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Molecular Typing of *Clostridium perfringens* Isolates from Faecal Samples of Healthy and Diarrhoeic Sheep and Goats in Kashmir, India

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A B S T R A C T

The current study reports the occurrence and molecular toxinotyping of *Clostridium perfringens* (*C. perfringens*) isolated from sheep and goat in Kashmir valley. A total of 462 faecal samples were collected from different organized and unorganized sheep and goat farms of the Kashmir valley. The samples consisted of 223 samples from sheep (adult-138, lamb-46) and goats (adult-15, kid-24) without diarrhoea (healthy) and 239 samples from sheep (adult-71, lamb-121) and goats (adult-17, kid-13) with diarrhoea. *C. pefringens* was isolated from 286 samples using Robertson's cooked meat media and Sulphite Polymixin Sulphadiazine (SPS) agar. The isolates were confirmed by 16S rRNA specific PCR. Out of the 138 adult sheep and 46 lamb samples without diarrhoea, *C. perfringens* was obtained from 63(45.6%) and 27(58.6%) samples, respectively. Similarly, 51(71.8%) and 91(76%) samples from 71 adult sheep and 121 lambs with diarrhoea, respectively yielded *C. perfringens.* Likewise, 8(53.3%) and 16(66.6%) isolates were recovered from 15 and 24 healthy adult goat and kid samples, respectively. The diarrhoeic samples yielded 17(58.6%) and 13(72.2%) isolates from adult goats and kids, respectively. Among the 286 isolates from 462 samples, 228(79.72%) were typed as toxinotype A, 197(86.4%) of which had only *cpa* gene and 31(13.6%) carried both *cpa* and *cpe* gene. Other 58(20.2%) isolates harboured both *cpa* and *etx* genes and thus were designated as toxinotype D. Majority of the type D isolates (58.6%) were from diarrhoeic lamb and this is followed by healthy lamb (15.5%), diarrhoeic adult sheep (8.6%) and others (3.5-5.2%). None of the isolates belonged to *C. perfringens* type B, C or E as they were negative for *cpb* or *cpi* genes.

Introduction

The Gram-positive bacterium *Clostridium perfringens* (*C. perfringens*) is an important human and veterinary pathogen, and it is most appreciated for its tremendous toxin-producing capability (Rood, 1998).

C. perfringens is believed to be the most wide-spread bacterial pathogen in nature (Quinn *et al.*, 2002), as it can be found in water, soil sediments, and also part of the normal intestinal flora of humans and

animals. As a member of the genus *Clostridium*, *C. perfringens* is, Gram-positive, anaerobic spore forming bacterium and able to produce various toxins and enzymes responsible for the associated lesions and symptoms (Varga *et al.,* 2006).*Clostridium perfringens* causes enterotoxaemia, which are acute, highly fatal intoxications that affect sheep, goats, lambs, calves, piglets and occasionally foals as well (Markey *et al.,* 2013).

C. perfringens strains are classified into five toxinotypes, A to E according to the production of four major extracellular toxins namely alpha (α), beta (β), epsilon (ε) and iota (ι), while various strains can also produce other toxins including β2, theta (θ) (perfringolysin O), kappa (κ), delta (δ), mu(μ), enterotoxin, necrotic enteritis B-like toxin (NetB), TpeL (toxin *C. perfringens* large cytotoxin) etc. Type A produces α toxin, 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 (Li *et al.,* 2013). The epsilon toxin (ETX)produced by both *C. perfringens* type B and D strains ranks as the third most potent clostridial toxin after the botulinum and tetanus toxin (Chen *et al.,* 2011). *C. perfringens* type D mainly affects sheep and goats and more rarely cattle and other animal species (Songer, 1996; Songer, 1997). The ETX produced by *C. perfringens* type D plays a major role in fatal enterotoxaemia or pulpy kidney disease in sheep and other small ruminants (Souza *et al.,* 2010). *C. perfringens* type B, which also produces β-toxin, is the etiological agent of dysentery in newborn lambs, but is also associated with enteritis and enterotoxaemia in calves, goats and foals (Songer, 1996; Songer, 1997). These isolates may be found in the intestine of normal animals, including sheep, goats, and cattle (Uzal and Songer, 2008). However, when the microbial balance of the gastrointestinal flora is disrupted, these bacteria proliferate in large numbers and produce disease, which is thought mainly to be mediated by toxins. Goats and lambs fed on high fiber diets are considered at a higher risk of epsilon toxin induced enterotoxemia (Smedley *et al.,* 2004).

Various PCR protocols including multiplex-PCR have been developed to detect the toxin genes to type *C. perfirngens* isolates (Ahsani *et al.,* 2010).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*. 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 (Elsify *et al.,* 2016). Typing of toxin gene by PCR has advantage of being practicable directly from primary culture colonies and hence is able to detect toxin genes, which are unstably maintained and might be lost during the cultivation process otherwise needed for the biological method (Kadra *et al.,* 1999). The present study was undertaken to isolate and identify *C. perfringens* from sheep and goats and to characterize them into different toxinotypes as there is no information available on *C. perfringens* of sheep and goats origin in the Kashmir valley.

Materials and Methods

Sampling

A total of 462 faecal samples were collected from different organized and unorganized sheep and goat farms of the Kashmir valley. The samples were comprised of 223 samples from sheep (adult-138, lamb-46) and goats (adult-15, kid-24) without diarrhoea (healthy) and 239 samples from sheep (adult-71, lamb-

121) and goats (adult-29, kid-18) with diarrhoea. The samples 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 *Clostridium 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 sub-cultured on the SPS agar plates until they were free from contaminating bacteria. The typical black and cream colonies, suggestive of *C. perfringens* were further streaked on the 5% sheep blood agar plates and egg yolk agar plates and incubated anaerobically for 24 hr. The colonies producing characteristic double zone of haemolysis around them on blood agar and producing zone of opalescence around the colonies on egg yolk agar were suggestive of *C. perfringens*. The isolates were also examined microscopically after staining with Gram's stain. The pure cultures of *C. perfringens* were kept as glycerol stock at -84°C and lyophilized using 0.5M sucrose solution for future use.

Extraction of DNA

Suspected isolated colonies of *C. perfringens* 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 10 min, cooled on ice for 10 min and centrifuged at 14,000×g in a table-top refrigerated microcentrifuge (Cooling Centrifuge, Eppendorf 5418R, Hamburg, Germany) for 10 min. Three microlitres (µl) of the supernatant was used as the template for PCR.

Amplification of 16S rRNA gene of *Clostridium perfringens* by polymerase chain reaction

Isolates of *C. perfringens* were confirmed by amplifying 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 95ºC for 5 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. A confirmed isolate of *C. perfringens* type A maintained in the Division was used as positive control while distilled water served as negative control.

Multiplex Polymerase chain reaction

All the *C. perfringens* isolates were screened for six different toxin genes using a multiplex PCR. These six toxin genes include α-(*cpa*), β-(*cpb*), ε-(*etx*), ί-(*cpi*), β2-(*cpb-2*) and enterotoxin (*cpe*). The primers used for the amplification of the genes are shown in Table 1. All the primers were procured from GCC Biotech, Kolkata, India.

All the PCR assays in this study were performed in 25 µl reaction volume in a thermal cycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany). 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 53ºC for 90 sec and extension at 72ºC for 90 sec. This was followed by final extension at 72ºC for 10 min (Van Asten *et al.,* 2008). Confirmed isolates of *C. perfringens* type A and D available in the Division and distilled water were used as positive and negative control, respectively.

Samples (6 µl) of PCR products were mixed with 6×loading buffer and loaded in separate wells on the submerged Agarose gel (1.5% w/v). Standard molecular weight marker (Fermentas Life Sciences) was also loaded in one well. The voltage 1-5 V/cm was applied across the gel until the bromophenol blue migrated to appropriate distance. The gel was removed and visualized under ultraviolet illumination and photographed with Gel Documentation System (Ultra Cam Digital Imaging, Ultra. Lum. Inc., Claremont, CA).

Results and Discussion

Isolation and identification of *C. perfringenes*

In the present study, sheep and goat populations from different regions of Kashmir valley were screened for the presence of *C. perfringens* toxinotypes. After enrichment in cooked meat medium, *C. perfringens* isolates produced typical black centered or creamish colour colonies on SPS agar (Figure 1).

The isolates also produced characteristics double zone of haemolysis and opalescence on sheep blood agar (Figure 2) and egg yolk agar (Figure 3), respectively.

Out of 462 faecal samples of sheep and goats, *C. perfringens* was isolated from 286 samples (Table 2). All the isolates, which were tentatively identified as *C. perfringens*, amplified 481bp product corresponding to16S rRNA gene of *C. perfringens* (Figure 4). Out of the 138 adult sheep and 46 lamb samples without diarrhoea, *C. perfringens* was obtained from 63(45.6%) and 27(58.6%) samples, respectively. Similarly, 51(71.8%) and 91(76%) samples from 71 adult sheep and 121 lambs with diarrhoea, respectively yielded *C. perfringens.*

Likewise, 8(53.3%) and 16(66.6%) isolates were recovered from 15 and 24 healthy adult goat and kid samples, respectively. The diarrhoeic samples yielded 17(58.6%) and 13(72.2%) isolates from adult goats and kids, respectively.

Multiplex polymerase chain reaction assay:

Toxinotyping of these isolates was done by multiples PCR (Figure 5). Among the 286 isolates from 462 samples, 228 (79.72%) were typed as toxinotype A, 197 (86.4%) of which had only *cpa* gene and 31 (13.6%) carried both *cpa* and *cpe* gene. Out of 228 *C. perfringens* toxinotype A, 61(26.7%) and 18 (7.8) isolates were obtained from healthy adult sheep and lambs respectively. Similarly, 46(20.1%) and 58(25.4%) isolates from adult sheep and lambs with diarrhoea, were typed as toxinotype A, respectively. Likewise, of the 228 *C. perfringens* toxinotype A isolates, 8(3.5%) and 12(5.2%) were recovered from healthy adult goats and kids, respectively. The diarrhoeic samples yielded 15(6.5%) and 10(4.3%) type A isolates from adult goats and kids, respectively.

Other 58 (20.2%) isolates harboured both *cpa* and *etx* genes and thus were designated as toxinotype D. Majority of the type D isolates (58.6%) were from diarrhoeic lambs and this is followed by healthy lamb (15.5%), diarrhoeic adult sheep (8.6%) and others (3.5-5.2%). Out of 58 *C. perfringens* toxinotype D, 2(3.4%) and 9 (15.5%) isolates were obtained from healthy adult sheep and lambs

respectively. Similarly, 5(8.6%) and 34(58.6%) isolates from adult sheep and lambs with diarrhoea, were typed as toxinotype D, respectively. Likewise, of the 58 *C. perfringens* toxinotype D isolates, 3(5.1%) were recovered from healthy kids and no type D toxinotype was found in healthy adult goat. The diarrhoeic samples yielded 2(3.4%) and 3(5.1%) type D isolates from adult goats and kids, respectively.

None of the isolates carried *cpb* or *cpi* genes indicating the absence of *C. perfringens* toxinotype B, C or E in sheep and goat samples. It was observed that most of the isolates belonged to the toxinotype A, followed by type D. Among the Sheep and goats, most of toxinotype A isolates were found in adults followed by the young stock. It was also observed that the toxinotype D was mostly detected in diarrhoeic animals especially lambs

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.	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	0.4	324	Van Asten *et al.*, (2008)
2.	*cpb*	F–GCGAATATGCTGAATCATCA R–GCAGGAACATTAGTATATCTTC	0.4	195	
3.	*etx*	F–TGGGAACTTCGATACAAGCA R–AACTGCACTATAATTTCCTTTTCC	0.4	376	
4.	*cpi*	F-AATGGTCCTTTAAATAATCC R-TTAGCAAATGCACTCATATT	0.4	272	
5.	*cpb2*	F–AAATATGATCCTAACCAACAA R-CCAAATACTCTAATYGATGC	0.4	548	
6.	*cpe*	F-TTCAGTTGGATTTACTTCTG R-TGTCCAGTAGCTGTAATTGT	0.4	485	

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

Species	Age group	Nature of samples	No. of samples	Total isolates (%)	Type A (%)	Type D (%)
Sheep	Adult	Healthy	138	63 (22.02)	61 (26.7)	2 (3.4)
		Diarrhoeic	71	51 (17.8)	46 (20.1)	5 (8.6)
	Lamb	Healthy	46	27 (9.4)	18 (7.8)	9 (15.5)
		Diarrhoeic	121	91 (31.8)	58 (25.4)	34 (58.6)
Goat	Adult	Healthy	15	8 (2.7)	8 (3.5)	0
		Diarrhoeic	29	17 (5.9)	15 (6.5)	2 (3.4)
	Kid	Healthy	24	16 (5.5)	12 (5.2)	3 (5.1)
		Diarrhoeic	18	13 (4.5)	10 (4.3)	3 (5.1)
Total			462	286 (61.9)	228 (79.72)	58 (20.2)

Fig.1 Cream colored colonies of *Clostridium perfringens* on SPS agar

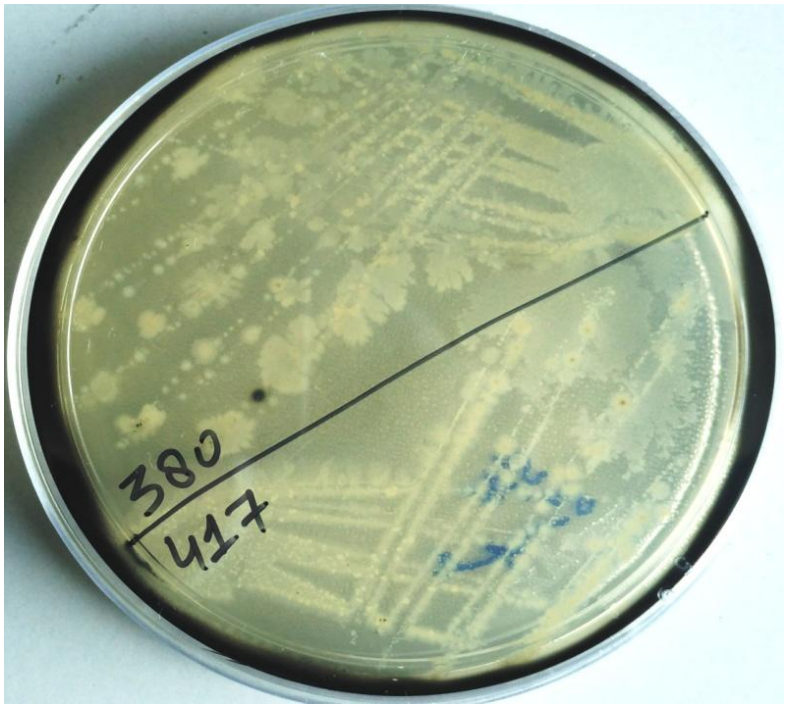


Fig.2 Double zone of hemolysis produced by *Clostridium perfringens* on sheep blood agar

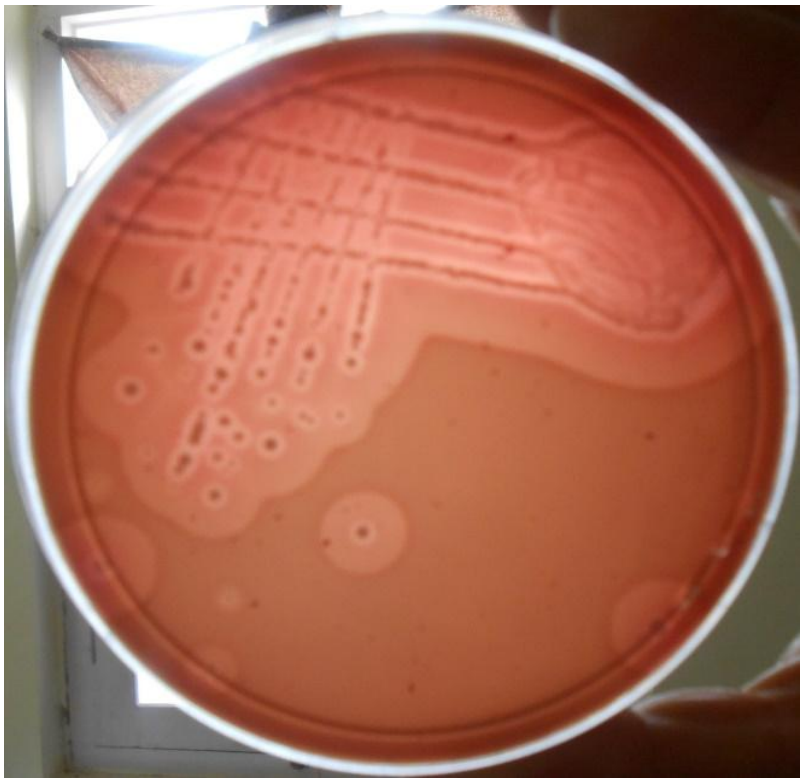


Fig.3 Lecithinase activity of *Clostridium perfringens* on egg yolk agar after 24 hrs of growth

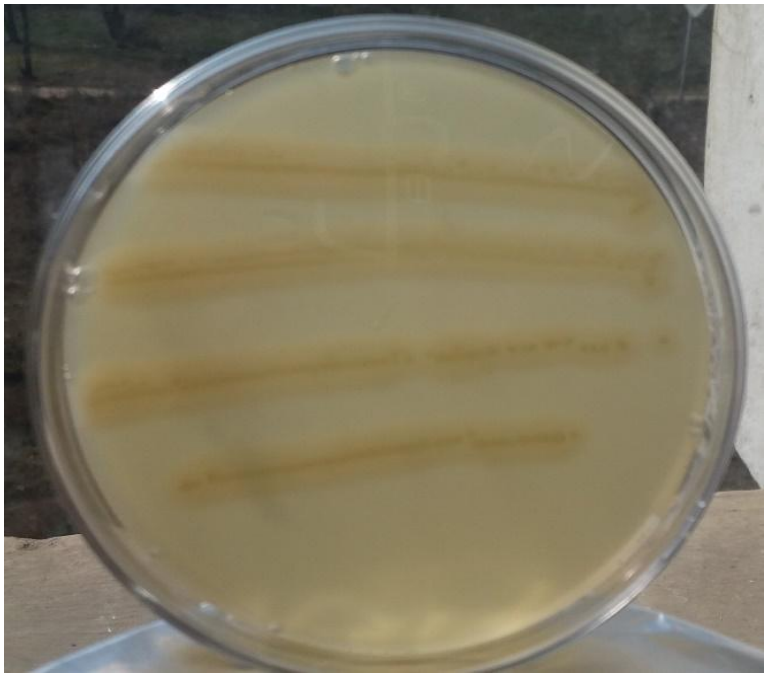
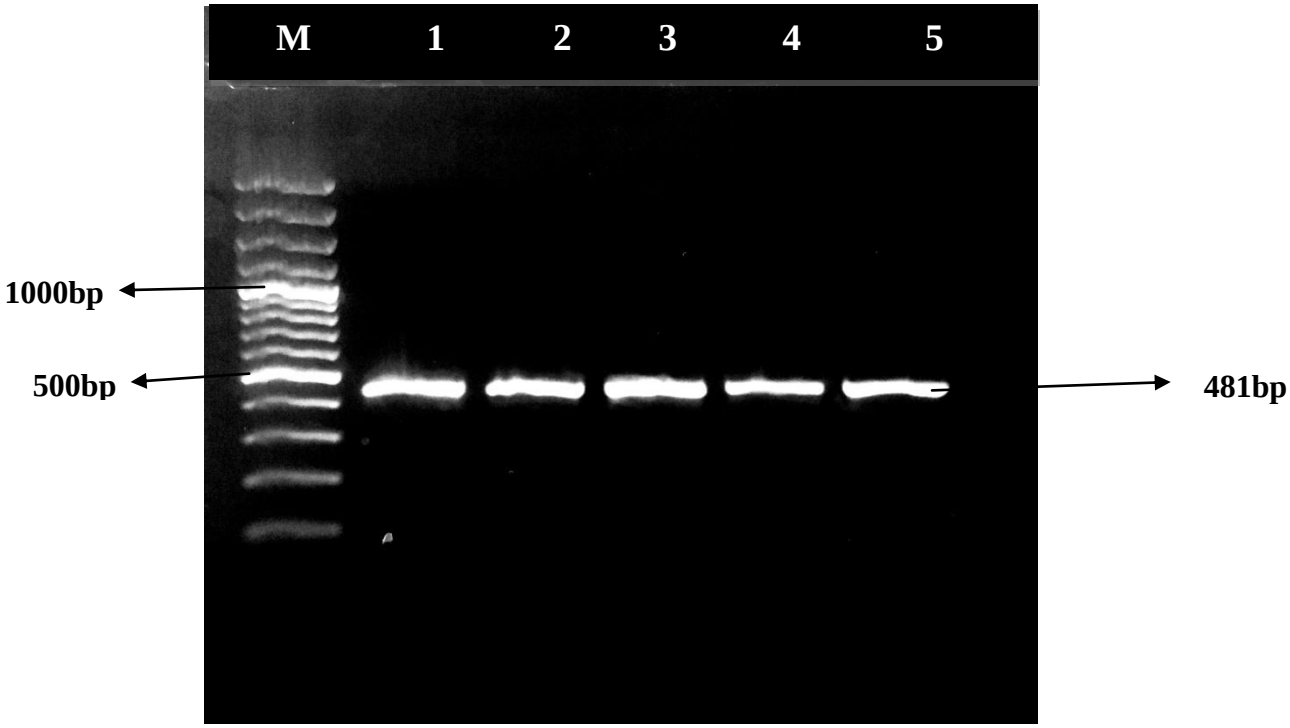
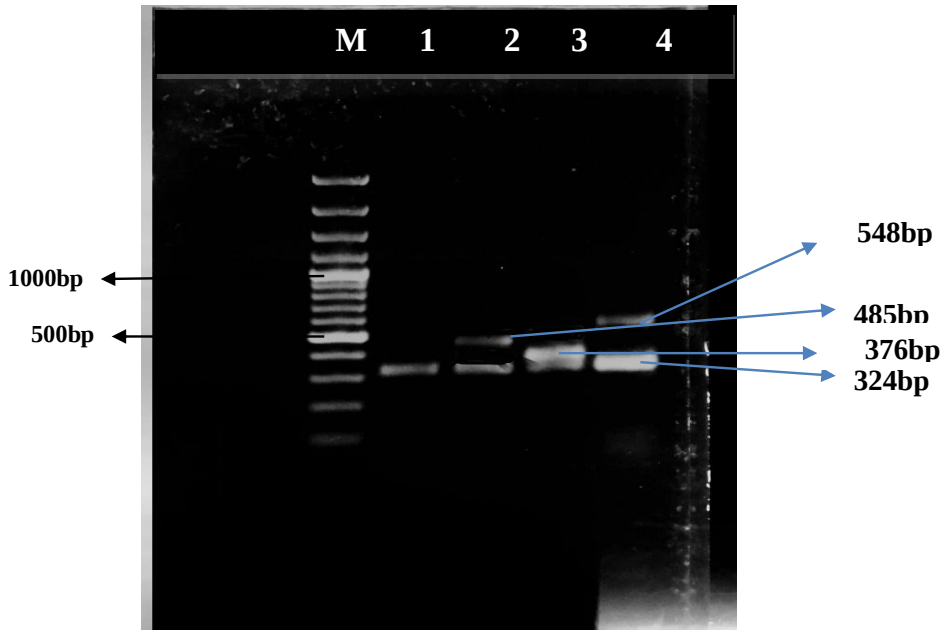


Fig.4 Agarose gel electrophoresis showing 481bp amplicon of *hsp*65 rRNA gene of *Clostridium perfringens*



M: 100bp plus marker
L1: Positive control
L2-5: Positive samples

Fig.5 Agarose gel electrophoresis of multiplex PCR amplicons of different virulence genes of *Clostridium perfringens*



M: 100bp plus marker
L1: *C. perfringens* Type A with amplified *cpa* only
L2: *C. perfringens* Type A with amplified *cpa and cpe* genes
L3: *C. perfringens* Type D with amplified *cpa and etx* genes
L4: *C. perfringens* Type A with amplified *cpa and beta-2* genes

Clostridium perfringens causes a wide range of enteric and other infections including fatal intoxications in different animal species (Songer, 1996, Songer, 1997; Markey *et al.*, 2013). These isolates may be found in the intestine of normal animals, including sheep, goats, and cattle (Uzal and Songer, 2008). Strains of *C. perfringens* have been classified into five toxigenic types (A through E) based on the production of the four commonly named major toxins-α, β, ε and ι (Hatheway, 1990; Badagliacca *et al.*, 2010).). Among the toxinotypes, type D, which are positive for ε (ETX), are responsible for fatal enterotoxaemia or pulpy kidney disease in sheep and other small ruminants (Souza *et al.*, 2010).

In the present study, we investigated the occurrence of *C. perfringens*, the causative agent of enteritis in sheep and goats of Kashmir valley as well as characterized their toxinotype. RCM medium was used for enrichment of samples. For further isolation, SPS agar was used and it supported appreciable luxuriant growth with creamish or black centered colonies after 24 hrs of incubation anaerobically. In a study, Shanmugasamy and Rajeswar (2012) reported that the frequency of isolation of *C. perfringens* was more with Robertson Cooked Meat Medium along with BHI broth than thioglycollate broth alone.The isolates produced double zone of haemolysis on sheep blood and lecithinase activity on egg yolk agar, the characteristics features of *C. perfringens* described by Quinn *et al.*, (2002).

In the present study, *C. perfringens* was isolated from 61.9% of total samples from sheep and goats. From healthy adults (without diarrhoea), the isolation rate was found to be

46.4%, while the rate was 61.4% from healthy lambs and kids. In case of diarrhoeic animals, isolation rate was found to be 68% in case of adults, while it was 74.8% in young stock.

No reports are available from Kashmir valley to compare the results. However, Phukan *et al.,* (1997) isolated *C. perfringens* from the diarrhoeic goats in Assam. Das *et al.,* (2012) also isolated *C. perfringens* from faecal samples of cattle with diarrhoea in Meghalaya. Recently, Kumar *et al.,* (2014) reported prevalence of *C. perfringens* toxin genotypes in enterotoxaemia suspected sheep flocks from Andhra Pradesh. *C. perfringens* was isolated from 69.29% enterotoxaemia suspected flocks and 39.71% from healthy flocks.

Typing of *C. perfringens* by multiplex PCR revealed that the isolates belonged to the toxinotypes A and D. Compared to conventional techniques, the PCR method has been shown to be much more rapid, giving results in a few hours and it is much more reliable for toxinotyping. In the present study, out of 114 isolates from healthy animals 100 (87.7%) isolates were found to be *C. perfringens* type A, while 14 (12.3%) were type D. Similarly, 129 (75%) and 43 (25%) out of 172 *C. perfringens* isolates from diarrhoeic animals were type A and type D, respectively. These findings were similar to Kumar *et al.,* (2014) who reported genotyping of the 97 isolates of *C. perfringens* by a multiplex PCR from enterotoxaemia suspected flocks of sheep and observed 67.01%, 11.34% and 21.65% isolates as type A, C and D, respectively. They also recorded 92.59% and 7.40% type A and D, respectively, from healthy flocks. Kalender *et al.,* (2005) also reported that 52 *C. perfringens* isolates from diseased sheep, 64% were type A, 21% were type D and 15% were type C, while types B and E were not identified. The type D isolates were significantly higher from the lambs with and without diarrhea and the reason for higher prevalence in healthy lambs may be due to close contact with diarrhoeic animals. Kumar *et al.,* (2016), recently reported that out of 42 isolates from enterotoxaemia suspected lambs, 30 (53.6 %) were type A, 9 (16.0 %) type D and 5 (8.9 %) were type C.

In conclusion, the rate of isolation of C. *perfringens* was more from diarrhoeic animals than the healthy ones and C. *perfringens* type A predominates in both healthy and diarrhoeic sheep and goats, irrespective of their age. However, type D isolates were significantly more from healthy and diarrhoeic lambs than other animals.

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