RESEARCH ARTICLE

Immunohistochemical Detection of Enteropathogens Associated with Calf Diarrhea

Raksha Suresh*, Apminder Pal Singh Brar, Geeta Devi Leishangthem, Bhupinder Singh Sandhu

Abstract

The objective of the study was to determine the infectious agents responsible for causing diarrhea in neonatal calves through immunohistochemistry. One hundred intestinal samples of dead calves with a history of diarrhea were collected from farms in and around Ludhiana and the postmortem hall of the Department of Veterinary Pathology, GADVASU, Ludhiana. All the samples were subjected to routine histopathology. Immunohistochemistry was employed to detect the presence of etiological agents in all of these samples. The primary gross lesions observed in the intestine were congestion, catarrhal enteritis, and fibrin on the mucosal surface. Common histopathologic observations were necrotic enteritis along with massive infiltration of mononuclear cells. 54 samples were found to be positive for *Salmonella* spp., 50 for *Clostridium perfringens*, 8 for *Cryptosporidium* spp., 4 for *E. coli* (K99), 4 for rotavirus. *Eimeria* spp. was also detected in 5 samples through immunohistochemistry. A total of 38 samples showed the involvement of multiple agents. The study concludes that calf diarrhea can be caused by multiple infectious etiological agents, either single or multiple, and that *Clostridium perfringens* and *Salmonella* spp were the main etiological agents responsible for causing diarrhea. Immunohistochemistry was found to be an effective tool for detecting the various etiological agents of calf diarrhea.

Keywords: *Clostridium perfringens*, Histopathology, Immunohistochemistry, Neonatal calf diarrhea, *Salmonella* spp.

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Introduction

Diarrhea is one of the important causes of mortality in neonatal calves. Diarrhea is usually multifactorial in nature. Important infectious agents implicated in bovine calf diarrhea are *Cryptosporidium parvum*, *bovine rotaviruses, bovine coronaviruses* (BCoV), *Salmonella* spp, *Clostridium perfringens type C*, *Escherichia coli* K99 (F5), and *Eimeria* spp. Multiple agents/factors are responsible for causing diarrhea, and thus difficult to diagnose the definitive causative agent. Hence, employing a technique that is both sensitive and specific is necessary for diagnosing the causative agent. This will help tailor the treatment effectively and avoid antibiotic resistance problems due to the extensive use of antibiotics. Immunohistochemistry (IHC) is a useful technique to detect infectious agents in the tissues and thereby help in the diagnosis of the causative agent of the diseases in animals (Haines and West, 2005; Eyzaguirre and Haque, 2008). This technique involves selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The convenience of submitting a single tissue sample for histopathology and identifying the organism without any temperature requirements makes the technique very ideal. IHC is also a handy tool to visualize the presence of the pathogen, its distribution in the tissue and the histological lesions it has caused. Therefore, the study's objective was to detect the causal organisms responsible for calf diarrhea through immunohistochemistry.

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Materials and Methods

Sample Collection

A total of 100 intestinal samples from dead bovine calves less than 2 months of age with a history of diarrhea were collected from dairy farms in and around Ludhiana and the postmortem hall of the Department of Veterinary Pathology, GADVASU. The samples were collected from August 2019 to December 2019 based the history of the calf and the gross lesions observed in the intestine. These samples were processed for histopathology and subjected to immunohistochemistry to detect etiological agents responsible for causing diarrhea.

Histopathology

Pieces of the intestine from the affected parts were collected in 10% neutral buffered formalin for fixation. After fixation for 2 days, tissue samples were given overnight washings under tap water, followed by dehydration and clearing in in ascending grades of alcohol and clearing in acetone and benzene. Tissues were embedded in paraffin wax (Leica Microsystem, Paraplast tissue embedding medium, 56°C) for further processing, and 4-5 µ thin sections were cut. The paraffin sections were stained with routine hematoxylin and eosin technique.

Immunohistochemistry Protocol

For immunohistochemical studies, 4-5 µ thin paraffin-embedded tissue sections were cut and mounted on positively charged microscopic slides. After deparaffinization and rehydration, antigen retrieval was done by keeping the slides in citrate buffer (pH 6) and heated to 95°C for 3 minutes, followed by 70°C for 7 minutes in a microwave oven. After cooling at room temperature, washing was done in PBS for 3 times of 3 minutes each. In order to quench the endogenous peroxides, slides were kept in 3% H_2O_2 for 30 minutes, followed by washing again in PBS for 3 times of 3 minutes each. 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 37°C 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).

Results and Discussion

Gross Findings

Grossly, the intestines of affected calves were congested and distended (Fig. 1). Mesenteric lymph nodes were enlarged in most of the cases. The intestinal mucosa was congested and covered with fibrin in some cases**.** Common gross changes observed in the intestine were hemorrhagic and catarrhal enteritis (Fig. 2). Thickening of intestinal mucosa and hemorrhages on the serosal surface of the intestine were also observed.

Histopathological Findings

The histopathological changes observed in the intestine revealed altered villi crypt ratio, desquamation of villous

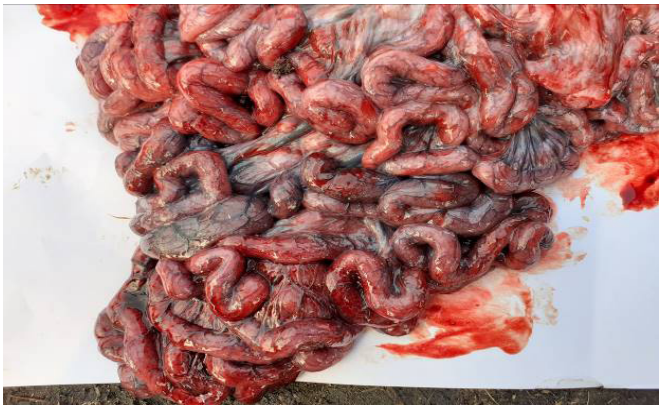

Fig. 1: Gross specimen of intestine showing severe congestion

Fig. 2: Gross specimen of intestine showing catarrhal enteritis

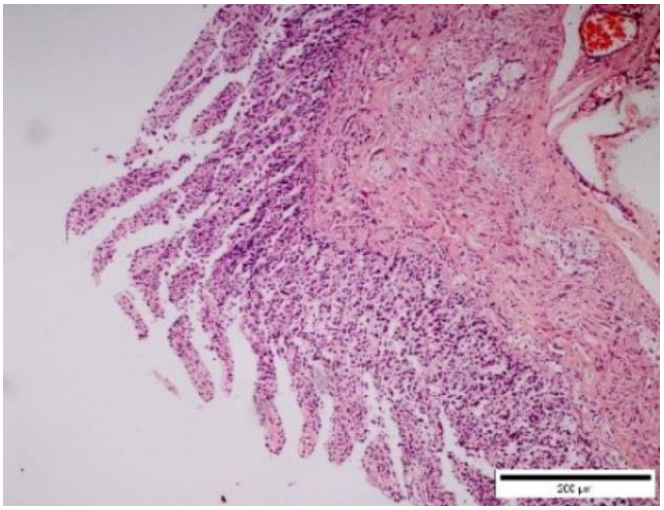

Fig. 3: Photomicrograph of intestine showing marked desquamation of villous epithelial cells (4X)

epithelial cells (Fig. 3), congestion, hyperplasia of crypt lining cells, mononuclear cell infiltration in mucosa often extending to submucosal layer (superficial and deep enteritis)**.** Similar observations were reported by other workers (Agrawal *et al.,* 2002; Jesse *et al*., 2016; Singh *et al*., 2020). The denudation of mucosa ranged from superficial to complete loss of villi, exposing the crypts and sometimes even muscularis with the destruction of muscularis mucosae. Another set of changes observed were a fusion of villi and fibrosis, crypt atrophy, and metaplasia of the epithelial cells of villi, indicating chronicity of the intestinal damage (Fig. 4). Necrotic enteritis was the most common type of change observed, which consisted of massive denudation of epithelial cells and necrotic debris in the lumen of the jejunum and ileum. Similar observations were reported by Valgaeren *et al*. (2013) and Awad *et al*. (2020).

During the histopathological study, 5 cases showed the presence of coccidia. Various developmental stages of *Eimeria* spp, including sexual and asexual phases, were observed mainly in crypts resulting in crypt hyperplasia, massive destruction of crypts and villi, along with massive infiltration of mononuclear cells (Fig 5).

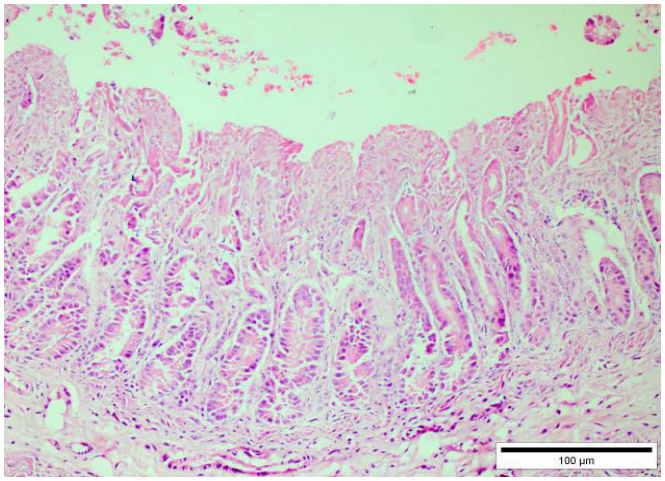

Fig. 4: Photomicrograph of intestine showing metaplasia of columnar epithelial cells (10X)

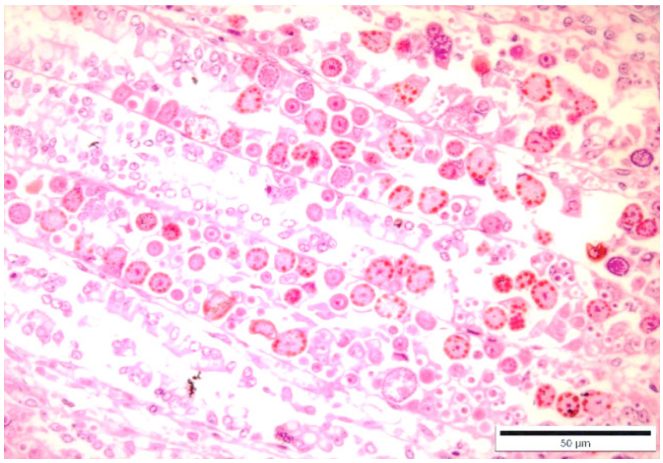

Fig. 5: Photomicrograph of intestine showing different developmental forms of coccidia (40X)

Immunohistochemical Localization of Infectious Agents Causing Calf Diarrhea

Out of 100 samples, *Salmonella* spp was detected in 54 samples; however, *Salmonella* alone was detected in 21 samples and the rest 33 samples were associated with another etiological agents/s. Similarly, out of 50 samples detected for *Clostridium perfringens,* 20 were *C. perfringens* alone, while the remaining 30 involved multiple agents.

The multiple agents detected in total 38 samples included *Salmonella* spp +*Clostridium perfringens (20), Salmonella* spp +*E. coli (1), Salmonella* spp +*Rota (1), Salmonella* spp +*Cryptosporidium (3), Salmonella* spp +*Eimeria* spp (3), *Salmonella* spp+ *Clostridium perfringens* +*Cryptosporidium (2), Salmonella* spp +*Clostridium perfringens* +*Eimeria* spp (1), *Salmonella* spp + *Clostridium perfringens* +*Rota (2), Clostridium perfringens* +*E. coli (1), Clostridium perfringens* +*Cryptosporidium* spp (2), *Clostridium perfringens* +*Eimeria* spp (1), *Clostridium perfringens* +*E. coli*+ *Cryptosporidium* spp (1). Some details of major pathogens were as under.

Cryptosporidium parvum

Immunoreactivity to *C. parvum* was demonstrated in 8 samples superficially in the intestinal tissue as golden yellow round bodies (Fig. 6). The morphology and diameter of these bodies matched *C. parvum* (Borah *et al*., 2013). It was also observed in necrotic debris in the intestinal lumen. A higher prevalence of *Cryptosporidium* spp has been reported by Singh *et al.* (2006) in the same region. The lower prevalence of *Cryptosporidium* spp can be attributed to the disappearance of the organism after the death of animals due to the presence of organism superficially on the mucosal layer. The nature of processing and sectioning might have also wiped off the organism from its superficial location in some of the cases.

Salmonella spp.

In the present study**,** immunoreactivity to salmonella was observed to be 54 % as yellow to brown coarse granules or bacilli in mucosal layer of the intestine (Fig. 7). A similar finding was observed by Desmidt *et al.* (1998) where *Salmonella enteritidis* adhered to the mucosa of ceca of chickens. In the present study, the positive reaction for salmonella was

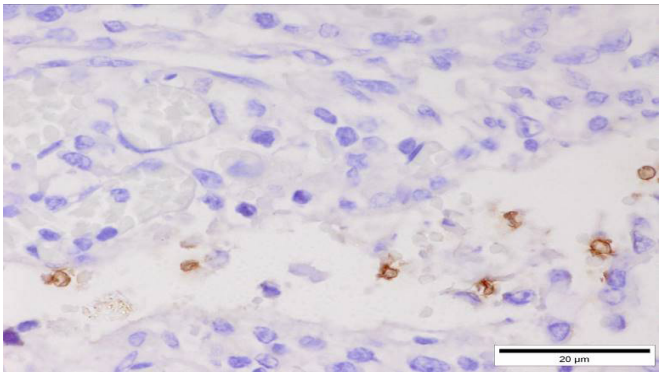

Fig. 6: Photomicrograph of intestine showing immunoreactivity for Cryptosporidium oocyst and other developmental forms (100X)

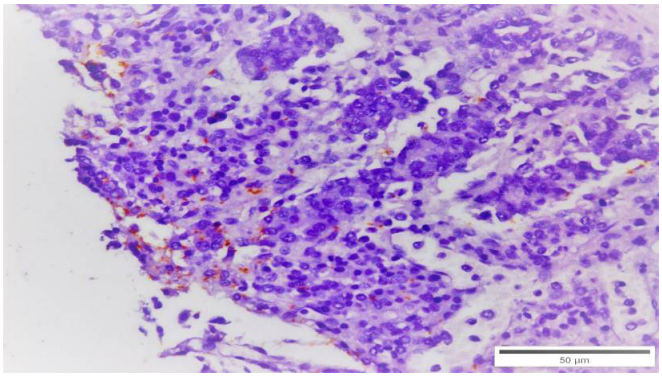

Fig. 7: Photomicrograph of intestine showing immunoreactivity to Salmonella spp in mucosa (40X)

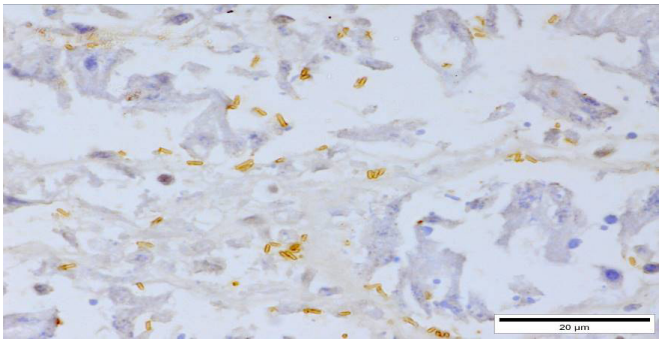

Fig. 8: Photomicrograph of intestine showing immunoreactivity to *Clostridium perfringens* in between and within crypts (100X)

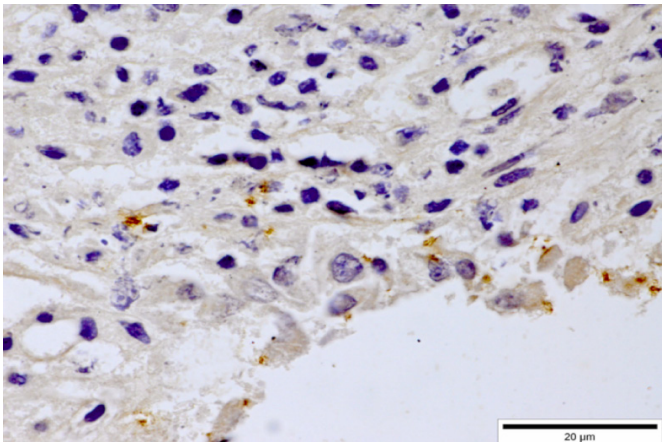

Fig. 9: Photomicrograph of intestine showing immunoreactivity to *E. coli* K99(100X)

also observed in the deeper intestinal mucosa, dilated microvasculature of the intestine, and occasionally in the mononuclear cells. Immunoreactivity in the blood vessels suggests the hematogenous spread of the bacteria to other visceral organs.

Clostridium perfringens

Immunoreactivity to *C. perfringens* was observed in 50 samples, mostly on superficial and deep mucosa. The bacteria were also demonstrated in between crypts and within intestinal crypts (Fig. 8). Also, the reactivity for *C. perfringens* was found in the deep microvasculature of the intestine in a few cases. Previous studies in northern India by Brar (2013) and Athira *et al.* (2018) have shown a prevalence rate of 62.79% and 37.2%, respectively, by IHC.

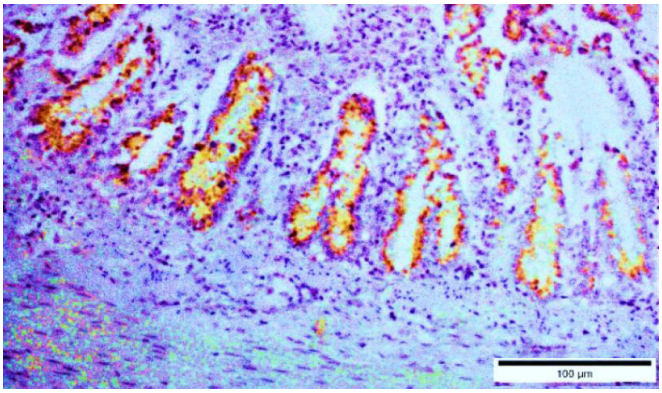

Fig. 10: Photomicrograph of intestine showing immunoreactivity to rota viral antigen in the crypts (10X)

Escherichia coli

In the present study, immunoreactivity for *E. coli* K99 was observed on the mucosal surface of the intestine (Fig. 9) in 4 samples. Though *E. coli* K99 is considered one of the crucial causes of neonatal diarrhea, its prevalence is relatively low. De Verdier *et al.* (2012) found that *E. coli* K99 virulence gene was not present in any Swedish dairy cattle. Coura *et al.* (2015) reported *E. coli* K99 in only 7 out of 1983 samples. This lower incidence can be attributed to the fact that *E. coli* K99 positive strains are usually isolated from the neonatal calves due to a reduction in the expression of *E. coli* K99 within few days of birth (Yadegari *et al.,* 2019).

Rotavirus

Cytoplasm of villus and/ or crypt epithelia showed moderate to strong immunoreactivity against bovine rotavirus (Fig. 10). The villi enterocytes were mainly sloughed off, and only the crypt remained, which showed strong immunoreactivity for the rotavirus. Ranganath (2013) demonstrated rotavirus antigen in the cytoplasm of intestinal villi epithelial cells, glandular lining cells of the small intestine, and Singh *et al.* (2020) demonstrated bovine rotavirus antigen in the jejunum, colon, ileum, Peyer's patches, and mesenteric lymph nodes of naturally infected calves.

In the present study, the prevalence of rotavirus was found to be 4%. Gill *et al.* (2017) reported the prevalence rate of 6.56%, 7.57%, and 9.59% by ELISA, RNA-PAGE, and RT-PCR, respectively. However, Varshney *et al.* (1995) observed that one-day-old calves were more susceptible to rotavirus than 10-day old calves as the reactivity in the immunostaining in the former was 3 times greater than the latter. Suresh *et al.* (2011[b]) observed that rotavirus was more prevalent in calves below 14-days of age. Although in the present study, no bovine coronavirus was detected in any of the samples, Björkman *et al.* (2003), Suresh *et al.* (2011[a]), Madesh *et al.* (2019), Singh *et al.* (2019) have reported a lower prevalence of Coronavirus.

Conclusion

This study concludes that calf diarrhea can be caused by multiple infectious etiological agents, either single or multiple. It was found that *Clostridium perfringens* 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. IHC can be employed to study the pathogenesis of various etiological agents affecting various tissue/organs.

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References

Agrawal, D.K., Singh, N.P., & Chauhan, R.S. (2002). Clinico-pathomorphological studies on rotavirus infection in neonatal calves. *Indian Journal of Veterinary Medicine, 22*(2), 85-88.

Athira, C.K., Milton, A.A.P., Reddy, A., Rajendrakumar, A.M., Verma, M.R., Kumar, A., Nagaleekar, V.K., & Agarwal, R.K. (2018). Diversity of toxin-genotypes among *Clostridium perfringens* isolated from healthy and diarrheic neonatal cattle and buffalo calves. *Anaerobe*, *49*, 99-102.

Awad, W.S., El-Sayed, A.A., Mohammed, F.F., Bakry, N.M., Abdou, N.E.M., & Kamel, M.S. (2020). Molecular characterization of pathogenic *Escherichia coli* isolated from diarrheic and in-contact cattle and buffalo calves. *Tropical Animal Health and Production*, *52*(6), 3173-3185.

Björkman, C., Svensson, C., Christensson, B., & De Verdier, K. (2003). *Cryptosporidium parvum* and *Giardia intestinalis* in calf diarrhea in Sweden. *Acta Veterinaria Scandinavica*, *44*(3), 1-8.

Borah, M.K., Sharma, R.L., Gogoi, R., Sharma, G.D., & Godara, R. (2013). Concurrent Cryptosporidium and Candida infections in a buffalo calf (*Bubalus bubalis*). *Comparative Clinical Pathology*, *22*(5), 895-898.

Brar, A.P.S. (2013). Studies on immunopathological and molecular diagnostics of infectious causes of diarrhea. *Ph.D. dissertation*. Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India.

Coura, F.M., Freitas, M.D., Ribeiro, J., de Leme, R.A., de Souza, C., Alfieri, A.A., Facury Filho, E.J., de Carvalho, A.Ú., Silva, M.X., Lage, A.P., & Heinemann, M.B. (2015). Longitudinal study of Salmonella spp., diarrheagenic *Escherichia coli*, Rotavirus, and Coronavirus isolated from healthy and diarrheic calves in a Brazilian dairy herd. *Tropical Animal Health and Production*, *47*(1), 3-11.

de Verdier, K., Nyman, A., Greko, C., & Bengtsson, B. (2012). Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Veterinaria Scandinavica*, *54*(1), 1-10.

Desmidt, M., Ducatelle, R., & Haesebrouck, F. (1998). Immunohistochemical observations in the ceca of chickens infected with Salmonella enteritidis phage type four. *Poultry Science*, *77*(1), 73-74.

Eyzaguirre, E., & Haque, A.K. (2008). Application of immunohistochemistry to infections. *Archives of Pathology and Laboratory Medicine*, *132*(3), 424-431.

Gill, G.S., Kaur, S., Dwivedi, P.N., & Gill, J.P.S. (2017). Comparative prevalence and molecular characterization of group A rotavirus in cow calves of Punjab, India. *Journal of Animal Research*, *7*(5), 927-933.

Haines, D. M., & West, K. H. (2005). Immunohistochemistry: forging the links between immunology and pathology. *Veterinary Immunology and Immunopathology*, *108*(1-2), 151-156.

Jesse, F.F.A., Sadiq, M.A., Abba, Y., Chung, E.L.T., & Adamu, L. (2016). Clinico-Pathological Findings of Septicaemic Colibacillosis in a Calf. *Journal of Dairy, Veterinary and Animal Research*, *4*(3), 124.

Madesh, A., Verma, A.K., Athira, C.K., Gupta, S., Athira, V., Verma, A., & Malik, Y.S. (2019). Antigenic detection of enteric pathogens associated with neonatal calf diarrhea. *Journal of Immunology and Immunopathology, 21*(1), 35-38.

Rangnath (2013). Molecular Epidemiology and Pathological studies on Rotavirus infection in Bovine calves. *PhD thesis*. Indian Veterinary Research Institute, Izatnagar, UP, India, pp 57.

Ramos-Vara, J. A. (2011). Principles and methods of immunohistochemistry. In: Drug Safety Evaluation, 2nd edn., Ed: Jean-Charles Gautier, Springer Nature, New York.pp115-128.

Singh, B.B., Sharma, R., Kumar, H., Banga, H.S., Aulakh, R.S., Gill, J.P.S., & Sharma, J.K. (2006). Prevalence of *Cryptosporidium parvum* infection in Punjab (India) and its association with diarrhea in neonatal dairy calves. *Veterinary Parasitology*, *140*(1-2), 162-165.

Singh, S., Singh, R., Singh, K.P., Singh, V., Malik, Y.P.S., Kamdi, B., Singh, R., & Kashyap, G. (2019). Prevalence of bovine coronavirus infection in organized dairy farms of Central and North regions, India. *Biological Rhythm Research*, 1-7 https://doi.org/10.1080/09291016.2019.1629093

Singh, S., Singh, R., Singh, K.P., Singh, V., Malik, Y.P.S., Kamdi, B., Singh, R., & Kashyap, G. (2020). Immunohistochemical and molecular detection of natural cases of bovine rotavirus and coronavirus infection causing enteritis in dairy calves. *Microbial Pathogenesis*, https: doi.org : 10.1016/j.micpath.2019.103814

Suresh, T., Rai, R.B., Dhama, K., Bhatt, P., Sawant, P.M., & Sharma, A.K. (2011[a]). Prevalence of rotavirus, coronavirus and *Escherichia coli*: the main agents responsible for calf diarrhea. *Veterinary Pactitioner, 13*(2): 160-65.

Suresh, T., Rai, R.B., Dhama, K., Rai, S., Sawant, P.M., & Sharma, A.K. (2011[b]). Pathology of rotavirus infection in calves and detection of viral antigen by ELISA and FAT. *Indian Journal of Veterinary Pathology, 35*(1): 1-3.

Valgaeren, B., Pardon, B., Goossens, E., Verherstraeten, S., Schauvliege, S., Timbermont, L., Ducatelle, R., Deprez, P., & Van Immerseel, F. (2013). Lesion development in a new intestinal loop model indicates the involvement of a shared *Clostridium perfringens* virulence factor in haemorrhagic enteritis in calves. *Journal of Comparative Pathology*, *149*(1), 103-112.

Varshney, K.R., Bridger, J.C., Parsons, K.R., Teucher, J., & Hall, G.A. (1995).The lesions of rotavirus infection in 1-10-day-old gnotobiotic calves. *Veterinary Pathology 32*: 619-627.

Yadegari, Z., Brujeni, G.N., Ghorbanpour, R., Moosakhani, F., & Lotfollahzadeh, S. (2019). Molecular characterization of enterotoxigenic Escherichia coli isolated from neonatal calves diarrhea. *Veterinary Research Forum*, *10*(1), 73.