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Necrotic Enteritis by Beta2toxin-Producing *Clostridium perfringens* in Doom Pigs of Assam, India

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A B S T R A C T

Piglet diarrhoea caused by beta2 toxin producing *Clostridium perfringens* is a serious problem to piggeries throughout the world. The newly recognized Doom breed of Assam, India can survive under poor rearing condition and is generally found to be disease resistant. Beta2 toxin was earlier reported to be associated with necrotic enteritis in piglets of different breeds but its role has not so far been reported in Doom breed. Two cases of piglet diarrhoea in their post mortem analysis were found to be havingsevere lesions of necrotic enteritis in their intestine. Bacterial isolation followed by biochemical and molecular analysis confirmed theirassociation with *C. perfringens* Type A possessing *beta2* gene. Further, to findout the total *C. perfringens* load under such severe condition, it was quantified by qPCR anda maximum of 2×10^6 fold incerease was observed compared to thereported load for healthy piglets.

Introduction

Clostridium perfringens is a gram positive, spore-forming anaerobic bacterium which causes a variety of enteric disorders in livestock leading to heavy economic loss. It produces an array of 17 different toxins across different hosts (Songer, 1996). It has been traditionally genotyped into five toxin types (A-E) based on the production of four major toxins α, β, ε and ι (Songer, 1996). Apart from these major toxins, *C. perfringens* may also produce important subsidiary toxins like *Clostridium perfringens* Enterotoxin (CPE) and β2 toxin which are highly correlated with enteric disease conditions in humans as well as animals (Miyamoto *et al.*, 2009; van Asten *et al.*, 2010).

The β2 toxin (27.6 kDa) was first reported in 1997 from a piglet suffering from necrotic enteritis (Gibert *et al.*, 1997) and since then presence of the gene (*cpb2*) coding for this toxin has been reported in isolates from pig,

horse, cattle and small ruminants (van Asten *et al.,* 2010). Presence of *cpb2* gene in isolates from piglets has particularly gained importance over the years due to its high correlation with piglet diarrhoea (Gibert *et al.,* 1997; Klaasen *et al.,* 1999; Bueschel *et al.,* 2003). Pigs contribute around 2.01% of total livestock population in India. It is one of the major animal commodities in Assam comprising highest (15.89%) population among the Indian states. Doom pigs are found in a few isolated pockets of Assam, which has been recognized as a breed very recently on 21st June, 2016 with accession number "INDIA_PIG_0200_ DOOM _09006" (New breeds registration, National Bureau of Animal Genetic Resources 2016). Doom pigs are known for its large body size, high prolificacy, disease resistance and sustenance in minimum input system (Zaman *et al.,* 2014; Devi *et al.,* 2017). Despite the high demand, the state has seen a decrease of 18.22% in the swine population from 2007 to 2012 (19th Livestock Census, 2012). This decrease in population may be attributed to poor healthcare and management practices in piglet raring (Roy *et al.,* 2014). Here, we are highlighting two severe cases of necrotic enteritis caused by β2 toxin of *C. perfringens* in piglets belonging to Doom breed.

Materials and Methods

Death of two piglets of around one month of age suffering from severe diarrhoea was reported from Kamrup district of Assam, India. Segments of their intestine collected at post-mortem were brought to the laboratory for further investigation. About 200mg of intestinal contents from both the samples were taken and treated with 50% ethanol for 30 min to remove any vegetative non-spore forming bacteria. *C. perfringens* was isolated on a blood agar plate with 5% defibrinated sheep blood in an anaerobic jar at 37ºC for 48 hrs. Colonies showing a clear zone of haemolysis were further purified by subculturing and analysed by biochemical tests. Sugar fermentation test was carried out for dextrose, lactose, maltose, sucrose, dulcitol and mannitol. The isolates were also tested for MR-VP, H_2S, catalase and indole production.

Toxin typing of the isolates was done by detection of four major toxin genes (*cpa, cpb, etx* and *iap*) and two subsidiary toxin genes (*cpb2* and *cpe*) by a multiplex PCR reaction (van Asten *et al.,* 2009). Bacterial DNA was isolated using UltraClean®Bacterial DNA isolation kit and 200ng of isolated DNA was used as template. Qiagen Multiplex PCR master mix (Qiagen, Germany) was used for preparation of the reaction mixture with a final concentration of 0.2 µM for all the primers, except cpb2, for which the primer concentration used was 0.4µM. Primer sequences and respective product sizes are listed in table 1. The PCR condition used was 15 min at 95 ºC followed by 40 cycles of 30s denaturation at 94 ºC, 90s annealing at 53 ºC and 90s extension at 72 ºC and a final extension step of 10 min at 72 ºC. Multiplex PCR results were analysed on 3% agarose gel by performing electrophoresis with 80V for 90 min.

For qPCR based enumeration of *C. perfringens*, total DNA was isolated from 200 mg of intestinal content using QIAamp® Fast DNA Stool Mini kit. In qPCR, 5µl of the isolated DNA was used as template in a reaction mixture containing 10µl of the Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, USA), 1µl each of forward primer (CP165F, 20 µM stock) and reverse primer (CP269R, 20µM stock), and 1µl of probe CP187F at 2µM concentration (Wise and Siragusa, 2005). The final reaction volume was made up to 20µl with NFW. Primers and probes used were: forward primer, CPerf165F (5´-CGCATAACGTTGA

AAGATGG-3´); reverse primer: CPerf269R (5´-CCTTGGTA GGCCGTTACCC-3´) and probe: CP187F (5´-[FAM]TCATCATTCAA CCAAAGGAGCAATCC [Iowa Black]-3´). The qPCR reactions were carried out in a Step One Plus real time thermal cycler (Thermo Fisher Scientific, USA) with the reaction condition as follows: initial denaturation 10min step at 94°C, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 70°C for 10 s. A standard curve was prepared using a serial dilution of known concentration of *C. perfringens* genomic DNA. The *C. perfringens* load was determined by calculating it from the DNA quantity considering the molecular weight of *C. perfringens* genome (Wu *et al.*, 2010).

Results and Discussion

Post-mortem examination of both the piglets showed intestinal necrotic lesions. Upon dissection, the internal lesions were found to be more severe (Fig. 1a,b). No other pathological alterations were reported in other vital organs.

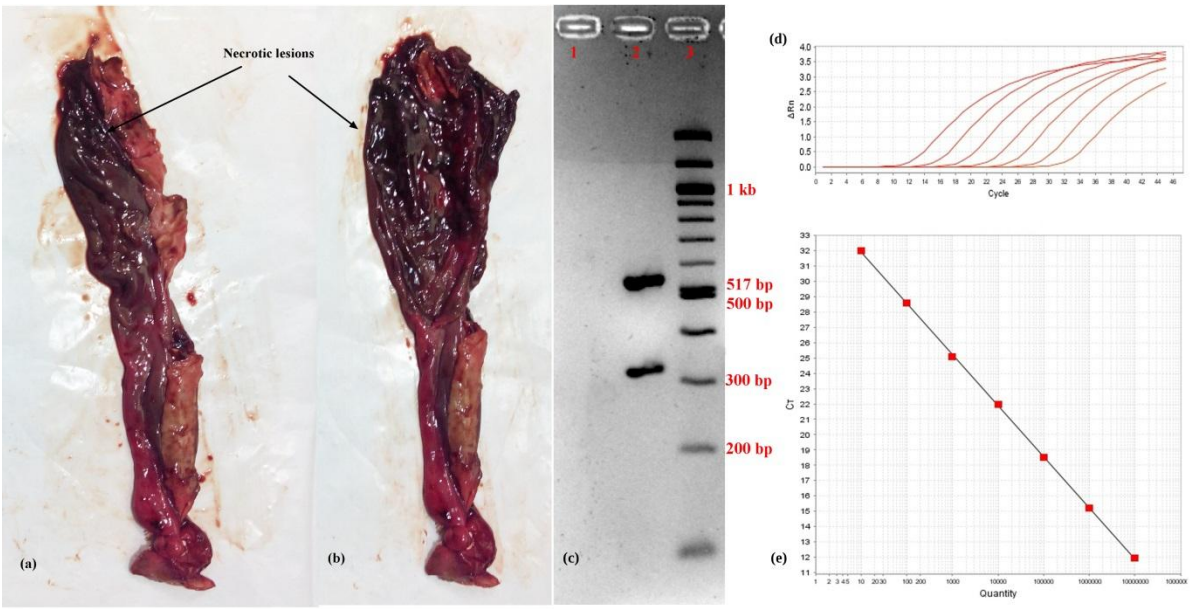
Colonies showing a clear zone of hemolysis from both the samples were further investigated by biochemical assays. Acid and gas production was observed in sugar fermentation test for dextrose, maltose, sucrose and lactose, whereas no changes were observed with dulcitol and mannitol.

Isolates were also found to be MR-VP, catalase and indole test negative. Upon toxin typing by multiplex PCR, the isolates were found to be of type A with *cpa*(324bp) and *cpb2*(548bp) genes (Fig. 1c). qPCR based C_t value determination and subsequent comparison with the standard curve (Fig. 1d,e) revealed a high load of *C. perfringens* (1.89 X 10^6 and 1.67 X 10^6per gram) in the intestinal content.

Table.1 Oligo nucleotide primers and respective product size (van Asten *et al.*, 2009)

Toxin	Primer	Sequence (5´-3´)	Product
α	cpa-F	GCTAATGTTACTGCCGTTGA	324
	cpa-R	CCTCTGATACATCGTGTAAG	
β	beta-F	GCGAATATGCTGAATCATCTA	195
	beta-R	GCAGGAACATTAGTATATCTTC	
β2	beta2-F	AAATATGATCCTAACCAAM[a]AA	548
	beta2-R	CCAAATACTY[b]TAATYGATGC	
ε	epsilon-F	TGGGAACTTCGATACAAGCA	376
	epsilon-R	AACTGCACTATAATTTCCTTTTCC	
ι	iota-F	AATGGTCCTTTAAATAATCC	272
	iota-R	TTAGCAAATGCACTCATATT	
Enterotoxin	entero-F	TTCAGTTGGATTTACTTCTG	485
	entero-R	TGTCCAGTAGCTGTAATTGT	

Fig.1 (a,b) Intestinal section showing necrotic lesions (c) Gel electrophoresis 1: No template control, 2: Multiplex PCR product with band for cpa (324 bp) and beta2 (548 bp), 3: 100 bp DNA ladder



Necrotic enteritis followed by diarrhoea is a serious problem in piglets. The present investigation demonstrated a classic case of β2 toxin pathogenesis by Type A *C. perfringens* causing fatal necrotic enteritis. This is the first report of fatal necrotic enteritis caused by β2 toxin producing *C. perfringens* in piglets of Doom breed from this region. Doom pigs appear to be disease resistant compared to other breeds of pig and can be reared with minimal input. Disease conditions caused by β2 toxin in such a naturally resistant breed are significant in terms of its pathogenesis. Although there have been several reports of β2 toxin producing *C. perfringens* causing up to 100% mortality in piglets, no report could be traced out in the available literature on quantification of *C. perfringens* in the intestinal content under such conditions. As β2 toxin positive Type A *C. perfringens* are commonly present in the piglet intestine, quantification carries distinctive diagnostic value. In this study, an increase of *C. perfringens* load up to 2.32 x 10^6 times was observed compared to that in healthy piglets reported earlier (Farzan *et al.*, 2013). This high load of *C. perfringens* could be related to production of high amount of beta2 toxin which led to severe necrotic enteritis.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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