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RESEARCH ARTICLE

MOLECULAR TYPING OF *Clostridium perfringens* FROM DIARRHOEIC CATTLE IN TAMIL NADU

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ABSTRACT

The present study aims to diagnose the predominant types of *Clostridium perfringens* from the cases of diarrhoea in cattle from Tamil Nadu. A total of 16 rectal swabs were aseptically collected from same number of cattle and were processed for the isolation and identification of *C. perfringens*. The molecular typing of the bacteria was carried out by the polymerase chain reaction assay, which revealed the identification of 14(87.5%) *C. perfringens* isolates from diarrhoeic cattle. The ultrastructure studies of the bacterial isolates in SEM were observed to be in clusters of thick rods with variable in length and mostly occurred in pairs. Out of six toxin genes namely alpha toxin (*cpa*), beta toxin (*cpb*), epsilon toxin (*etx*), iota toxin (*i*A), enterotoxin (*cpe*) and beta2 toxin (*cpb*2) used for typing the isolates, only *cpa* of 324bp fragment were detected from all the 14 isolates. The PCR result suggested that isolates from the diarrhoeic cattle belonged to the *C. perfringens* genotype A, which is also the most frequently isolated genotype of *C. perfringens* in cattle. PCR has established a sensitive and reliable investigative tool for the rapid detection of *C. perfringens*.

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INTRODUCTION

In India, the cattle population is approximately 15% of total cattle population in the world and is mainly reared for milk production apart from meat and leather. Tamilnadu being 10th in milk production contributes 5.13% of total milk production in India, estimates over 145.88 lakh litres per day. Perhaps the most important problem that plagues the cattle production sector is the occurrence of various diseases, contributing significantly to the loss of the cattle population as well, which in turns poorly hits to our countries economy. Among the various diseases that cause a threat to the cattle population is the occurrence of infectious diseases like gastroenteritis caused by the *Clostridium perfringens*. *C. perfringens* is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium of the genus Clostridium (Songer, 1996). It is widely distributed in the environment and foods, and forms part of the normal gut flora in man and animals (Baldassi *et al*., 2003). Domestic animals are known to be sources of human food poisoning and to decrease or eliminate this risk, strategies must be developed to prevent infected animals from entering the food chain.
Isolates of *C. perfringens* are classified into five genotypes (A–E) depending on the differential production of four major (i.e. lethal) exotoxins (alpha, beta, epsilon and iota). In addition, isolates of *C. perfringens* may also produce a number of other toxins, including: beta2 toxin (CPB2) and enterotoxin (CPE), also be directly involved in the enteritis of cattle (Manteca *et al*., 2002) and other animals. Among all the toxins, alpha toxin is produced by all the types of *C. perfringens* and is involved in pathogenesis of various diseases both in human and animal (Petit *et al*., 1999). Classically, typing of *C. perfringens* is expensive and time consuming. To avoid these problems, molecular techniques like PCR have been used to type *C. perfringens* into its five toxin types (Songer and Meer, 1996; Yoo *et al*., 1997). In this present study, PCR has been employed to detect toxin types of *C. perfringens* isolated from cattle suffering from diarrhoea.

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MATERIALS AND METHODS

History of animals and sample collection

Sixteen cross breed cattle of age between 3.6-4.8 years suffering from the diarrhoea maintained in semi-intensive rearing system of an organized dairy farm located in Erode District of Tamil Nadu, India were investigated. Infected animals showed poor growth, anorexia, pyrexia, rough hair coat and prolonged episodes of diarrhoea. The atmospheric temperature and humidity was recorded between 30-38°C and 60-75% respectively with rare rainfall during that period. The rectal swabs from the diarrhoeic cattle were aseptically collected in the properly sterilized sample collecting vials (Himedia, Mumbai), leveled immediately and were processed for the isolation of *C. perfringens*.

Isolation and identification of *C. perfringens*

Samples Rectal were inoculated in Robertson's cooked meat (RCM) medium with neutral oil overlay and incubated at 37°C for 24hr. The inoculums from the RCM media was seeded onto 10% sheep blood agar and incubated anaerobically for 24 hr at 37°C. Bacterial colonies were purified individually based on the size, shape, color and hemolysis. All these were subjected to Gram's and malachite spore staining followed by array of biochemical tests such as gelatinase, deoxyribonuclease (DNase), lecithinase, fermentation of glucose, lactose and skim milk and were identified as per Holt *et al.* (1994). Scanning electron micrography of the isolated bacteria was performed according to standered protocol. The photography was done under the SEM (JEOL JSM-6360, Tokiyo, Japan) and was analyzed with the help of the specific software.

Table 1. Oligonucleotides and the PCR cycle conditions used for the detection of *cpa, cpb, etx, iA, cpb2* and *cpe* genes of *C. perfringens*

Toxin genes	*Primer sequences*	Primer Concentr -ation (µM) each	Amplicon Size(bp)	Initial Denatura-tion (^0C)/4 min	Amplification (35 cycles)			Final Extension (^0C)/10 min	Reference
					Denatur-ation (^0C)/ min	Annealing (^0C)/ min	Extension (^0C)/ min		
cpa	For 5′ -gctaatgttactgccgttga- 3′ Rev 5′ -cctctgatacatcgtgtaag- 3′	0.50	324	94	94	55	72	72	
cpb	For 5′ -gcgaatatgctgaatcatcta- 3′ Rev 5′ -gcaggaacattagtatatcttc- 3′	0.50	180	94	94	55	72	72	Das *et al*. (2012)
etx	For 5′ -gcggtgatatccatctattc- 3′ Rev 5′ -ccacttacttgtcctactaac- 3′	0.50	655	94	94	55	72	72	
iA	For 5′ -actactctcagacaagacag- 3′ Rev 5′ -ctttccttctattactatacg- 3′	0.34	446	94	94	55	72	72	
*cpb*2	For 5′ -agattttaaatatgatcctaacc- 3′ Rev 5′ -caataccettcaccaaatactc- 3′	0.36	567	94	94	55	72	72	
cpe	For 5′ -ggagatggttggatattagg- 3′ Rev 5′ -ggaccagcagttgtagata- 3′	0.36	233	94	94	55	72	72	

For: Forward primer; Rev: Reverse primer; bp: Base pair

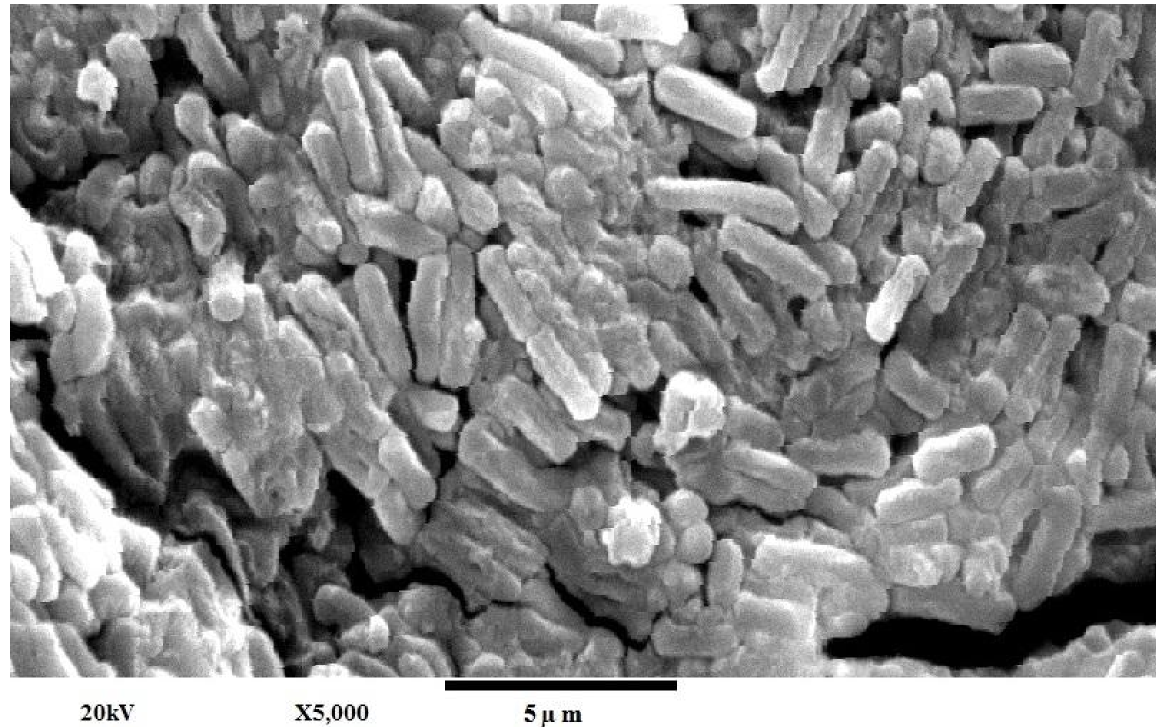

Figure 1. Ultra-structure of *C. perfringens* type A under SEM

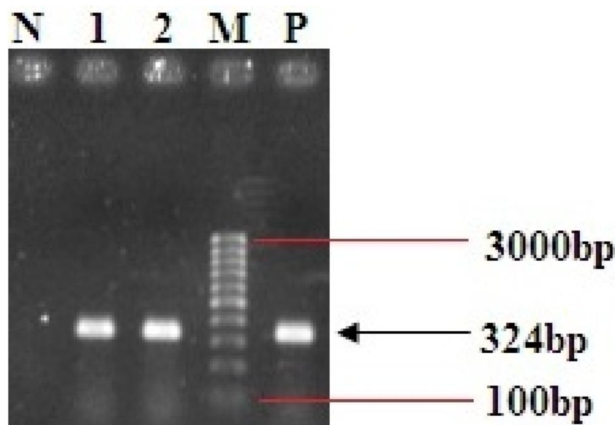

Figure 2. Detection of *C. perfringens* alpha toxin gene (*cpa*, 324 bp) fragment by PCR

P: Positive control
N: Negative control
1-2: Field isolates of positive for *cpa*
M: 100 bp marker DNA

Template DNA preparation for PCR

A total of 250µl of sterile Milli-Q water were taken in 1.5µl of micro centrifuged tubes. Loop full colonies of freshly grown suspected *C. perfringens* pilled from the sheep blood agar plate were taken in the micro centrifuged tube, gently vortexed and boiled at 100°C for 10 min in water bath. The cell debris was removed by centrifugation at 10,000 rpm for 5 min at 4°C. The top clear supernatant was used as source of template DNA.

PCR assay

The PCR amplification was carried out in 25µl reaction volume 12.5µl of 2× PCR master mix (Promega, USA) [4mM $MgCl_2$; 0.4mM of each dNTPs (dATP, dCTP, dGTP, dTTP); 0.5units/µl of Taq DNA polymerase; 150mM Tris-HCl PCR buffer (pH 8.5)]; desired µM of each (Forward and Reverse) primers (Table 1) and 2.5µl of template DNA. The primer pairs for cpa, cpb, etx, iA, cpb2 and cpe genes of C. perfringens were commercially synthesized (Eurofins Genomic India Ltd., Bangalore). The detailed amplification conditions in Thermal Cycler (Eppendorf, USA) and primers used in this study were described in Table 1. C. perfringens (ATCC 13124) and Escherichia coli (MTCC 723) were used as positive and negative controls respectively in PCR.

Agarose gel electrophoresis

The PCR amplicons (5µl) mixed with 6× sample loading dye (Genei, Bangalore) were separated by electrophoresis in 1.5% Agarose (Promega, USA) gel with 1× TAE (Tris-Acetate-EDTA; pH 8.0) as running buffer at 60V till the front dye moved 2/3rd of the gel. The gel was stained with 0.4µg/ml ethidium bromide (Genei, Bangalore), visualized and photographed in gel documentation system (Universal Hood, BIORAD, Italy).

RESULTS AND DISCUSSION

In the present study, a total of 14(87.5%) *C. perfringens* cultures were recovered from 16 samples processed, which showed double hemolysis on blood agar, gram positive rods with sub-terminal spore, production of gelatinase, DNase, lecithinase and fermentation of glucose, lactose, galactose and skim milk. Similar observations and identification criteria were followed in the previous studied (Shome *et al*., 2006; Das *et al*., 2012). The ultrastructure study of *C. perfringens* in SEM was observed to be in clusters of thick rods (Figure 1). The rods were observed to be variable in length; sometimes occur either single or in pairs and occasionally in short chains. In PCR, the positive control all the 14 clinical isolates of *C. perfringens* amplified the primer specific for alpha toxin (*cpa*) genes of the product 324bp (Figure 2). The other toxin specific primers of *cpb*, *etx*, *iA*, *cpb*2 and *cpe* genes did not amplify with any of the clinical isolates. The PCR result suggested that the isolated bacteria were *C. perfringens* type A. Very similar to the present study, Das *et al*. (2012) reported the detection of *cpa* from 22 isolates of *C. perfringens* type A from diarrhoeic cattle by PCR. Piatti *et al*. (2004) detected the presence *cpa* gene from 89 isolates of *C. perfringens* from bovine enterotoxaemia by PCR. This also suggested the importance of PCR in the epidemiological survey and diagnosis of clinical diseases, its use in molecular typing of *C. perfringens* (Songer and Meer, 1996; Shome *et al*., 2006) over the expensive, time-consuming toxin neutralization test performed in laboratory animals. The *C. perfringens* type A from diarrhoeic cattle is not safe for human consumption and has a crucial impact on public health, also it is highly lethal, hemolytic and well known cause of gas gangrene in human and myonecrosis, enteric diseases like enterotoxaemia in calves (Hatheway, 1990; Ginter *et al.*, 1995). In some cases, the enterotoxaemia and severe enteritis in calves is consistent with the action of alpha toxin in the circulation, causing massive intravascular hemolysis and capillary damage, inflammation, platelet aggregation, shock, and cardiac effects, culminating in death. A large amount of type A alpha toxin can be found in the feces of natural cases of diarrhoea or even in the GI tract without causing any clinically apparent disease (Songer, 1996) in cattle.

Conclusion

Prophylaxis of enteric diseases in diary calves can be achieved by vaccination; thus, PCR technique can become a first choice tool for identification and typing of *C. perfringens* strains that cause disease. The present findings suggested the occurrence and predominance of alpha toxin positive *C. perfringens* type A in the cases of diarrhoea in cattle in Tamil Nadu, India. The bacteria is spore forming and the alpha toxin positive strains of type A is highly toxigenic and lethal than any other types and can also cause diseases in animal and human by entering in to the food chain. Since, the disease is a public health importance, good management practices, awareness regarding the disease is in first priority. Further, molecular analysis of the pathogen is also required for undertaking the development of control measures, especially for the formulation of cost effecting vaccine.

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