

Seromonitoring, Serotyping and Histopathology of Foot and Mouth Disease in Mithun (*Bos Frontalis*) in the Northeastern Hilly Terrain of India

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ABSTRACT

Frequent FMD outbreaks have been one of the major challenges faced by the farmers and breeders of Mithun animals in Nagaland, owing to a lack of adequate knowledge and proper vaccination programs for the disease. Therefore, this study aimed to determine the extent of FMD serotype O virus infection, which is the most common serotype causing FMD specifically in the Mithun population in the northeastern state of Nagaland, India. In-vitro screening of serotype O virus antibodies in the blood serum of the Mithun population was carried out by ELISA kit. A total of 213 blood samples of Mithun animals were collected and screened where 67% of the samples tested were found to be positive and only 32% of the samples were found to be negative for the virus. All the samples collected from the neighbouring states of Mizoram and Arunachal Pradesh were found to be negative for the antibodies against the serotype O virus. Through m-RT PCR, O serotype was detected from all the clinically affected animals from the cells of the tongue epithelium, blood, and saliva of animals. During the histopathological studies, in the pericardium, severe interstitial myocarditis and liver showed focal areas of coagulative necrosis, focal necrosis, and pulmonary oedema were prominent and ballooning degeneration of epithelium in the tongue was evident in all cases. In conclusion, we were able to show the prevalence of FMDV serotype O antibodies in a very high percentage of the Mithun population that were tested along with pathological alterations, which should be addressed immediately with proper vaccination programs and by informing and educating the farmers and breeders about the disease before an outbreak occurs.

Keywords: Food and Mouth Disease, Serotype, ELISA, Vaccination

1. Introduction

Foot and Mouth Disease Virus (FMDV) is a highly infectious viral pathogen capable of causing persistent infections and long-term effects on the condition and productivity of all cloven-hoofed domestic and wild animals including Mithun [3, 19]. FMDV is a *Picornavirus* belonging to the *Aphthovirus* genus [27], which exists in seven major serotypes: O, A, C, Asia 1 and Southern African Territories (SAT) 1, SAT-2

and SAT-3 and multiple subtypes [11]. They are endemic to India, occurring throughout the year and causing severe economic losses to the livestock industry as well as to the poor farmers rearing these animals for their livelihood. In India, three (O, A, and Asia1) of the seven serotypes are prevalent [32] of which, type O has been the most responsible (i.e., about 80%) of the cases of the confirmed outbreak in India and is also the most prevalent type in the northeast region of India [35] where Mithun are reared. Mithun plays a crucial role in the socio-economic and cultural life of the tribal people. It is the State animal of Nagaland and Arunachal Pradesh. As per the 20th Livestock census, the total Mithun population in the country is 3.9 Lakhs, with a 30.6% increase in the population from the last census. Arunachal Pradesh has the highest number of Mithun at 89.7%, 5.98% in Nagaland, 2.36% in Manipur, and 1.02% in Mizoram. Nagaland is one of the regions in northeast India that has often been affected by frequent outbreaks of Food and Mouth Disease (FMD) [7, 35] causing major health issues for the infected Mithun animals. The spread of FMD not only affects the Mithun animals but also severely affects the socio-economic life of the tribal community as they depend on the animal for their livelihood. The symptoms shown by the infected Mithun animals are found to be similar to those found in cattle like lameness, drooling of saliva, erosion of the oro-nasal area, and lesions on inter-digital cleft [7] including myocardial and petechial haemorrhages on the kidney [16]. FMD in Mithun is a major hurdle that needs to be overcome to establish and popularize Mithun farming as an economic and profitable venture among the farmers. The virus spreads via all secretions and excretions (saliva, urine, faeces, etc.) of the affected animals. Transmission occurs through ingestion, inhalation, and contact with contaminated materials, fomites, and other inanimate objects. The disease is characterized by the formation of vesicles on the muzzle, lips, gums, teat, cleft of the feet, and other non-hairy regions of the body. Excessive salivation with the smacking of the lips, sloughing of the hoofs, and severe diarrhoea are the common clinical signs. Young calves are severely affected to death due to myocarditis. Diagnosis is based on clinical signs followed by the lesions and virus isolation and serotyping.

In Nagaland, animals like cattle, goats, Mithun, etc. are all reared together in the same grazing area having a common water source and are often co-housed under the same shed. This close proximity rearing and sharing common air with susceptible animals pose a potential risk of the virus spreading among the Mithun population in the area. Ruminant animals persistently infected with FMDV can act as asymptomatic carriers even after recovery from infection [34, 38]. Mithun, being a ruminant animal also may act as a potential source of FMDV and contribute to spreading the disease, which raises a serious concern about the control of FMD in the population. To counter this, retrospective analysis, epidemiological information [26, 28], implementation of regular, proper, and systematic vaccination policy [24], regular monitoring of disease outbreaks and sero-surveillance of the FMD-affected regions, are important as they can aid in devising appropriate vaccination choice and help to eradicate FMD successfully.

Even though FMDV is an important veterinary disease, the studies of its prevalence, especially in the Mithun population are very limited. Therefore, in this study, we tried to determine the infection status of FMDV serotype O circulating in the Mithun population of Nagaland, by in-vitro screening of antibodies against the FMDV serotype O and also studies on different pathology of the affected organs.

2. Materials and methods

A total of 213 Mithun blood samples were collected randomly from different parts of Nagaland and 21 samples from the neighbouring states of Mizoram and Manipur during the period of 2010-2017. After collection, the blood samples were allowed to clot for about 30 minutes at room temperature and then

centrifuged at 3000 rpm for 15 minutes. The supernatant (serum) was transferred to a new tube and the pellet was discarded. The serum sample was stored at -20 °C until further use.

The in-vitro detection of antibodies against FMDV serotype O in serum was carried out using the commercially available PrioCHECKFMDV type O ELISA kit. Briefly, 10 µl undiluted samples and buffer were added to each of the 96 well plates coated with non-infectious FMDV type O antigen. The plate is then incubated for one hour at room temperature (22±3 °C). After washing, the mAb-HRPO conjugate is added to the plate and the plate is incubated for one hour at room temperature (22±3 °C). The plate is then washed and the chromogen (TMB) substrate is added. After incubation colour development is stopped by adding a stop solution and the optical density (OD) is measured at 450 nm. The OD₄₅₀ values of all samples are expressed as percentage inhibition (PI) relative to the OD₄₅₀ max.

$$PI = 100 - \left[\frac{\text{Corrected } OD_{450} \text{ test sample}}{\text{Corrected } OD_{450} \text{ max}} \right] \times 100$$

PI less than 50% was taken as negative and PI equal to or more than 50% was taken as positive for FMDV type O antibodies in the test serum. All the experiments were carried out in duplicates.

Serotyping of FMD virus from clinically affected Mithun

A total of 11 numbers (n=11) of samples (Blood: 4, Epithelial tissue: 3, and Saliva: 4) collected from clinically affected Mithun with FMD were subjected to mRT-PCR for typing of the virus as per standard procedure [31]. The mRT-PCR was done as per available standard primers.

Histopathological study

The materials for the present study were collected from the six carcasses of the Mithun (n=6) that died due to Foot and Mouth diseases in the Phek district of Nagaland. At the time of post-mortem examination, visible gross lesions were recorded and representative tissue samples were collected in 10% formalin solution. Histopathological studies were carried out by routine hematoxylin and eosin (H&E) staining methods [22].

3. Results and discussion

In this present study, *in vitro* detection of antibodies against FMDV serotype O was carried out by blocking ELISA method. Out of the total 213 Mithun animals screened for the virus in Nagaland, 144 (67%) of the animal populations were found to be positive for the antibodies against FMDV serotype O and only 69 (32%) were found to be negative (Figure 1). According to the test results from different villages of Nagaland (Figure 2), NRCM farm, Medziphema and Porba village under Phek district showed 66% of the Mithun population tested positive for the presence of antibodies against FMDV serotype O, whereas in Mussolomi Village 64% of the animals tested were found to be positive. While Doimukh Village in Arunachal Pradesh and Khuwanglang village in Mizoram show no presence of the antibodies against the serotype in the sample tested.

Figure 1: Screening of Mithun population for the presence of FMD serotype O virus

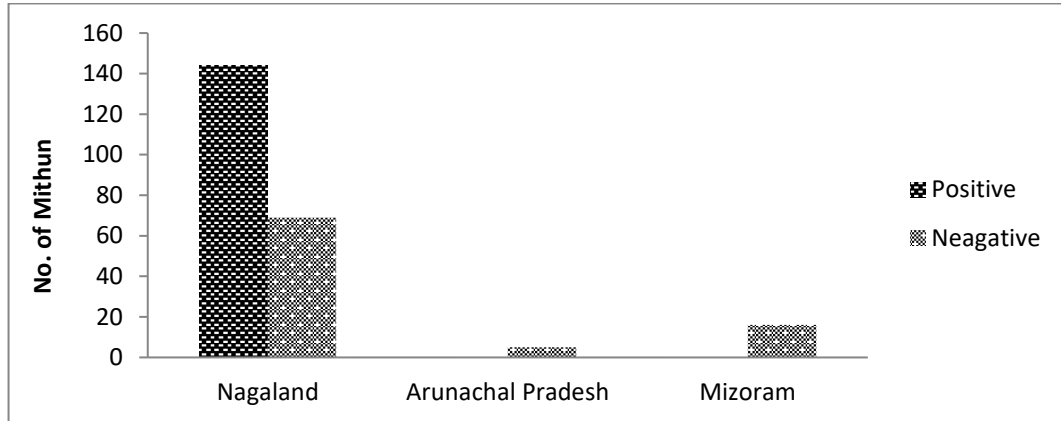
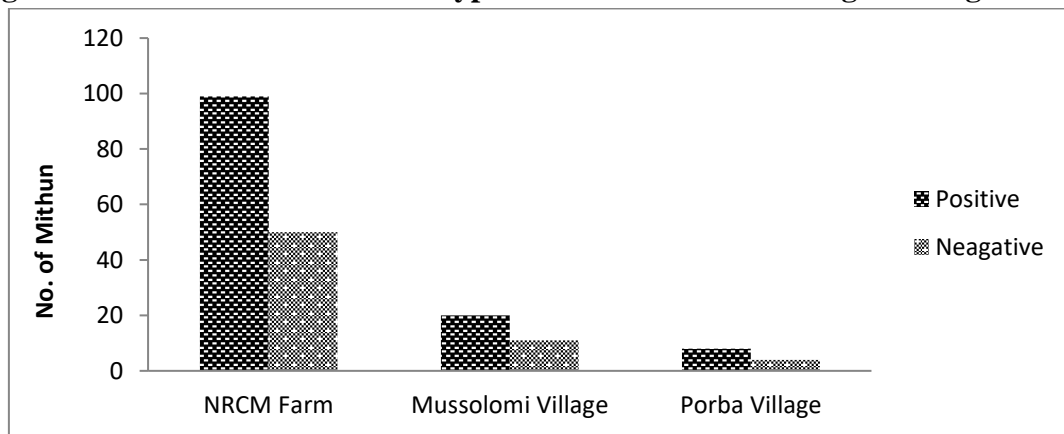


Figure 2: Prevalence of FMD serotype O virus in different villages of Nagaland



Serotyping of the FMD virus from clinically affected Mithun from Porba village, Phek district of Nagaland was also recorded. A total of 11 numbers of samples (Blood: 4, Epithelial tissue: 3, and Saliva: 4) collected from clinically affected Mithun with FMD (Figures 3, 4 and 5) were subjected to Sandwich ELISA/ mRT-PCR for typing of the virus. Out of the 11 samples, 3 epithelial tissue samples were found to be positive for FMD virus serotype “O” by sandwich ELISA, whereas 5 samples (3 epithelial tissue and 2 saliva) were found to be positive for FMD virus serotype “O” by mRT-PCR.

Figure 3: Ulceration in the dental pad of a Mithun (*Bos frontalis*) infected with Foot and mouth disease



Figure 4: Drooling of saliva from a Mithun (*Bos frontalis*) infected with Foot and mouth disease



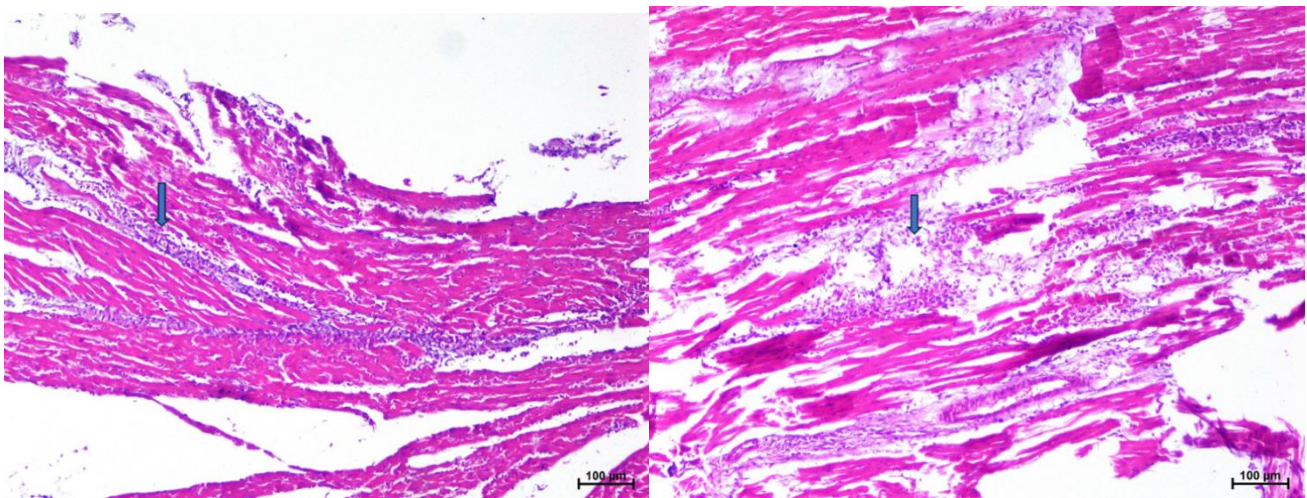
Figure 5: Dental pad affection in a Mithun (*Bos frontalis*) infected with Foot and mouth disease



To know the pathology of the affected organs, six animals were necropsied (n=6), that died due to FMD in the Phek district of Nagaland. As per standard methods, histopathological slides were prepared as per routine H&E methods. In the heart, severe interstitial myocarditis was observed. Necrosed myocardial fibres with intense leucocytic infiltration (prominently lymphocytic and occasional neutrophils.) were noted in Mithun died of FMD. Hyaline degeneration and necrosis of myocytes (hyalinization) with

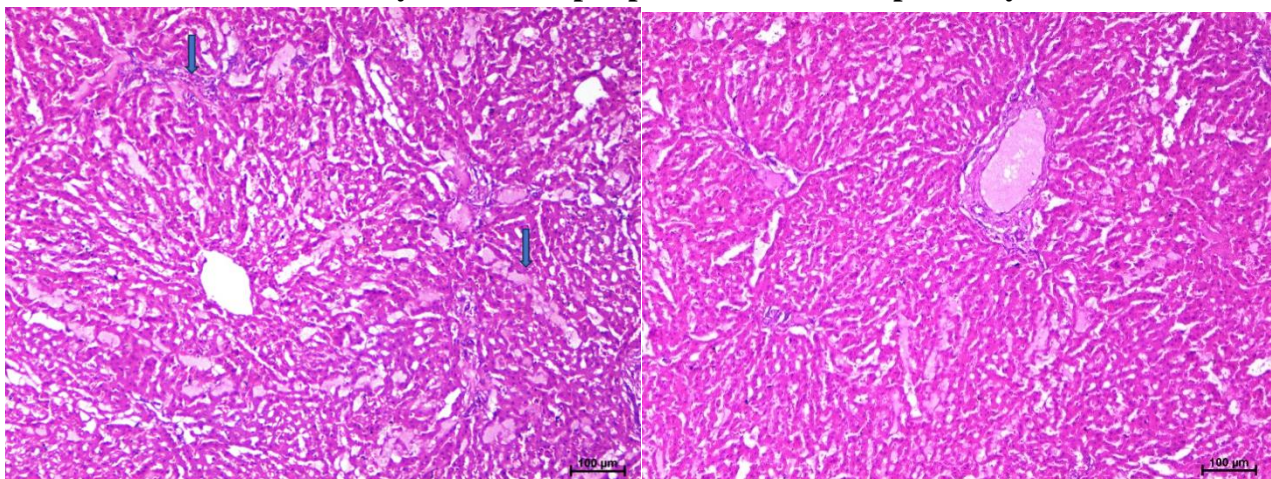
mononuclear cell infiltrations were also observed (Figures 6 and 7). The cardiomyocytes exhibited a hyper eosinophilic cytoplasm with a loss of striation. As evident from histopathology, the focal areas of the myocardium were replaced with lymphocytes (Figures 6 and 7). Alexandersen, et al., 2003 noted that in young animals dying from acute disease, there was lymphohistiocytic myocarditis with hyaline degeneration, necrosis of myocytes, and infiltration with mononuclear cells [4].

Figures 6 and 7: Severe interstitial myocarditis is observed. Necrosed myocardial fibres with intense leucocytic infiltration (prominently lymphocytic and occasional neutrophils). Hyaline degeneration and necrosis of myocytes (hyalinization) with mononuclear cell infiltrations (figure 7) was a prominent feature in all cases. The cardiomyocytes exhibit a hyper eosinophilic cytoplasm with a loss of striation. A focal area of the myocardium is replaced with lymphocytes. H&E 100X



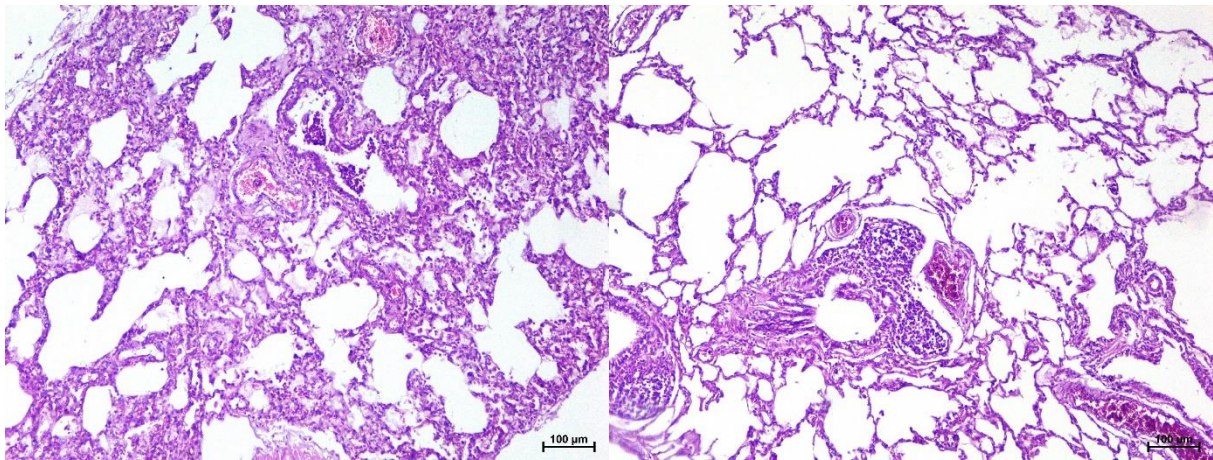
The liver showed a focal area of coagulative necrosis during the present observation. Congestion and Infiltration of inflammatory cells in the periportal area of liver parenchyma were evident (Figures 8 and 9). In the liver, El-Amir, et al., 2014 noted that there were focal areas of necrosis with the presence of clostridial bacilli in buffaloes. However, in the present cases, no clostridial organisms were detected [14].

Figures 8 and 9: Liver showing the focal area of coagulative necrosis. Congestion and Infiltration of inflammatory cells in the periportal area of liver parenchyma. H&E 100X



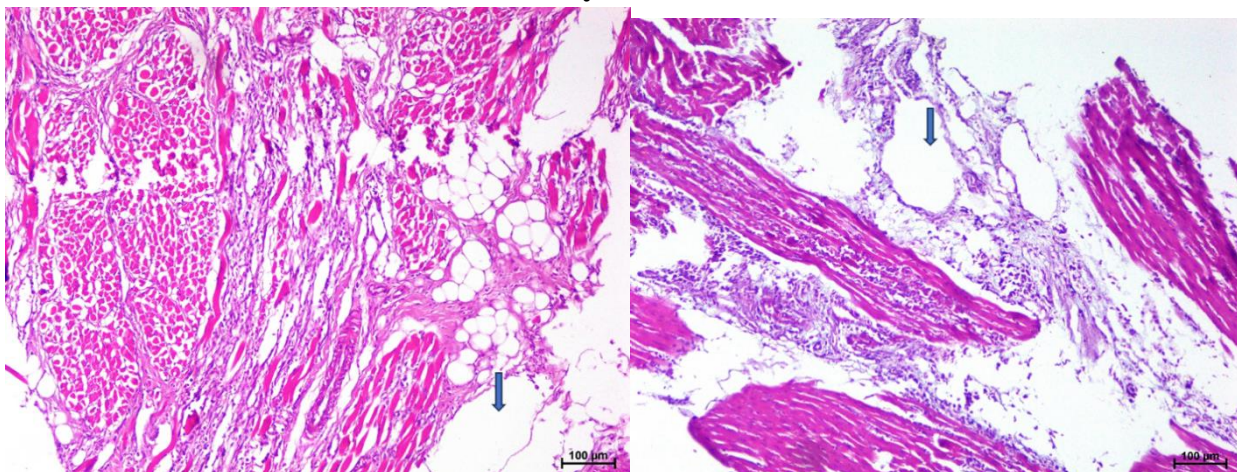
Congestion of interalveolar capillaries was also observed in the lungs. Interstitial infiltrates of mononuclear cells (mainly lymphocytes and plasma cells) with thickening of interalveolar septa were seen. Focal necrosis and pulmonary oedema were prominent (Figures 10 and 11). Similar lesions were also noted by [33] where lungs showed severe engorgement, oedema, and inter-alveolar mononuclear cell infiltration in buffalos.

Figures 10 and 11: Congestion of interalveolar capillaries. Interstitial infiltrates of mononuclear cells with thickening of interalveolar septa (mainly lymphocytes and plasma cells). Focal necrosis and pulmonary oedema were noted. H&E 100X



Ballooning degeneration of epithelium in the tongue was evident in all the cases in the present investigation. High infiltration of inflammatory cells in the dermis was noted (Figures 12 and 13). Singh, et al., 2008 also observed microvesicles in the mucosal epithelium and necrotizing inflammation [33]. The underneath dermis had mononuclear cell infiltration around the engorged blood vessels and oedema of the connective tissue in buffaloes.

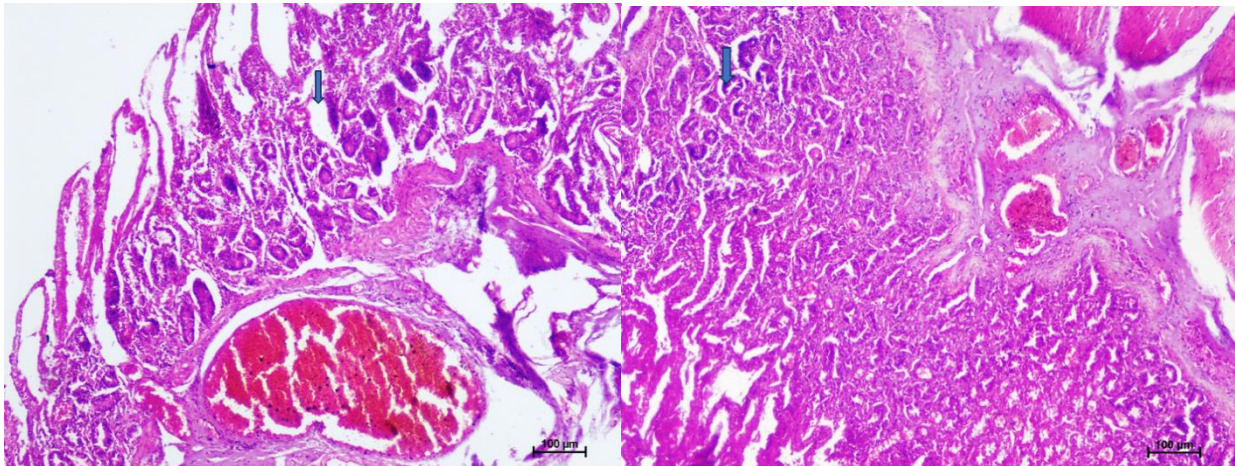
Figures 12 and 13: Ballooning degeneration of epithelium on the tongue. High infiltration of inflammatory cells is noted. H&E 100X



In the gastrointestinal tract, the duodenum showed severe mucosal changes including widespread villous

atrophy, increased crypt depth, and villous epithelial desquamation. Necrosis and severe infiltration of mononuclear inflammatory cells in the submucosa were evident. Similar lesions were also observed in the jejunum and ileum (Figures 14 and 15). Sporadic cases of examined buffalo and cattle displayed lymphocytic enteritis in the form of leukocytic cell infiltrations of the intestinal villi [1].

Figures 14 and 15: Duodenum shows severe mucosal changes including widespread villous atrophy, increased crypt depth, and villous epithelial desquamation. Necrosis and severe infiltration of mononuclear inflammatory cells in the submucosa are evident. Similar lesions were also observed in jejunum (figure 15) and ileum. H&E 100X



In the northeastern region of India, foot-and-mouth disease (FMD) in Mithun is commonly found in various areas of Nagaland, Arunachal Pradesh, Manipur, and Mizoram. The prevalence of FMD is particularly high in the state of Arunachal Pradesh (personal communication). During our present study, the serological prevalence of 'O' antibodies in the Mithun population was found to be quite high (66%), confirming their exposure to the Mithun population and the circulation of FMDV in the studied area. Although the investigation of other serotypes was not conducted, proper research is necessary to fully comprehend and assess the extent of FMDV infection. From 1974 to 1997, during FMD outbreaks in Arunachal Pradesh, Nagaland, and Assam, a morbidity rate of 22.90% and a mortality rate of 6.5% in Mithun were recorded [5]. Moreover, during the 1994 to 1995 FMD outbreak in Arunachal Pradesh, a total of 6239 Mithun from various villages were affected, with a mortality rate of 13%, attributed to FMD virus type Asia 1 [37]. Recently, Rout, et al., 2017 reported the prevalence of FMDV Non-Structured Protein-Antibodies in semi-domesticated Mithun, yak, and their hybrids in Arunachal Pradesh [29]. Additionally, FMDV infection in Mithun has been reported from Bannerghatta Biological Park, Bangalore, and Calcutta Zoo in India [8, 16]. In neighbouring countries like Bangladesh, FMD viral serotypes O, A, and Asia 1 were reported in 62.2%, 14.4%, and 17.8% of cases, respectively, using multiplex PCR [18]. The FMDV serotype O found in Nagaland was reported to have a close relationship with the Bangladesh isolates [7].

The study [30] investigated serotyping and genomic detection methods, specifically genomic RT-PCR and multiplex-PCR, in cattle and buffalo calves naturally infected with the Foot-and-Mouth disease virus. Necropsy examinations revealed myocardial lesions resembling a 'tigroid heart appearance' in all cases. Additionally, various organ-specific lesions were observed, including vesiculo-ulcerative stomatitis, pulmonary oedema, petechial haemorrhages, endocrine oedema, and gastroenteritis. Histopathological

analysis uncovered vesicles and ulcerations in the stratified squamous epithelium of the tongue, acute necrotizing myocarditis, lymphoid depletion in lymphoid tissues, hepatitis, pancreatitis, thymic hyperplasia, thyroiditis, adenitis, and enteritis. Between December 2016 and January-March 2017, Egypt experienced outbreaks of Foot-and-Mouth Disease (FMD). To understand the serotypes responsible for these outbreaks and gather information on the virus's effects, a cross-sectional study was conducted. Postmortem tissue and clinical samples were collected from deceased and infected animals, including oral swabs, vesicular fluids, and blood. Pathological examination revealed typical FMD lesions such as vesicular and erosive lesions on epithelial tissues, along with non-suppurative lymphoplasmacytic myocarditis. Phylogenetic and sequencing analyses identified FMDV serotype O, EA-3 topotype, VP1, as the prevalent serotype responsible for the observed pathological alterations and high mortality rates in young calves, adult cattle, and water buffalo [17]. Myocarditis is associated with foot-and-mouth disease in suckling calves [2]. They recorded that all the calves with myocarditis were younger than 2 months old, suggesting that myocarditis caused by FMD is mainly found in very young suckling calves. Another study supports the existence of neurodegeneration induced by FMDV infection in buffalo calves [6].

Presently FMD diagnosis is being carried out using techniques such as virus isolation (VI), Sandwich-ELISA (S-ELISA), Liquid-Phase Blocking ELISA (LPBE), Multiplex-PCR (m-PCR), and indirect ELISA (DIVA), and real time-PCR can be used for detection of antibody against nonstructural proteins. Nucleotide sequencing for serotyping, microarray as well as recombinant antigen-based detection, biosensor, phage display, and nucleic-acid-based diagnostic are on the way for rapid and specific detection of FMDV. Various pen side tests, namely, lateral flow, RT-LAMP, Immunostrip tests, and so forth. are also developed for the detection of the virus in field conditions [21]. Foot-and-mouth disease (FMD) is endemic in India, where the circulation of serotypes O, A, and Asia1 is frequent [15]. Based on novel multiplex real-time RT-PCR, a study detected and conducted serotyping of foot-and-mouth disease virus serotypes O, A, and Asia1 from the field samples [9]. In central Ethiopia, From the outbreak investigation, 28.8% (n = 378) of cattle showed signs and lesions suggestive of FMD, and 34 samples were subjected to virus isolation. Twenty-eight of these cultures exhibited cytopathic effect (CPE) and were serotyped as O, A, and SAT 2 FMD viruses [13, 36]. A 2019 study detected antibodies against five FMDV serotypes (O, A, SAT1, SAT2, and SAT3) by solid phase competitive ELISA with combinations of two or more serotypes being common [12]. Of the 21 FMDVs that could be isolated 19 were sequenced and 18 were confirmed as SAT2 (lineage VII) while one was characterized as serotype O (EA-3 topotype). Molecular Detection of Bovine FMD Virus from Outbreak Cases in the Aba'ala District of Afar Region, Ethiopia by universal primer was also done [13] and the serotype identified was SAT-2 FMD virus. Out of 27 clinical samples tested by conventional RT-PCR, only 12 FMDV samples were found to be FMDV positive by universal primers [13].

Though veterinarians of these regions have carried out several rounds of FMD vaccination in Mithun, with different formulations of the virus by different companies, still vaccination was not successful because of the difficulty in vaccinating all the animal population in a particular area. This is because the Mithun are reared in a semi-wild manner and spend most of their time foraging in the jungle, so it becomes very difficult to catch and administer the vaccine. Besides FMD is spread through direct contact or inhalation of virus aerosols, which ultimately leads to difficulty in controlling the disease. However, with proper treatment, and hygienic and quarantine measures, controlling the disease may be possible in future.

4. Summary and conclusion

In this study, 213 Mithun animals in Nagaland were tested for FMDV serotype O antibodies using blocking ELISA, with 144 (67%) testing positive and 69 (32%) negative. Serotyping of FMD virus from clinically affected Mithun in Phek district revealed positive results for FMDV serotype O using both sandwich ELISA and mRT-PCR methods. Post-mortem examinations on six deceased Mithun revealed significant pathology associated with FMD. Severe interstitial myocarditis, necrosis, and inflammation were observed in the heart, while the liver showed focal coagulative necrosis and inflammatory infiltration. Lung examination showed congestion and interstitial infiltrates of mononuclear cells, and the gastrointestinal tract displayed severe mucosal changes.

The problems in the control of disease in Mithun largely depend on the system of rearing. Since the animals are reared in a free-range system, health care and management are the difficult task. And since Mithun is not included in the nationwide disease control program, the vaccine coverage is still low. In many instances, the animals are found dead in the jungle before care can be taken. Therefore, it can be assumed that if proper healthcare management can be improvised at the time, the problems may be minimized. Maintenance of cold chain in handling and storing of vaccine is also a challenge. Moreover, this is also the first report on the pathology of FMD in Mithun. The genetic and molecular basis of the immune response against FMD infection and other microbial diseases in Mithun is largely unknown; even though Mithun is considered to be sturdy and immunologically strong against many of the diseases. Hence, studies on the molecular level concerning immunity and its related gene expression during the course of infection/vaccination may help in a better understanding of the disease. In addition, a comparative study on the vaccine delivery system and the vaccine preparation will help formulate an effective vaccination strategy for Mithun.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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