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Detection of *Clostridium perfringens* toxinotypes, enteropathogenic *E. coli*, rota and corona viruses in the intestine of neonatal goat kids by molecular techniques

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ABSTRACT

The role of various pathogenic agents affecting gastro-intestinal tract of neonatal goat kids was studied. Intestinal tissue samples of 142 necropsied kids died due to enteritis, pneumo-enteritis, tapeworm's infestations and septicemia were collected for detection of toxinotypes of *Clostridium perfringens*, enteropathogenic *E. coli* (EPEC), Group-A rotavirus (GARV) and bovine coronavirus (BCV). *C. perfringens* toxinotyping was done by multiplex PCR using primers for *cpa*, *cpb*, *cpb2*, *etx* and *iap* gene. Further, identification of EPEC was done using SYBR green based real-time PCR assay targeting *bfp*A gene. Detection of GARV and BCV was done by one-step RT-PCR (osRT-PCR). The incidence of *C. perfringens* was 16.20% (47.83% toxinotype A and 52.17% toxinotype D), with b2-toxin present in 30.43% of the samples. Incidence of EPEC in 0-1 and 1–3 month diarrhoeic kids was 36.62% and 25.35%, respectively. For viral aetiology, 11.97% were positive for GARV and 9.86% positive for BCV. Mixed infection of *C. perfringens* and EPEC was seen in 12.68% cases, while it was 2.11% for *C. perfringens* + GARV and 1.40% for *C. perfringens* + BCV. Similarly, the mixed occurrence of EPEC+GARV was 10.56% and that of EPEC+BCV was 7.04%. The combined infection of EPEC+GARV+BCV was 0.70%. The results suggested that *C. perfringens* type A and type D are the common toxinotypes affecting neonatal goat kids, with β2 toxin being an additional potential virulence factor. SYBR green based real-time PCR assay can be used as quick lab-based technique for efficient screening of EPEC from enteritis affected kids.Group-A rotavirus and bovine coronavirus appeared to be instrumental in causing mixed infection enteritis in kids.

Key words: BCV, *Clostridium perfringens*, EPEC, Goat, GARV, Neonatal diarrhoea, Toxinotypes

Role of goats in human food chain offers great potential due to the popularity of goat rearing in the developing economies and the demand for goat milk and meat in nutritional security. The incidence of diseases is one of the major constraints in the development of goat enterprise, contributing towards substantial losses to the goat keepers. Diseases in goats result in mortality which ranges from 10 to 40% in kids (Rekib and Vihan 1997). *Clostridium perfringens* is considered as part of normal flora in different animal species including sheep and goats (McClane *et al*. 2005), but leads to enterotoxaemia caused by massive toxin production due to slowdown of intestinal peristalsis and proliferation of *C. perfringens* in that environment (Songer 1996). Enterotoxaemia is an economically important and devastating disease of sheep and goats (Niilo 1980), and most important cause of sudden death in goats of different ages. The late log phase of bacterial growth is optimum for all toxin production such as α, β, β2, ε and ι by *C. perfringens* (Sayeed *et al.* 2005). Enteropathogenic *Escherichia coli* (EPEC) are a major cause of infantile diarrhoea and mortality in developing countries (Donnenberg and Kaper 1992). In the recent past, studies envisaged the presence of one or more virulent genes including *stx*1, *stx*2, *eaeA*, and *hlyA* in the field isolates from goats (Wani *et al*. 2006), and a 12.5% incidence of EPEC isolates in diarrhoeic lambs (Bhat *et al*. 2008) are the major concerns leading to neonatal mortality. Bundle forming pilin (BFP) protein encoded by bundle forming pilin (*bfp*) gene plays a significant role in adherence and micro-colonization in the small intestine that culminates into clinical diarrhoea (Giron *et al.* 1991), and in this study it has been used as a candidate to differentiate the EPEC from non-EPEC isolates. Rotavirus gastroenteritis is a

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worldwide disease affecting primarily infants, young children and young ones of wide variety of mammalian and avian species. Diarrhoea in goat kids is most frequently found associated with GroupA rotavirus (GARV) (Dey *et al.* 2007) and another enteric pathogen bovine coronavirus (BCV) is a major viral pathogen associated with neonatal calf diarrhoea (NCD) (Mebus *et al.* 1973). Enteric BCV replicates in epithelial cells of gut, destroying villi, resulting in severe, often bloody diarrhoea in calves (Clark 1993). Here we report an organized farm-based study of BCV in case of neonatal goat kids in India. In view of scarce literature and information on mixed infections in neonatal goat kids, the present study was undertaken on detection of toxinotypes of clostridial toxins, EPEC, GARV, and BCV using specific cum targeted molecular techniques.

MATERIALS AND METHODS

Sample collection: During the period from January, 2015 to January, 2017; population of 227 necropsied neonatal goat kids of 0–3 month's age of both sexes were examined for enteritis, pneumo-enteritis, tapeworm's infestations and septicemia. A total of 142 samples were collected from Postmortem House, Division of Animal Health, ICAR-Central Institute for Research on Goats (CIRG), Makhdoom, Farah, Mathura (Uttar Pradesh) as well as field outbreaks in the villages of Uttar Pradesh and Rajasthan. After postmortem, the intestinal loops of neonatal goat kids were collected and transported on ice for molecular and culture studies.

Isolation of bacterial DNA from intestinal tissue: DNA extraction was done from intestinal tissues for *C. perfringens* and EPEC using commercially available kit (QIAamp® DNA Mini Kit) following manufacturer's instructions. The quantity and quality of DNA was assessed at A260nm and A260/280 using biophotometer plus (Eppendorf, USA).

Toxinotype multiplex PCR (*TmPCR*)*:* The multiplex PCR kit (Qiagen, USA) was used for amplifying array of toxin genes for molecular toxinotyping. Published primers for *cpa*, *cpb*, *cpb2*, *etx* and *iap* were used (Van Asten *et al.* 2009) (Table 1).

SYBR green real time PCR for EPEC detection: Primers were designed for the amplification of *bfp*A gene, viz. *bfp*A F: 5'-ATGGTGCTTGCGCTTGCTGC-3', *bfp*A R: 5'-AATCCACTATAACTGGTCTGC-3', for diagnosing EPEC isolates of *E. coli* using BioEdit-v.7.2.5 software (Hall 1999)

Table 1. Toxinotyping primers

Toxin	Primer	Primer sequence (5´-3´)	Amplicon size	Reference
Alpha	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	324 bp	Van *et al*. (2009)
Beta	*cpb*	F-GCGAATATGCTGAATCATCTA R-GCAGGAACATTAGTATATCTTC	195 bp	
Beta 2	*cpb2*	F-AAATATGATCCTAACCAAMAAA R-CCAAATACTYBTAATYGATGC	548 bp	
Epsilon	*etx*	F-TGGGAACTTCGATACAAGCA R-AACTGCACTATAATTTCCTTTTCC	376 bp	
Iota	*iap*	F-AATGGTCCTTTAAATAATCC R-TTAGCAAATGCACTCATATT	272 bp	

F, Forward primer; R, Reverse primer

Table 2. Primers used for GARV and BCV in osRT-PCR

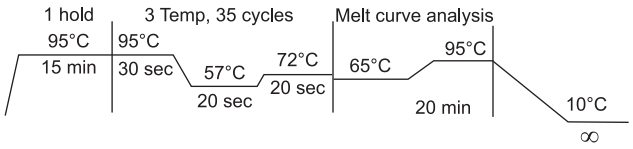
Virus	Target gene	Primer	Sequence (5'–3')	Product length (bp)	Reference
GARV	VP6	GEN_VP6F GARVP6-928R	GGCTTTWAAACGAAGTCTTC GGYGTCATATTYGGTGG	928	Matthijnssens *et al*. (2008)
BCV	Nucleocapsid	BCV-N-F BCV-N-R	GCCGATCAGTCCGACCAATC AGAATGTCAGCCGGGGTAT	407	Tsunemitsu *et al*. (1999)

Table 3. Incidence of various post-mortem affections used as basis for sampling and detection of enteropathogenic agents including *C. perfringens*, EPEC, rota and corona virus infection

Disease	0–1 month			1–3 month			Total
	Male	Female	Total	Male	Female	Total	
Enteritis	18 (7.93%)	10 (4.40%)	28 (12.33%)	25 (11.01%)	39 (17.18%)	64 (28.19%)	92 (40.53%)
Pneumo-enteritis	05 (2.20%)	02 (0.88%)	07 (3.08%)	07 (3.08%)	08 (3.51%)	15 (6.61%)	22 (9.69%)
Tapeworms	-	01 (0.44%)	01 (0.44%)	05 (2.20%)	07 (3.08%)	12 (5.28%)	13 (5.72%)
Septicemia	-	06 (2.64%)	06 (2.64%)	04 (1.74%)	05 (2.20%)	09 (3.96%)	15 (6.61%)
Total	23 (10.13%)	19 (08.37%)	42 (18.50%)	41 (18.06%)	59 (25.97%)	100 (44.05%)	142 (62.55%)

with the nucleotide database sequences from NCBI database. A conventional gradient PCR was conducted to check the quality of the reaction and amplification of 158 bp amplicon. This technique was further standardized for efficient differentiation of EPEC isolates from that of non-EPEC isolates.

The reaction was carried out with 2× Universal SYBR green master mix (Roche Diagnostics, Switzerland) with 5 picomole concentration of each primer along with 1 µl of template DNA per reaction, with the cycling conditions as illustrated below.



RNA extraction for GARV and BCV: RNA isolation was done from intestinal tissues by adding TRI-reagent (Sigma-Aldrich, USA) method essentially following manufacturer's protocol. For GARV detection, the RNA was further denatured by adding 1µl of DMSO and incubated at 95°C for 5 min due to its double stranded RNA genome.

One-step RT-PCR (osRT-PCR) for GARV and BCV: osRT-PCR amplification of fecal RNA was done as per manufacturer manual by using GARV and BCV primers by SuperScript® III One Step RT-PCR system with Platinum®Taq High Fidelity kit (Invitrogen, USA). Published primers (Matthijnssens *et al.* 2008, Tsunemitsu *et al.* 1999) used in the osRT-PCR are described below (Table 2).

RESULTS AND DISCUSSION

Out of 227 necropsied cases of goat kids of 0–3 month's age, 62.55% (142/227) died due to either of the lesions described below (Table 3). The gross lesions including enteritis, pneumo-enteritis, cestodes infestation and septicaemic lesions were used as criteria for screening the diarrhoeic/intestinal affections.

The listed cases (n=142) containing any of these gross lesions were used for sampling and the intestinal loops were collected for toxinotyping multiplex PCR (TmPCR) for *C. perfringens*, osRT-PCR (GARV and BCV) and SYBR green based real-time PCR for EPEC detection. Based on TmPCR,16.20% (23/142) samples were positive for *C. perfringens* as presented in Table 4. Among positive samples, 47.83% (11/23) isolates were *C. perfringens* toxinotype A and 52.17% (12/23) were *C. perfringens* toxinotype D (Fig. 1). β2-toxin gene was present in 30.43% of the samples toxinotyped. Incidence rate of EPEC in 0–1 and 1–3 month diarrhoeic kids was 36.62% and 25.35% respectively. Based on osRT-PCR, 11.97% were positive for GARV and 9.86% for BCV. Mixed infection was also another important finding and various combinations of

Table 4. Incidence (%) of positive samples with multiplex PCR for *C. perfringens,* Real Time PCR for EPEC and osRT-PCR for GARV and BCV and their mixed infection in intestinal samples of goat kids.

Pathogen	No. of 0–1 month age positive samples and incidence (%)			No. of 1–3 month age positive samples and incidence (%)			Total no. and incidence of positive samples
	Male	Female	Total	Male	Female	Total	
C. perfringens	05 (3.52%)	04 (2.81%)	09 (6.34%)	05 (3.52%)	09 (6.34%)	14 (9.86%)	23 (16.20%)
EPEC	24 (16.90%)	28 (19.72%)	52 (36.62%)	21 (14.79%)	15 (10.56%)	36 (25.35%)	88 (61.97%)
GARV	06 (4.22%)	04 (2.82%)	10 (7.04%)	03 (2.11%)	04 (2.82%)	07 (4.93%)	17 (11.97%)
BCV	05 (3.52%)	03 (2.11%)	08 (5.63%)	02 (1.40%)	04 (2.82%)	06 (4.22%)	14 (9.86%)
C. perfringens + EPEC	04 (2.82%)	03 (2.11%)	07 (4.93%)	03 (2.11%)	08 (5.63%)	11 (7.74%)	18 (12.68%)
C. perfringens + GARV	02 (1.41%)	-	02 (1.41%)	-	01 (0.70%)	01 (0.70%)	03 (2.11%)
C. perfringens + BCV	-	01 (0.70%)	01 (0.70%)	-	01 (0.70%)	01 (0.70%)	02 (1.40%)
EPEC + GARV	05 (3.52%)	04 (2.81%)	09 (6.33%)	03 (2.11%)	03 (2.11%)	06 (4.22%)	15 (10.56%)
EPEC + BCV	04 (2.81%)	03 (2.11%)	07 (4.93%)	01 (0.70%)	02 (1.40%)	03 (2.11%)	10 (7.04%)
GARV + BCV	-	-	-	-	-	-	-
C. perfringens + GARV + BCV	-	-	-	-	-	-	-
EPEC + GARV + BCV	-	01 (0.70%)	01 (0.70%)	-	-	-	01 (0.70%)
C. perfringens + EPEC + GARV + BCV	-	-	-	-	-	-	-

Table 5. Results of TmPCR and proportion of various toxin genes of *C. perfringens* used for toxinotyping in neonatal goat kids.

Toxin gene	Toxinotypes	No. of isolates from 0–1 month age kids		No. of isolates from 1–3 month age kids		Total no. of isolates
		Male	Female	Male	Female	
cpa	A	03	02	02	04	11
etx	D	02	02	03	05	12
cpb2	A, B, C, D and E	01	01	01	04	07

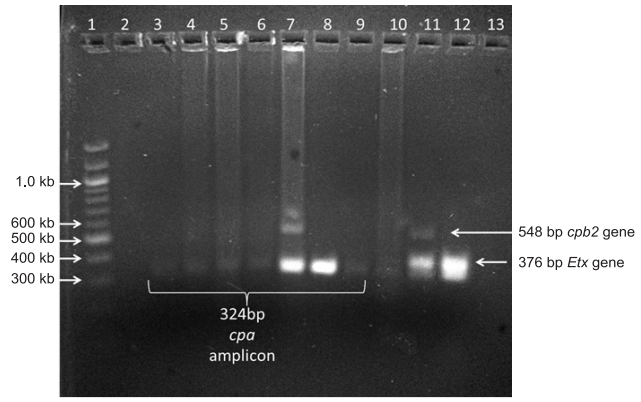


Fig. 1. Gel picture showing multiplex toxinotyping PCR with various amplicons of genes including *cpa*, *etx* and *cpb2* in enteric diseases samples of goat kids. Lane 1, 100 bp ladder; lanes 3, 4, 5, 6, 7, 8, 9, alpha toxin gene with an amplified 324 bp amplicon; lane 12, showing epsilon toxin gene with 376 bp product; lanes 7 and 11, showing beta two toxin gene amplicon with a size of 548 bp and lanes 2 and 13, negative samples.

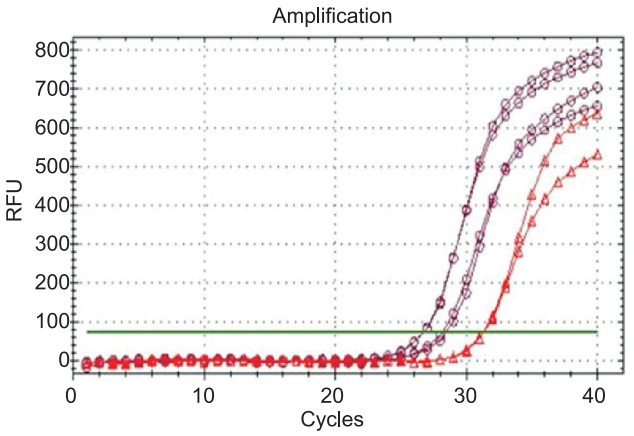


Fig. 2. SYBR green-chemistry based real time PCR assay targeting *bfpA* gene for EPEC showing Cq (cycle quantification) or cycle threshold (Ct) of positive control (Brown) and negative control (Red) in enteric diseases samples of goat kids.

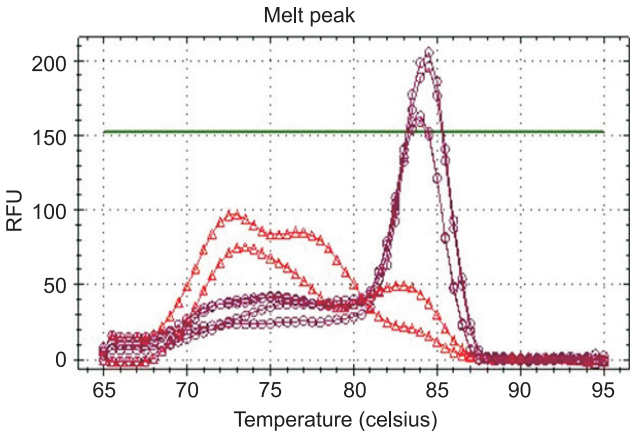


Fig. 3. SYBR green-chemistry based real time PCR assay for EPEC in melting peak of approximately 85°C (Brown) above the threshold 150 are positive amplicons. Whereas, the other noisy peaks less than 85°C and threshold line are primer dimers and non-specific amplicons in enteric diseases samples of goat kids.

pathogens could be detected. The *C. perfringens* + EPEC incidence was 12.68%, while it was 2.11% for *C. perfringens* + GARV and 1.40% for *C. perfringens* + BCV combined infections. Similarly, the mixed occurrence of EPEC + GARV was 10.56% and that of EPEC + BCV was 7.04%. The combined infection of EPEC, GARV and BCV was 0.70%. Further data on gender-wise as well as age-wise incidence of toxinotypes is presented in Table 5.

Neonatal infectious pathologies in goat may be caused by multiple etiologies including bacteria, viruses, parasites and other non-infectious causes, often in synergy and are usually characterized by high morbidity and mortality. *C. perfringens* is responsible for several forms of enterotoxaemia, which differs in clinical manifestations and in severity according to toxigenic type involved and specific toxins produced (Songer 1996). The bacteria produce several toxins which play key roles in pathogenesis of disease (Songer 1996) and are classified into five toxinotypes (A, B, C, D, and E) according to production of 4 major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) (Niilo 1980). However, *C. perfringens* can produce up to 15 toxins in various combinations, including lethal toxins such as perfringolysin 'O' (PFO), enterotoxin (CPE), and β2 toxin (CPB2) (Garmory *et al.* 2000).

Based on findings of the current study, *C. perfringens* types A and D were the common causes of enterotoxemia in neonatal goat kids, which corroborate with earlier reports (Greco *et al.* 2005). *C. perfringens* type D, that harbours the genes *cpa* (α toxin) and *etx* (ε toxin), was isolated from 12 samples during our study.

Virulence factor, β2-toxin gene was present in 30.43% (7/23) of the isolates toxinotyped (Fig. 1) including 28.57% (2/7) male and 71.43% (5/7) females. Among the isolates that have the potential to express β2 toxin, 2 were detected in 0-1 month age group and 5 in 1–3 month age (Table 5).

Among the major toxigenic factors in various isolates of *C. perfringens*, the β2 toxin could be considered as highly virulent due to its association with severe clinical diarrhoea (Gibert *et al.* 1997). It is also imperative that the association of *C. perfringens* type A is not clearly understood, although some studies reported fatal hemolytic disease in lambs and associated gas-gangrene lesions in adult sheep (Niilo 1986, Songer 1996), but its association with β2-toxin gene could have aggravated the pathogenesis in goat kids of the current study. The contact with spores and carrier animals with virulent toxinotypes maintains the vicious cycle adding to risk of further disease caused due to predisposing factors leading to change in conditions of gut micro-environment precipitating enterotoxaemia.

The TmPCR could detect 7 isolates belonging to both *C. perfringens* type A and D carrying the additional virulent β2-toxin gene. Here we report based on gross pathology of intestinal lesions as well as TmPCR, that type A in association with β2-toxin could prove more fatal to neonatal goat kids as observed in the current study. On contrary,

earlier reports also explored the role of the β2 toxin in causing clinical diarrhoea and other associated pathogenesis caused due to enterotoxaemia in goats but their relationship could not be firmly established (Greco *et al.* 2005). However, in the piglets with severe diarrhoea, there are reports that relate the pathogenicity of *cpb2* expressing *C. perfringens* with intestinal lesion during necropsy (Waters *et al.* 2003). With these findings related to our contention on virulence and pathogenicity of β2-toxin, it can be re-iterated that β2 toxin has a larger role to play in neonatal diarrhoea especially in goat kids.

The role of EPEC and its association with enteritis has been studied using intestinal samples collected from necropsied goat kids. EPEC are an important cause of neonatal kid's mortality (Nataro and Kaper 1998) with colonization of small intestinal epithelial lining leading to 'attaching and effacing' (A/E) lesions in the microvilli characterized by intimate aggregation and attachment of bacteria (Knutton *et al*. 1989, Moon *et al*. 1983).There are several different well characterized and putative adhesive factors involved in this process including bundle forming pilin (BFP) (Cleary *et al*. 2004). Therefore this pathogenic gene representing enteropathogenic *Escherichia coli* (EPEC), viz. *bfpA* gene based SYBR-green real time PCR was conducted to screen the samples (Figs 2 and 3). The incidence of EPEC in the clinically affected kids was 61.97% with 36.62% in 0–1 month age and 25.35% in 1–3 month age group (Table 4).

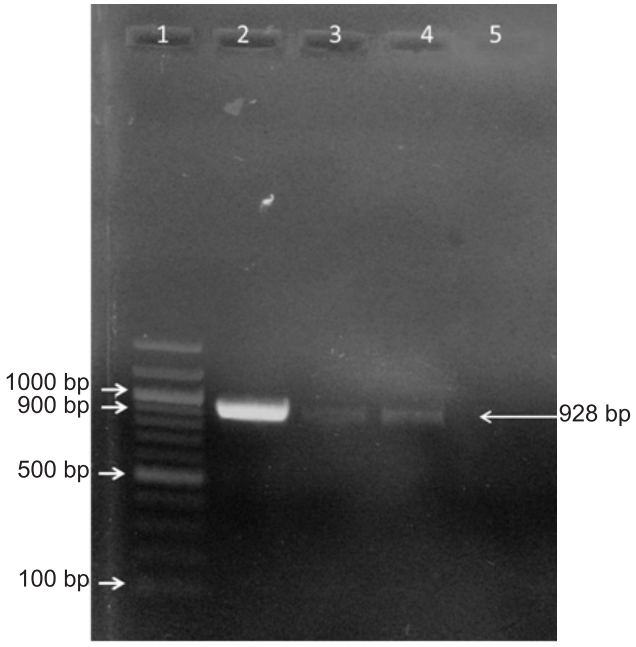


Fig. 4. Gel picture showing the one step RT-PCR of denatured RNA samples obtained from enteric diseases samples amplification of VP6 gene of GARV of goat kids. Lane 1, 100 bp ladder; lanes 2, 3, 4, VP6 gene with an amplified 928 bp amplicon; lane 5, negative sample.

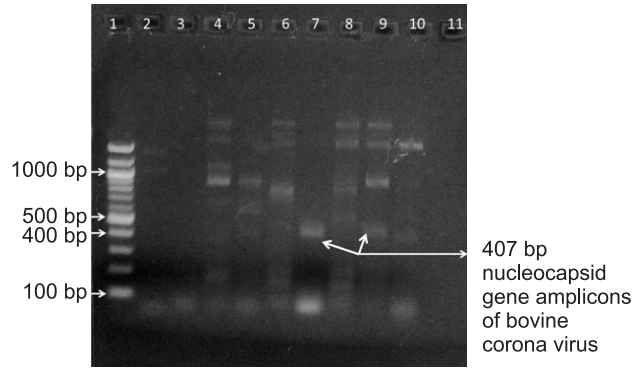


Fig. 5. Gel picture showing the one step RT-PCR of denatured RNA samples obtained from enteric diseases samples amplification of Nucleocapsid gene of BCV of goat kids. Lane 1, 100 bp ladder; lanes 7 and 9, nucleocapsid gene of BCV with amplified 407 bp amplicon; lanes 2, 3, 4, 5, 8, 10 and 11, negative sample.

In this current study, we report a SYBR Green real time PCR technique for differentiation of EPEC and non-EPEC isolates (target organisms responsible for intestinal lesions) in neonatal goat kids targeting the virulent *bfp*A gene. In present study, the incidence of EPEC (61.97%) was higher than the earlier findings of Wani *et al*. (2003) who reported 26.6% incidence of EPEC whereas the findings of Bhat *et al*. (2008) portrayed a much less prevalence of 12.5% EPEC in diarrhoeic lambs. The variations in the prevalence of EPEC could be attributed to the determinants like micro-environment, farm ecosystem and management, seasonal variations, host-species etc. However, to arrive at the optimal data, it is essential to conduct prevalence studies by continuous surveillance mechanisms.

PCR-based methods are more sensitive and rapid than phenotypic tests performed on individual colonies (Rich *et al*. 2001). Among the several chemistries available for real-time PCR assays, SYBR Green based assays are the most widely used. Real-time PCR based techniques have been reported for detection of many pathogens of veterinary importance (Aguero *et al.* 2007). In earlier reports also, SYBR green based real time PCR assay was used earlier to differentiate EPEC, verotoxic *E.coli* and other enteroaggregative *E. coli* more effectively (Bischoff *et al*. 2005), but domestic lab-based screening tests (as developed in the current study) are also the need of the hour for effective screening of EPEC strains.

Out of total samples, 11.97% were positive for GARV (Table 4 and Fig. 4), with 7.04% in 0–1 month age and 4.93% in 1–3 month age. Nucleocapsid gene was targeted for amplifying BCV and 9.86% samples showed positive result (Fig. 5; Table 4) with 5.63% in 0–1 month and 4.22% in 1–3 month old kids. Rotavirus is responsible for causing economically significant malady in neonates of many domestic animals (Kapikian and Chanock 1996) and reported in sheep as a cause of enteritis and diarrhoea (Wani *et al*. 2004). Wani *et al*. (2004) recorded GARV association with lamb diarrhoea in an outbreak in Kashmir, India in 25% diarrhoeic lambs. Previous study (Dey *et al*. 2007) showed an incidence of rota virus at 8.68% in Black Bengal goats, which is a notch lesser than our incidence (11.97%). It was also reported (Dey *et al*. 2007) in that study that kids of 7 days to 1-month age group were frequently found

associated with rotavirus infection (13.63%). Another study (Khafagi *et al*. 2010) presented a similar picture with 12.3% prevalence of rotavirus associated with diarrhoea in lambs and kids in samples. It is true that the incidence of rotaviral diarrhoea is increasing as evidenced by these reports, but the association with other pathogens could not be established based on the clinical signs and gross lesions. In the current study, the mixed infections were studied to identify the inter-pathogenic interaction in relation with the diarrhoea (Table 4). Most of the studies assessed the relevance of BCV as a primary pathogen in neonatal calf diarrhea (NCD) and associated mortality (Ammar *et al*. 2014). The prevalence rate of 3–20% had been recorded for coronavirus in calf diarrhoea (Mayameei *et al*. 2009). In present study, the incidence of BCV was 9.86% with slightly low incidence (5.63%) in 0–1 month than 1–3 months (4.22%) kids. The previously reported prevalence for BCV was 11.76% from clinical diarrhoeic calves aged below 3 months (Rai *et al*. 2011). However, to the best of knowledge, no organized study was found on BCV infection in goats. This makes room that, the possibility of BCV as a pathogen in neonatal enteritis of goats require further push to relate the symptoms, season and the disease on a single platform to control the spread of BCV-induced enteritis.

In present study, mixed infections of the various enteric pathogens were studied to identify their synergistic role in causing neonatal mortality in goat kids. The results of mixed infection incidence are presented in Table 4. The mixed infections in necropsied kids were 12.68% positive for *C. perfringens* and EPEC and in positive, 38.89% were 0–1 month and 61.11% of 1–3 month age. Another combination including *C. perfringens* and GARV was detected in 2.11% samples with 66.67% in 0–1 month age and 33.33% in 1–3 month age. Mixed infection of *C. perfringens* and BCV was 1.40%. The mixed infection of EPEC and GARV was 10.56% with 60.0% of 0–1 month and 40.0% kids of 1–3 month age group. The combination of EPEC and BCV detected in 7.07% goat kid samples including 70.0% of 0–1 month of age and 30.0% were 1–3 months of age. Only 0.70% samples were positive for infection of EPEC, GARV and BCV together (Table 4). As such type of study has not been conducted previously on neonatal goat kids, no literature could be traced for comparative studies on mixed infections based on necropsy lesions in goats. However, Bok *et al*. (2015) detected a high percentage of samples (34.79%) infected with both BCV and GARV in calves. This was somewhat expected, as GARV is responsible for the majority of neonatal calf diarrhoea worldwide (Kapikian and Shope 1996). The findings of Ammar *et al.* (2014) showed that prevalence of GARV and BCV infection at 14.63% and 20.73%, respectively.

With the experience of the current work on neonatal enteritis and its etiological agents, it is concluded that neonatal enteritis and the associated mortality patterns incur severe monetary risks to goat keepers. The major lacunae in addressing these diseases are the lack of availability of resources like veterinary aid, vaccines, and proper medicines besides poor upkeep of farms in terms of hygiene. The present study envisages occurrence of enteric diseases of mixed etiologies which is major cause of mortality in neonatal kids under field conditions and its diagnosis by specific molecular assays.The multiplex PCR technique aided in the characterization the *C. perfringens* toxinotypes as well of detection of virulence factors like β2 toxin gene. The availability of method to define exact toxinotype of *C. perfringens* may shorten and simplify development of adequate vaccines fitting the epidemiological situation. Real time PCR gives accurate and authentic diagnosis of EPEC causing severe mortality in young goat kids. Detection of GARV and BCV in diarrhoeic neonatal goat kid's samples using VP6 gene and nucleoprotein gene primers respectively by osRT-PCR will help in early diagnosis and optimal managemental interventions like proper hygiene, sanitation and biosecurity measures during neonatal period. Early diagnosis will help in treatment and in devising a kid-cum-dam hood vaccination for healthy neonates to protect them which will eventually prevent the economic losses to the farmers.

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