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Molecular detection of *Clostridium perfringens* in diarrhoea cases of neonatal piglets

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Abstract
One of the main causes of neonatal death in pigs is neonatal diarrhoea. *C. perfringens* type A is thought to be the primary cause of neonatal diarrhoea in piglets, which primarily affects newborns in their first few weeks of life. Gram-positive, spore-forming, anaerobic bacillus known as *C. perfringens* is divided into seven kinds (A-G).

In the present study total of 25 faecal samples were collected from organized pig farms around Shirwal, Maharashtra from severe diarrheic neonatal piglets. The diarrhoeal faecal samples were processed for anaerobic cultivation by inoculating in RCM broth and further streaked on Clostridial agar with Tryptose sulphite cycloserine supplements (TSC) which showed black colour colonies. Further DNA was extracted from the isolates and was subjected for confirmation of *C. perfringens* by carrying out 16S rRNA PCR (species specific PCR). Isolates confirmed by species specific PCR were further used for toxinotyping.

Molecular characterization revealed that, total 16 isolates were positive for *C. perfringens* by 16S rRNA PCR (species specific PCR) showing an amplicon size of 481 bp on 1.5% agarose gel electrophoresis. Toxinotyping of the 16 isolates, revealed the presence of *cpa* gene in all the isolates showing an amplicon size of 324 bp which is one of the major toxins of *C. perfringens* and is the suspected cause of neonatal diarrhoea.

Keywords: *C. perfringens*, *cpa* gene, genotyping, piglets

Introduction

Among the diseases, some pathogens are associated with enteric form causing diarrhoea. This usually harbour the newborn causing neonatal diarrhoea. *Clostridium perfringens* causes diarrhea mostly in the first week of new born life and observed very less after 7 days of age. Neonatal enteritis related with *C. perfringens* type A infection is mostly associated with pre-weaning mortality (Lehe K.2009) [7]. *C. perfringens* strains are classified into seven toxinotypes, A to G according to the production of four major extracellular toxins namely, alpha (α), beta (β), epsilon (ε) and iota (ι). Among this α toxin is produced by Type A, type B produces α, β and ε toxins, type C produces α and β toxins, type D produces α and ε toxins and type E produces α and ι toxins (Dar *et al*., 2017) [3]. Type F isolates are those that produces *C. perfringens* enterotoxin (CPE), which causes food poisoning in human and diarrhoea due to over use of antibiotics. The second new strain of *C. perfringens* type G produces NetB toxin that is responsible for causing of necrotic enteritis in poultry birds (Rood, J. I., 2018) [10].

C. perfringens type A most commonly affects neonates in the first few week of life and mostly considered as the main cause of neonatal diarrhoea in piglets (Songer & Uzal 2005 and Chan *et al*., 2012) [12, 2]. Along with type A, piglets are more commonly affected by type C (Fitzgerald *et al*., 1988) [5] and Beta toxin is one of the major lethal toxin produced by type C strains along with Alpha toxin (*cpa*) of *C. perfringens.* Type C produces Beta 2 toxin and its encoding gene (cpb2) was first isolated from piglet suffering with necrotizing enterocolitis (Tolooe *et al.* 2011) [13].

Typing of toxin is considered as an important method for detecting the toxin genes associated with the diarrhoeal cases of piglets which will helps for the epidemiological study and in detection of virulence factors of the pathogen. The objectives of the present study was to identify the toxin type A and Type C which is mainly associated with the diarrhoea in piglets.

Materials and Methods

In the present study, total 25 faecal samples from severe diarrhoeic neonatal piglets aged between 25 to 40 days were collected from organized pig farms around Shirwal. The samples were collected in sterile containers which were immediately transported to the laboratory in ice cold container.

Isolation and identification of the *Clostridium perfringens*

All the samples that were received were individually infected in RCMM (Robertson Cooked Meat Media). After inoculation, the media was heated at 80 °C for 10 minutes to kill any organisms in their vegetative condition. The entire batch of RCMM tubes was then incubated anaerobically for 48 hours at 37 °C using an anaerobic jar and anaerogas pack (of the Himedia brand). A culture of RCMM broth was streaked on a perfringens agar base (T.S.C Supplement) plate and was incubated anaerobically in a jar at 37 °C for 24 hours in order to isolate *C. perfringens* (Udhayavel et al., 2017) [16]. *Clostridium perfringens* growth on plates was monitored. Following incubation, the agar colony types, sizes, and shapes were noted. Egg streaking was used to validate the isolates one more time.

Extraction of bacterial DNA

Pure colonies of *C. perfringens* from the perfringens agar base (T.S.C Supplement) plate were selected for DNA extraction. Extraction of bacterial genomic DNA was performed with phenol: chloroform method as described by Sambrook *et.al* (1989) [11] with slight modifications. Further the extracted genomic DNA was used as a template DNA.

Confirmation of *C. perfringens* by species specific PCR (16S rRNA)

The identity of the extracted DNA templates was further subjected to 16srRNA for the confirmation of *C. perfringens* using specific published primers (Table 1). PCR was performed by preparing a final reaction volume of 20 µl in 0.2 ml thin walled PCR tubes. It was prepared by taking 3.3 µl master mix supplied with Taq DNA, $MgCl_2$ and dNTPs; adding 2 µl each of forward and reverse primers, 2 µl template DNA and 10.7 µl nuclease free water. The reaction was carried out in a thermo cycler as follows: initial denaturation of 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec, 72 ºC for 1 min and a final extension at 72 ºC for 2 min.

Toxinotyping of *C. perfringens* by Multiplex PCR

Following species-specific PCR confirmation, all the isolates were subjected to multiplex PCR for toxin types A and C, targeting the cpa, cpb, and cpb2 genes as previously mentioned by Brady et al. (2010) [1] and Dar et al. (2017) [3]. (Table 1). The multiplex PCR experiments in this work were carried out in a 25 l reaction volume in an Eppendorf AG Mastercycler gradient. For all species-specific verified isolates, a multiplex PCR approach was employed to check for the presence of the cpa, cpb, and cpb2 toxin genes. Three pairs of unique primers were used for each toxin. For the PCR of these three genes (cpa, cpb, and cpb2), in 0.2 ml thin-walled PCR tubes a final reaction volume of 25 µl was prepared. It was prepared by taking 3.5µl master mix with Taq DNA, $MgCl_2$ and dNTPs; adding 1.5µl each of forward and reverse primers (each *cpa*, *cpb*, and *cpb2 gene*), 2 µl each template DNA and 10.5 µl nuclease free water. Samples were subjected to 35 PCR cycles, each consisting of Initial denaturation of 15 min at 95 °C; 30 sec of denaturation at 94 °C; 90 sec of annealing at 53 °C, 90 sec of elongation at 72 °C and final extension of 10min at 72 °C.

Table 1: PCR primers used for detection of *C. perfringens* and its toxin *cpa*, *cpb* and *cpb2* gene

Gene	Primer and oligonucleotide sequence	Product length	Reference
16S rRNA	F-TAACCTGCCTCATAGAGT R-TTTCACATCCCACTTAATC	481bp	Tonooka *et al.*, 2005 [4]
Cpa	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324 bp	Brady *et al*., 2010 [1]
Cpb	F: GCGAATATGCTGAATCATCA R: GCAGGAACATTAGTATATCTTC	198 bp	Dar *et al*., 2017 [3]
cpb2	F AAATATGATCCTAACCAACAA R-CCAAATACTCTAATYGATGC	548 bp	Dar *et al*., 2017 [3]

Results and Discussion

Phenotypic characterization of the *Clostridium perfringens*

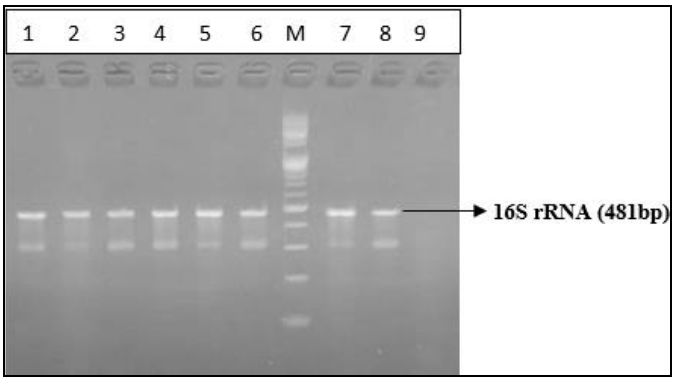
For the present study, total 25 diarrhoeal faecal samples were collected from neonatal piglets. Out of the 25 samples, 16 samples were found phenotypically positive as *C. perfringens*. The prevalence of the occurrence of the neonatal diarrhoea in piglets due to *C. perfringens* was recorded to be 64%. For cultural examination, all the collected faecal samples were inoculated in RCMM. After incubation of 48 Hrs. RCMM showed turbidity with slight gas production turning the meat particle pink in colour. Malmurugan *et al.* (2012) [8] also reported RCMM the best medium for the initial enrichment of the Clostridium spp. Further on selective agar, the colonies of *C. perfringens* were black and rough colonies with sulphite reduction. Pure colonies were picked from perfringens agar plates and further streaked on egg yolk agar medium, which displayed the distinctive diffused opalescence brought on by alpha toxins lecithinase activity of. On Biochemical examination, all the culturally positive organisms, showed the oxidase and Catalase test negative.

Molecular detection and toxinotyping of C. perfringens

PCR can be considered as a useful assay for rapid detection of *C. perfringens*. Multiplex PCR for identification of genes which codes for the production of various toxins in *C. perfringens* species was done for several years. In present study, total 16 isolates of *C. perfringens* were confirmed by 16S rRNA (species specific PCR), showing an amplicon size of 481bp on 1.5% agarose gel electrophoresis. (Fig.1). Total percentage positivity of isolates by 16s rRNA was 100%.

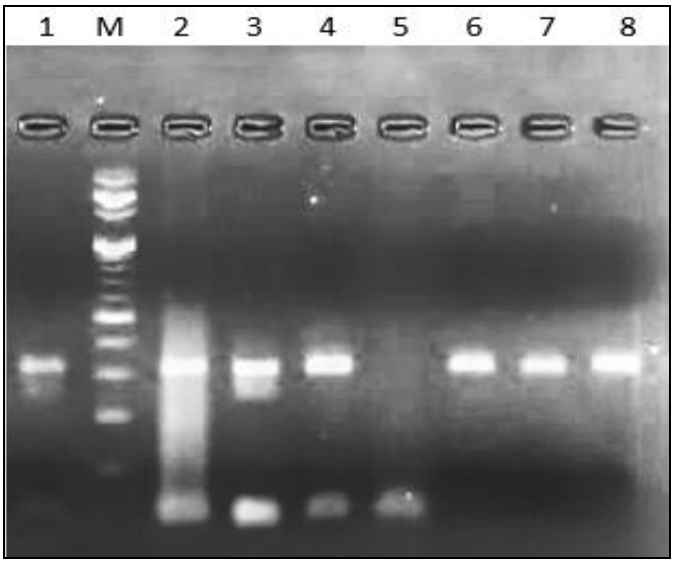
Further toxinotyping for different types, solely revealed positive for one major toxin gene (*cpa*) and therefore assigned to type A of *C. perfringens* showing an amplicon size of 324bp (Hussain M 2017) [6], but failed to reveal types C from the isolates (Fig. 2). *Clostridium perfringens* type A is speculated to be an emerging pathogen associated with neonatal piglet diarrhoea (Waters M 2003) [17] and all the isolates in the present study was denoted in type A toxin. High genetic variety among the various strains of this bacterial species may make it difficult to identify the type C genes, which include the cpb and cpb2 genes (Tsutsui, K., 1995) [15]. According to Nilo (1993) [9], the jejunum and ileum, where large numbers of *C. perfringens* type A organisms accumulate, produce an alpha (a) toxin that causes enterocyte necrosis and a profuse loss of both electrolytes and fluids that result in diarrhoea.

Piglet neonatal morbidity and mortality continue to be largely attributed to newborn diarrhoea. Although *C. perfringens* type A was shown to predominate among the diarrhoeal cases in neonatal pigs in the current investigation, there may be strains with unique traits that the genotyping technology in use is unable to identify. The several toxins connected to neonatal piglet diarrhoea need to be identified through additional research.



Lane M-Molecular weight marker (100bp ladder)
Lane-1, 2,3,4,5,6, & 8 = Positive samples,
Lane-7=Positive control, Lane 9=Negative control

Fig 1: Agarose gel electrophoresis showing481bp amplicon of *C. perfringens* (Species specific PCR)



Lane M-Molecular weight marker (100bp ladder)
Lane-1,2,3,4,6,7& 8-positive samples,
Lane-5=Negative control,

Fig 2: Agarose gel electrophoresis showing 324bp amplicon of cpa gene of *C. perfringens*

Conclusion

Based on the result of the present study conducted, our findings allows us to say that the *Clostridium perfringens* Type A was the major cause of neonatal diarrhoea in piglets and showed PCR as a rapid and valuable diagnostic method for confirmation and toxinotyping of *C. perfringens*. Further studies need to be designed to know the role of the different type of toxin associated with diarrhoea in pigs.

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References

1. Brady J, Hernandez-Doria JD, Bennett C, Guenter W, House JD, Rodrıguez-Lecompte JC. Toxinotyping of necrotic enteritis-producing and commensal isolates of Clostridium perfringens from chickens fed organic diets. Avian Pathol. 2010;39(6):475-481.
2. Chan G, Farzan A, Soltes V, Vovihan MN, Yanlong P, Roert F, *et al*. The epidemiology of Clostridium perfringens type A on Ontario swine farms, with special reference to cpb2-positive isolates. BMC Vet Res. 2012;8:156.
3. Dar PS, Wani SA, Wani AH, Hussain I, Maqbool R, Ganaie MY, *et al*. Isolation, identification and molecular characterization of Clostridium perfringens from poultry in Kashmir valley. J Entomol. Zool. Stud. 2017;5(5):409-414.
4. Datta S, Rakha NK, Narang G, Arora D, Mahajan NK. Prevalence of α, β and netb toxin producing strains of Clostridium perfringens in broiler chickens in Haryana. Haryana Vet. 2014;53(1):39-42.
5. Fitzgerald GR, Barker T, Welter MW, Welter CJ. Diarrhoea in young pigs: comparing the incidence of the five most common infectious agents. Vet Med. 1988;83(1):80-86.
6. Hussain M, Borah P, Hussain I, Sharma RK, Kalita MC. Necrotic Enteritis by Beta2 toxin-Producing Clostridium perfringens in Doom Pigs of Assam, India. Int. J Curr. Microbiol Appl. Sci. 2017;6(5):1872-1876.
7. Lehe K. Successful control of clostridial enteritis: A report from the back forty. Am Assoc. Swine Pract. c2009. p. 413-414.
8. Malmarugan S, Boobalan A, Dorairajan N. Necrotic Enteritis in Broiler and Layer Farms in Tamil Nadu, India. Indian J Agro Vet Med Sci. 2012;6(4):241-249.
9. Gyles NCL, Thoen CO, editors. Pathogenesis of Bacterial Infections in Animals. Ames: Iowa State Univ. Press; c1993. p. 114-123.
10. Rood JI, Adams V, Lacey J, Lyrad D, McClane AB, Melvillie SB, et al. Expansion of the Clostridium perfringens toxin-based typing scheme. Anaerobes. 2018;53:5-10.
11. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning-A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press; c1989. p. 1641-1708.
12. Songer JG, Uzal FA. Clostridial enteric infections in pigs. J Vet Diagn Invest. 2005;17(6):528-536.
13. Tolooe A, Shojadoost B, Peighambari SM. Molecular detection and characterization of cpb2 gene in Clostridium perfringens isolates from healthy and

diseased chickens. J Venom Anim Toxins Trop Dis. 2011;17(1):59-65.
14. Tonooka T, Sakata S, Kitahara M, Hanai M, Ishizeki S, Takada M, Sakamoto M, Benno Y. Detection and Quantification of Four Species of the Genus Clostridium in Infant Feces. Microbiol Immunol. 2005;49(11):987–992.
15. Tsutsui K, Minami J, Matsushita O, Katayama S, Taniguchi Y, Nakamura S, *et al*. Phylogenetic analysis of phospholipase C genes from Clostridium perfringens types A to E and *Clostridium novyi*. J Bacteriol. 1995;177(24):7164-7170.
16. Udhayavel S, Ramsamy GT, Gowthaman V, Malmarugun S, Senthivel K. Occurrence of Clostridium perfringens contamination in poultry feed ingredients: isolation, identification and its antibiotic sensitivity pattern. Anim Nutr. 2017;3(3):309-312.
17. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer GJ, *et al*. Genotyping and phenotyping of beta2-toxigenic Clostridium perfringens fecal isolates associated with gastrointestinal diseases in piglets. J Clin Microbiol. 2003;41:3584-3591.
18. Yoo HS, Lee SU, Park KY, Park YH. Molecular Typing and Epidemiological Survey of Prevalence of Clostridium perfringens Types by Multiplex PCR. J Clin Microbiol. 1997;35(1):228-232.