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ELISA and RT-PCR Based Detection of Bovine Coronavirus in Northern India

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ABSTRACT

Bovine coronavirus (BCoV) is widespread in cattle population, resulting in heavy economic losses to both dairy and beef industry throughout the world. The syndromes associated with BCoV include winter dysentery in adult dairy cattle and respiratory and intestinal tract infections in young calves. The virus has specific tropism for intestinal and pulmonary epithelial cells. Reports regarding prevalence and molecular detection of BCoV from India are scarce. In this study, 101 fecal samples were collected from clinical cases of diarrheic calves from North Indian region covering three dairy farms of Uttar Pradesh and dead calves of post mortem facility of Indian Veterinary Research Institute. Fecal samples from all the cases were screened for the presence of BCoV by commercially available ELISA kit. Furthermore, all samples were subjected to RT-PCR for detection and confirmation BCoV. RT-PCR was carried out using two different sets of primers to amplify the conserved nucleocapsid (N) gene of the virus targeting a 407 and 730 bp fragments. An incidence rate of more than 14% (15/101) was observed with ELISA and about 20% (20/101) by RT-PCR. The present report is first in its nature regarding the detection of BCoV at molecular level in India. In conclusion, RT-PCR was found more sensitive than commercial ELISA kit for detecting BCoV in fecal samples. Further extensive epidemiological studies are suggested for the virus in the country to know the magnitude of BCoV infection in dairy calves along with isolation of viral strains and to investigate their antigenic and genetic properties.

Key words: Bovine coronavirus, calf diarrhea, ELISA, prevalence, RT-PCR

INTRODUCTION

Diarrhea remains an important cause of illness and death in young calves and the economic losses associated with the disease include decreased performance, high morbidity and mortality and the expenses of medication and labor to treat the sick animals. It has been recognized as one of the main six causes of all deaths from infectious diseases, thus is of much concern regarding animal health and for dairy industry (Kapikian, 1996; Murray and Lopez, 1997; Dhama *et al.*, 2009). The major infectious agents associated calf diarrhea include Rotavirus, Coronavirus,

Cryptosporidium parvum, enterotoxigenic *Escherichia coli* and *Salmonella* spp., which are collectively responsible for 75-95% of infection in neonatal calves worldwide (Moon *et al.*, 1978; Snodgrass *et al.*, 1986; Bendali *et al.*, 1999; Hoet *et al.*, 2003; Gumusova *et al.*, 2007; Dhama *et al.*, 2009; Malik *et al.*, 2012). Among all these pathogenic agents Rotaviruses are the leading cause and Coronaviruses are a major contributor of calf diarrhea (Gumusova *et al.*, 2007; Uhde *et al.*, 2008; Dhama *et al.*, 2009). The infections associated with bovine coronaviruses (BCoV) are more severe because they affect both the small and large intestines (Gunn *et al.*, 2009; Boileau and Kapil, 2010). The other syndromes associated with BCoV include Winter Dysentery (WD) in adult dairy cattle and respiratory tract infections in calves and feedlot cattle (Saif *et al.*, 1991; Storz *et al.*, 2000; Saif, 2010).

Bovine coronavirus, first reported by Mebus *et al.* (1972), is a single-stranded, non-segmented, RNA virus of 32 kb genome and belongs to group 2 corona viruses of the family Coronaviridae, order Nidovirales (De Vries *et al.*, 1997; Van Regenmortel *et al.*, 2000). The virus affects both gastrointestinal and respiratory tracts (Clark, 1993). Generally it affects calves ranging in age from day 1 to 3 months 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). The primary sites of BCoV replication are epithelial cells in the respiratory tract, enterocytes in the distal ileum and colon, thus virus being excreted with the respective excretions. In early years, the association of BCoV with pneumoenteritis had been investigated by many works indicating its importance (Thomas *et al.*, 1982; Reynolds *et al.*, 1985, 1986; Saif *et al.*, 1986; Heckert *et al.*, 1989, 1991). 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 has worldwide distribution and being reported from several countries (Jeong *et al.*, 2005; Khalili and Morshedi, 2006; Takiuchi *et al.*, 2006; Traven *et al.*, 2006; Gumusova *et al.*, 2007; Park *et al.*, 2007; Boileau and Kapil, 2010; Dash *et al.*, 2012). However, epidemiological data on BCoV in India is scarce. Therefore, the aim of the present study was to screen the fecal samples for BCoV collected from clinical cases and dead calves with the history of diarrhea from North Indian region covering three dairy farms of Uttar Pradesh and post mortem facility of Indian Veterinary Research Institute, respectively. ELISA being a sensitive method for antigen detection was used for determining BCoV incidence. Furthermore, virus confirmation was done at genomic level by the molecular method of RT-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: During the period from September 2009 to March 2010, faecal samples were collected from 101 calves of below 3 months of age, reported to be clinical cases with the history of diarrhea, from two dairy farms of Bareilly district (Military Dairy Farm, 38; and LPM farm, IVRI, 30), one dairy farm of Raebareli district (22) of Uttar Pradesh State and from 11 spontaneous dead calf cases from the post-mortem (PM) facility, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly (U.P.).

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

Table 1: Oligonucleotide primers used in the present study for amplification of N gene of BCoV

Primer sequence (5'-3')	Position	Product size (bp)	Reference
TF-5' GCCGATCAGTCCGACCAATC 3'	91-111	407	Tsunemitsu *et al.* (1999)
TR-5' AGAATGTCAGCCGGGGTAA-3'	498-480		
CF-5' GCAATCCAGTAGTAGAGCGT-3'	21-40	730	Cho *et al.* (2001)
CR-5' CTTAGTGGCATCCTTGCCAA-3'	731-750		

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 assays. Generally, the processing of fecal samples was done within 24 h of collection of samples.

Screening of fecal samples: All the 101 fecal samples were subjected to ELISA for detecting the presence of BCoV employing commercially available ELISA kit (Bio-X Diagnostics, Belgium kit-BIO K 151) and following the protocol as per 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)
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 101 faecal samples from broth live and dead diarrheic calves. Both the sets of primers were used to amplify the N gene which is the conserved region in the genome of Mebus strain virus.

RNA extraction: Viral RNA was extracted from the clinical samples with TRIzol LS reagent (Invitrogen, USA) as per the manufacturer's instructions.

RT-PCR: cDNA was prepared from the extracted viral RNA by reverse transcription reaction and used for PCR amplification of the N gene generating expected amplicons of 407 and 730 bp sizes, as per protocol described previously by Tsunemitsu *et al.* (1999) and Cho *et al.* (2001), respectively. The amplified products were visualized on 1.2% agarose gel (Amresco, USA) stained with ethidium bromide (0.5 µg mL^{-1}).

RESULTS
Screening of faecal samples by ELISA: The screening of fecal samples, collected from diarrheic calves below three months of age from Northern state of India-Uttar Pradesh, by commercially available ELISA kit indicated high prevalence of BCoV. Out of the total of 101 fecal samples including 90 diarrhoeic samples from clinical cases and 11 intestinal contents from dead calves, 15 (14.85%) samples were found positive for BCoV (Table 2). The BCoV incidence rate in clinical cases in Military dairy farm and LPM farm, IVRI, both in Bareilly district was found to be 15.79% (6/38) and 10% (3/30), respectively. The overall prevalence of BCoV in Bareilly district was found to be 13.23% (9/68) and 27.27% (3/11) in clinical cases and dead cases, respectively. In dairy farm of Raebareli district, 13.64% (3/22) virus incidence rate was recorded (Table 2).

Detection of BCoV by RT-PCR: Standardized RT-PCR was applied for detection and confirmation of BCoV in all the 101 fecal samples collected from clinical cases of diarrheic and dead calves (Fig. 1, 2).
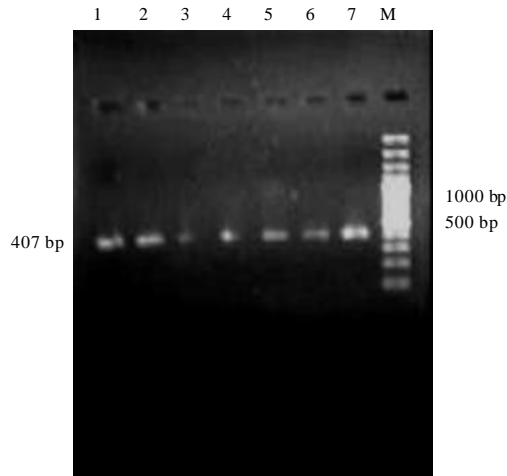
Fig. 1: RT-PCR detection of BCoV, 407 bp amplified product of N gene using established primer, Lane M: DNA ladder of 100 bp, Lane 1-7: RT-PCR amplified products (407 bp) of BCoV N gene in faecal samples
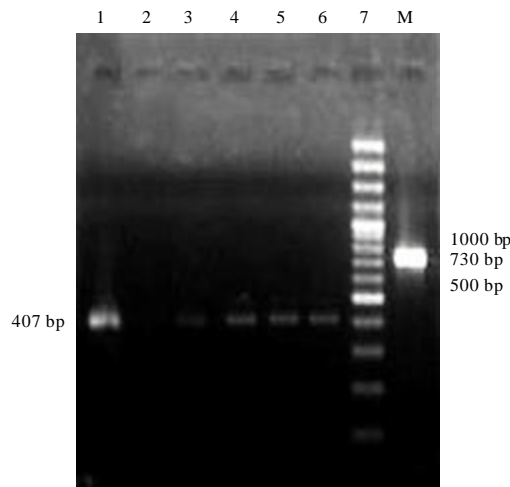

Fig. 2: RT-PCR detection of BCoV, 407 bp and 730 bp amplified product of N gene using established primer, Lane M: DNA ladder of 100 bp, Lane 2: Control, Lane 1 and 7: RT-PCR amplified products (407 bp) and 730 bp of BCoV N gene in diarrhea faecal samples, Lane 3 to 6: RT-PCR amplified products (407 bp) of BCoV N gene in post mortem faecal samples

Out of total 101 samples screened by RT-PCR, 20 (19.80%) samples were found positive for BCoV. The virus incidence rate in clinical cases at military dairy farm and LPM farm, IVRI, both in Bareilly district was found to be 21.05% (8/38) and 16.67% (5/30), respectively, with an overall prevalence of 19.11% (13/68). In dead cases from Post Mortem Facility, IVRI, Bareilly, the incidence rate of 27.27% (3/11) was observed. At dairy farm of Raebareli district, an incidence rate of 18.18% (4/22) was recorded by RT-PCR (Table 2).

Table 2: Comparison between ELISA and RT-PCR for the detection of BCoV infections in the faecal samples

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	38	6	15.79	8	21.05
LPM farm, IVRI	30	3	10.00	5	16.67
Total clinical cases (A)	68	9	13.23	13	19.11
Post mortem facility, IVRI (dead cases) (B)	11	3	27.27	3	27.27
Raebareli					
Dairy farm (clinical cases) (C)	22	3	13.64	4	18.18
Total (A+B+C)	101	15	14.85	20	19.80

DISCUSSION

Neonatal calf mortality is one of the most common animal health concerns for dairy industry. The increased mortality in young calves during early days of life, adversely affects the economic stability of most of the livestock farming ventures. It has been estimated that neonatal diseases resulting in calf mortality reduce farm net profit by 38% (Martin and Wiggins, 1973; Khan and Khan, 1991). Among the various neonatal diseases of calves, diarrhea is of major health concern causing high economical losses. Diarrhea affects young calves at an age when they have immature immune status, lacks specific antibody, illustrate high metabolism with added stresses imposed by weaning and sometimes deprivation of immune colostrum feeding. Among the various etiological agents of calf diarrhea, viral pathogens viz., rotavirus and coronavirus are the most important ones (Jeong *et al.*, 2005; Gumusova *et al.*, 2007; Uhde *et al.*, 2008; Dhama *et al.*, 2009; Boileau and Kapil, 2010; Malik *et al.*, 2012). However, the pathology of BCoV associated diarrhea is often more severe compared to rotavirus and others and it often results in mucohemorrhagic enterocolitis (Gunn *et al.*, 2009; Boileau and Kapil, 2010). The reports regarding Coronavirus prevalence in dairy calves of India are limited; therefore, the present study was carried out to investigate the incidences of BCoV in dairy farms of Northern Indian state employing ELISA kit and RT-PCR detection of the virus. 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 is reported to cause winter dysentery in adult cattle during winter season (Cho *et al.*, 2000; Boileau and Kapil, 2010).

In the present study, a total of 101 clinical samples comprising of 90 fecal samples from calves with clinical presentation of diarrhea and intestinal contents of 11 dead cases were screened for BCoV antigen by commercially available ELISA kit. The enzyme linked immunosorbent assay, ELISA was used owing to the facts that most of the other immunodiagnostic tests developed for detection of enteric pathogen have either low sensitivity and/or specificity. ELISA also has ability to detect enteric pathogens even at low concentrations (Selim *et al.*, 1991; Kelkar *et al.*, 2004). The antigen capture ELISA type is regarded as diagnostic test of choice for BCoV detection (Boileau and Kapil, 2010). The prevalence rate, as tested by commercially available ELISA kit, at Raebareli and Bareilly districts of Uttar Pradesh state was recorded for bovine coronavirus as 13.64 and 13.23%, respectively, in clinical cases and 27.27% in dead cases from Bareilly region. The overall prevalence of BCoV in both Raebareli and Bareilly district as detected by ELISA was 14.85%, which is similar

with earlier reported virus prevalence of 14% (Reynolds *et al.*, 1986), 20% (Bordas *et al.*, 1985) and 20% (Vanamayya, 1990). 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 our study, a higher incidence of 27.27% 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 pathological evaluation of these dead calves also confirmed that BCoV affects multiple organ systems (Hansa, 2010).

In the present investigation, molecular tool of RT-PCR was also employed further for rapid detection and confirmation of BCoV by targeting N gene. RT-PCR was standardized selecting viral N gene based primers as it is conserved among BCoV strains. The expected specific viral amplicons with sizes of 407 and 730 bp 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 101 samples, 20 (19.80%) were found positive for BCoV as compared to 15 (14.85%) samples found positive by ELISA. It is of significant importance to mention that all the ELISA tested fecal samples were found positive by RT-PCR also. It is expected that RT-PCR assays can be used for sensitive detection of BCoV in clinical samples as RT-PCR may detect BCoV-positive animals that might otherwise be classified as BCoV-negative by ELISA or other methods. The results support that RT-PCR is highly applicable in detecting BCoV in clinically diarrheic calves and is more sensitive than ELISA for detecting BCoV in fecal samples. A rapid, sensitive, confirmatory and timely detection of BCoV infection by RT-PCR in calves, detecting even subclinical cases or within their early or late course of illness or after re-infection when low level of virus is being shed from intestines into the feces, is an essential approach concerning epidemiological surveillance as it allows for the application of suitable preventative measures prior to the emergence of diarrhea on a farm (Cho *et al.*, 2001; Boileau and Kapil, 2010). To the best of our knowledge this report is first in its nature regarding the molecular detection and screening of fecal samples for BCoV by RT-PCR in India. However, RT-PCR needs to be applied at a large scale for studying the BCoV detection both in diarrheic calves (clinical cases) as well as in non-diarrheic calves (subclinical infections) so as to know the magnitude of BCoV infection and its molecular epidemiology, thereby adding more information to the available data on this virus.

It is to be kept in mind that BCoV genome being the longest (approximately 32,000 RNA bases) among animal viruses, the virus has chance of further evolution. It is still unclear whether isolates/strains of BCoV having different tropism for various organs like affecting the respiratory and enteric system can be distinguished antigenically by ELISA (Boileau and Kapil, 2010). Nowadays, the molecular tools and techniques are being widely used in animal disease diagnosis including PCR and allied techniques viz., PCR-RE/RFLP, RT-PCR, Q-PCR, RRT-PCR, real-time PCR, LAMP, nucleotide sequencing and phylogenetic analysis. These have strengthened detection of animal pathogens in terms of reliability and rapidity and also for characterizing and monitoring various pathogens, which are altogether very helpful for an effective disease control programme. For BCoV detection and characterization, several workers have recently developed and applied molecular diagnostics viz., RT-PCR, nested PCR, multiplex semi-nested RT-PCR, *Taq*Man-based real-time RT-PCR, SYBR Green real time RT-PCR (Cho *et al.*, 2001, 2010; Escutenaire *et al.*, 2007; Park *et al.*, 2007; Decaro *et al.*, 2008; Klein *et al.*, 2009; Asano *et al.*, 2010). Techniques like multiplex real-time PCR assay have the advantage of simultaneous detection and quantification of major pathogens causing calf diarrhea.

CONCLUSION

BCoV is prevalent and likely endemic in northern India and has high correlation with neonatal calf diarrhea and associated mortality. For screening of large number of fecal samples ELISA was found very convenient; however, RT-PCR was found to be more sensitive than ELISA for BCoV detection. 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 number of samples, which would highlight the epidemiological significance of this important virus and would be helpful for devising appropriate and timely prevention and control measures, thereby reducing economical losses. For this purpose, the use of molecular based diagnostic methods needs to be exploited to their full potential for continuous virus monitoring and surveillance. Further studies are also needed to isolate BCoV strains in India and to characterize them at genomic levels investigating their antigenic and genetic properties.

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