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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Detection of bovine coronavirus in calf diarrheic samples by indirect antigen capture ELISA and RT-PCR

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Abstract

In the present study, 160 fecal samples were collected from clinical cases of diarrheic calves from different Indian states covering eight dairy farms of Uttar Pradesh, Tamil Nadu, and Karnataka and dead calves of Bareilly region. The fecal samples from all these cases were screened for the presence of bovine coronavirus (BCoV) by using commercially available indirect antigen capture ELISA kit. Furthermore, all samples were subjected to RT-PCR for detection and confirmation of BCoV. RT-PCR was done by using two different types of primers to amplify the conserved nucleocapsid (N) gene of the virus targeting a 407 bp and 730 bp gene fragments of BCoV. Out of 160 cases, 5.63% (9/160) and 9.38% (15/160) of cases were positive for BCoV by ELISA and RT-PCR, respectively. Further, all the reported cases are from the Bareilly region of Uttar Pradesh with incidence rate of 9.38% (11/65). In conclusion, indirect antigen capture ELISA can be employed for the rapid detection of bovine coronavirus in diarrhoeic cases in fecal samples; however RT-PCR is more sensitive than ELISA for detection of coronavirus in diarrheic cases. Further studies are needed on bovine coronavirus to isolate from different parts of our country, to characterize them at genomic levels thereby investigating their antigenic and genetic properties and to check strain variations.

Keywords: Neonatal calf diarrhea; Bovine coronavirus; ELISA; RT-PCR; FAT; electron microscopy; prevalence; India.

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Introduction

Infectious diarrhoea is responsible for major economic losses and neonatal calf mortality in dairy and beef herds (Oxender et al., 1973). It has been recognized as one of the main six causes of all deaths from infectious diseases; hence it is more significant in animal health and for dairy industry (Kapikian, 1996; Dhama et al., 2009). Although there are several infectious agents involved in the etiology of neonatal calf diarrhoea, bovine group A rotavirus, bovine coronavirus, *E. coli*, *Salmonella* spp., and *Cryptosporidium* are the major agents and responsible for 75-95% of infection in neonatal calves worldwide (Moon et al., 1978; Snodgrass et al., 1986; Radostits et al., 1994; Bendali et al., 1999; Hoet et al., 2003; Gumusova et al., 2007; Dhama et al., 2009; Malik et al., 2012). Out of these pathogenic agents, Rotaviruses are the leading cause and Coronaviruses are a major contributor of calf diarrhea (Marsolais et al., 1978; Gumusova et al., 2007; Uhde et al., 2008; Dhama et al., 2009). The diseases associated with bovine coronaviruses (BCoV) are more severe because they affect both the small and large intestines (Clark, 1993; Gunn et al., 2009; Boileau and Kapil, 2010). The other infections associated with BCoV include winter dysentery (WD) in adult dairy cattle and respiratory tract infections in calves and feedlot cattle (Saif et al

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1991; Saif, 2010). Recent reports indicate that it is second only to bovine herpesvirus affecting the respiratory system and the serologic incidence suggests that most cattle become exposed to BCoV during their lifetime (Fukai et al., 1998; Boileau and Kapil, 2010).

Bovine coronavirus is a member of group 2 coronaviruses of the family *Coronaviridae*, order *Nidovirales* and possess a single-stranded, enveloped, non-segmented RNA of 32 kb genome with positive polarity (De Vries et al., 1997; Van Regenmortel et al., 2000). Virion contains five structural proteins: the nucleocapsid (N), the transmembrane (M), the haemagglutinin/esterase (HE), the spike (S) and the small membrane (E) proteins (Saif, 1993). Generally, it affects calves from day 1 to 3 months of age and diarrhea typically occurs between 2-8 weeks of age (Mebus et al., 1973; Langpap et al., 1979; Mostel and Burki, 1987; Boileau and Kapil, 2010). These viruses were reported to be frequently found in both normal and diarrhoeic faeces of calves (Snodgrass et al., 1986). The primary sites of BCoV replication are enterocytes of the distal ileum and colon, epithelial cells in the respiratory tract, thus virus being excreted with the respective excretions. BCoV has worldwide distribution and being reported from several countries (Jeong et al., 2005; Khalili and Morshedi, 2006; Takiuchi et al., 2006; Gumusova et al., 2007; Park et al., 2007; Boileau and Kapil, 2010; Dash et al., 2012; Hansa et al., 2012). However, epidemiological and pathological data on BCoV in India are limited. So, the aim of the present study was to screen the fecal samples for BCoV collected from clinical cases and dead calves under 3 months of age with the history of diarrhea from Bareilly (65) and Lucknow (25) regions of Uttar Pradesh, Vellore (9) and Chennai (13) regions of Tamil Nadu, and Mysore (17) and Bangalore (20) regions of Karnataka during November 2011 to February 2012. Indirect antigen capture ELISA, being a sensitive method for antigen detection, was used for determining BCoV prevalence. Furthermore, virus confirmation was done at genomic level by the molecular method of RT-PCR, nested PCR and its sensitivity was compared with that of ELISA for detecting virus in clinical samples.

Materials and Methods

Collection and processing of faecal samples: A total of 160 diarrhoeic fecal samples were collected from both cattle and buffalo calves under 3 months of age, with the history of diarrhea, from two dairy farms of Bareilly district (65 samples) (LPM Cattle and Buffalo farm, IVRI, 54; Military Dairy Farm, 11), one dairy farm from Lucknow district (12) and one dairy farm from Barabanki district (13) of Uttar Pradesh State, Vellore (9) and Chennai (13) regions of Tamil Nadu state, and Mysore (17) and Bangalore (20) regions of Karnataka state during November 2011 to February 2012. Spontaneous dead calf cases (11) from the post-mortem (PM) room, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly (U.P.) were also included in the study.

All the fecal samples were obtained in sterile collection vials and kept at -20°C till use. Fecal suspensions (v/v; 50%, watery feces; 20%, loose feces; or 10%, normal feces) were prepared with ultra-pure water treated with 0.1% diethyl-pyrocarbonate (DEPC water). Samples were centrifuged at 14000 x g for 10 min at 4°C and supernatants were collected and used for screening of BCoV by ELISA and RT-PCR. The processing of fecal samples was done within 24 hr of collection of samples.

Screening of fecal samples: All the 160 fecal samples were subjected to an indirect antigen-capture ELISA employing monoclonal antibodies (mAbs) to detect the presence of BCoV employing commercially available ELISA kit (Bio-X Diagnostics, Belgium kit - BIO K 151) following the manufacturer's instructions. The results were interpreted by using the OD values obtained at 450 nm using ELISA Reader (BioTec, USA).

Reverse Transcription - Polymerase Chain Reaction (RT-PCR):

RNA Extraction: Viral RNA was extracted from the clinical samples using TRIzol® LS reagent (Invitrogen, USA) as per the manufacturer's instructions and the extracted RNA was resuspended in 20µl of nuclease free water and preserved at -70° C until used.

RT-PCR primers: Two distinct sets of established primers were used for confirmation of BCoV (Tsunemitsu et al., 1999; Cho et al., 2001) (Table 1). The RT-PCR was performed on all the 160 faecal samples from both live and dead diarrheic neonates. Both the sets of primers were used to amplify the N gene which is the conserved region in the genome of Mebus strain virus.

RT-PCR and nested PCR (N-PCR): Complementary (c) DNA was prepared from the extracted viral RNA by reverse transcription reaction and used for PCR amplification of the conserved region, N gene of the virus. Two distinct sets of established primers were used for the amplification of cDNA generating expected amplicons of 407 bp and 730 bp sizes, for confirmation of BCoV (Tsunemitsu et al., 1999; Cho et al., 2001) (Table 1). The amplified products were visualized on 1.2% agarose gel (Amresco, USA) stained with ethidium bromide (0.5 µg/ml).

Table 1: Oligonucleotide Primers used for amplification of N gene of bovine coronavirus

Target gene	Primer sequence (5'-3')	Position	Product size	Reference
N gene	F-5' GCCGATCAGTCCGACCAATC-3'	91-111	407 bp	Tsunemitsu *et al.* 1999.
	R-5' AGAATGTCAGCCGGGGTAA-3'	498-480		Cho *et al.*, 2001
	F-5' GCAATCCAGTAGTAGAGCGT-3'	21-40	730 bp	
	R-5' CTTAGTGGCATCCTTGCCAA-3'	731-750		

Direct Fluorescent Antibody Technique (d FAT)
Direct Fluorescent Antibody Technique (d FAT) was used to determine the BCoV at antigenic level in paraffin embedded sections of intestine, brain, lung, liver, spleen, mesenteric lymphnodes, heart and kidney as per the standard protocol. Briefly, slides were coated with 3-aminopropyle-triethoxysilane (APES) (Sigma Aldrich, USA). The paraffin sections of tissue samples were deparaffinised and rehydrated. The slides were treated with 0.3% H_2O_2 in methanol solution for 20 min to quench the endogenous peroxidases, thoroughly rinsed thrice with PBS (pH 7.2), 5 min each. Later, the slides were kept in Couplin jar, having antigen unmasking solution and boiled for 15 min in microwave oven for antigen retrieval. The sections were cooled to room temperature, and then incubated with normal goat serum for 20 min. Then after thorough washing with PBS, the slides were incubated with 50 µl of FITC conjugated anti-coronavirus monoclonal antibodies (1:20) (Bio-X Diagnostics, Belgium), at 4^oC for overnight. The slides were finally washed thrice with PBS (5 min each) and then mounted with glycerol aqueous to be viewed under the fluorescent microscope (40X) for the presence of specific apple green fluorescence signals.

Transmission electron microscopy (TEM): Final confirmation of BCoV positive faecal samples was done by transmission electron microscopy. Briefly, the suspension of the sample in 10 % PBS was sonicated for 1 minute and then clarified at 14,000 g for about 30 minutes. Then they were negatively stained with 2 % phosphotungstic acid (PTA). Thereafter, 20 µl of the suspension was nebulized on a 200 mesh carbon coated grid. The suspension was then viewed under a transmission electron microscope at a magnification of 40,000 to 80,000 at G.B. Pant Agriculture University, Pantnagar, Uttarakhand, India.

Results

Screening of faecal samples by ELISA: The screening of fecal samples, collected from diarrheic calves below three months of age from Bareilly (65) and Lucknow (12) regions of Uttar Pradesh, Vellore (9) and Chennai (13) regions of Tamil Nadu, and Mysore (17) and Bangalore (20) regions of Karnataka, by commercially available ELISA kit indicated high prevalence of BCoV. Out of the total of 160 fecal samples, 149 samples were from diarrhoeic faecal samples from clinical cases and 11 were as intestinal contents of necropsied cases from the post-mortem (PM) room, IVRI, Izatnagar, Bareilly (U.P.). Out of which, 9 (5.63%) samples were positive for bovine coronavirus i.e., 7 diarrhoeic faecal samples of clinical cases and 2 intestinal contents of necropsied cases were shown positive for bovine coronavirus (Table 2). The BCoV incidence rate in clinical cases in LPM farm, IVRI and Military dairy farm, both in Bareilly district was found to be 11.11% (6/54) and 9.09% (1/11), respectively. Diarrhoeic faecal samples collected at LPM (C&B) dairy farm, I.V.R.I., Bareilly showed the prevalence of Coronavirus, Rotavirus, *Eschericia coli*, Cryptosporidium and *Eimeria spp.* as 8.96%,16.41%, 14.93%, 80.60% and 4.48%, respectively (data not shown). The overall prevalence of BCoV in Bareilly district was found to be 10.77% (7/65) and 18.18% (2/11) in clinical cases and necropsied cases, respectively. None of the diarrhoeic faecal samples from Lucknow, Barabanki areas of Uttar Pradesh, Chennai, Vellore regions of Tamil Nadu and Mysore, Bangalore regions of Karnataka were found positive for bovine coronavirus by ELISA (Table 2).

Detection of BCoV by RT-PCR: Standardized RT-PCR was applied for detection and confirmation of BCoV in all the 160 fecal samples collected from clinical cases of diarrheic and dead calves (Fig.1). Out of total 160 samples screened by RT-PCR, 15 (9.38%) samples were found positive for BCoV i.e., 11 diarrhoeic faecal samples of clinical cases and 4 intestinal contents of necropsied cases were shown positive for BCoV. The virus incidence rate in clinical cases at LPM (Cattle & Buffalo) dairy farm, IVRI and military dairy farm, both in Bareilly district was found to be 18.52% (10/54) and 9.09% (1/11), respectively, with an overall prevalence of 9.38% (11/65). In dead cases from PM room, IVRI, Bareilly, the incidence rate of 27.27% (4/11) was observed. None of the diarrhoeic faecal samples from Lucknow, Barabanki areas of Uttar Pradesh, Chennai, Vellore regions of Tamil Nadu and Mysore, Bangalore regions of Karnataka were found positive for bovine coronavirus by RT-PCR (Table 2).

Data regarding overall prevalence of BCoV as determined by ELISA and RT-PCR and the comparison of sensitivity of these tests with regard to age and breed are given in Table 2-4 and Figure 2-4. In this study, bovine coronavirus infections were observed during 7

Table 2: Comparison between ELISA and RT-PCR for the detection of bovine coronavirus infections in the faecal samples of calf origin

Place of Collection	No. of Samples Tested	ELISA		RT-PCR	
		No of positive samples	Prevalence rate (%)	No of positive samples	Prevalence rate (%)
Bareilly					
Military Dairy Farm	11	1	9.09	1	9.09
LPM farm, IVRI	54	6	11.11	10	18.52
Post Mortem Facility, IVRI (Dead cases)	11	2	18.18	4	36.36
Total Clinical cases (A)	76	9	11.84	15	19.74
Lucknow					
Dairy Farm (Clinical cases) (B)	12	0	0	0	0
Barabanki					
Dairy Farm (Clinical cases) (C)	13	0	0	0	0
Tamilnadu					
Vellore	9	0	0	0	0
Chennai	13	0	0	0	0
Total Clinical cases (D)	22	0	0	0	0
Karnataka					
Mysore	17	0	0	0	0
Bangalore	20	0	0	0	0
Total Clinical cases (E)	37	0	0	0	0
Total (A+B+C+D+E)	160	9	5.63	15	9.38

Table 3: Age wise prevalence studies of bovine corona virus in calves

Age of calf	Total numbers of cases	No. of BCoV positive cases
0-10 days	28	4
11-20 days	22	3
21-30 days	31	3
1-2 months	55	5
2-3 months	24	0

Table 4: Breed wise prevalence studies of bovine corona virus in calves

Breed	Total numbers of cases	No. of BCoV positive cases
Vrindhavani	49	10
Murrah	14	4
Friesian cross	24	1
Tharparkar	6	0
Jersey cross	37	0
Non descript	30	0

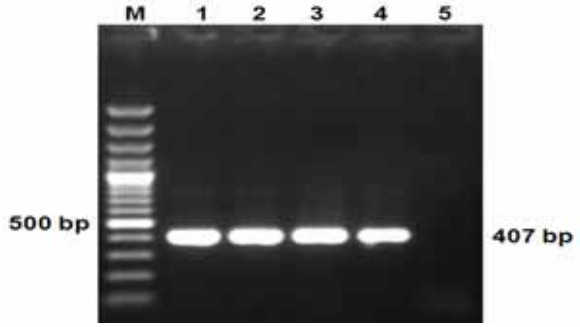

Fig.1: RT-PCR detection of BCoV: Lane 1-5: RT-PCR amplified product of 407 bp of BCoV N gene in faecal samples of calves; Lane M: DNA ladder of 100 bp plus

to 50 days of age (1-7 weeks). The present study, also showed that incidence of bovine coronavirus was higher during winter season.

Conformation of BCoV by nested PCR (N-PCR):

All the 15 bovine coronavirus positive samples by RT-PCR (11 faecal samples of clinical cases and 4 intestinal contents of dead cases) were subjected to nested PCR (N-PCR) first by amplifying the 730bp RT-PCR product and subsequently 407 by using N gene (Fig. 5).

Direct Fluorescent Antibody Technique (dFAT)

Bovine coronaviral antigen was demonstrated by dFAT mainly in distal small intestine, lungs, brain and liver. Specific apple green fluorescence was noticed in villous enterocytes of distal small intestine, alveolar epithelial cells of the lungs, glial cells of brain and liver (Fig. 6).

Transmission electron microscopy

Faecal samples examined for bovine coronavirus by transmission electron microscopy revealed viral presence with the diameter ranging from 72-206 nm with an average of 140 nm (Fig. 7). Bovine coronavirus in faecal samples showed crown like appearance during TEM analysis.

Discussion

Neonatal calf diarrhoea is responsible for major economic losses in many dairy and beef herds (Oxender et al., 1973). The increased mortality in young calves during early days of life, adversely affects the

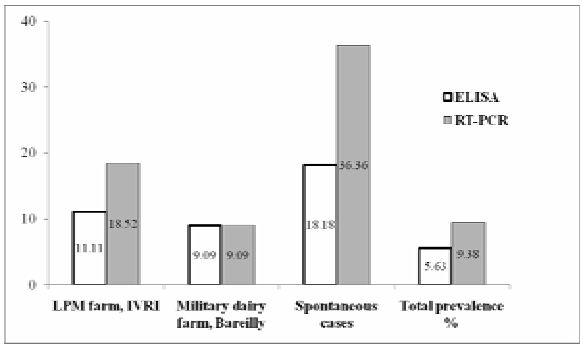

Fig. 2: Histogram showing comparison of prevalence percentage of bovine corona virus in calves by ELISA and RT-PCR

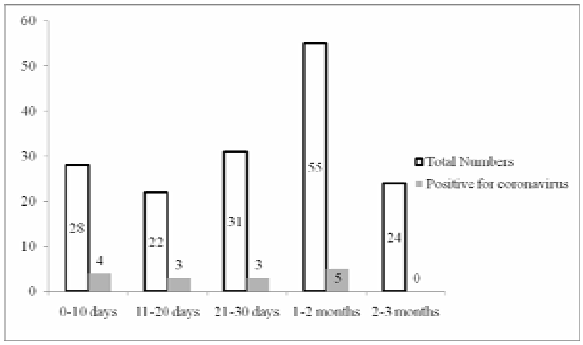

Fig. 3: Histogram showing age wise prevalence of BCoV in calves

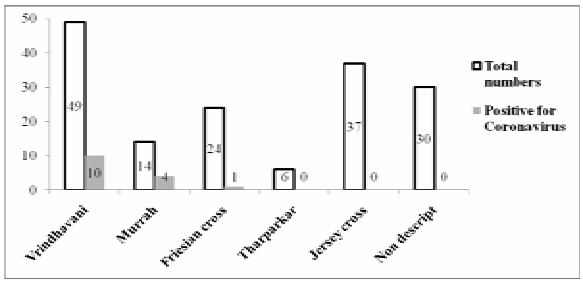

Fig. 4: Histogram showing breed wise prevalence studies of bovine corona virus

profitability of most of the livestock farming systems. It has been calculated that neonatal diseases resulting in calf mortality reduce farm net profitability by 38% (Martin and Wiggins, 1973; Khan and Khan, 1991). The major causes of calf mortality are gastro-enteritis, pneumonia and septicaemia. Among these, up to 60% total calf mortality is attributed to gastro-enteric conditions (Swain and Dhama, 1999; Smith, 2002). Calves are at greatest risk of developing diarrhoea during the first month of life, when they have immature immune status, lacks specific antibody, stresses forced by weaning and deprivation of immune colostrum feeding. Calf diarrhea is associated with various etiological agents, among which viral etiology viz., rotavirus and coronavirus are the most important ones (Marsolais et al., 1978; Clark, 1993; Jeong et al., 2005; Gumusova et al., 2007; Uhde et al., 2008; Dhama et al., 2009; Boileau and Kapil, 2010; Malik et al., 2012). BCoV is the second major cause of viral diarrhoea in calves, with rotavirus being the first (Craig and Kapil, 1994). BCoV causes a more severe disease and higher mortalities than those caused by the bovine Rotavirus because it multiplies in both the small intestine and the large intestine, whereas the rotavirus infects only the small intestine (Torres-Medina et al., 1985). The reports regarding Coronavirus prevalence in dairy calves of India are limited; however, its prevalence in India was reported by few workers in limited regions by electron microscopy, virus isolation or ELISA (Rai and Singh, 1983; Rai et al., 2011; Suresh et al., 2011). Only recently, Hansa et al. (2012) reported BCoV detection in Northern region of India. So, the present study was carried out to investigate the incidences of BCoV in dairy farms of Indian states, covering Northern and Southern states, employing ELISA kit and RT-PCR detection of the BCoV. In the present study, screening of the fecal samples was carried out both on live and dead animals during the winter months because BCoV is more stable during colder climates due to its enveloped nature (Evermann and Benfield, 2001) and it also causes winter dysentery in adult cattle during winter season (Cho et al., 2000; Boileau and Kapil, 2010).

A total of 160 clinical samples comprising of 149 fecal samples from calves with clinical presentation of diarrhea and intestinal contents of 11 dead cases were screened for BCoV antigen by commercial ELISA kit. The ELISA, enzyme linked immunosorbent assay was used due to the facts that most of the other immunodiagnostic tests developed for detection of enteric pathogen have either low specificity and/ or sensitivity. ELISA also has ability to detect enteric pathogens even at low concentrations (Selim et al., 1991; Kelkar et al., 2004). Antigen capture ELISA type is regarded as the diagnostic test of choice for BCoV detection (Schoenthaler et al., 1999; Boileau and Kapil, 2010). All the positive cases were reported only in the farms of Bareilly region with incidence rate of 10.77% in clinical cases, as tested by commercially available ELISA kit and 18.18% in dead cases from Bareilly region. None of the diarrhoeic faecal samples from Lucknow, Barabanki areas of Uttar Pradesh, Chennai, Vellore regions of Tamil Nadu and Mysore, Bangalore regions of Karnataka were found positive for bovine coronavirus by ELISA. The overall prevalence of BCoV in Bareilly district as detected by ELISA was 5.63%, which is less than with earlier reported virus prevalence of 14% (Reynolds et al., 1986), 20% (Bordas et al., 1985) and 20% (Vanamayya, 1990).

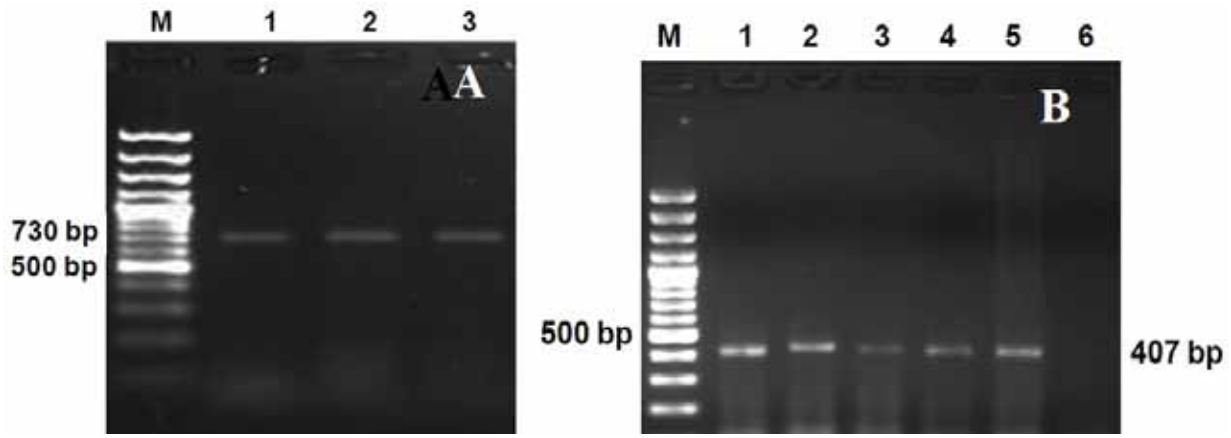

Fig. 5: Nested-PCR (N-PCR) detection of BCoV N gene (full length) in faecal samples of calves. (A): Lane 1-3: Nested -PCR amplified product of 730 bp; (B): Lane 1-5: Nested -PCR amplified product of 407 bp; Lane M: DNA ladder of 100 bp plus

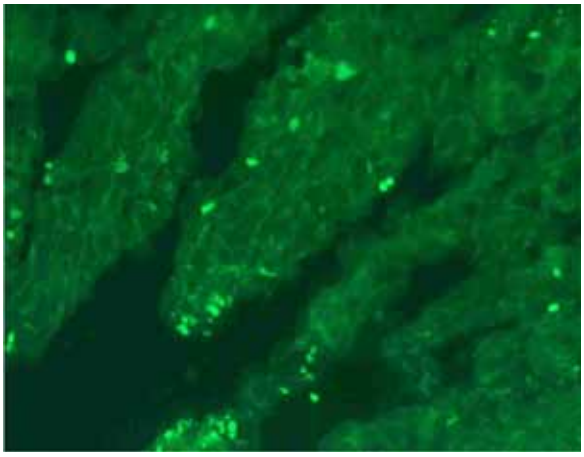

Fig. 6: Calf Intestine showing specific apple green fluorescence of coronaviral antigen in villous epithelium. dFAT x 400

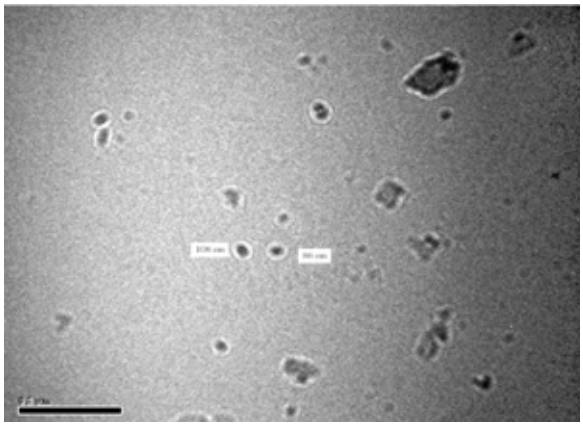

Fig. 7: Transmission electron microscopy showing coronavirus particles of size around 86 and 108 nm diameter from the sample of calf faecal material, Negative stain x 60000.

However, Dash et al. (2012) recently reported a comparatively lower BCoV prevalence rate of 4.76% in Mathura and its adjacent regions of Uttar Pradesh. In India its prevalence rate was reported from 3.7 to 14.0% by earlier workers (Suresh et al., 2011; Rai et al., 2011; Hansa et al., 2012). In our study, a higher incidence of 18.18% was recorded in dead cases from post mortem facility indicating that BCoV plays an important role in causing the calf diarrhea and the associated mortality as suggested by several workers (Rai and Singh, 1983, 1986; Khan and Khan, 1991; Boileau and Kapil, 2010). The variations in the incidence rate of coronavirus in different regions may be attributed to managemental practices followed and climatic conditions of that particular region. The pathological evaluation of these dead calves also confirmed that BCoV affects multiple organs (Hansa, 2010).

In the present investigation, molecular tool RT-PCR by targeting N gene was done for rapid detection and confirmation of BCoV. Standardization of RT-PCR was done by selecting viral N gene based primers as it is conserved among BCoV strains. The estimated specific viral amplicons with sizes of 407 bp and 730bp were obtained confirming virus detection in clinical samples and were in agreement with earlier reports (Tsunemitsu et al., 1999; Cho et al., 2001; Khalili and Morshedi, 2006). Out of total 160 samples, 15 (9.38%) were found positive for BCoV as compared to 9 (5.63%) samples found positive by ELISA. However, only samples from Bareilly regions gave positive amplification for N-gene specific primers with incidence rate of 16.92% (11/65), the results are shown in Table 2. The inability to detect the presence of bovine coronavirus in the diarrhoeic fecal samples from the southern region of India could be due to either less prevalence of virus in that region or as the coronavirus

is highly fragile might have degraded during transportation and storage. The results suggest that RT-PCR is more sensitive than ELISA to detect BCoV, in diarrhoeic fecal sample. This is because RT-PCR assay can detect even few viral particles in fecal sample if otherwise BCoV-positive animals then might be classified as BCoV-negative by ELISA or by other methods.

In the present study, bovine coronavirus infection was observed during the age of 7 to 50 days (1 to 7 weeks) in calves which found to be in accordance with the earlier reports of Mebus et al. (1973); Langpap et al. (1979) and Vanamayya (1990), since they observed bovine coronavirus infection in 2-8 weeks age group. The present study also showed that incidence of BCoV was higher in winter season. Stair et al. (1972) also reported an increased frequency of neonatal calf diarrhoea in beef type calves during a relatively short period of time in the late winter and early spring when inclement weather cause stress. High prevalence of enteric infection especially those of viral infection in winter months were in agreement with earlier reports (Singh et al., 1985).

Bovine coronaviral antigen was demonstrated by dFAT mainly in distal small intestine and transverse colon, lungs, brain and lung in the present study. The fluorescence observed was concordant with the findings of Rai and Singh (1983). Transmission electron microscopy although is not being used for disease diagnosis now, was used only for virus confirmation in this study. In the present study, it was found that the size of bovine coronavirus ranging from 72–206 nm with an average of 140 nm which was in harmony with the reports (Tyrell et al., 1968; Stair et al., 1972; Langpap et al., 1979; Patel et al., 1982; Rai, 1983; Rai and Singh, 1986).

RT-PCR detecting subclinical cases or early or late course of infection or after re-infection when low level of virus is being excreted from intestines into the feces, is an important approach concerning epidemiological surveillance as it allows for the application of suitable preventative measures prior to the emergence of diarrhea at a farm. RT-PCR is a rapid, sensitive, confirmatory method for timely detection of BCoV infection in diarrhoeic calves (Cho et al., 2001; Boileau and Kapil, 2010). By the best of our knowledge, this is second report regarding the molecular detection and screening of fecal samples for BCoV by RT-PCR in India covering wider region compared to previous report by Hansa et al. (2012). Thus the data obtained herein adds to the earlier epidemiological data of BCoV prevalence worldwide. However, RT-PCR needs to be applied at a large scale for screening the BCoV infection both in clinical diarrheic calves as well as in subclinical non-diarrheic calves so as to know the degree of BCoV infection and its molecular epidemiology and virus distribution thereby putting more information to the existing data on BCoV.

Extremely large bovine coronavirus genome, approximately 32,000 RNA bases and being the longest among animal viruses, are thought to mutate at high frequency as a consequence of high error rates of the RNA polymerase that are predicted to accumulate several base substitutions per round of replication. Changes in virulence, tissue tropisms and interspecies transmission of BCoVs occur through genetic variations in structural and non-structural proteins (Schoenthaler et al., 1999; Boileau and Kapil, 2010). Recently, the molecular tools and techniques are being widely used in animal disease diagnosis including PCR and allied techniques viz., RT-PCR, RRT-PCR, Q-PCR, real-time PCR, PCR-RE/RFLP, LAMP, nucleotide sequencing and phylogenetic analysis. All these techniques have strengthened detection of animal pathogens in terms of rapidity and reliability, and also for characterizing and screening various etiological agents, which are altogether very helpful for an effective disease control programs. Several workers have recently developed the applied molecular diagnostics for BCoV detection and characterization, viz., RT-PCR, multiplex PCR, nested PCR, semi-nested RT-PCR, real-time RT-PCR (TaqMan-probe based or SYBR Green based) (Cho et al., 2001, 2010; Escutenaire et al., 2007; Park et al., 2007; Decaro et al., 2008; Klein et al., 2009; Asana et al., 2010). Techniques like multiplex real-time PCR assay have the advantage of simultaneous detection and quantification of major pathogens causing calf diarrhea.

The results of the present study and the earlier reports indicate that bovine coronavirus is one of the important etiological agents of neonatal calf diarrhea in India. The ELISA, RT-PCR, TEM, virus isolation and dFAT were used for detecting the presence of bovine coronavirus. Rapid screening of large number of samples for BCoV by indirect ELISA is useful tool, but for more sensitive detection RT-PCR seems to be the best method. It is suggested that more investigations should to be carried out for BCoV detection in Indian dairy herds covering different geographical regions and with large quantity of samples, which would highlight the real epidemiological significance of this important virus and would be helpful for planning suitable and timely prevention and control programs, thereby reducing economical losses and increasing the profitability of dairy farms. So, the use of molecular based diagnostic methods needs to be applied to their full potential for continuous virus monitoring and surveillance. Further studies are needed for bovine coronavirus to isolate from different parts of our country, to characterize them at genomic levels and to check strain variation. Complete sequencing of Indian isolate has to be done, since no published sequence have been imparted still now in our country. The extent

of virus distribution and damage to other organs has to be further evaluated and the relation of virus tropism with strain variation will be of great importance.

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