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Occurrence of *Clostridium perfringens* β2 toxin in healthy sheep from Kashmir valley

Nazima Nazir, Shakil Ahmed Wani, Qazi Nyrah, Sabia Qureshi, Zahid Amin Kashoo, Shaheen Farooq, Qusina Beigh, Sumayah Zari and Shafqat Majeed Khan

Abstract
*Clostridium perfringens*is a rod shaped, Gram-positive, anaerobic spore forming bacterium, able to produce various toxins which are responsible for various lesions and clinical symptoms in livestock. *Clostridium perfringens* has the ability to produce many toxins viz., alpha, beta, theta, iota, epsilon and kappa. In addition to the above mentioned toxins, *C. perfringens* can also produce a novel beta2 (β2) toxin. In the present study 415 samples were collected from healthy sheep from Kashmir valley. The isolates were confirmed by 16S rRNA specific PCR that revealed, 265 (63.85%) samples positive for *C. perfringens*. These 265 isolates were further screened for four toxin genes viz., *cpa, cpb, etx* and *cbp2* using a multiplex PCR. All *C. perfringens* isolates were positive for α toxin gene, but not forβ, orε, therefore, all strains were identified as type A *C. perfringens.*72.17% of the isolates harboured both α &β2 toxin gene. The high (100%) occurrence of *C. perfringens* was observed, even in day-old lambs. These findings clearly suggest that α- toxin and β2- toxin gene harbouring *C. Perfringens* genotype are widely distributed among animal species.

Keywords: *clostridium perfringens,* toxinotyping, β2 toxin, sheep

Introduction
*Clostridium perfringens*is a Gram-positive, spore-forming, ubiquitous, anaerobic bacterium found widely in the environment and is a normal inhabitant of the intestines of humans and animals [1]. Undercertain circumstances, *C. perfringen*s is able to cause severe diseases by the production of a variety of toxins [2] On the basis of four major toxins, namely, alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*itx*), the *C. perfringens* is divided into five toxinotypes, i.e. A, B, C, D, and E [3]. The specific toxins are responsible for the clinical signs and a syndrome attributable to each type [4]. Among the different *C. perfringens* types, type A is the most common type isolated from the intestine of healthy domestic animals. This toxin playsanimportant role in several diseases of animals including enterotoxaemia in calves [5], clostridial dysentery in lambs [6] and haemorrhagic sudden death in veal calves during the feeding period [7] Apart from the above toxins *C. perfringens* also produces a minor toxin CPB2. It is a newly known toxin and its encoding gene has been characterized [8]. Although the amino acid sequence of β2-gene has no noteworthy similarity to β-toxin but both of them have similar lethal effect on mice and have cytotoxic effect on intestinal cells. In this way, they do have analogous activity [9]. It may be assumed that β2-toxin does have a role in causing disease [10] The CPB2-producing type A strains of *C. perfringens* may cause enteric problems in different species of animals, including sheep and goats [11].
Different PCR protocols including multiplex PCR have been developed to detect the toxin genes to type *C. perfirngens* isolates [12]. The multiplex PCR method may provide a more sophisticated approach, enabling a simultaneous and specific detection of all the toxinotypes of *C. perfringens*. Many studies have been carried out for the detection of *C. perfringens*toxin genotypes by multiplex PCR using primers specific for each of the toxin genes present [13] Compared to conventional methods, these protocols provide rapid and sensitive detection of the organisms. The multiplex PCR method may provide a more sophisticated approach, enabling a simultaneous and specific detection of all the toxinotypes of *C. perfringens* [14].

Materials and Methods
Samples: A total of 415 fecal samples were collected from organized and unorganized farms.

The fecalsamples were collected with sterile swabs and carried on ice to the laboratory, where they were processed immediately for isolation of *C. perfringens.*

Isolation and identification of *C. perfringens*

For isolation of *C. perfringens*, samples were inoculated in DifcoTMCooked meat medium (Becton, Dickinson and Company, Sparks, MD, USA) and incubated anaerobically in 3.5 litre anaerobic jar (Oxoid Limited, UK) with GasPakTM Anaerobe Container System (Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C for 24 hrs. Enriched samples were streaked on Sulphite PolymixinSulphadiazine agar plates (SPS HiVegTM Agar, Modified; Hi-Media laboratories, Mumbai, India) and the plates were incubated anaerobically at 37 °C for 24 hrs. After incubation suspected colonies were subcultured on the SPS agar plates until they were free from contaminating bacteria.

Confirmation of the isolates was done by demonstration of the typical cellular morphology in Gram's stained smear, standard biochemical tests and molecular confirmation was done byamplifying 16S rRNA gene of the *C. perfringens*as per Tonooka *et al.,* (2005). The primers used in the experiment have the following sequences forward-TAACCTGCCTCATAGAGT and reverse-TTTCACATCCCACTTAATC. The PCR conditions consisted of initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 49 ºC for 90 sec and extension at 72 ºC for 90 sec. This was followed by final extension at 72 ºC for 10 min. A confirmed isolate of *C. perfringens*type A maintained in the Division was used as positive control while distilled water served as negative control

Molecular characterization of *C. perfringens* isolates bacterial DNA isolation

Suspected isolated colonies from agar plates were suspended in 1.5 ml micro centrifuge tubes containing 100 µl of distilled water by gentle vortexing. The samples were boiled for 5 min, cooled on ice for 10 min and centrifuged at 10,000×g in a table-top micro centrifuge (Cooling Centrifuge, Eppendorf 5418R, Hamburg, Germany) for 1 min. Three microlitres (µl) of the supernatant was used as the template for PCR.

Polymerase chain reaction

All the PCR assays in this study were performed in 25 µl reaction volume in Master cycler gradient (Eppendorf AG, Hamburg, Germany). The reaction consisted of 3.0 µl template DNA, 2.5 µl of 10X buffer, 0.2 µl of 25mM dNTP mix, 1 U of Taq DNA Polymerase (Fermentas Life Sciences) and sterile distilled water. The MgCl2 was used at 2.0 mM concentration, unless otherwise indicated. Sterilized distilled water was used as negative controls. All the primers were acquired from GCC Biotech, Kolkata, India.

Multiplex PCR of virulent genes All the *C. perfringens* isolates were screened for four different toxin genes using a multiplex PCR. These includedα-toxin (*cpa*), β-toxin (*cpb*), εtoxin (*etx*) and beta2 toxin (beta 2). The primers used for the amplification of the genes are shown in Table 1. The PCR conditions were similar to that used for amplification of 16S rRNA gene except for the annealing temperature that was set at 53ºC. The amplified products were electrophoresed in 1.5% agarosegel (Sigma Aldrich, St. Louis, USA) and stained with ethidium bromide (0.5 µg/ml). Amplified bands were visualised and photographed under UV illumination (Ultra Cam Digital Imaging, Ultra. Lum. Inc., Claremont, CA).

Results and Discussion

From 415 samples collected from sheep 265 were positive for *C. perfringens*. All the positive isolates were morphologically and biochemically identified by Gram staining, (Figure1) showingtypical black centered or creamish colour colonies on SPS agar (Figure 2) and formation of double zone of haemolysis on 5% sheep blood agar (Figure. 3). These isolates amplified 481bp product (Fig. 4) corresponding to *C. perfringens.* All the positive samples from 16SrRNA were subjected to PCR toxinotyping and screened for four toxin genes viz., *cpa, etx, cpb and cbp2* using a multiplex PCR.The results of multiplex PCR indicated that all samples were positive for *cp*a gene and none was positive for *etx* or*cpb*. Thus all were identified as toxinotype A and none of the isolates were found positive for toxinotype B or D. However, *(cpb2)* was identified in 72.17% of the isolates. (Figure.5).

Table 1: The primers used for the amplification of the genes

S. No.	Target gene	Primer Sequence (5′-3′)	Primer conc. (µM)	Product size (bp)	Reference
1.	16S rRNA	F-TAACCTGCCTCATAGAGT R-TTTCACATCCCACTTAATC	0.4	481bp	[15]
2	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	0.4	324	[16]
3	*cpb*	F–GCGAATATGCTGAATCATCA R–GCAGGAACATTAGTATATCTTC	0.4	195	
4	*etx*	F–TGGGAACTTCGATACAAGCA R–AACTGCACTATAATTTCCTTTTCC	0.4	376	
6	*cpb2*	F–AAATATGATCCTAACCAACAA R-CCAAATACTCTAATYGATGC	0.4	548	

*Clostridium perfringens*is an important cause of enteric disease in humans and domestic animals. In particular, *C. Perfringens*is responsible for several forms of enterotoxaemia, which differs in clinical manifestation and severity according to the toxigenic type involved and specific toxins produced [17]. In the present study, we investigated the occurrence of *C. perfringens* and β2 toxin from healthy sheep from Kashmir valley.The results of the present study demonstrate a high prevalence of *C. Perfringens* in healthy sheep. The bacterium was isolated from lambs as young as one day old.The results of our study also revealed a high prevalence and distribution of *C. perfringens*beta2 toxin encoding strains and among the five toxinotypes, toxinotypeA was widely distributed. Our findings revealed that 265(100%) out of 265isolatesweretoxinotype A, based on PCR amplification of *cpa* gene. These findings are in agreement with the observations reported from Argentina in which 100% of *C. perfringens* isolated from the lambs is oftoxinotype A [18]. Similarly, Kumar *et al.,* (2014) recorded 92.59% type A from healthy sheep flocks [1].

Similar studies in this region by Rasool. S *et al*., (2017) and Nazki *et al*. (2017) reported 87.7% and 60.90% of isolates from sheep to be *C. perfringens* type A respectively. [19], [20]. Over the past decade a variant of the beta toxin known as beta2 (*cpb2*) has been associated with enteric diseases in a wide range of animals including swine, cattle, poultry, sheep, horses, dogs, avian and aquatic species [21]. Beta2 toxin has been implicated as an accessory toxin in *C. perfringens* mediated antibiotic associated diarrhea and sporadic diarrhea [22]. This study showed a high prevalence i.e., 72.17% of beta2 gene in sheep. Mohiuddin. M *et al*., (2016) found 72% of the isolates carrying beta 2 genes in sheep and goat population [10]. Similarly, Singh *et al.* (2018) found 61.11% of isolates carrying the β2-toxin gene. These findings clearly suggest that α- toxin and β2- toxin gene harbouring *C. Perfringens* genotype are widely distributed among animal species [23]. As a major toxigenic factor in various isolates of *C. perfringens*, the β2 toxin could be considered as highly virulent due to its association with severe clinical diarrhea [21]. It is also important that the association of *C. perfringens* type A is not clearly understood, although some studies have reported fatal hemolytic disease in lambs and associated gas-gangrene lesions in adult sheep [24] and enteritis in calves [25] The presence of *C. perfringens* strains harbouring *cpb2* toxin gene in healthy animals may not be considered risk by itself, but can be considered an emerging threat to animal health if the physiological equilibrium of the intestine and resident Microbiolflora is disturbed due to change in feed, antibiotic therapy [26].

Conclusion

A high prevalence of *C. perfringens* was detected, in sheep in Kashmir Valley. The majority of the isolates were characterized as toxinotype A. Compared to earlier studies done in Kashmir Valley beta 2 occurrence was found to be high. Although studies on association between β2-toxin and gastrointestinal diseases in humans or other animal species is identified, still future research is essential to understand the role of beta2 toxin in the induction of enteric diseases, the capability of the *cpb2* gene to produce toxin and the regulatory mechanisms involved in the expression of beta2 toxin.

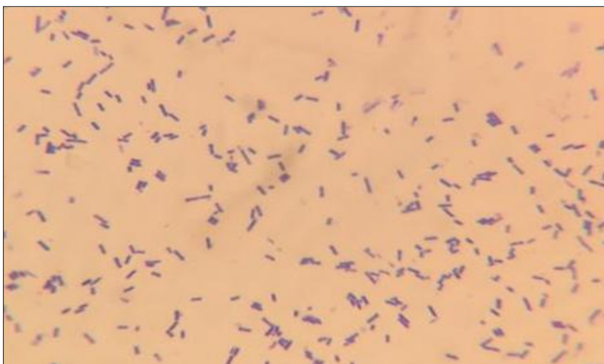

Fig 1: Smear of *C. perfringens* showing Gram positive rods

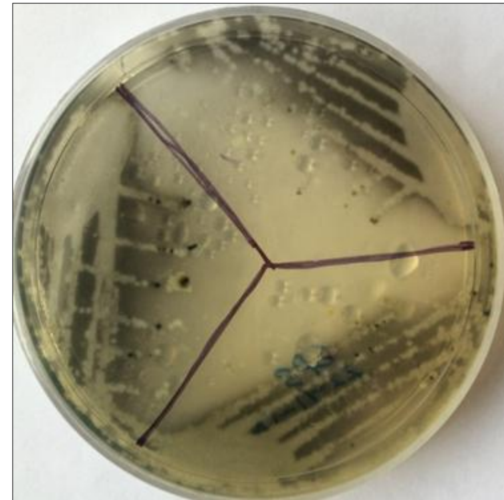

Fig 2: Blackcentered and Creamcolored colonies of *Clostridium perfringens* on SPS agar

Fig 3: Double zone of hemolysis produced by *Clostridium perfringens* on sheep blood agar

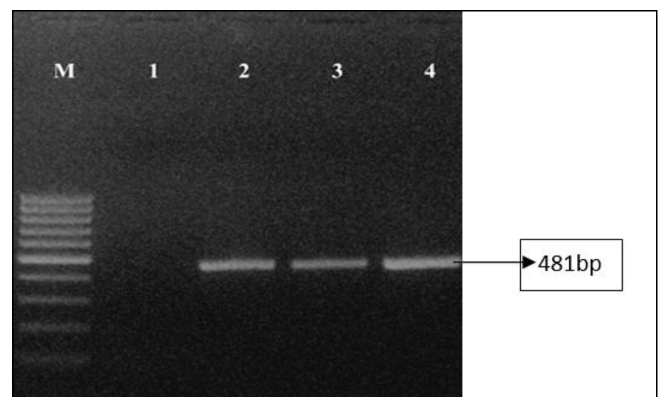

Fig 4: Agarose gel electrophoresis showing 481bp ampliconr RNA gene of *Clostridium perfringens*.

Lane M: 100 bpladder, Lane 1: Negative control, Lane 2: Positive control Lane 3 and 4: Samples

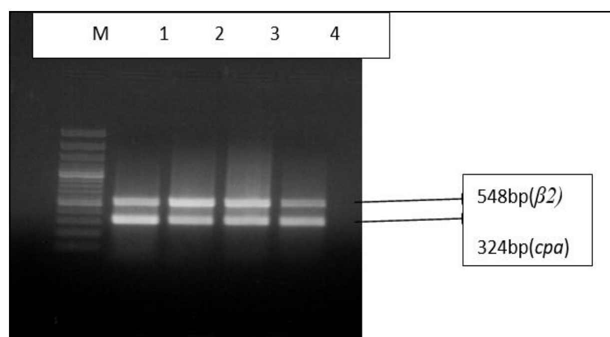

Fig 5: Agarose gel electrophoresis of multiplex PCR amplicons of *cpa* and *β2* toxin gene of *Clostridium perfringens*

Lane M: 100 bp ladder, Lane 1: Positive control Lane 2, 3 and 4: Samples.

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