 72°C for 5 min. Then, 10 μL of the amplified product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

Table 2: Relationship between age and prevalence of *C. perfringens* onto Neomycin sulphate sheep blood agar medium

		Positive cases give double zone of haemolysis onto Neomycin sulphate sheep blood agar medium						
		1st	2nd	3rd	4th	5th	6th	Percentage
Cases	No. of cases	----------weeks----------						total
Apparently healthy broilers	50	-	-	-	-	1	-	2
Diseased broilers	50	-	-	1	1	2	4	4
Total	100	9 isolates						

Table 3: Results of multiplex PCR and detection of *netB* gene, enterotoxin (cpe)

			Type of *Clostridium perfringens* toxins			
Application	No. of samples	No. of positive isolates	Alpha (α)	Beta (β)	Epsilon (ε)	Type of *C. perfringens*
Multiplex PCR	8	1	+	-	+	Type D
		6	+	-	-	Type A
		1	+	+	+	Type B
Uniplex PCR for *netB* gene	8	3				Type A
Uniplex PCR for cpe gene	8	-				

RESULTS

Prevalence of *Clostridium perfringens* onto neomycin sulphate sheep blood agar medium: The prevalence of NE was 8 isolates (16%) out of fifty diseased intestinal samples. The isolates produced double zone of haemolysis onto neomycin sulphate sheep blood agar medium and the relation between age and prevalence of NE was observed, a higher prevalence was noticed with old age. While, in apparently healthy was (2%) as shown in Table 2.

Biochemical and virulence activities of *Clostridium perfringens* isolates: All the isolates were positive for lecithinase activity, sugar fermentation, stormy fermentation, while catalase and oxidase tests negative.

Dermonecrotic reaction in guinea pig for typing of *Clostridium perfringens*: Six isolates out of 9 isolates of *C. perfringens*, were identified as type A, one isolates type D and one isolates type B from diseased broilers and the only isolate from apparently healthy didn't show any reaction in guinea pig.

Results of Multiplex PCR and uniplex PCR for detection of *netB* gene, enterotoxin (cpe): All isolates from diseased broilers, subjected to molecular detection. Through application of multiplex PCR, Six isolates out of eight isolates, were identified as *C. perfringens* type A, one isolate as type D and one isolate type B as shown in Fig. 1. Uniplex PCR for detection of *netB* gene and enterotoxin (cpe), showed three isolates of type A proved *netB* gene as illustrated in Fig. 2 and none of the isolates carried the enterotoxin gene (cpe) as shown in Table 3.

DISCUSSION

Clostridium perfringens type A, a bacterial pathogen causing necrotic enteritis in broiler chickens results in both visible and invisible economic losses. In the present finding, the prevalence rate of *C. perfringens* was 16% out of 50 diseased broiler chickens as shown in Table 2. Previous studies by Miah *et al.*[17] and Kalender and Ertas[18] reported lower prevalence rate of necrotic enteritis 8 and 5% from intestinal broiler chickens, respectively, while a higher prevalence rate (58.40%) was recorded[19]. This variation may have related to the different methodologies used for isolation, classifying the microorganism as well as poultry farms management used such as using of growth promoting[20]. The present finding summarized that the incidence of *C. perfringens* increased with age and this come in agreement with Osman *et al.*[21] realized that the infection of necrotic enteritis increased with old age. The pathogencity of *C. perfringens* is associated with their ability to secrete major and minor toxins which play important role in pathogenesis and induction of the disease. Molecular typing of *C. perfringens* by multiplex PCR is rapid and effective method for typing of *C. perfringens* toxins. The present finding revealed that type A was the most predominant among tested isolates as illustrated in Fig. 1, this agree with Shanmugasamy and Rajeswar[22] and Doosti *et al.*[23] *C. perfringens* type A was the most predominant isolated strain and proved cpα gene. Moreover, Thomas *et al.*[5] used multiplex PCR for detection of α toxin (cpα) and β2 toxin of *C. perfringens*. For long time a phospholipase C enzyme called α-toxin was considered the main virulence factor in necrotic enteritis caused by *C. perfringens*. Recent studies have now discovered a virulence determinant (*netB*) toxin

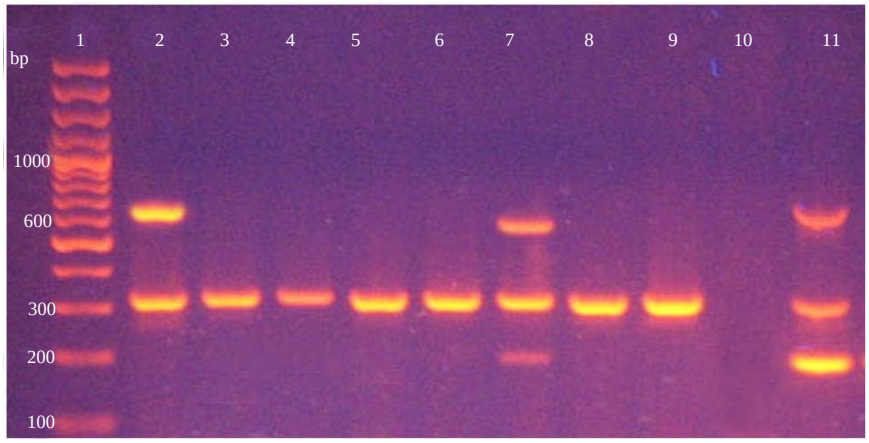

Fig. 1: Multiplex PCR for toxins typing of *C. perfringens* field isolates, Lane 1: 100 bp pair DNA ladder, Lane 2-9: Samples (lane 2 *C. perfringens* type D, lane 7 *C. perfringens* type B and lanes 3, 4, 5, 6, 8 and 9 *C. perfringens* type A), Lane 10: Control negative and Lane 11: Control positive

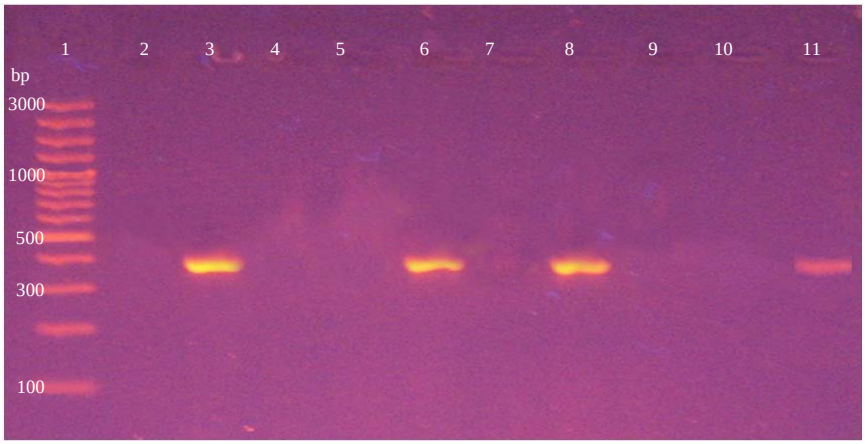

Fig. 2: Agarose gel electrophoresis of *C. perfringens* field isolates in a PCR with *netB* gene, Lane 1: 100 bp pair DNA ladder, Lane 2: Control negative, Lane 3: Control positive and Lane 4-11: Samples (3 isolates type A, lane 6, 8 and 11 were positive for *netB* gene)

which has a role in pathogensis of NE[24]. Our result revealed that three strains of *C. perfringens* type A isolated from Necrotic Enteritis (NE) were proved *netB* gene by PCR as shown in Fig. 2. This finding correlate with Keyburn *et al.*[8] identified and characterized of NetB, a novel *C. perfringens* toxin in *C. perfringens* type A and realized that *netB* toxin is critical for the ability of *C. perfringens* to cause NE in chickens. Moreover, Johansson *et al.*[3] reported more than 90% of *C. perfringens* isolates from NE-specific carried *netB* gene which codes for a recently described pore-forming toxin. Other finding by Timbermont *et al.*[25] who recovered *netB* toxin gene from both healthy and diseased birds. In our result, none of the isolates carried the enterotoxin gene (cpe). Similar result reported by Bailey *et al.*[26] recognized that all isolated strains of *C. perfringens* were type A and not carried cpe gene. Future investigations should focus on the regulatory mechanisms involved in the expression of *netB* and potentially also other toxins and its implications for the virulence of individual *C. perfringens* strains.

CONCLUSION

Multiplex PCR is rapid and effective method for typing of *C. perfringens* even correlated with traditional methods. Phospholipase (alpha toxin) and *netB* toxin are have critical role in pathogenesis of Necrotic Enteritis (NE) in broilers chickens enterotoxin has no role in the occurrence of this diseased.

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