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Rotavirus A associated pathology of intestine and mesenteric lymph nodes and occurrence in bovine calves of Gwalior and Bareilly regions

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ABSTRACT

To understand the pathology of natural cases of rotavirus (RVA) in bovine calves, a total of 40 cases below 6 months died due to diarrhoea were studied, out of which 7 cases (17.5%) turned positive for RVA by RT-PCR. Histopathology of small intestine showed loss of villous enterocytes, blunting and fusion of villi, elongation of crypts and mononuclear cells infiltration in the lamina-propria. The mesenteric lymph nodes were severely depleted of lymphocytes. These changes were corroborated with presence of RVA antigen in sections by dFAT and nucleic acid by RT-PCR. The fluorescent signals were more in mesenteric lymph nodes than in intestine. Besides, 115 rectal fecal samples were also collected from calves for RVA detection by RT-PCR using VP6 gene specific sets of primers. Dead carcasses of calves (n= 40) belonged to organized dairy farm of Bareilly, while rectal fecal samples belonged to both organized (n= 38) and unorganized farms (n= 77) of Bareilly and Gwalior. The overall occurrence of RVA was 19.3% (30/155), comprising 5/37 cases (13.5%) from Gwalior (MP) and 25/118 cases (21.1%) from Bareilly (UP). These findings suggest the infection of RVA widely prevalent in calves and have potential to escape from the intestinal site to mesenteric lymph nodes.

Key words: Bovine Calves, dFAT, Diarrhoea, Histopathology, Mesenteric Lymph Node, Rotavirus, RT-PCR.

INTRODUCTION

Rotavirus A (RVA) is a major cause of severe diarrhoea in infants and young mammalian animals worldwide (Estes and Greenberg, 2013). RVA is responsible for an estimated 4,53, 000 deaths annually in children <5 years of age and 57% of weaning calf mortality (Tate *et al*., 2012) (NAHMS, 2007 report). The prevalence rate of bovine rotavirus infection ranges from 7% to 94% worldwide (Falcone *et al.,* 1999). In India, the incidence of infection of RVA ranges from 10% to 52% in cattle calves and 11 % to 24% in buffalo calves (Malik *et al*., 2012; Naveen *et al.,* 2015). In a study by Rangnath *et al*. (2013), the prevalence of RVA has been reported to 19.5% in diarrhoeic samples and 4.4% in non diarrhoeic samples in bovine calves.

The RVA is a species of the genus *Rotavirus*, within the family *Reoviridae*. The 11 segments of dsRNA are encased in a triple-layered capsid. The genome encodes six structural proteins (VP1–VP4, VP6 and VP7) and five or six non-structural proteins (NSP1–NSP5/6) (Matthijnssens *et al*., 2012). Amongst these proteins, the outer capsid proteins VP7 and VP4 elicit the protective immune response and virulence, respectively. The VP6 protein is the group-specific antigen. The segmented nature of the virus favours genetic reassortment and generates progeny of new strains, which may differ in pathogenicity, virulence and interspecies transmission (Estes and Kapikian, 2007). Usually, RVA infections restrict to the small intestine, but lately also reported in other non-intestinal sites, both in natural (children) and experimental animals (Feng *et al*., 2008; Craford *et al*., 2006). Recently, the persistence of the RVA genome has been demonstrated higher in the mesenteric lymphnodes (MLN) than in intestinal contents in calves by RT and semi-nested PCR in natural cases of calf diarrhoea (Hiromichi *et al*., 2015). Further, it was opined that, RVA genome could persist in MLNs even after viral clearance from the gut.

Rotavirus targets the mature villous enterocytes, for their efficient colonization and infectivity. The RVA needs outer capsid to be cleaved to VP8 and VP5 by proteases, which facilitates receptor mediated endocytosis by the cells (Desselberger *et al.,* 2005). Mature epithelial cells of the villi in duodenum are the first to become infected and they releases significant number of virions, to favor more severe attack on enterocytes of mid and distal portion of small intestine and cause reduction in epithelial surface area thereby resulting in malabsorption syndrome (Ramig, 2004). It is

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suggested that sialic acid orgala ctose-based receptors may have some role in endocytosis, along with co-receptors like integrins (Murphy *et al.,* 1999; Desselberger *et al.,* 2005). Due to limited number of studies available on natural cases of RV induced pathology in calves, mesenteric lymphnode spread and its occurrence in different geographical regions of the country (India), the present work was taken up.

MATERIALS AND METHODS

During the period from September, 2015 to April, 2016, a total of 155 samples (faecal / intestinal contents) were collected from bovine calves (cattle-115, buffalo- 40), which had either diarrhoea or recovered from diarrhoea. These calves belonged to organized (n= 77) and unorganized (n= 38) dairy farms situated in and around Gwalior (MP) and Bareilly (UP). The calves were below three months of age and were non-descript (65), Vrindavani (13) and Sahiwal (3) cattle, Murrah (31) and Bhadavari (3 cases) buffalo. Besides, a total of 40 dead calves (< 6 months) of Murrah (8) and Vrindavani (32) of cattle and buffalo from the institute were examined for the study. Many of these cases had history of diarrhoea, dehydration and fever. The intestinal contents, pieces of small intestine and mesenteric lymph nodes were sampled. Fecal samples and intestinal contents were dissolved separately in 1X PBS **(**with pH 7.4**)** to make 10% suspension, vortexed and centrifuged at 750g for 10 min. The supernatants were taken in sterilized eppendorf tubes and kept at -80 ^{0}C for RNA extraction. Thin pieces of small intestine and mesenteric lymph nodes of 40 dead calves were collected at the time of necropsy in RNA Later and in NBF.

Standardization of RT-PCR reaction: The RNA was extracted from the jejunum/ileum contents and intestinal and MLN tissues using isopropyl alcohol method and Quigen RNA easy kit, respectively. The cDNA synthesis was performed as described earlier (Malik *et al*., 2012). The amplification of cDNA was carried out using published primers RVA_D_VP6_F **5'**TTTGATCACTAAYTATTCA CC**3'** and RVA_F_VP6_R **5'**GGTCACATCCTCTCACTA**3')** **(**Mondal *et al.,* 2013) of VP6 gene. The PCR reaction was carried out in 0.2 ml PCR tubes containing reaction mixture of 6 µL PCR Master Mix 2x, 1 µL each of forward primer and reverse primer (10 pmol/µL) and 1 iL of cDNA (200ng/µL). The contents were mixed thoroughly and spun briefly. The tubes were then placed in a thermocycler and PCR cycling conditions were standardized with initial denaturation of 95^{o}C for 5 min, followed by 39 cycles of 94^{o}C for 10 sec, 48^{o}C for 20 sec, 72^{o}C for 30 sec, and a single cycle of final extension at 72^{o}C for 7 min. Amplified products were resolved by agarose gel electrophoresis (1% w/v) at 100V for 1 h in 1X TAE buffer with 0.5 µg/mL ethidium bromide and viewed under UV transilluminator.

Histopathology and direct immuno-florescence test: The NBF fixed paraffin embedded tissues were processed for histopathology. The haematoxylin and eosin stained sections were examined under microscope using different magnification. The dFAT method was carried out in paraffin sections after deparaffinisation and rehydration. 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 1X PBS (pH7.4), 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 by incubating with 5% normal goat serum for 20 min, and rinsed thrice with 1X PBS (pH- 7.4). For immunostaining, the primary antibody-FITC conjugated anti Rotavirus monoclonal antibody was used at a dilution rate of 1:20.The sections were incubated overnight at 4^{0}C in a humidified chamber. The slides were rinsed thrice (3 min each) using PBS (pH- 7.4) The cryo- sections of mesenteric lymphnodes were treated as mentioned in datasheet of

Table 1: Occurrence of rotavirus A in bovine calves

Type of distribution		**No. of sample**	**No. of PositiveSample**	**Occurrence(%)**
Total no. of sample		**155**	**30**	**19.3**
Geographical distribution	Bareilly	118	25	22.5
	Gwalior	37	5	13.5
Location	Intestinal content	40	7	17.5
	Fecal sample	115	23	20
Species	Cattle	75	14	18.6
	Buffalo	80	16	20
Organization of farm	Unorganized	38	14	36.8
	Organized	117	15	12.8
Season	Sep-Oct	35	5	14.2
	Nov-Feb	96	23	23.9
	Mar-Apr	24	2	8.3
Age (days)	01-20	45	13	28.8
	21-30	52	11	21.1
	31-60	37	3	8.1
	61-180	21	2	9.5
Consistency	Diarrheic	115	27	23.4
	Recovered diarrhea	40	3	7.5

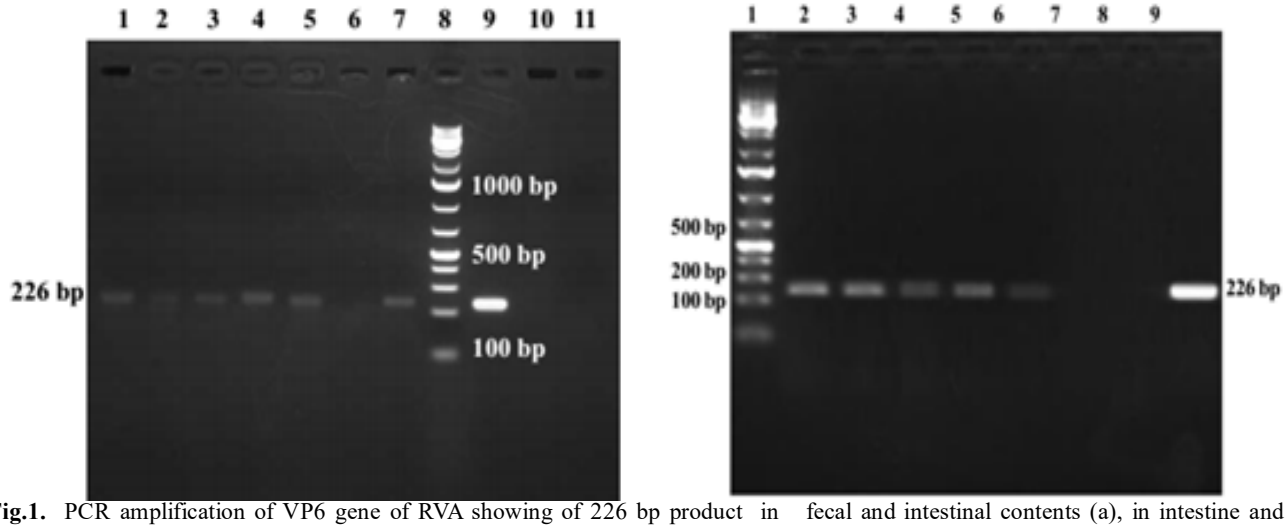

Fig.1. PCR amplification of VP6 gene of RVA showing of 226 bp product in fecal and intestinal contents (a), in intestine and mesenteric lymphnode (b)

Antibody. The slides were immediately mounted with fluorescent anti-bleaching mounting fluid and viewed under fluorescent microscope for positive signals, using 10X and 40X objectives.

RESULTS AND DISCUSSION

The RV diarrhoea is the major concern of death and economic losses in young animals worldwide. To access the status of RV in livestock farms, a survey was conducted in which samples (115 rectal, 40 intestinal contents) showed 30 / 155 (19.3%) cases of RVA positive, comprising 5/37 cases (13.5%) from Gwalior (MP) and 25/118 cases (22.5%) from Bareilly (UP). The samples tested by RT-PCR showed RVA positivity in 20% (23/115) fecal samples, 17.5% (7/40) in intestinal content (Fig. 1a), and 42% (3/7) in mesenteric lymph nodes (Fig. 1b). The overall RVA occurrence, ranging from 10.15% to 27.10%, has been reported in calves from districts of UP (Mathura and Bareilly) (Dash *et al*., 2011), Uttarakhand (Pantnagar, Mukteswar and Dehradoon) (Naveen *et al.,* 2015), Tamil Nadu (Namakkal) (Ghosh *et al*., 2007) and West Bengal (Kolkata) (Basera *et al*., 2010). The RVA positivity was found in 13.5% (5/37) cases (2 cattle calf and 3 buffalo calf) from Gwalior (MP) and 21.5% (25/118) cases (12/65 cattle calf, 13/53 buffalo calf) from Bareilly (UP). In the present study, occurrence of

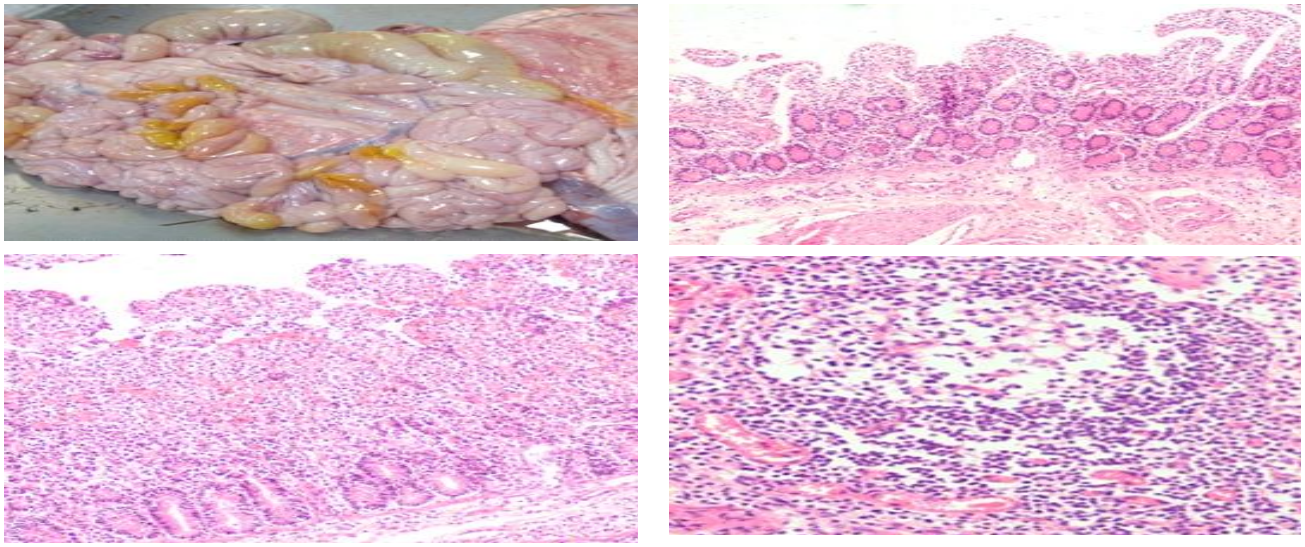

Fig. 2. Rotavirus enteritis in cattle calves: Grossly, the small intestines are distended with yellowish and catarrhal contents (a). Histopathology of the jejunum, showing loss of villous epithelium, blunting of villi and their fusion and the lamina-propria infiltrated moderate numbers of mononuclear cells (b, c arrows).The mesenteric lymphnode showing lymphoid tissue depletion (d, arrow). H & E. 10X.

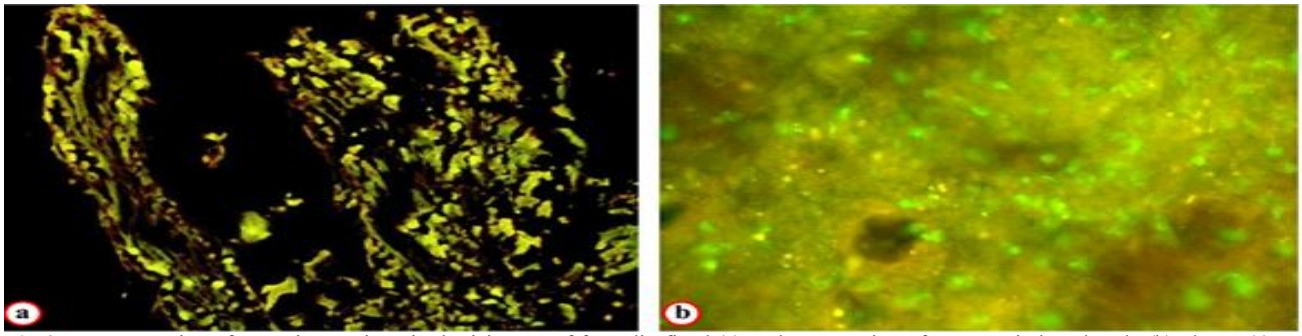

Fig 3: Demonstration of Rotavirus antigen in the jejunum of formalin fixed (a) and cryo-section of mesenteric lymphnode (b). dFAT, 40X.

RV infection was slightly higher in buffalo calves (16/80, 20%) compared to cattle calves (14/75, 18.6%). Similar observations have been made by earlier workers (Malik *et al.,* 2012; Sravani *et al*., 2015). The occurrence of RVA was apparently higher in unorganized (14/38) than in organized (15/117) farms. The higher occurrence of RVA in fecal samples in unorganized calves in the present study was akin to the findings reported by Jindal *et al*. (2000). The reasons for the same could have been due to poor management, colostral feeding and seasonal change as described by Dhama *et al*. (2009). The fecal samples collected during the winter season (November to February) showed higher RVA positivity (23.9%) than in other months (8 to 14%). The reason for higher occurrence of RVA diarrhoea in winter season has been described due to cold stress in north India (Sravani *et al*., 2015). Furthermore, the age-wise occurrence of RVA was higher (28.8%) in both species of calves below 3-weeks of age, which declined with advancing age (Table 1). The earlier workers also reported higher occurrence of RVA diarrhoea in neonatal calves from 1st week to 8th week of age (Dash *et al*., 2011). The reason for high occurrence of RV diarrhoea could be due to less developed immune system in neonates and lack of adequate amount of maternal antibodies in the colostrum (Windeyer *et al*., 2014). The inadequate maternal antibodies may result in the RV attachment to the available free sites to sialic acid or galactosidase receptors of enterocytes for initiating disease process. The receptors on the enterocytes usually disappear with advancing age in neonates, though not proven (Sissons, 1981). The same may be true in our study, wherein calves suffered less with RVA with advancing age. In the present study, a total of 16.5% (27/115) RVA positive cases were associated with diarrhoea and 7.5% (3/40) were associated with recovered cases of diarrhoea. The non – diarrhoeic calves were also found positive for RVA, though less as compared to the diarrhoeic calves, and this could be due to fast turnover of the enterocytes and shedding of virus harbouring enterocytes previously in the feces (McGavin *et al*., 2007).

Out of 17.5% (7/40) RVA positive cases from intestine, 6/32 cases were from cattle calf and 1/8 case were from buffalo calf. The intestine (7/40) RVA positive cases were also corroborated with histopathological lesions and presence of RVA antigen and nucleic acid. Grossly, the positive cases showed mildly congested small intestines filled with yellowish and catarrhal contents (Fig.2a). Histopathology of the jejunum showed loss of villous enterocytes, shortening and fusion of villi, flattening of mucosa, cryptal hyperplasia and elongation (Fig.2b) . The lamina-propria infiltrated with moderate number of mononuclear cells (lymphocytes and macrophages) and capillaries engorgement (Fig.2c). In spontaneous cases of RV enteritis, the lesions such as shortening and fusion of villi, denudation, and the presence of cuboidal enterocytes were very similar to those described by earlier workers in rotavirus infected calves (Gruenberg, 2014). The histopathological changes might have occurred due to increased rate of enterocytes loss compared with replacement of enterocytes from crypts, fusion of villi due to sticky naked basement membrane and stunting of villi due to contraction of muscles of lamina propria (McGavin *et al*., 2007). The lesions of enteritis due to RV were confirmed by presence of RVA antigen by immuno-florescence labelling (Fig.3a) and RVA nucleic acid detection by RT-PCR.

Grossly, associated MLN were slightly enlarged and congested. The RVA in 3 out of 7 MLN cases was detected by dFAT (Fig.3b) and RT-PCR along with histopathological lesions like depleted lymphocytes from germinal centre (Fig.2d). The presence of RVA in extra-intestinal site has been reported in natural and experimental cases of mice, calf and pigs (Crawford *et al.,* 2006; Kim *et al*., 2012). In the present study, 4 MLNs were found negative though the corresponding intestines and contents were positive for RVA both by RT-PCR and dFAT. This observation suggests that RVA can transmit from the lesion site of intestine to nearby MLNs. In natural cases of RV diarrhoea in calves, the RVA spread from the intestine to MLNs has been reported by Hiromichi *et al*. (2015). They further reported RVA persistence in MLNs even after absence/ clearance from the digestive tract. Notably, in the present study most of the RVA-positive cattle calves; RVA was detected in intestine, but not in MLNs. This could be due to initial stage of RVA infection, and death might have caused by another pathogen. The virus might have reached to the MLNs by macrophages from the propria via lymphatic. However, cell-free RVA transmission via blood stream could be a major path in a mouse model

(Fenaux *et al*., 2006), which needs further investigation. The RVA antigen signals were more in number in MLNs than in intestines, indicating possibility of long persistence of RVA in these sites as stated by others (Park *et al*., 2014; Hiromichi *et al.,* 2015).

Overall, the findings suggest that, the RVA caused calf diarrhoea with specific lesions in the intestines and mesenteric lymph nodes. These lesions might have resulted into morbidity and mortality in bovine calves below 3 weeks of age. There is a need to further elucidate pathogenesis of RVA diarrhoea in calves with respect to extra-intestinal spread. Understanding the immuno-pathogenesis at subtle level would further enhance our knowledge in adopting better measures to control the RV associated diarrhoea in bovine calves.

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