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Abha Tikoo
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

J S Soodan
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

N K Sood
Adjunct Professor, Department of Veterinary pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Gagandeep Singh
Animal Health Specialist, KVK Talwada, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Rajiv Singh
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

Rajesh Agrawal
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

Savleen Kour
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

Corresponding Author:
Savleen Kour
Division of Veterinary Medicine, F.V.Sc.& A.H., SKUAST-J, R.S. Pura, Jammu, UT of Jammu & Kashmir, India

Immuno-histochemical alterations in diarrhoeic calves affected with mixed aetiological infection

Abha Tikoo, JS Soodan, NK Sood, Gagandeep Singh, Rajiv Singh, Rajesh Agrawal and Savleen Kour

Abstract
Neonatal calf diarrhoea (NCD) is the most important disease of neonatal calves which results in the greatest economic losses in both beef and dairy calves. Multiple agents are responsible for diarrhea, and thus difficult to diagnose the definitive causative agent. Immuno-histochemistry involves selective antigens detection in tissue section by binding of antibodies specifically to antigens in tissues. It is a handy tool to visualize the presence of the pathogen. Hence, employing a technique that is both sensitive and specific is necessary for diagnosing the causative agent. The faecal samples were collected from 485 neonatal bovine calves below 30 days of age in and around Jammu region; UT J&K. Nine calves died due to diarrhea and were observed for various gross, histopathologic and immuno-histochemistry studies in different organs. Gross changes were noticed in rumen, omasum, intestines, liver, lungs and spleen. Major histopathologic lesions were found in intestines, liver, kidney, lungs, spleen and trachea. Immunohistochemical studies showed positive results for *Rotavirus* infection in intestine, *Clostridium sp* and *Salmonella sp.* in intestine and liver.

Keywords: diarrhea, antibodies, *Clostridium sp*

1. Introduction
Neonatal calf diarrhoea (NCD) is the most important disease of neonatal calves which results in the greatest economic losses in this age group in both beef and dairy calves (Millemann, 2009) [13]. Diarrheal etiology being a complex syndrome is associated with both infectious and non infectious causes. The infectious diarrhea is caused by varied etiological agents namely, bacterial, viral and parasitic. Among the bacteria *E.coli* and *Salmonella spp.* are notable while *Cryptosporidium* and *Rotaviruses* are the common protozoan and viral agents, respectively encountered in diarrhoea. (Radostits *et al*., 2007) [14]. The cardinal hemato-biochemical features of calf diarrhea are higher packed cell volume, leukopenia, lymphopenia, hyponatraemia, pre-renal failure and metabolic acidosis (Michell, 1994; Grove-White and White, 1998; Alsaad *et al*., 2012) [2]. Metabolic acidosis is a well-recognised potentially life-threatening consequence of diarrhea in calves regardless of the causal agents which contributes to cardiac arrhythmia or death by decreasing myocardial potassium, elevating extracellular potassium and changing membrane potential (Schumann *et al.*, 1990; Grove-White and Michell 2001; Lorenz, 2004b) [17, 8, 11]. However, these changes are n ot specefic of causative agent causing diarrhoea. Multiple etiological factors are responsible for causing neonatal diarrhea in calves, and thus difficult to diagnose the definitive causative pathogen. Hence, a technique that is both sensitive and specific for diagnosis the causative agent is necessary. It will help in tailoring the treatment effectively and prevention of antimicrobial resistance due to antibiotics misuse. Immunohistochemistry (IHC) is a useful technique to visualize the presence of the pathogen, its distribution in the tissue and the histological lesions it has caused thereby help in the diagnosis of the causative agent (Haines and West, 2005; Eyzaguirre and Haque, 2008) [9, 6]. Therefore, the study's objective was to detect causative pathogen for calf diarrhea through immunohistochemistry.

2. Materials and Methods
The study was carried out in farms of diverse management systems *viz*., organized and unorganized dairy farms which were visited periodically and faecal samples were collected from 485 neonatal bovine calves below 30 days of age in and around Jammu region (UT J&K) from October, 2009 to January, 2012.

During the study period, nine calves died due to diarrhea and were observed for various gross and histopathologic changes in different organs. The tissue samples from intestines, heart, liver, kidney, brain, spleen, gallbladder, abomasum from animals which died due to diarrhea were collected in 10 per cent formalin solution for histopathology. Paraffin embedded tissue were cut out at 4 to 6µ and stained with haematoxylin and eosin stain and examined under microscope. Immunohistochemical studies were done as per the method described by Stevens and Palmer (1996) [19]. A thin paraffin embedded tissue sections of 4-5 µ size were cut and mounted on microscopic slides. After deparaffinization and rehydration, antigen retrieval was done in citrate buffer, thereafter heated at 95ºC for 3 minutes and 70ºC for 7 minutes. After that, cooling at room temperature and washing with PBS for 3 times was done. In order to suppress the endogenous peroxides, slides were kept in 3% H2O2 for 30 minutes, followed by washing again in PBS for 3 times. 2.5% normal horse serum was used for protein blocking to avoid non-specific binding. It was kept for 30 minutes in a humidified chamber at room temperature. Primary antibodies were diluted to 1:500 for polyclonal rabbit Salmonella and rabbit anti–Clostridium perfringens and 1:50 for monoclonal Mouse Anti-E. coli K99 pili, Mouse anti-cryptosporidium, Rotavirus p42 antibody, and Mouse anti-bovine coronavirus surface antigen in PBS. Tissue sections were covered with primary antibodies and incubated at 37oC for 2 hours in an incubator. Slides were washed three times in PBS for three minutes each. One drop of universal secondary antibody (Imm PRESS® HRP Universal Antibody-Horse Anti-Mouse IgG/ Anti-Rabbit IgG, Peroxidase Polymer Detection Kit was put on the tissue and incubated for 30 mins at room temperature. Washing was done in PBS for 3 times of 3 minutes each. Freshly prepared 3,3'-diaminobenzidine (DAB) (ImmPACT® DAB Peroxidase (HRP) Substrate) solution was put on the tissue sections until color developed and counterstained in Gill's Haematoxylin. Negative control of each tissue was run by incubating with PBS instead of primary antibody (Ramos-Vara, 2011) [15].

The data was analyzed statistically by applying student's t-test at 5 and 1 per cent level of significance using SPSS (Statistical Package for Social Sciences Software (version 14.0-SPSS Inc.)

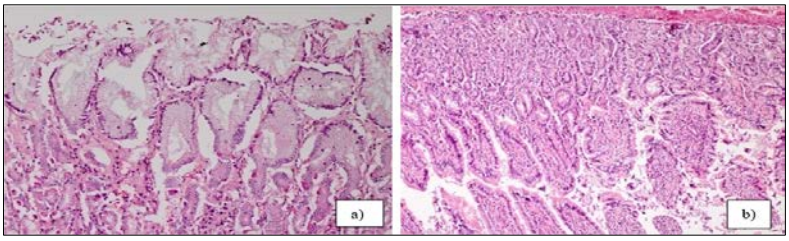

Fig 1: a) Enteritis with sloughing and atrophy of villous epithelium (100X, H & E). b) Chronic superficial gastritis. Severe congestion (200X,H & E)

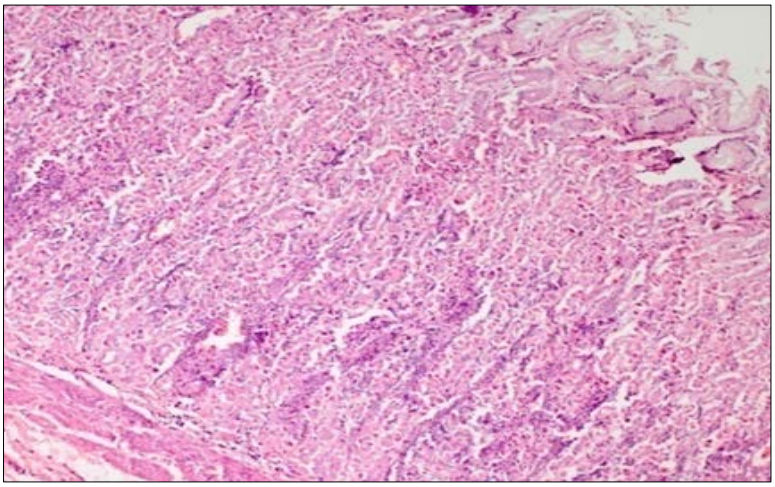

Fig 2: Chronic suppurative catarrhal gastropathy with erosion of villous epithelium, pycnotic nuclei, excessive mucoid discharge (100 X, H & E)

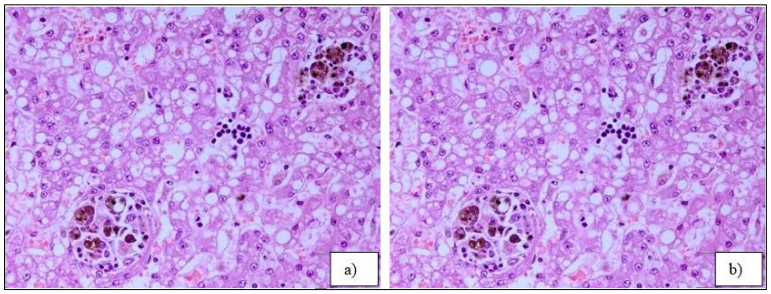

Fig 3: a) Chronic venous congestion with atrophy of hepatocytes, moderate to severe fatty changes mononuclear cell infiltration, Hemosiderosis (400X, H & E). b) Chronic venous congestion, atrophy of hepatocytes, vacoulation, hemosiderosis

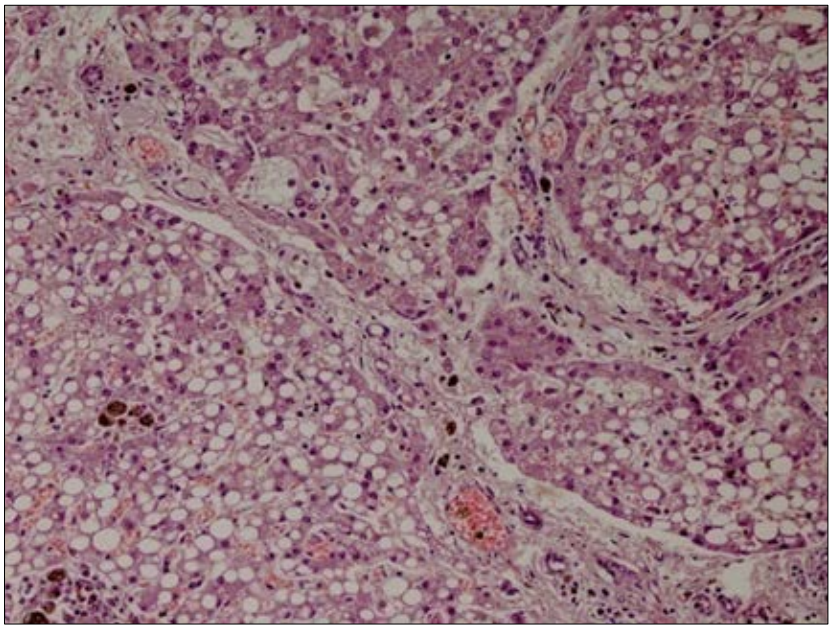

Fig 4: Chronic venous congestion with atrophy of hepatocytes and disintegration of central vein, mono nuclear cell infiltration, pseudolobulation of hepatocytes (400X, H & E).

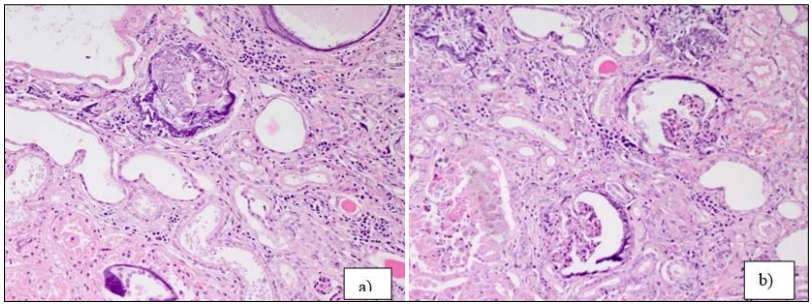

Fig 5: a) Damaged tubules, missing glomeruli, laden with hyaline casts (1000X, H & E). b) Severe congestion, necrosis of glomeruli (1000X,H & E)

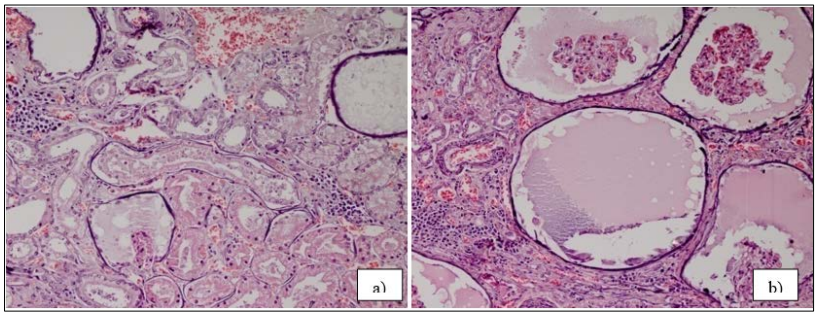

Fig 6: a) Severe congestion, missing glomeruli, Chronic infiltration of lymphocytes (1000X, H & E). b) Missing glomeruli, glomerulosclerosis, chronic congestion of tubules. (400X, H & E)

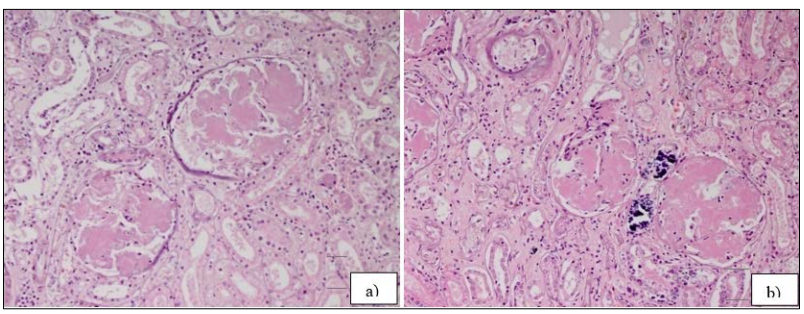

Fig 7: a) Marked chronic interstitial nephritis, chronic glomerulitis, deposition of calcium laden casts in tubules (400X, H & E). b) Metastatic calcification in some of the glomeruli, focal area of marked interstitial chronic hepatitis (400X, H & E).

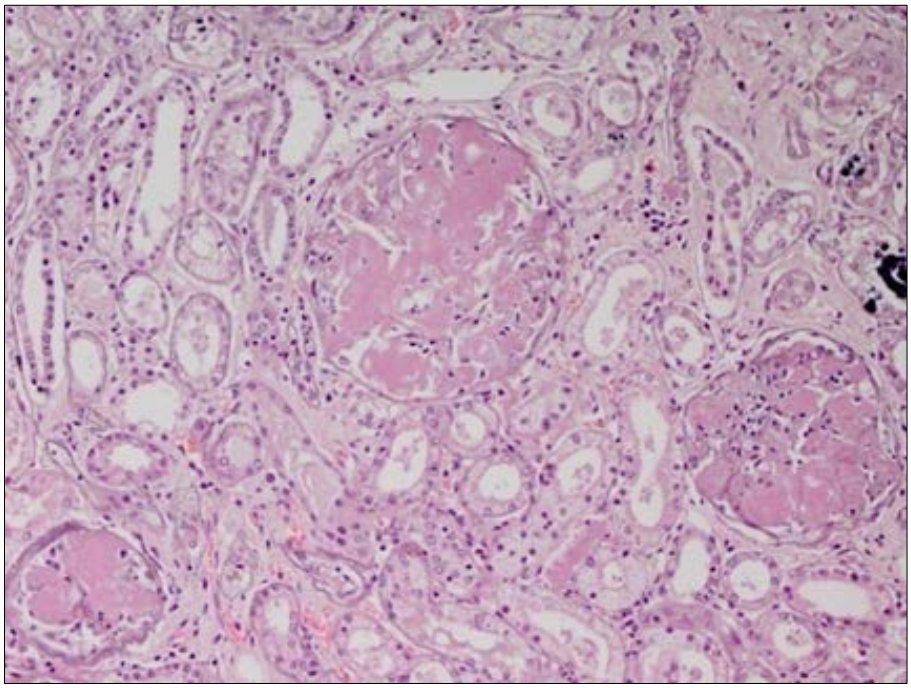

Fig 8: Focal interstitial nephritis, proliferative glomerulitis (400X, H & E)

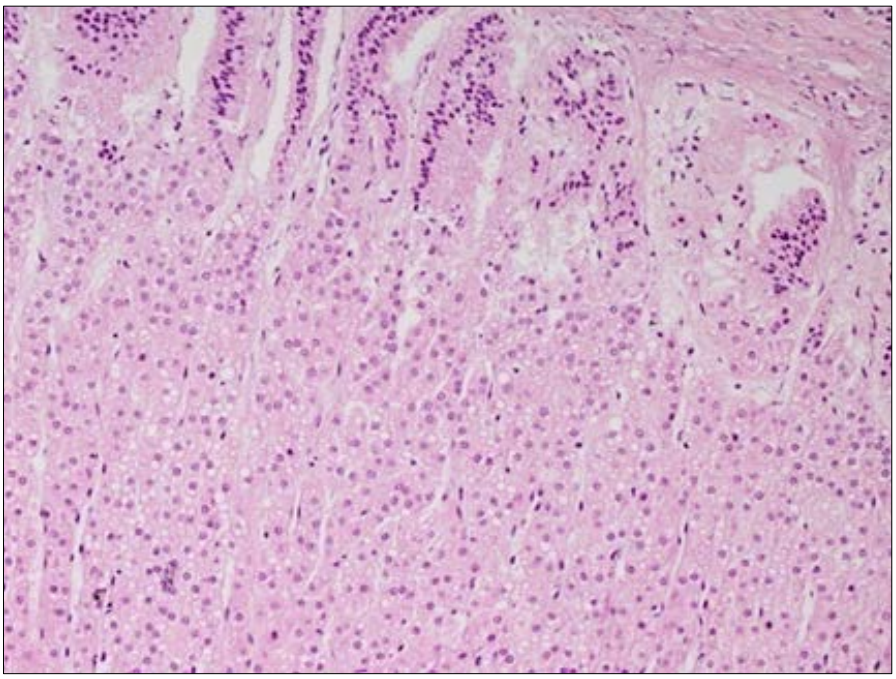

Fig 9: Superficial hemorrhages in adrenal with fatty changes (200X, H & E)

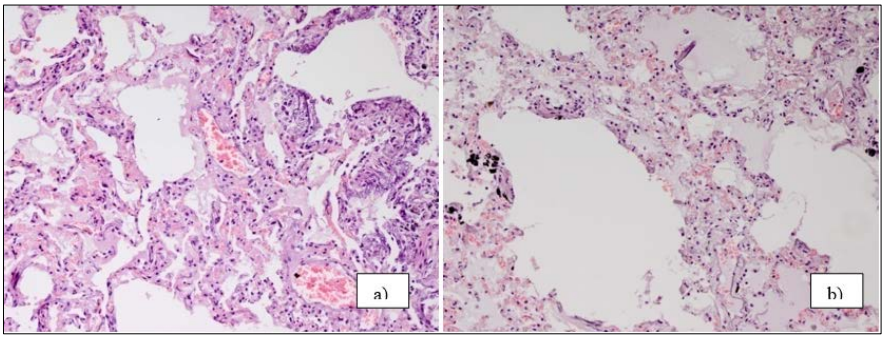

Fig 10: a) severe marked interstitial pneumonia, severe congestion in lungs (1000X, H&E). b) Interstitial pneumonia with congestion and Severe lymphocyte infiltration (1000X, H & E)

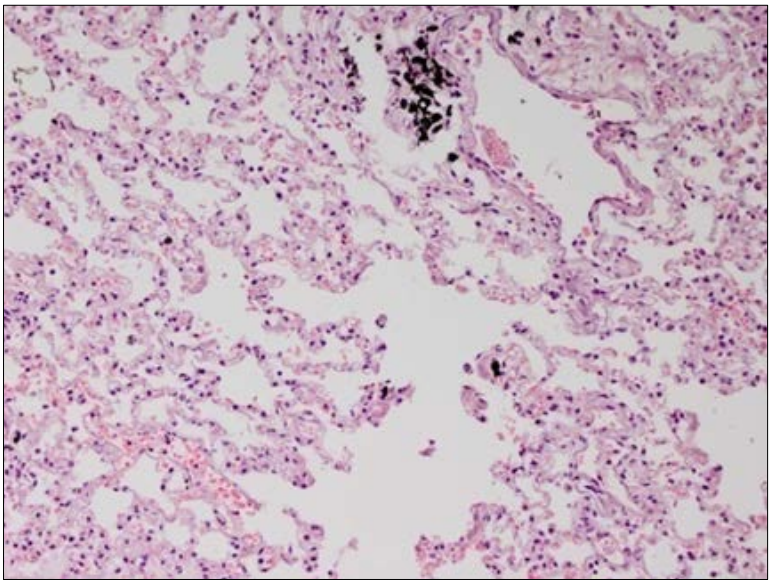

Fig 11: Chronic interstitial lung disease, anthracosis (400X, H & E)

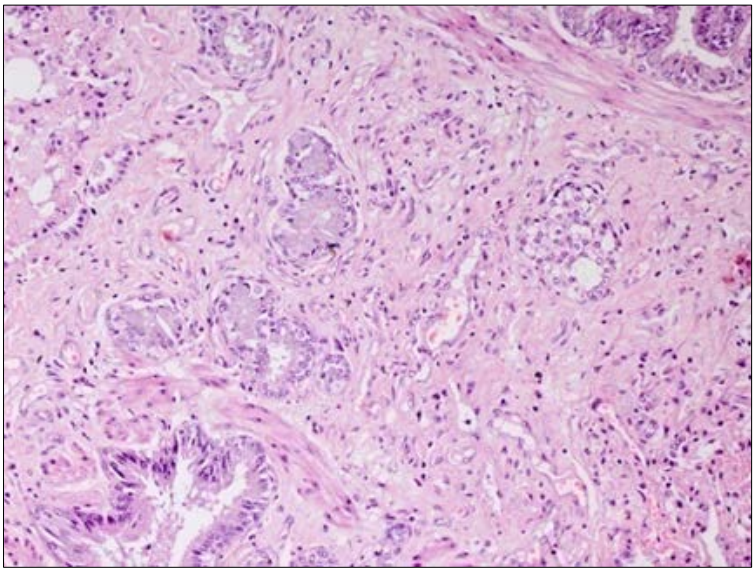

Fig 12: Sloughing of tracheal epithelium (200X, H & E)

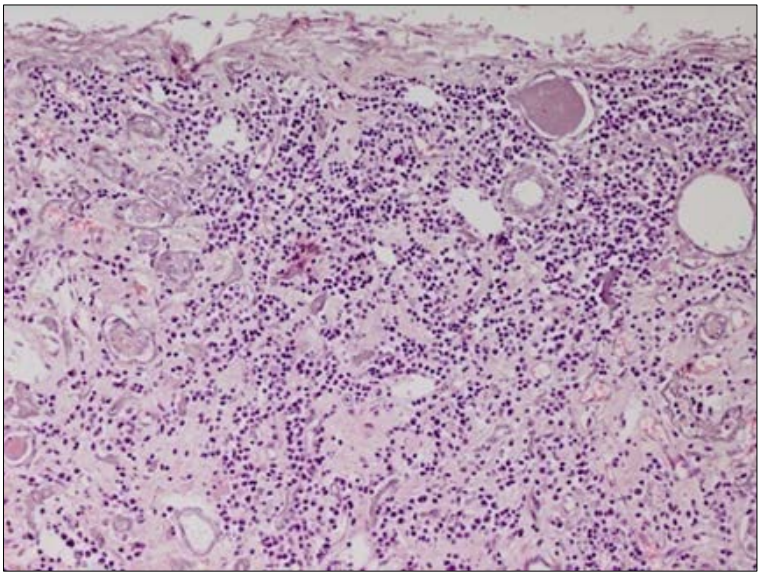

Fig 13: Marked depletion of red pulp (400X, H & E)

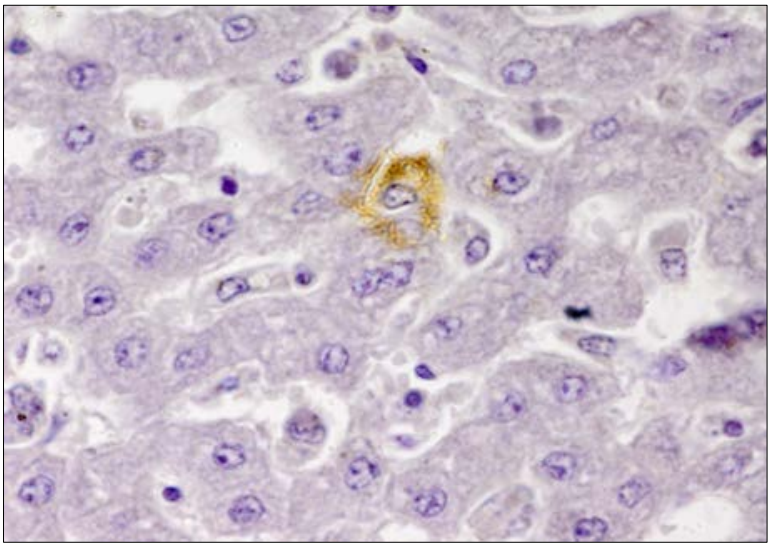

Fig 14: *Clostridia* in liver (1000X)

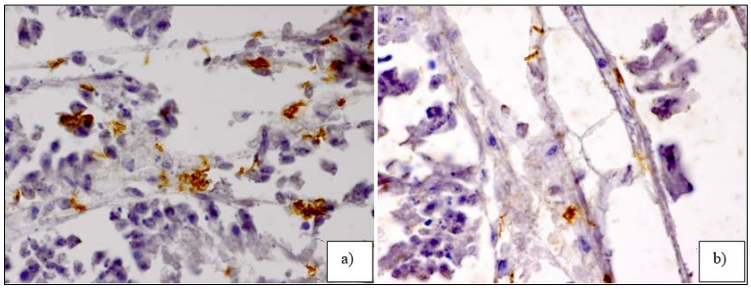

Fig 15: a) *Clostridia* in intestine (1000X), b) *Clostridia* in intestine (1000X)

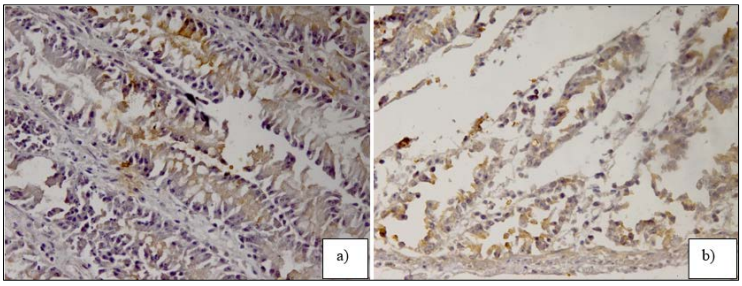

Fig 16: a) *Rotavirus* in intestine (400X), b) *Rotavirus* in intestine (400X)

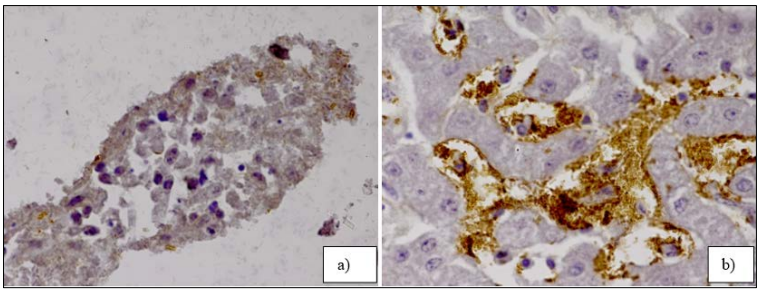

Fig 17: a) *Salmonella* in intestine (1000X), b) *Salmonella* in liver (1000X)

3. Results and discussion

3.1 Gross findings: Gross changes were noticed in rumen, omasum, intestines, liver, lungs and spleen. The characteristic changes were sloughing off of surface of rumen, congestion and haemorrhages in the omasum, severe haemorrhages and congestion in intestines, congestion rounding of the edges and focal necrotic areas in the liver, hemorrhages in the lungs, ecchymotic haemorrhages in the spleen and hemorrhages and focal necrotic areas in the kidney. No gross abnormalities were detected in heart. Mesenteric lymph nodes were enlarged in most of the cases. The main gross lesions observed in the intestine by Suresh *et al*. (2021) [20] were haemorrhagic catarrhal enteritis with thickened serosal layer of intestine.

3.2 Histopathologic Observations

3.2.1 Gastric and intestinal changes: Intestinal sections revealed chronic enteritis with sloughing and atrophy of

villous epithelium with chronic superficial gastritis (Figure 1a and 1b). In addition to these changes, chronic suppurative catarrhal gastropathy with erosion of villous epithelium, pycnotic nuclei with excessive mucoid discharge was also observed (Figure 2). Similar observations were documented by other workers (Agrawal *et al*., 2002; Jesse *et al*., 2016; Singh *et al*., 2020) [1, 10, 18] the intestine that revealed altered villi crypt ratio, desquamation of villous congestion, hyperplasia of crypt lining cells, mononuclear cell infiltration in mucosa.

3.2.2 Liver: Hepatocytes revealed chronic venous congestion with atrophy of hepatocytes and disintegration of central vein, mono nuclear cell infiltration. Pseudolobulation and atrophy of hepatocytes with moderate to severe fatty changes and mononuclear cell infiltration with hemosiderosis were also observed (Figure 3a, 3b and 4).

3.2.3 Kidneys: Kidney sections revealed severe congestion, necrosis of glomeruli damaged tubules, missing glomeruli laden with hyaline casts (Figure 5a and 5b), missing glomeruli, chronic infiltration of lymphocytes (Figure 6a), missing glomeruli, glomerulosclerosis, and chronic congestion of tubules (Figure 6b), marked chronic interstitial nephritis, chronic glomerulitis, deposition of calcium laden casts in tubules (Figure 7a), metastatic calcification in some of the glomeruli, focal area of marked interstitial chronic hepatitis (Figure 7b); proliferative glomerulitis (Figure 8). Adrenals revealed superficial hemorrhages with fatty changes (Figure 9).

3.2.4 Lungs: Sections of the lungs revealed severe congestion, marked interstitial pneumonia lungs and severe lymphocyte infiltration (Figure 10a and 10b). In one of the calves, chronic interstitial lung disease and anthracosis was also recorded (Figure 11).

3.2.5 Trachea: Severe sloughing of tracheal epithelium was also recorded (Figure 12).

3.2.6 Spleen: Sections of spleen revealed marked depletion of red pulp (Figure 13).

3.3 Immuno-histochemistry: Immunohistochemistry observations recorded in nine calves revealed presence of spectra of bacterial and viral organisms in various organs affected with these pathogens.

3.3.1 *Clostridia*: *Clostridial* organisms were observed in liver (Figure 14), in intestine of superficial brown colour, intestinal villi, villous epithelium (Plate 15a and 15b). Similar observations were found in the study of Suresh *et al*. (2021) [20] in which immunoreactivity of bacteria were demonstrated within crypts and in between intestinal crypts. Also, studies of Brar (2013) [4] and Athira *et al*. (2018) [3] showed similar observations of *Clostridium infection* in intestines by immuno-histochemistry (IHC).

3.3.2 *Rotavirus*: *Rotavirus* was observed in various organs affected due to this pathogen *viz*., in intestines and peripheral surface of villi, in cytoplasm of crypts (Figure 16a and 16b). It showed extensive intra-cytoplasmic fluorescence associated with rotavirus infection. The lesions described were similar to the observation of Tzipori *et al*. (1983) [21] and Suresh *et al*. (2021) [20]. Ranganath (2013) [16] observed antigen against rotavirus in the cytoplasm of intestinal villi epithelial cells, glandular lining of the small intestine, and Singh *et al*. (2020) [18] demonstrated antigen against rotavirus in the jejunum, colon, ileum, peyer's patches, and mesenteric lymph nodes of the calves.

3.3.3 *Salmonella*: *Salmonella* organisms were observed in sinusoids of liver and in intestines (Figure 17a and 17b). The immunoreactivity to salmonella was observed as yellow to brown coarse granules or bacilli in mucosal layer of the intestine. The positive reaction to salmonella infection was observed in the form of lesions in deep intestinal layer with dilated vasculature. Similar finding were seen in the observations of Desmidt *et al*. (1998) [5] and Suresh *et al*. (2021) [20].

4. Conclusion

This study concludes that calf diarrhea can be caused by either single or multiple infectious etiological agents. It was found that *Clostridium perferinges*, *Rotavirus* and *Salmonella spp* were the main etiological agents responsible for causing diarrhea. Immunohistochemistry (IHC) was an effective tool for detecting the various etiological agents of calf diarrhea and can be employed to demonstrate the Pathophysiology of agents affecting various tissue or organs.

5. Acknowledgment

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