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**Immunohistochemical and molecular detection of natural cases of bovine rotavirus and coronavirus infection causing enteritis in dairy calves**

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## Abstract

Bovine rotavirus (BRoV) and bovine coronavirus (BCoV) are major enteric viral pathogens responsible for calve diarrhoea. They are widespread both in dairy and beef cattle throughout the world and causing huge economic losses. The diagnosis of these agents is very difficult due to non-specific nature of lesions and the involvement of some intrinsic and extrinsic risk factors. We performed postmortem of 45 calves, which was below three months of age. Out of 45 necropsied calves, three (6.66%) cases were positive for BRoV and four (8.88%) cases were found positive for BCoV, screened by reverse transcriptase polymerase chain reaction (RT-PCR). Further RT-PCR positive cases were confirmed by immunohistochemistry (IHC) in paraffin-embedded intestinal tissue sections. Three cases of enteritis caused by BRoV showed the hallmark lesions of the shortening and fusion of villi, denudation and infiltration of mononuclear cells in the lamina propria. The BRoV antigen distribution was prominent within the lining epithelium of the villi, peyer's patches in the ileum and strong immunoreactions in the lymphocytes and some macrophages of the mesenteric lymph nodes. Four cases in which BCoV was detected, grossly lesions characterized by colonic mucosa covered with thick, fibrinous and diphtheritic membrane. Histopathologically, jejunum showed skipping lesion of micro-abscesses in crypts. The BCoV antigen distribution was prominent within the necrotic crypts in the jejunum and cryptic micro-abscesses in the colon and ileum. It is the first report of BRoV and BCoV antigen demonstration in the jejunum, colon, ileum, Peyer's patches and mesenteric lymph nodes of naturally infected calves from India by using IHC.

**KEY WORDS:** Rotavirus, coronavirus, RT-PCR, Immunohistochemistry

## 1. Introduction

Livestock sector in India plays a pivotal role in supporting the livelihood of rural masses. This sector alone contributes 4.11% of the total GDP. Economics of any livestock farm dependent on

the health of its neonates, which are the future herd (Razzaque *et al.*, 2009). Despite the fact, neonatal calves are abysmally cared, with the result significant morbidity and mortality of these calves incurred huge economic consequences. Amongst various causes, the calf diarrhoea (enteritis) is a commonly reported disease worldwide in cattle. Diarrhoea is a multi-factorial disease, which accounts for 57% of mortality in < 1 month old calves (USDA, Dairy, 2007). In diarrhoeic calves, although co-infection observed frequently, but at times a single etiology can also be the cause in some cases. Major enteric viral pathogens responsible for calf diarrhoea are mainly Rotavirus and Coronavirus. Of late, newer emerging enteric pathogens such as Torovirus, Norovirus, Nebovirus, Enterovirus, Calcivirus and Parvovirus have also been added to the list of diarrhoea causing agents (Cho and Yoon, 2014). Co-infection with more than one pathogen is more frequent and often worsens the symptoms. The tetrad of Rotavirus, Coronavirus, *Cryptosporidium*, and *Escherichia coli* accounts for 75-95% of infection in neonatal calves worldwide, of which especially rotavirus and coronavirus account for 27-36% and 20-26% infections, respectively (Malik *et al.*, 2005; Gumusova *et al.*, 2007; Dhama *et al.*, 2009; Singh *et al.*, 2018). Among all pathogens, rotaviruses are the leading cause of calf diarrhoea, and coronaviruses are a major contributor to it (Gumusova *et al.*, 2007; Uhde *et al.*, 2008; Dhama *et al.*, 2009; Kashyap *et al.*, 2018). The coronavirus infect both small intestine and large intestine to cause severe disease (Boileau and Kapil, 2010). In calves, group A rotavirus and coronavirus, either single or in combination, are predominately associated with neonatal (mostly up to 5-15 day old) diarrhoea (Barry *et al.*, 2009). These pathogens, if not causing death in calves, warrant extra care and sometimes intensive care of calves (Gay *et al.*, 2012). The diagnosis of diarrhoea (enteritis) cases is cumbersome due to non specific nature of clinical signs/ lesions, interaction of polymicrobial agents and involvement of intrinsic and extrinsic risk factors (Athanasios *et al.*, 1994).

Rotaviruses mainly replicate in the mature villous enterocytes. Triple protein coat of virus helps them to escape unaffected from the acidic pH of the stomach and the digestive enzymes in the gut. The mature enterocytes of duodenum villi are the first to become infected to release significant number of virions, to favor more severe attack on enterocytes of mid and distal portion of small intestine (Ramig, 2004). Bovine coronavirus is capable of infecting mature epithelium of small intestine and large intestine. Villi of the affected small intestine and colonic crypts become atrophic, and the lamina propria becomes necrotic and lumen of hyperplastic crypts filled with necrotic debris

(Schultze *et al.*, 1991). Necrosis of mesenteric lymph node, Peyer's patches of ileum and formation of cryptic abscesses in colon of calves also the histopathological lesions detected in coronavirus infection (Jubb, *et al.*, 2007). The pathological data on bovine coronavirus and rotavirus in India are very limited. We used RT-PCR and IHC to investigate the pathological changes in the of natural cases of bovine rotavirus and coronavirus infected dairy calves

## **2. Material and methods**

### **2.1 Tissue samples**

During the period from November, 2016 to February, 2018, total of 45 carcasses of calves (Vrindavani- 36, Tharparker – 4 and HFX -5) were necropsied at Post-mortem Facility of Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, India. These calves have history of enteritis. The calves were below three month of age (up to one month -20 calves, two months – 20 calves and up to three month -5 calves). Samples of intestinal contents and different tissues viz. intestine, mesenteric lymph nodes, spleen, liver, lungs, kidneys and heart were collected in 10% neutral buffered formalin (NBF) and in RNA later. The samples collected in RNA later were stored at -20°C for molecular study.

### **2.2 Reverse transcriptase Polymerase chain reaction (RT-PCR)**

Total RNA was extracted from collected tissue (small intestine with content) samples using commercial TRIzol® Reagent (Thermo Fisher Scientific, USA) as per manufacture's protocol. All extracted RNA samples were quantified by NanoVue plus (Thermo Fisher Scientific, USA) and the purity of RNA was checked by A260/280 and A260/230 ratio. The cDNA was synthesized from total RNA by using High-capacity cDNA reverse transcription kits (Applied Biosystems). The synthesized cDNA was stored at -20°C till further use. The amplification of N gene of coronavirus and VP6 gene of rotavirus was carried out *via* RT-PCR. The self designed primer Sequence(5'--3') for bovine coronavirus BCoV; F-TGACGAGCCCCAGAAGGATGT and BCoV; R- GACCACCTGACGCTGTGGTT have amplicon size 127 bp and the primer Sequence(5'--3') for rotavirus A,RVA; F-TTTGATCACTAATATTCACC and RVA; R-GGTCACATCCTCTACTA have amplicon size 227 bp was used in the study (Mondel *et al.* 2013). PCR reaction was carried out in 0.2 ml PCR tubes containing reaction mixture of 6.0 µL of PCR Master Mix 2× (Takara, City), 0.5 µL of forward primer (10 pmol/µL) and reverse primer (10 pmol/µL), 1 µL of cDNA (200 ng/µL) 12.00 µL of Nuclease-free water. The tubes

were then placed in a thermocycler and PCR cycling conditions were used with initial denaturation of 95 °C for 5 min, followed by 35 cycles [denaturation(94 °C ,10 s); annealing (48 °C , 20 s) , extension on (72 °C, 30 s)] and a single cycle of final extension at 68 °C for 7 min. Amplified products were resolved by agarose gel electrophoresis (1% w/v) at 100 V for 1 h in TAE buffer with 0.5 µg/mL ethidium bromide and viewed under UV transilluminator (Geldoc, USA).

#### ***2.4 Histopathological examination***

After proper fixation in 10% NBF, tissues were cut into small sections with thickness of 2-3 mm and embedded in the paraffin by standard procedures. The paraffin embedded tissues were cut into 5-6 micron thick section and stained with hematoxylin and eosin (Luna, 1972).

#### ***2.5 Immunohistochemistry***

The immunohistochemistry (IHC) was carried out in paraffin sections after deparaffinisation and rehydration. The slides were treated with 0.3% Hydrogen peroxide in methanol solution for 20 min to quench the endogenous peroxides, thoroughly rinsed thrice with 1X PBS (pH7.2), 5 min each. Antigen unmasking was performed by subjecting the tissue sections to microwave irradiation in a coplin jar containing 0.01M citrate buffer (pH 6.0) for 15 min. Non-specific binding was blocked with 5% normal goat serum (200-400µl) (Sigma Chemicals,USA) in PBS for 30 min at room temperature in a humidifier chamber. The slides were washed thrice (5 min each) using PBS (pH 7.2). For immunostaining, antibody was titrated for optimum concentration and sections were covered with 100-150 µl of primary monoclonal antibodies (1:100, mouse monoclonal IgG2a RVA capsid protein and 1:100, mouse monoclonal IgG2a coronavirus capsid antigen, Santa Cruz Biotechnology, USA), in PBS containing 1% BSA (Sigma Chemicals, USA). The negative controls were covered with 1% BSA in PBS only. The sections were tilted in two different directions before being incubated overnight at 4<sup>0</sup>C in a humid chamber. The slides were washed with PBS for 3 times (5 min each) with slow and continuous stirring. After wiping around the section carefully, Goat anti-mouse IgGs secondary antibodies HRP conjugated (Invitrogen) diluted at 1:500 were applied to cover the moist section and incubated for 1 h in a humid chamber. Following three PBS (pH 7.2) washing of 5 min each, the section was covered with 3, 3-diaminobenzidine (DAB, Sigma Chemicals, USA) as a substrate, which gave brick red (reddish brown) colour. DAB substrate solution was prepared by adding 1 drop of DAB

chromogen to 1 ml of DAB buffer. The reaction was terminated before generalized background staining appeared in the negative controls by rinsing in double DW. Sections were then counter stained for 1-2 min with Mayer's haematoxylin. After dehydrating in ascending grades of alcohol, sections were mounted with cover slips using DPX mountant.

### 3. Results

In 45 necropsicid calves, three (6.66%) cases were positive for rotavirus and four (8.88%) cases were found positive for coronavirus by employing RT-PCR (Fig. 1 and 2 respectively) and IHC. All seven cases (rotavirus-3 and coronavirus-4) were found positive in below one month of age of calves. In three cases, where rotavirus was identified, grossly the serosa of small and large intestine was severely congested and the lumen filled with blood mixed catarrhal contents. The pin head sized hemorrhagic spots were found on the mucosal surfaces of the jejunum and the ileum. Mesenteric lymph nodes were swollen, severely congested and edematous. Histopathological examination of the ileum showed fusion and sloughing of villi enterocytes and severe engorgement of capillary plexus and mononuclear cells (mainly lymphocytes and few macrophages) infiltration in the lamina propria (Fig 3, a). The crypt epithelium was found proliferated. The Peyer's patches were edematous and congested and lymphoid cells were depleted and necrosed (Fig. 3, b). The mesenteric lymph nodes showed engorged vessels and depletion of lymphoid follicles and with moderate deposition of fibrin in the cortex and the medulla (Fig.3, c). We observed immunohistochemical staining for rotavirus in all three calves which were positive from RT-PCR. The rotavirus antigen distribution (dark brown signals) was prominent within the lining epithelium of the villi along with peyer's patches of the ileum (Fig. 4c). We also detected strong immunohistochemical staining for rotavirus in the lymphocytes and some macrophages of mesenteric lymph nodes (Fig. 4b).

Four cases in which coronavirus was detected, grossly reddish white necrotic ulcers were seen on serosal surface of the colon. The ileum and mesenteric lymph nodes were markedly congested and swollen. The colonic mucosa was covered with thick, fibrinous diptheric membrane (Fig. 5, a). Microscopically, the villi of the ileum were sloughed off at several places and their lamina propria was severely engorged / hemorrhagic and infiltrated with mononuclear cells. There was thick homogeneous necrotic material adhered upon upper mucous surface of the colon (Fig. 5, b). Mesenteric lymph nodes showed moderate depletion of many cortical follicles (Fig. 3, d). In other one case, grossly, whole small and large intestinal tract was moderately congested and

filled with yellowish faecal material. Mesenteric lymph nodes were juicy, enlarged and slightly congested. Histopathological examination of the jejunum showed severe infiltration of mononuclear cells in the lamina propria and crypts showed skipping lesion of microabscesses (Fig. 5, c). Crypt lining cells were slightly hyperplastic. The capsule of mesenteric lymph nodes was thick and lymphoid follicles were necrosed. We observed immunohistochemical staining for coronavirus antigen in all four calves which was positive from RT-PCR. The BCoV antigen distribution was prominent within the lining epithelium of the villi, necrotic crypts in the jejunum and cryptic microabscesses in the colon and ileum (Fig. 6). We also detected strong immunoreactions in the mononuclear cells of mesenteric lymph nodes (Fig. 6d).

#### 4. Discussion

Diarrhoea in calves is a multifactorial disease that results into hypersecretion of fluid, malabsorption due to disruption of enterocytes, exudation of protein due to increased capillary/epithelial permeability or hypermotility leading to approximately 75% of the calf mortality under 3-weeks-age (McGavin *et al.*, 2007). The recovered cases of diarrhoea might suffer with production losses even more than anticipated. The diarrhoea due to rotavirus and coronavirus in infants of humans, neonates of mammalian and avian species is a worldwide problem. The age has been found to be the greatest influencing factor in causing rotavirus and coronavirus infections. In our study calves below 1-month of age suffered with these pathogens. The same has been described by previous workers for occurrence of rotaviral and coronavirus diarrhoea in neonatal calves (Bellinzoni *et al.*, 1987; Dash *et al.*, 2011; Radostits *et al.*, 2007). The reason for high occurrence of these pathogens below one-month of age could be due to less developed immune system in neonates and the lack of adequate amount of maternal antibodies in the colostrum (Windeyer *et al.*, 2012), so the free sites are available for attachment of virus to sialic acid or galactosidase receptors of enterocytes. These receptors disappear with the increasing age of neonates (Chauhan *et al.*, 2008).

Among 45 deaths of cattle calves, 3 cases of enteritis were of due to rotavirus as demonstrated by the hallmark lesions of the shortening and fusion of villi, denudation, and the presence of cuboidal enterocytes, these lesions were very similar to those described by earlier workers in rotavirus infected calves (Reynolds *et al.*, 1985; Gruenberg, 2014; Kashyap *et al.*, 2018). Similarly, the extra-intestinal lesions in mesenteric lymph nodes (lymphocytes depletion and antigen detection by IHC) were also matched with the findings of previous workers, who



described the depletion of lymphocytes from the germinal centers of lymphoid follicles of mesenteric lymph nodes and detection of rotavirus antigen by direct immunofluorescence technique in calves (Kim *et al.*, 2011; Kashyap *et al.*, 2018; Crawford *et al.*, 2006). The rotavirus replication in macrophages have also been demonstrated in such studies, indicating a possible mechanism of rotaviral spread via the lymphatic of lamina propria to extra-intestinal organs.

In four cases of coronavirus necrotic enteritis, the jejunum lesions included severe capillary bed engorgement/ hemorrhage, chronic cells infiltration in villi, scattered crypt microabscesses, crypt lining cells hyperplasia, and lymphoid necrosis/depletion in draining mesenteric lymph nodes were akin to the lesions described by others in coronavirus enteritis in calves (Schultze *et al.*, 1991; Jubb, *et al.*, 2007; Park *et al.*, 2007). The histopathological lesions of coronavirus were colocalized with viral capsid protein in the jejunum and mesenteric lymph nodes, indicated that the lesions observed were due to coronavirus infection and replication. In present study the bovine coronavirus antigen distribution was prominent within the lining epithelium of the villi, jejunum, ileum, colon and mesenteric lymph nodes which was accordance with Field, 2015, who demonstrated coronavirus antigen in intestine of calves by immunohistochemistry, which was negative for RT-PCR. It is the first report of BRoV and BCoV antigen demonstration in the jejunum, colon, ileum, Peyer's patches and mesenteric lymph nodes of naturally infected calves from India by using immunohistochemistry. Overall, the findings suggest that, the BRoV and BCoV caused calf diarrhoea with specific lesions in the intestines (enteritis) and mesenteric lymph nodes. These lesions might have resulted into morbidity and mortality in bovine calves below 3 weeks of age. Further studies are needed to elucidate the pathogenesis by which BRoV and BCoV spread beyond the intestine and cause disease other than enteritis. Understanding the immuno-pathogenesis at subtle level would further enhance our knowledge in adopting better measures to control the BRoV and BCoV associated diarrhoea in bovine calves.

### **Conflict of interest**

All the authors declare no conflict of interest.

### **Acknowledgements**

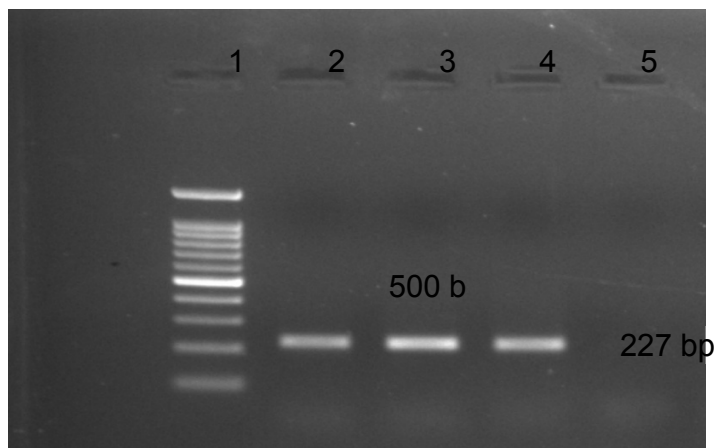
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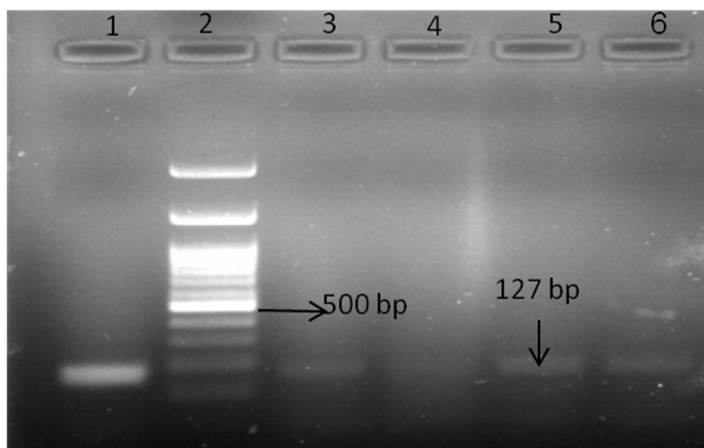
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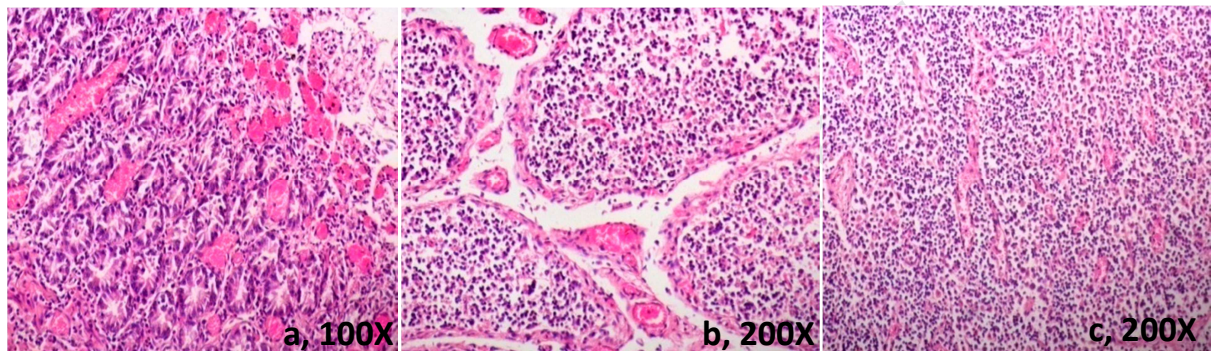


**Fig. 1:** PCR amplification of VP6 gene of RVA showing 227 bp size amplicon in 1.5% agarose gel. Lane 1 loaded 1kb plus DNA ladder; samples in lane 2, 3 and 4 are positive samples, lane 5 as non template control.

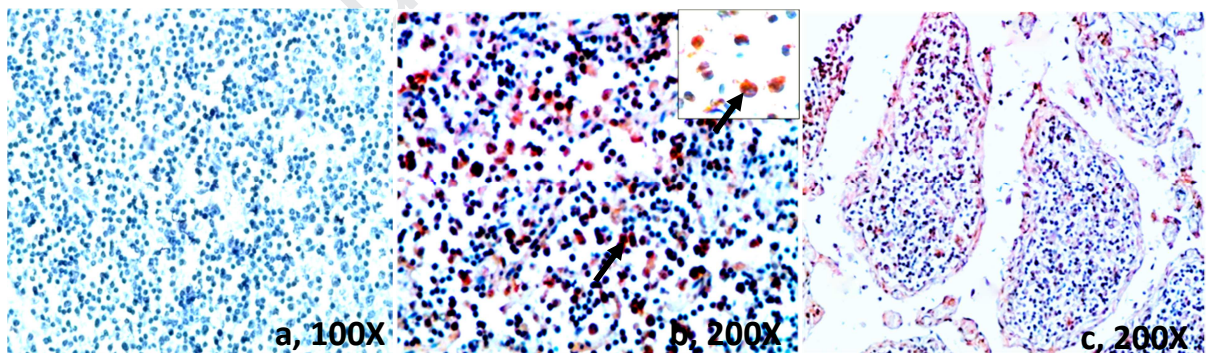


**Fig. 2:** PCR amplification of N gene of bovine coronavirus showing 127 bp size amplicon in 1.5% agarose gel. Lane 1 as positive control plasmid 172 bp. Lane 2 loaded 1kb plus DNA ladder, samples in lane 3, 4, 5 and 6 are positive samples.

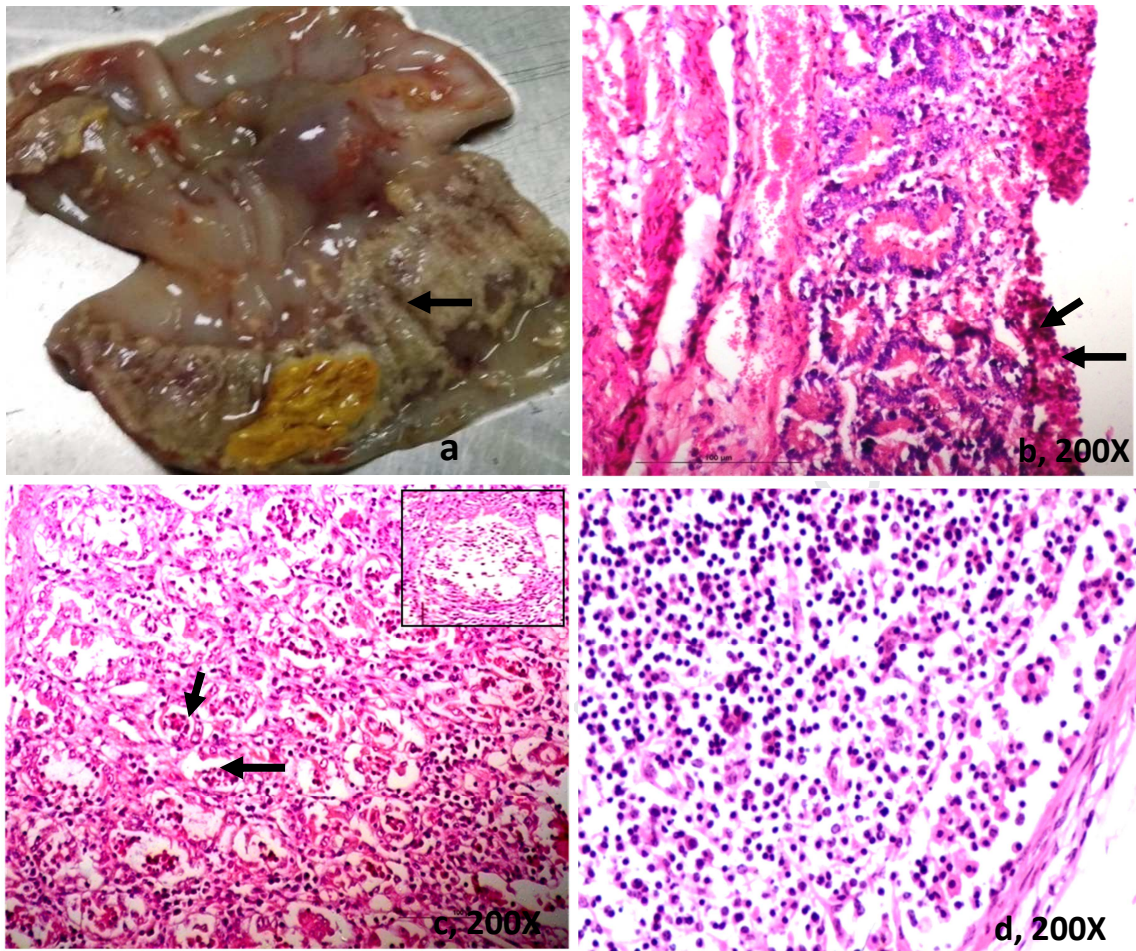




**Fig.3. Histopathology of rotavirus infected calves:** Paraffin sections of a calf (41A/17, 537A/16) stained with H & E stain showing exfoliation of villi, capillary plexus engorgement, moderate infiltration in the lamina propria (a) and Peyer's patches depletion (b) in the ileum. The section of mesenteric lymph node showing edema/ congestion, medullary cords and sinuses infiltrated with MNCs (c). H&E.

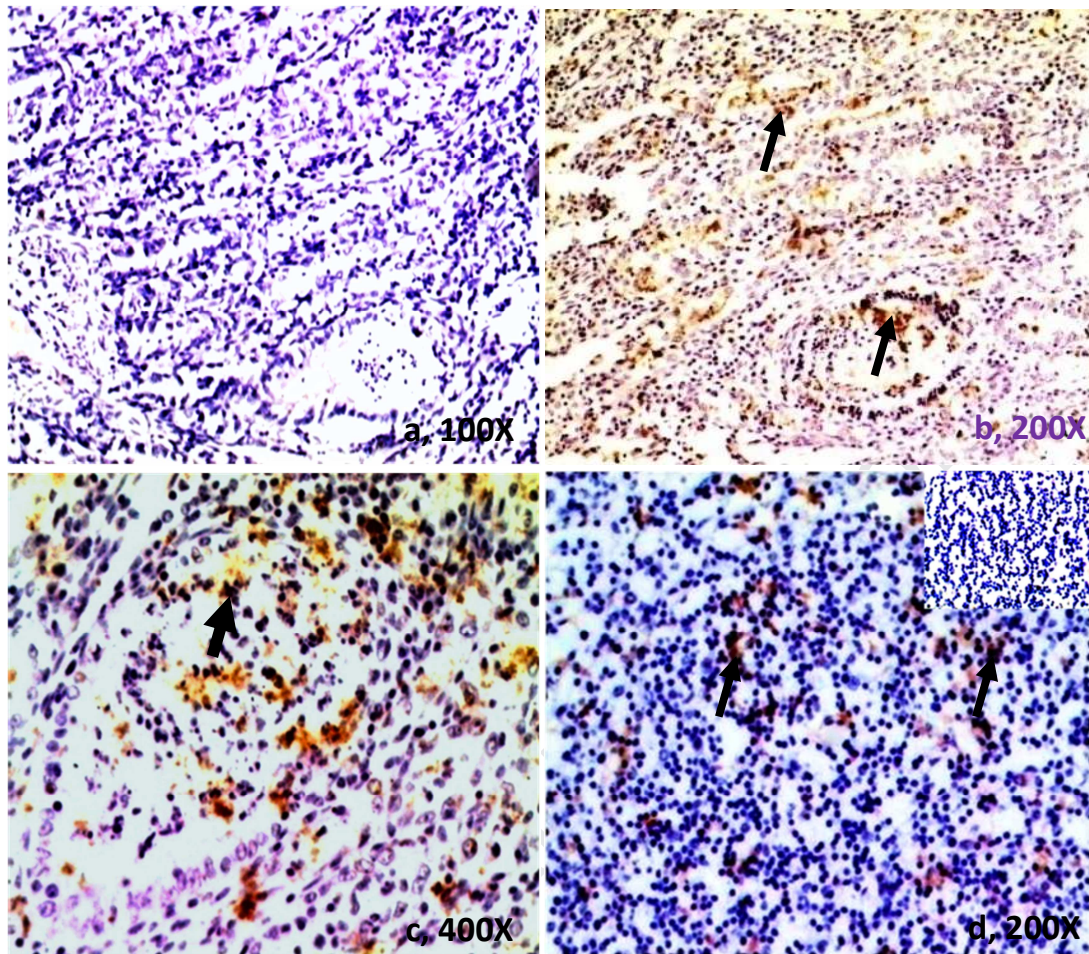


**Fig. 4: Immunohistochemistry of bovine rotavirus infection in bovine calves:**a) Antibody control; b)The RVA antigen distribution in calves (41A/17, 537A/16) is seen as reddish brown signals (DAB as chromogen, arrows ) in paraffin sections of mesenteric lymph nodes(b) (MLN) ( Inset, 400X) and c) Peyer's patches (PP) , Mayer's haematoxylin used as counter stain.



**Fig. 5: Gross and histopathological lesions of corona virus infected calf:** Gross image of the colon (40A/18) showing thick necrotic bran-like diphtheritic deposits over the mucosa (a, arrow). Microscopically, thick homogeneous necrotic material is seen over the ulcerated mucosa of the colon (b, arrows). Crypt lining cells are necrotic (c, arrows) and the lamina propria infiltrated with MNCs. Microabscesses in the crypt (c, inset). Mesenteric lymph nodes showed moderate depletion of many cortical follicles with thick capsule (d). H & E.





**Fig .6 Immunohistochemistry of bovine coronavirus in tissues section of calves: a)** antibody control). The bovine coronavirus (BCoV) antigen distribution is seen as dark brown (DAB as chromogen) positive signals in lining epithelial cells of villi and necrotic crypts in the jejunum (thin arrows, b) and microabscesses (thick arrow, c) respectively and **d)** In MNCs of mesenteric lymph nodes (MLN) also showed positive signals of BCoV antigen (arrows) (inset, antibody control no signals).



### Highlights

- The present study was to investigation of natural cases of BRoV and BCoV infection causing enteritis in dairy calves.
- Out of 45 necropsicid calves, 6.66% cases for BRoV and 8.88% cases for BCoV were found positive.
- BRoV and BCoV antigen demonstration in the jejunum, colon, ileum, Peyer's patches and mesenteric lymph nodes of infected calves by using IHC.