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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Isolation of Bovine Coronavirus (BCoV) in Vero Cell Line and its Confirmation by Direct FAT and RT-PCR

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Abstract: Bovine Coronavirus (BCoV) is widespread both in dairy and beef cattle throughout the world. The virus is one of the largest RNA virus and has specific tropism for intestinal and pulmonary epithelial cells. It is responsible for huge economic losses by causing winter dysentery in adult dairy cattle and respiratory and intestinal tract infections leading to pneumo-enteritis in young calves. Isolation of BCoV has been reported to be difficult. Studies regarding epidemiology, virus isolation and molecular detection from India are very few. In the present study Vero cell line was used for isolation of the BCoV from Enzyme Linked Immunosorbent Assay (ELISA) positive samples. Direct fluorescent antibody technique (dFAT) and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to confirm the isolated virus strains at antigenic and genomic levels, respectively. Out of the 15 positive fecal samples, virus from only seven was able to infect vero cell line. Subsequently BCoV got adapted to the vero cell line upto three passages, which was confirmed both at genomic and antigenic levels by dFAT and RT-PCR testing. It can be concluded that vero cell line can be used for isolation of BCoV, however due to the enormous strain diversity of the virus it is possible that many strains can't grow and get adapt in this cell line. Further studies are required for isolation of different viral strains, finding the susceptible cell lines and also to confirm the variations among the BCoV isolates at antigenic/genomic levels.

Key words: Bovine coronavirus, calf diarrhea, isolation, FAT, RT-PCR, vero cell line

INTRODUCTION

Bovine coronavirus (BCoV) is an important pathogen affecting cattle population with worldwide distribution. The virus is responsible for causing winter dysentery in adult dairy cattle and respiratory and intestinal tract infections in younger calves which accounts for high economic sufferings to both dairy and beef industry (Clark, 1993; Storz *et al.*, 2000; Saif, 2010; Hansa *et al.*, 2012). BCoV affects both the small and large intestines thereby causes severe hemorrhagic enteritis in young calves of up to 3 months of age (Gunn *et al.*, 2009; Boileau and Kapil, 2010). Structurally the virus contains single non-segmented 32 kb RNA strand and has been classified as Group 2 Coronavirus under the family Coronaviridae (De Vries *et al.*, 1997; Van Regenmortel *et al.*, 2000). The virus is being reported to be emerging from several countries (Jeong *et al.*, 2005; Khalili and Morshedi, 2006; Traven *et al.*, 2006; Gumusova *et al.*, 2007; Park *et al.*, 2007; Boileau and Kapil, 2010; Hansa *et al.*, 2012). Due to its large genome size, high frequency of recombination

and lack of proofreading RNA polymerase, defective interfering RNA's are frequently generated in coronaviruses and thus the virus is said to be having chance of further evolution (Lai *et al.*, 1985; Boileau and Kapil, 2010; Hansa *et al.*, 2012). It is unclear at this moment that the various strains affecting different body organs can be differentiated antigenically by commonly available protein techniques. Also limited reports are available regarding the prevalence and molecular detection of BCoV from India (Hansa *et al.*, 2012). The aim of present study was to isolate BCoV by using Vero cell line and confirmation of the virus detection by direct fluorescent antibody technique (dFAT) and reverse transcriptase-polymerase chain reaction (RT-PCR) at antigenic and genomic levels, respectively.

MATERIALS AND METHODS

Collection of samples: Fecal samples (n = 101) were collected in sterile conditions from both live and dead calves below 3 months of age having history of diarrhea

during September 2009 to March 2010 from different farms [Military Dairy Farm, Bareilly (38); Livestock Product Management farm (LPM) (30) and Postmortem Facility, IVRI (11); Dairy Farm, Raebareilly (22), IVRI, Bareilly. All the clinical samples were stored at -20°C till use.

Isolation and Propagation of virus in Vero cell line: All the fecal suspensions (v/v; 50%, watery feces; 20%, loose feces; or 10%, normal feces) were prepared with sterile phosphate buffered saline (PBS) solution (pH 7.2). Samples were centrifuged at 14,000×g for 10 min at 4°C and supernatants were collected, filtered through 0.45 µm membrane syringe filter (Genetix, India) and screening for the presence of BCoV antigen by commercially available BoCV ELISA kit (Bio-X Diagnostics, Belgium kit-BIO K151). The ELISA positive samples were further used for attempting virus isolation studies in vero cell line. All the positive filtered supernatant fluids were mixed separately with an equal volume of DMEM maintenance medium containing 2% of Fetal Calf Serum (FCS) and 10 µg mL⁻¹ crystalline trypsin (Amersco, USA) and were incubated at 37°C for 30 min. After incubation, 1 mL of the mixture was inoculated into the 25 cm² culture flasks of confluent monolayer of Vero cell line for adsorption of virus particles at 37°C for 1 h, followed which the cells were washed thrice with plain DMEM and then over layered with DMEM maintenance medium containing 10% FCS, crystalline trypsin (1 µg mL⁻¹), antibiotics and buffer and incubated at 37°C. The cells were incubated for 5 days post infection (p.i) and daily observed for the development of BCoV specific cytopathic effects (CPE). When the characteristic CPE were observed, the cells were harvested by freezing and thawing thrice and centrifuged at 12,000 rpm for 20 min at 4°C for the removal of cell debris. The supernatant containing the virus was collected and stored at -20°C for further passages in Vero cell lines.

Staining of infected Vero cells by H and E: After the characteristic CPE were observed in vero cells, the culture flasks were washed with sterile 1% PBS solution and were fixed with 2:1 methanol and acetone for 10 min. Thereafter, the cells were brought to water by passing through descending grades of alcohol. Cells were then stained first with Mayer's Hematoxylin (about 2 mL was added to each flask and kept for 2 min), washed with plain water thrice, then Eosin (about 2 mL was added and kept for 30 sec) and finally washed with plain water and dried. After drying, cells were examined under inverted microscope for development of characteristic CPE and eosinophilic intracytoplasmic inclusion body.

Confirmation of the establishment of virus infection in Vero cells by dFAT and RT-PCR

Direct Fluorescence antibody test (dFAT): Direct fluorescence antibody test (dFAT) was employed on infected Vero cells for confirmation of the isolated virus at antigenic level. Cells showing the characteristic CPE in the culture flasks were washed first with sterile 1% PBS solution and then were fixed with 2:1 methanol and acetone for 15 min as described above. The cells were then again washed with 1% PBS solution and FITC anti-corona virus monoclonal antibody (1:20 dilution-Bio-X Diagnostics, Belgium) was added and cells incubated at 25°C for 1 h for immunological (antigen-antibody) reaction to take place. Then the cells were finally washed with 1% PBS and mounted with aqueous glycerol mountant and examined under UV fluorescent microscope.

Reverse transcription-polymerase chain reaction (RT-PCR): Reverse transcription-polymerase chain reaction (RT-PCR) was employed to confirm the isolated virus at genomic level as per the method described previously (Tsunemitsu *et al.*, 1999). Viral RNA was extracted with TRIzol LS reagent (Invitrogen, USA) as per the manufacturer's instructions followed by first strand cDNA synthesis using random hexamers, dNTP, RNase inhibitor and RT enzyme (all reagents from Fermentas, USA). For confirmation of BCoV, established oligonucleotide primer sequences [Primer 1 (TF-5' GCCGATCAGTCCGACCAATC 3', genomic position 91-111) and Primer 2 (TR-5' AGAATGTCAGCCGGGGTAA-3', genomic position 498-480)] were used to amplify a specific product size of 407 bp of the viral N gene, which is the conserved region in the genome of Mebus strain.

RESULTS

Isolation of bovine coronavirus in Vero cell line: Isolation of coronavirus was attempted with 15 clinical samples (12 clinical cases and three dead calves) found positive for BCoV antigen out of a total of 101 as screened by ELISA kit. Out of 15 positive samples, only seven were adapted in Vero cells as evinced by characteristic cytopathic effect (CPE) at second passage level and which continued up to three passages in Vero cells in the present study. The infected vero cells at 24 h p.i, became rounded, at 48 h p.i, showed vacuolations and clumping which was more prominent at 72 h p.i. At 96 h p.i, the cells became small showing tendency to form syncytia and

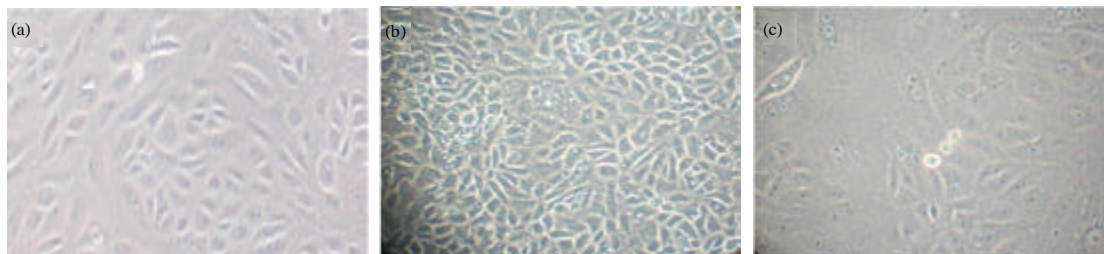


Fig. 1(a-c): Vero cell line monolayer infected by ELISA positive BoCV sample - (a): Uninfected control Vero monolayer, unstained $\times 200$, (b): Vero cells showing vacuolation and rounding of cells (arrow) at 48 h p.i., unstained $\times 200$, (c): Detached floating infected cells becoming round throughout the monolayer and aggregating with each other, also showing tendency to form syncytia in the media at 96 h p.i., unstained $\times 200$

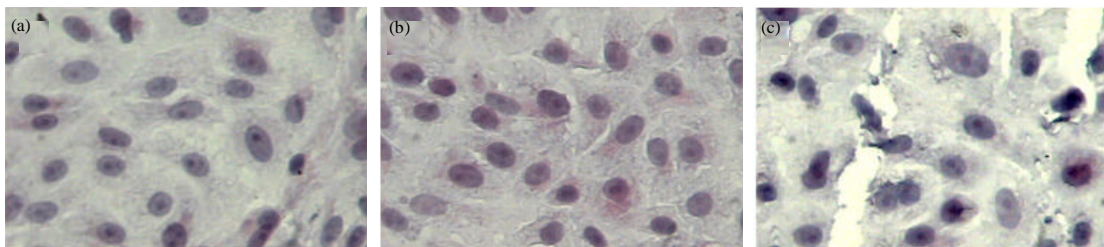


Fig. 2(a-c): Vero monolayer cells stained with H and E stain $\times 400$ -(a): Normal monolayer (b): Infected monolayer cells showing the intracytoplasmic inclusion, (c): Infected monolayer showing the syncytia formation 96 h p. i.

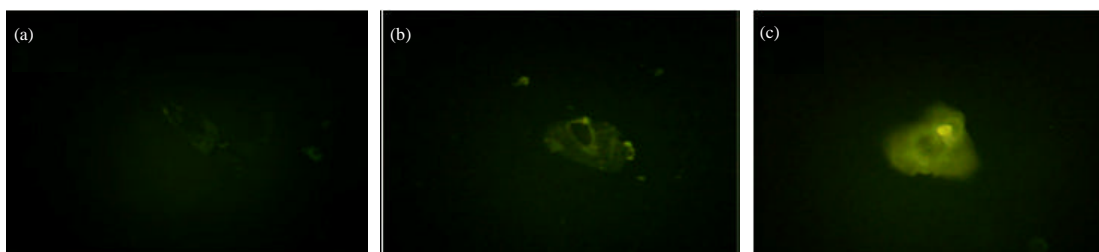


Fig. 3(a-c): Vero cell monolayer infected with BCoV and stained with coronavirus specific FITC conjugated monoclonal antibodies -(a): Control (Uninfected) cells, $\times 400$ (b): Infected cells showing specific apple green fluorescent, $\times 400$. (C): Infected cells showing specific apple green fluorescence by intracytoplasmic inclusion body of coronavirus at 96 h p.i., $\times 1000$

rounding in majority of monolayer. Detached cells showed tendency to form clumps and finally the monolayer possessed the impression of typical moth eaten appearance (Fig. 1). The Vero infected cultures were stained by standard H and E staining method. The infected cells showed syncytia formation, presence of eosinophilic intracytoplasmic inclusion body indicative of viral replication (Fig. 2).

Confirmation of BCoV by direct FAT: The infected vero cell cultures were stained for BCoV antigen using specific

coronavirus FITC conjugated monoclonal antibodies for confirming the presence of viral replication and establishment of infection. Specific granular, diffuse apple green fluorescence was seen in the cytoplasm of the infected cells. In few cases inclusion body and syncytia of infected cells were also observed (Fig. 3).

Confirmation of BCoV by RT-PCR: RT-PCR testing of the infected vero cell cultures revealed amplification of an expected product size of 407 bp (Fig. 4), which confirmed the presence of viral genome in infected cells.

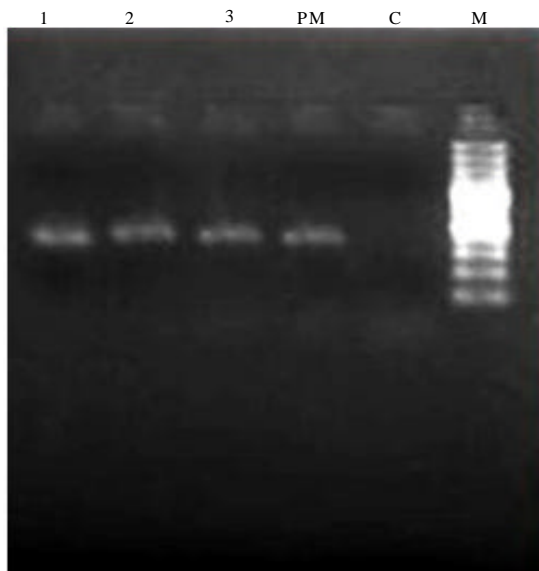


Fig. 4: RT-PCR detection BCoV in infected Vero cell cultures during virus passaging/isolation: Lane M: DNA ladder of 100 bp, Lane 1, 2, 3, PM-RT-PCR positive with amplified products of 407 bp of N gene of virus.; C: Control (Uninfected cultures)

DISCUSSION

Diarrhea remains a significant cause of clinical illness and death in young dairy calves, the monetary losses associated with it are mainly due to decreased performance, high morbidity and mortality and the expenses of medication and labor to treat the sick animals. The major infectious agents associated with calf diarrhea include rotavirus, coronavirus, *Cryptosporidium parvum*, enterotoxigenic *Escherichia coli* and *Salmonella* spp (Snodgrass *et al.*, 1986; Bendali *et al.*, 1999; Gumusova *et al.*, 2007; Uhde *et al.*, 2008; Dhama *et al.*, 2009; Hansa *et al.*, 2012; Malik *et al.*, 2012). Among all these pathogenic agents, Coronaviruses are regarded as major contributor of calf diarrhea as these cause more severe diarrhea because they affect both the small and large intestines (Gumusova *et al.*, 2007; Gunn *et al.*, 2009; Boileau and Kapil, 2010). The other syndromes associated with BCoV includes 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).

Isolation of virus in tissue culture is rarely used as a means of diagnosis as BCoV is difficult to isolate. However, it has been found that BCoV can grow in tracheal and gut organ cultures and other large number of

cell lines including Human rectal tumour-18 (HRT-18), Vero (African green monkey kidney), BEK-1 (Bovine embryonic kidney), D2BFS (Bovine fetal spleen), BEL 14 (Bovine embryonic lung), Madin Darby bovine kidney (MDBK) and Madin Darby canine kidney 1 (MDCK1) (Clark, 1993; Benfield and Saif, 1990; Tsunemitsu and Saif, 1995). In this study, Vero cell line was used to cultivate corona virus, as it is said to produce consistent CPE (Tektoff *et al.*, 1983). Isolation of coronavirus was done in a total of 15 cases found positive for BCoV by ELISA testing, from 12 clinical cases and 03 dead (calves) cases. Out of fifteen samples, only seven samples were able to get adapt and replicate in Vero cells as evinced by characteristic CPE at second passage level and which continued upto three passages in Vero cells in the present study. Pre-treatment with crystalline trypsin ($10 \mu\text{g mL}^{-1}$) and its incorporation ($1 \mu\text{g mL}^{-1}$) in maintenance medium produced distinct CPE after 48 h was previously reported by Albert (1990) as it is said to enhance the infectivity by facilitating the greater fraction of corona virus to uncoating the infected cell. At 48 h p.i, the infected cells showed vacuolation and clumping which were more prominent at 72 h p.i. At 96 h p.i, the cells became small showing tendency to form syncytia and becoming round in majority of monolayers. Detached cells showed property to aggregate with each other and finally the monolayer possessed the impression of typical moth eaten appearance. The CPE produced in this study were in agreement with those of the earlier reports (Tektoff *et al.*, 1983). The virus multiplied/replicated in endoplasmic reticulum and the cluster of viruses were seen as intra-cytoplasmic inclusion bodies. Infected cells were stained with H&E staining method and showed syncytia of infected cells and eosinophilic intracytoplasmic inclusion bodies.

BCoV genome being one of the animal viruses with longest genome (approximately 32,000 RNA bases) has chance of further evolution. At this point of time it is still unclear whether isolates/strains of BCoV having different tropism for various organs can be distinguished antigenically by ELISA (Boileau and Kapil, 2010). Also, detection of the virus at antigenic level by FAT can't suffice the need of using genomic based methods for its detection and confirmation. In the present study, the adaptation of BCoV in the vero cells was confirmed by FAT testing revealing specific granular, diffuse apple green fluorescence in the cytoplasm and the presence of inclusion body was also seen at few places. In RT-PCR testing for the presence of viral genomic DNA, however conserved region was selected for confirmation at genomic level in this study. The expected product size of 407 bp obtained was similar to earlier reports of

Tsunemitsu *et al.*, 1999), Cho *et al.* (2001) and Khalili and Morshedi (2006), which further confirmed the adaptation of BCoV in the vero cells.

It can be concluded from the present study that besides HRT-18 cell line, Vero cell line could be used for BCoV isolation in which coronavirus produced distinct CPE and intracytoplasmic inclusions post infection at upto 96 h post infection. However, not all the ELISA positive virus samples were able to get adapted or multiply/replicate and infect the vero cells, which indicate towards some kind of the strain variation. Also, BCoV could be detected by RT-PCR and direct FAT in Vero infected cells. Further, research is however required for isolation of different strains and determining the susceptible cell lines and to confirm the variations among the isolates both at antigenic and genomic levels.

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