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Molecular Characterisation of *Clostridium perfringens* Type D Isolated from Sheep in Kashmir Himalayas, India

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The molecular characterization of *Clostridium perfringens* in sheep for various toxinotypes was investigated in this study. A total of 147 samples collected from healthy, diarrhoeic animals and morbid material of animals suspected to have died of enterotoxaemia were screened for *Clostridium perfringens* (*C. perfringens*) toxinotypes. The polymerase chain reaction (PCR) amplification of 16S rRNA gene revealed that out of 147 samples collected, 92 (62.58%) were found positive for *C. perfringens*. All the 92 isolates were screened for three toxin genes viz., *cpa, cpb* and *etx* using a multiplex PCR. Toxinotyping revealed that 65 (72.65%) were positive for *Clostridium perfringens* Type A and 27 (29.34%) were that of *Clostridium perfringens* Type D None of the isolates was found to be toxinotype B or C.

Introduction

Clostridium perfringens is one of the ubiquitous organisms among clostridial species. It is the common inhabitant of gastrointestinal tract of humans and animals and also occurs in the soil. It is relatively aero-tolerant, spore forming, non-motile, Gram-positive rods (0.6-0.8 × 2-4 µm). The spores are oval, sub-terminal and bulge from the mother cell (Prescott *et al.* 2016). On the basis of four major toxins viz., 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. The toxinotypes were distinguished using mouse lethality tests and checking sero-protection with neutralizing antibodies raised against culture supernatants of the representative *C. perfringens* toxinotype (Sterne and Batty, 1975). The toxin production

depends on the specific toxinotype while all isolates of *C. perfringens* from animals produce alpha-toxin (CPA), and more than 98% produce theta toxin, also known as perfringolysin O (Songer, 1996). The specific toxins are responsible for the clinical signs and a syndrome attributable to each type. The specific enteric infections of various animal species are associated to different toxinotypes (Ohtani and Shimizu. 2016, Ashgan. 2013)

The *C. perfringens* toxinotype D produces alpha and epsilon toxins and is responsible for ovine enterotoxaemia and caprine enterocolitis. Enterotoxaemia is an acute, highly fatal intoxication that affects sheep, lambs, kids and goats. Sheep of all ages are affected by enterotoxaemia, but lambs under 10 weeks of age are most susceptible as they are nursed by heavy-lactating ewes and the weaned lambs on lush pasture or in feedlots (Songer, 1996).

In 2010-11, livestock generated a total of 4% of the GDP and 26% of the agricultural GDP in India. Sheep rearing is considered to be one of the major contributors to the livestock sector. The economics of sheep farming depends largely on the survival of the lambs and later lambing percentage of adult stock. A study showed that enterotoxaemia (incidence rate-1.5%, death rate-2.4% and case fatality rate-30.8%) comes next to diseases like blue tongue, PPR, and anthrax with respect to incidence and death rate in India (Singh and Prasad. 2009). The prevalence rates of enterotoxaemia due to *C. perfringens* toxinotype D ranging between 24.13% and 100% have been reported and the disease is considered one of the most frequently occurring diseases of sheep and goats worldwide (El Idrissi and Ward. 1992, Greco. 2005).

The present study investigated the prevalence of *Clostridium perfringens* (*C. perfringens*), in sheep and goats of Kashmir valley as well as characterized the genotype of its isolates. This study documented the presence of *C. perfringens* toxinotype A and D in sheep and goats in Kashmir valley.

Materials and Methods

Samples

A total of 147 samples comprising of faecal material, intestinal contents, kidneys and abomasum pieces were collected from sheep. Out of the 147 samples, 105 faecal materials were from healthy, 36 faecal materials from diarrhoeic animals and 6 were from carcasses. The samples were collected in sterile vials from animals of different age groups.

Isolation and identification of *C. perfringens*

For isolation of *C. perfringens*, samples were inoculated in Difco™Cooked meat medium (Becton, Dickinson and Company, Sparks, MD, USA) and incubated anaerobically in 3.5 litre anaerobic jar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) with GasPak™Anaerobe Container System (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C for 24 hrs. Enriched samples were streaked on Sulphite Polymixin Sulphadiazine agar plates (SPS HiVeg™ 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. The pure cultures of *C. perfringens* toxinotypes were lyophilized for future use in the laboratory using 0.25M sucrose as cryoprotectant.

Confirmation of the isolates was done by demonstration of the typical cellular morphology in Gram's stained smear, standard

biochemical tests and detection of *C. perfringens* by species specific polymerase chain reaction (PCR) using 16S rRNA gene primers.

Molecular characterization of *C. perfringens* isolates

Bacterial DNA isolation

Suspected isolated colonies from agar plates were suspended in 1.5 ml microcentrifuge 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 microcentrifuge (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 Mastercycler 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 $MgCl_2$ 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.

16S rRNA gene amplification

After identification of *C. perfringens* by phenotypic characteristics like colony characteristics, Gram's staining, the isolates were further confirmed using species-specific primers (Table 1) targeting 16S rRNA gene of the *C. perfringens*. The PCR conditions consisted of initial denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 49ºC for 90 sec and extension at 72ºC for 90 sec. This was followed by final extension at 72ºC for 10 min. The DNA of *C. perfringens* Type D isolate obtained from Sheep Husbandry Department was used as positive control.

Multiplex PCR of virulent genes

All the *C. perfringens* isolates were screened for three different toxin genes using a multiplex PCR. These three toxin genes include α-toxin (*cpa*), β-toxin (*cpb*) and ε-toxin (*etx*). 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% agarose gel (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 147 samples collected from sheep, 92 (62.58%) carried *C. perfringens.* All the 92 isolates were morphologically and biochemically identified by Gram staining, capsular staining, lecithenase activity on egg yolk agar media, triple sugar iron (TSI) test and formation of double zone of haemolysis on 5% sheep blood agar (Fig. 1) as *C. perfringens.* These isolates amplified 481bp product (Fig. 2) corresponding to *C. perfringens.*

Out of a total of 92 isolates from sheep, 65 (70.65%) were found to carry *cpa* gene alone as a major toxin gene, thus were designated as toxinotype A. While the remaining 27 (29.34%) harboured both *cpa* and *etx* genes, thus were designated as toxinotype D. None of

the isolates carried *cpb* gene indicating the absence of *C. perfringens* toxinotype B or C in sheep samples (Fig. 3). The *C. perfringens* isolates were characterized for important virulence factors including *cpa, cpb* and *etx.* Among lambs the occurrence of toxinotype D (55.76%) was higher than that of toxinotype A (Table 2). Among adult sheep occurrence of toxinotype A (60%) was higher than that of toxinotype D. *Clostridium perfringens* toxinotypes are responsible for varied disease syndromes in livestock animals and poultry. In the present study, healthy as well as suspected sheep populations from different regions of Kashmir valley were screened for the presence of *C. perfringens* toxinotypes. Our findings revealed that 92 (62.58%) of 147 samples from sheep were positive for *C. perfringens* based on isolation and PCR amplification of 16S rRNA gene. In accordance with our study, a lower occurrence of 24.13% of *C. perfringens* in sheep of Morocco (el Idrissi and Ward. 1992) while as a higher prevalence of 100% of *C. perfringens* in sheep of Italy (Greco. 2005) has been recorded. Similarly, prevalence of 59.62% of *C. perfringens* in sheep was reported in Andhra Pradesh, India (Kumar. 2014)[9]. The prevalence of 96.92% of *C. perfringens* in sheep and goats in Switzerland has been reported (Miserez. 1998). Recent reports by Rasool *et al.* (2017) reported prevalence of *C. perfringens* to the tune of 44.94% from sheep in Kashmir valley where type A was most prevalent corroborating with our study. Similar study in this region by Nazki *et al*. (2017) reported prevalence of 72.36% from sheep.

Table.1 List of primers used in PCR for amplification of *Clostridium perfringens* toxin Genes

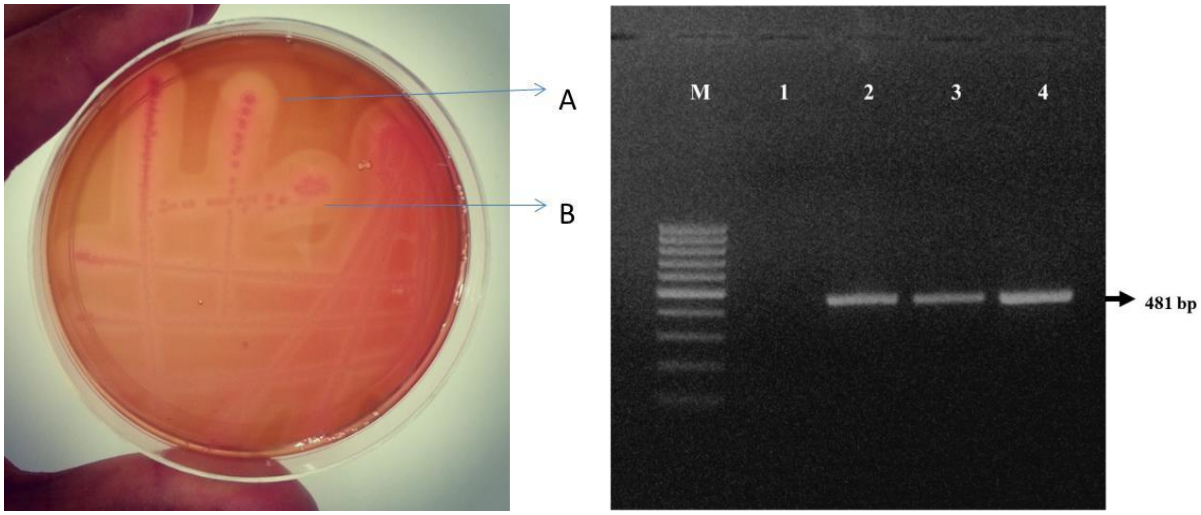
S. No.	Target gene	Primer Sequence (5′-3′)	Primer conc. (µM)	Product size (bp)	Reference
1.	16S rRNA	F-TAACCTGCCTCATAGAGT R- TTTCACATCCCACTTAATC	0.4	481	Tonooka*et al.* (2005)
2.	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	0.4	324	van Asten*et al.* (2008)
3.	*cpb*	F–GCGAATATGCTGAATCATCA R–GCAGGAACATTAGTATATCTTC	0.4	195	
4.	*etx*	F–TGGGAACTTCGATACAAGCA R–AACTGCACTATAATTTCCTTTTC C	0.4	376	

Table.2 Details of the isolates of *C. perfringens* from adult sheep and lambs

Age Group	Healthy/ Diarrhoeic	No. of samples screened	Number positive for *C. perfringens*	Type A	Type D
Adult	Healthy	59	33	21	12
	Diarrhoeic	10 +1*=11	7	3	4
Young	Healthy	46	28	15	13
	Diarrhoeic	26 + 5* =31	24	8	16
		147	92	47	45

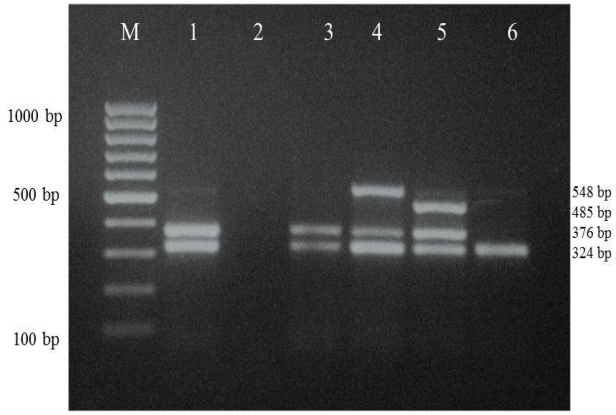
*caracass samples

Fig.1&2 Double zone of hemolysis produced by *C. perfringens* on sheep blood agar & Amplicons of 16S rRNA gene based PCR



Lane M: 100bp DNA ladder
Lane 1: Negative control
Lane 2: Positive control
Lanes 3-4: Isolates positive for *C. perfringens*

Fig.3 Multiplex PCR amplicons of different virulence genes of *Clostridium perfringens*



Lane M: 100bp DNA ladder
Lane 1: Positive control of *C. perfringens*Type D with amplified *cpa*(324bp) and *etx*(376bp) genes. **Lane 2**: Negative control, **Lane 3**: *C. perfringens*Type D,
Lane 4: *C. perfringens*Type D with amplified *cpa*,*etx* and *beta2*(548bp) genes
Lane 5: *C. perfringens*Type D with amplified *cpa*,*etx* and *cpe*(485bp) genes
Lane 6: *C. perfringens* Type A with *cpa*gene amplification

In the present study, *C. perfringens* toxinotype D was more prevalent among lambs (56.16%) than adult sheep. Our findings are in agreement with Redostitis *et al*. (2007), who reported that enterotoxaemia is more prevalent in lambs aged between 3-8 weeks, in fattening lambs in United Kingdom. The authors attributed it to the heavy feeding and milking of lambs by ewes that are grazed on lush pastures. However, they also observed

its higher prevalence in adult animals grazed on luxurious pastures. The spillover of the carbohydrate and protein rich nutrients into the small intestine from the abomasum encourages rapid multiplication of organisms and production of ETX. The increased prevalence of *C. perfringens* Type D (21.65%) in lambs than healthy adult sheep (3.7%) has been reported in Andhra Pradesh, India (Kumar. 2014). Recent studies by Rasool *et al*. (2017) and Nazki *et al*. (2017) also reported that majority of type D isolates were from diarrhoeic lambs occurrence of 58.6% and 56.16% respectively of type D isolates which endorse our results. *Clostridium perfringens* isolates obtained in this study were screened for presence of three toxin genes viz., *cpa, cpb* and *etx*. Out of 92 isolates from sheep, 65 (70.65%) were found positive only for *cpa* toxin gene, thus belonged to toxinotype A, while the 27 (29.34%) carried both *cpa* and *etx* toxin genes thus belonged to toxinotype D. None of these isolates possessed *cpb* toxin gene, indicating absence of *C. perfringens* toxinotype B or C. These findings are in agreement with the observations reported from the Italy in which 84% of *C. perfringens* isolated from the lambs and kids in Italy is toxinotype A and the remaining 16% as toxinotype D and none belonged to type B, C or E (Greco. 2005). In India 69.29% prevalence of enterotoxaemia from suspected sheep flocks and 39.71% from healthy sheep flocks has been reported (Kumar. 2014). Genotyping of the isolates from healthy animals indicated the presence of toxinotype A and D to be 45.56% and 31.64%, respectively. Although, toxinotype C has not been reported from sheep in the present study, the presence of toxinotype B or C cannot be ruled out owing to the fact the study being preliminary and based comparatively on small sample size.

In conclusion, this study documents the prevalence, isolation and characterization of *C. perfringens* toxinotype D in sheep of Kashmir valley. The study concluded that, *C. perfringens* was prevalent among lambs in Kashmir valley and toxinotype A being most prevalent toxinotype in sheep. Absence of toxinotypes B and C in this study does not indicate the absence of these toxinotypes in the sheep population as the number of samples was comparatively less. The present work also made local strains of *C. perfringens* available for formulation of vaccine, to effectively control the menace in the state.

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