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Original Article

Molecular characterization and pathology of field isolates of foot-and-mouth virus in Swiss albino mice

Caracterização molecular e patologia de isolados de campo do vírus da febre aftosa em camundongos albinos suíços

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Abstract

Foot-and-mouth disease is responsible for severe economic losses to the livestock industry of Pakistan. This study aimed to use Swiss albino mice as a cost-effective experimental animal model to study different immunological and histopathological aspects of FMDV instead of natural targeted species like cattle. After isolation of field isolates FMDV on BHK-21 cell line, biological titer of the virus and mice infectious $dose_{50}$ was calculated. Virus was injected in 45 Swiss albino mice (group A) through intraperitoneal route. The gross, histopathological and immunopathological lesions in heart, trachea and lungs were recorded at different day's intervals. Histopathologically, the heart showed congestion, hemorrhages and necrosis of cardiac muscles. Trachea showed deciliated epithelium and lungs showed hemorrhages, bronchial edema and alveolar emphysema. Immunohistochemical studies revealed the presence of virus in cardiac muscles, tracheal and bronchial epithelium and alveolar lumen. The findings evoked a thought that laboratory animals could be an alternative to large animals to meet budget limitations for further research on foot-and-mouth-disease.

Keywords: BHK-21 cell line, histopathology, immunohistochemistry, serotype O, Swiss albino mice.

Resumo

A febre aftosa (FMD) é responsável por graves perdas econômicas para a indústria pecuária do Paquistão. Este estudo teve como objetivo usar camundongos albinos suíços como um modelo animal experimental de baixo custo para estudar diferentes aspectos imunológicos e histopatológicos do FMDV em vez de espécies naturais como o gado. Após o isolamento dos isolados de campo do FMDV na linhagem celular BHK-21, calculou-se o título biológico do vírus e a dose infecciosa dos $camundongos_{50}$. O vírus foi injetado em 45 camundongos albinos suíços (grupo A) por via intraperitoneal. As lesões macroscópicas, histopatológicas e imunopatológicas no coração, traqueia e pulmões foram registradas em diferentes intervalos de dias. Histopatologicamente, o coração apresentava congestão, hemorragias e necrose dos músculos cardíacos. A traqueia apresentava epitélio deciliado e os pulmões apresentavam hemorragias, edema brônquico e enfisema alveolar. Estudos imuno-histoquímicos revelaram a presença de vírus em músculos cardíacos, epitélio traqueal e orçamentárias para pesquisas adicionais sobre a febre aftosa

Palavras-chave: linhagem celular BHK-21, histopatologia, imuno-histoquímica, sorotipo O, camundongos albinos suíços.

1. Introduction

Livestock is considered poor man's wealth in Pakistan and this sector plays a pivotal role in poverty reduction. The role of different infectious and non-infectious diseases cannot be negated, affecting the functioning of livestock. Foot-and-mouth disease (FMD) is one of those problems responsible for a significant loss to the cattle industry. The disease is highly contagious to split-hooved animals including cattle, water buffalo, sheep, goats, pigs, and wild animals. It is characterized by high fever and the development of vesicles in the oral cavity, hooves and teats of the affected animals. Rupture of these vesicles may induce severe pain and lameness in animals. It spreads rapidly to the affected animals through contact with contaminated farming equipment, vehicles, clothing, and feed. The effective control of the disease is vaccination, trade limitations, quarantines, authoritarian surveillance

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and culling of both infected and healthy (uninfected) animals (Stenfeldt et al., 2018).

The causative agent of Foot- and- mouth- disease is Aphtho virus, an RNA virus belonging to Picornaviridae family. In Pakistan, A, O and Asia 1 are reported out of seven serotypes. These serotypes have more than 100 sub-serotypes having no or little cross-protection against each other. The mutation is also a predominant character of FMDV. That is why vaccination program often fails to give the desired results (Jamal et al., 2021). Being endemic, per annum losses due to FMD in Pakistan are more than USD 692 million, exhibiting the ruinous effects of disease on the country's economy (Abubakar et al., 2022). The pathogenesis of FMD has been widely studied in cows, buffaloes and pigs. Laboratory mice, rats, guinea pigs and chickens have been infected experimentally but naturally, they are not the targeted species (Alexandersen and Mowat, 2005). Studies are available on phylogenetic analysis of virus in different areas of Pakistan but much research has to be done on virus-host interaction (Habiela et al., 2014). For many viral diseases like Ebola (Bray et al., 1998), Hantaviruses (Wichmann et al., 2002), Herpes Simplex (Hayashi et al.,1986) and Venezuelan Equine Encephalitis (Jackson et al., 1991), mice had been proven to be equally efficient experimental animals to study these diseases and ultimately their treatment and vaccination.

This study aimed to molecular characterizes the field circulating FMD virus and its subsequent cytopathic effect was observed by *in vitro* adaptation on BHK-21 cell line. Histopathological alterations and tissue tropism of FMDV in mice's vital organs were also observed through immunohistochemistry to manifest that the virus would not change its dynamics in an unnatural host and mice can be a cost-effective part of research activities leading to the development of different vaccines, testing their safety, efficacy and potency, which is laborious and expensive in large animals.

2. Materials and Methods

2.1. Sample collection and processing

In the present study, the samples of epithelia of oral cavity and vesicular lesions of interdigital spaces of feet of affected cattle (n=17) were collected from different areas of Lahore and Kasur districts, Punjab province, Pakistan from October 2020-April 2021. The samples were placed in the transport medium containing equal amounts of glycerol and 0.04 M phosphate buffer (pH 7.2-7.6) for further processing in the cell culture laboratory of Foot and Mouth Disease Research Center, Lahore, Pakistan. For virus isolation, 20% tissue homogenates were prepared in Dulbecco's Modified Eagles Medium (DMEM, Caisson Labs, USA) in sterile pestle and mortar. The tissue suspensions were centrifuged at 3000 rpm for 20 minutes and supernatant was collected in 15 ml falcon tubes containing 1× Antibiotic-Antimycotic solution (Penicillin, Streptomycin and Amphotericin-B) and stored at -80^{0}C for further use (Rafique et al., 2020).

2.2. Growth of FMD virus on BHK-21 cell line

BHK-21 cell line was maintained in Glasgow Eagle's Medium (GMEM, Caisson Labs, USA) with 10% fetal calf serum (Capricorn Scientific, Germany) in 25 ml cell culture flasks at 37 ^{o}C. After formation of a confluent monolayer of cell line in 24 hours, 2% of virus stock prepared in DMEM was spread homogenously on the cell line and placed in incubator at 37^{o}C. The cytopathic effects (CPE) were noted for 24-72 hours. After observing 90% cytopathic effects on BHK- 21 cell line, the virus culture was harvested and after 10 continuous passages the virus was stored at -80°C for further use. In case, no CPE was found, the cells were frozen and thawed three times. Then the supernatant was again inoculated to the new cells and examined for CPE. Negative samples were discarded (Shahiduzzaman et al., 2016)

2.3. Molecular identification of FMDV

The positive harvested material was subjected to reverse transcription polymerase chain reaction (RT-PCR) for confirmation of FMD virus serotypes. The extraction of viral RNA from the cell-isolated virus was done using Accuzol™ Extraction Kit, Bioneer, Korea (k-3090). The positive samples were subjected to RNA extraction, cDNA synthesis and RT-PCR described by Reid et al. (2014). RNA extraction was done using TRIzol method. The extracted RNA was reverse transcribed for the synthesis of cDNA. RT-PCR was performed in thermocycler (Applied Biosystems, United States) with previously reported primer set SA (F) (5´-ACC ACC TCC ACA GGT GA-3´) and SA (R) (5´-CAA AAG CTG TTT CAC AGG TGC-3´) were used to amplify VP1 region of FMDV genome (Kanwal et al., 2014). 13 µl of Master mix was added in thin walled PCR tubes with 5 µl of cDNA. Added 1ul of forward and reverse primers each and finally added 5µl of Nuclease Free Water. PCR was performed in thermocycler with conditions of 95C^o for 4 min (Hot Start), 95^{o}C for 45 sec (Denaturation), 56^{o}C for 45 sec (Annealing), 72C^o for 55 sec (Extension) followed by 30 cycles and 72^{o}C for 4 mins (Final Extension). After confirmation of RT-PCR products according to Sanger dideoxy sequencing method, the nucleotide sequences were submitted to National Center for Biotechnology Information, a public database (http/www.ncbi.nlm.nih.gov/GenBank) to retrieve genome sequences. The nucleotide sequences were aligned by ClustalW using BioEdit version 7.2.5. MEGA-6 software was used for the evolutionary analyses of VP1 coding sequences (Tamura et al., 2013). The Neighbor-Joining method was applied to construct a phylogenetic tree using the VP1 coding sequences of BHK-21 adapted field isolates from the GenBank database. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. For Gaps/missing data "pairwise deletion" option was chosen.

The virus was titrated using two-fold dilution in 96-well flat-bottom cell culture plates and Reed and Muench method (1938) was used to calculate the tissue culture infective $Dose_{50}$ ($TCID_{50}$). Different dilutions of the virus i.e., 10^{-2}, 10^{-3}, 10^{-4} 10^{-5} were injected intraperitoneally in 2 weeks old Swiss albino mice (n=20) to calculate Mice Infectious $dose_{50}$ (MID_{50}). The observations were recorded

till 50% of animal infected showed oral and feet lesions. MID_{50} was calculated following Reed and Muench (1938).

2.4. Experimental design

Experimental Swiss albino mice, each 2-3 weeks old (n=90), were obtained from the experimental animal's colony of Foot-and-Mouth Disease Research Center, Lahore. These animals were reared under standard conditions with *ad libitum* supply of food and water. The animals were divided in two different groups A & B (45 animals per group). The mice of group A were inoculated with 0.1 ml of $10^{6.0}$/ml MID_{50} of FMD virus, serotype O intraperitoneally and the control animals of the B group were injected with PBS at the same dose rate.

2.5. Clinical and histopathological observation

The development of the lesions was recorded for 28 days. The necropsy of diseased or dead mice was performed on 1, 2, 3, 14 and 28 days post-inoculation. The organs i.e., heart, trachea and lungs were collected and preserved in 10% neutral buffered formalin for histopathology (Suvarna et al., 2019) and immunohistochemistry (Imran et al., 2019).

3. Results

3.1. Adaptation of virus on BKH-21 cell line

Twelve tongue epithelial tissues and five feet tissues samples were collected from different outbreaks. The naturally affected cattle showed representative clinical symptoms of FMD like the presence of vesicles on the oral cavity, teats, lameness and a decrease in milk production. Out of twelve tongue epithelial samples, eight were successfully adapted on the BHK-21 cell line. They showed typical cytopathic effects like cell swelling, rounding and detachment from the surfaces of the culture flasks (Figure 1b-1c). Only one sample from the feet lesions was adapted on BHK-21 cell line (Table 1).

3.2. RT-PCR and phylogenetic analysis

The results of RT-PCR were found positive for four isolates adapted on BHK-21 cell line by amplifying the product size (639bp) (Figure 1a). Four positive samples were grouped into two isolates based on high similarities of VP1 coding regions. The partial genome sequences of the current study were submitted to the NCBI-GenBank database with the accession numbers MW588802 and MW057925. Using nucleotide sequences (639bp), the phylogenetic tree analysis suggested that the FMD viral isolate were classified as lineage O/ME-SA/Ind-2001e. The variation in the 1D of O/ME-SA/Ind-2001e showed a 98% to 100% similarity index with the isolates of pool 2 and 3 countries (Figure 2).

3.3. Biological titration and calculation of mice infectious dose$_{50}$ (MID_{50})

The obtained biological titer of the FMD virus was $10^{6.3}$/mL and MID_{50} of the virus in mice was recorded as $10^{6.0}$/ml.

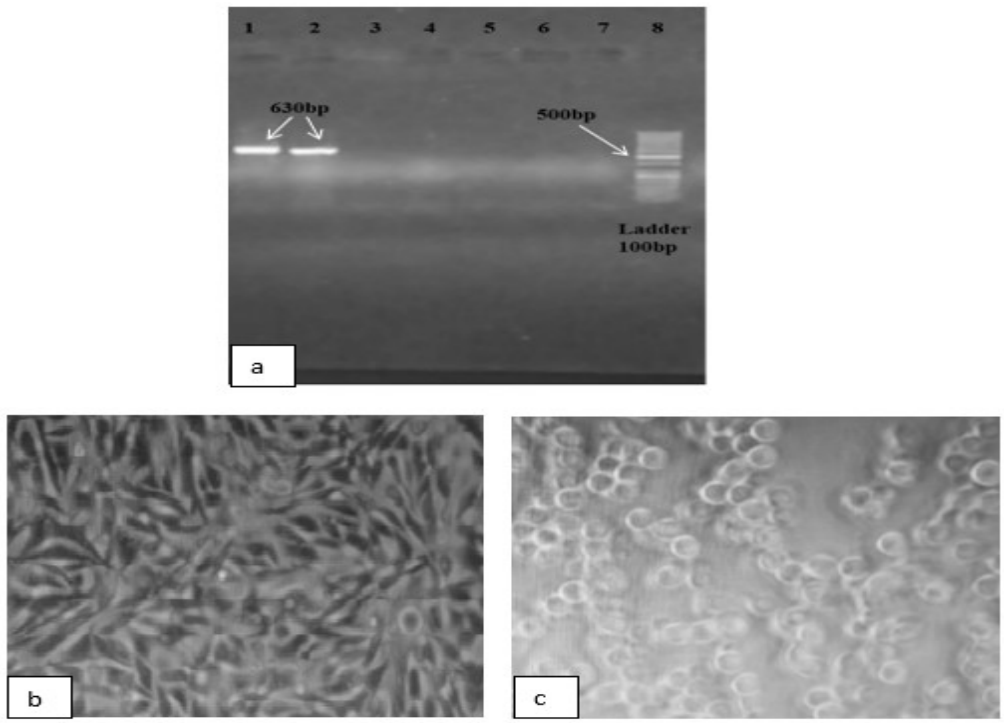

Figure 1. a: PCR positive bands of foot-and-mouth disease virus Serotype O; b: normal BHK-21 cell line; c: cytopathogenic effects of FMDV on BHK-21 cell line.

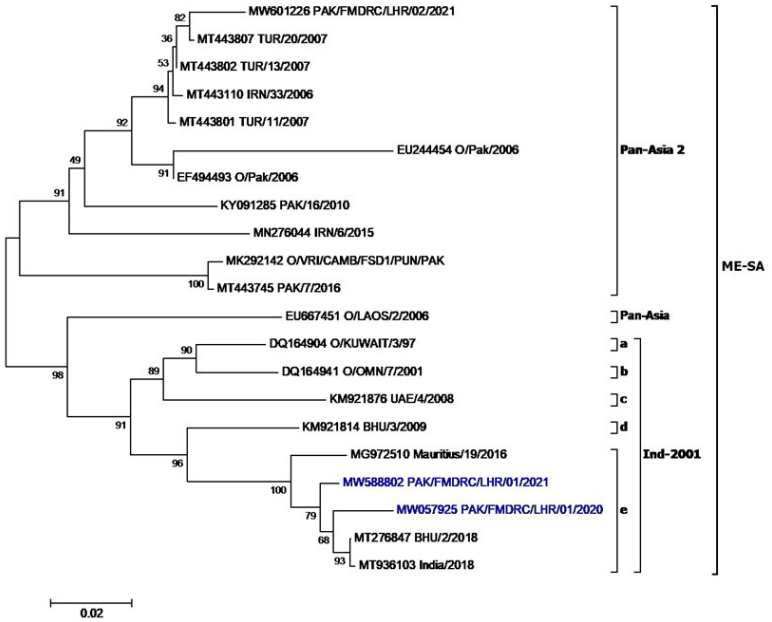

Figure 2. FMDV O gene based phylogenetic analysis using neighbor-joining method with 1,000 bootstrap replicates with the help of Tamura model through MEGA 6 software. Blue font isolates belonged to current study.

3.4. Clinicopathological observations

After 24 hours of experimental infection, feet and oral lesions were observed in 3 mice. After two days post-infection (dpi), the death of seven mice were observed having paralysis and vesicles on the feet. Upon necropsy of dead mice, organs were collected. Grossly, the heart of the affected animals was enlarged and hemorrhagic. The trachea and lungs of the affected animals were found congested. After three days, five mice died. The vesicles and erosive epithelium were present on the feet and around the lips of the affected animals, respectively (Figure 3a-3b). Tissue samples were collected from the animals that died after 3rd dpi. The heart, trachea and lungs showed severe hemorrhages (Figure 4 a-4c). Next tissue sampling was done on 14th and 28th dpi. During the third week, only one mouse died and after that, up to 28 days, no death was recorded. The clinical signs (Table 2) and gross changes in the organs were recorded during the whole period of the experiment. All the mice in the control group remained normal throughout the study.

3.5. Histopathological observations

The heart showed slight congestion on 24 hours post-challenge. On the 2nd dpi, severe congestion and haemorrhages in the heart muscle were observed. After the 3rd dpi, necrosis of the cardiac muscle fibers was observed in the animals of group A (Figure 5a). After 14th and 28th dpi, no definite lesion was recorded in the heart compared to the control group except for some slight congestion. (Table 3). The trachea was also affected and on the 3rd dpi, the tracheal epithelium was sloughed off (Figure 5b). The severity of the lesions in the trachea decreased with time but congestion and haemorrhages were observed even on 28th dpi (Table 4). After two days of the experimental challenge, the lungs of dead animals showed interstitial pneumonia and emphysema. Necrotic changes were present in bronchi. Mononuclear cells infiltration was present in the alveolar epithelium. The same lesions persisted in mice that died at 3rd dpi (Figure 5c). During the study until 28 days, the lung was found congested with emphysema

Table 1. Virus isolation from FMDV field isolates on BHK-21 cell line.

No. of Samples	Source	Samples adapted on BHK-21 cell line	%age Adaptation
12	tongue epithelia	8	66%
5	feet tissues	1	20%

Table 2. Symptoms recorded in mice at different days intervals.

Days post infection	Symptoms recorded in no. of mice		No. of deaths
	Paralysis	Foot and oral cavity abscesses	
1	0	3	0
2	7	2	7
Up to 4th	0	5	5
Up to 14th	0	1	1
Up to 28th	0	0	0

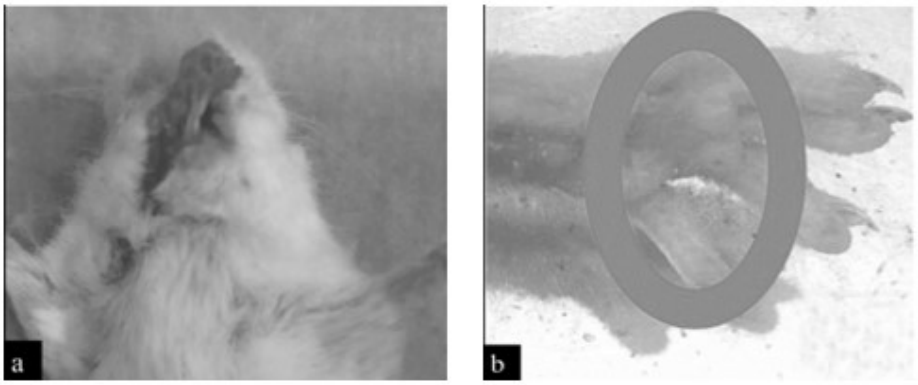

Figure 3. Oral erosive epithelium (a) and foot vesicles (b) of mouse at 3rd day post challenge.

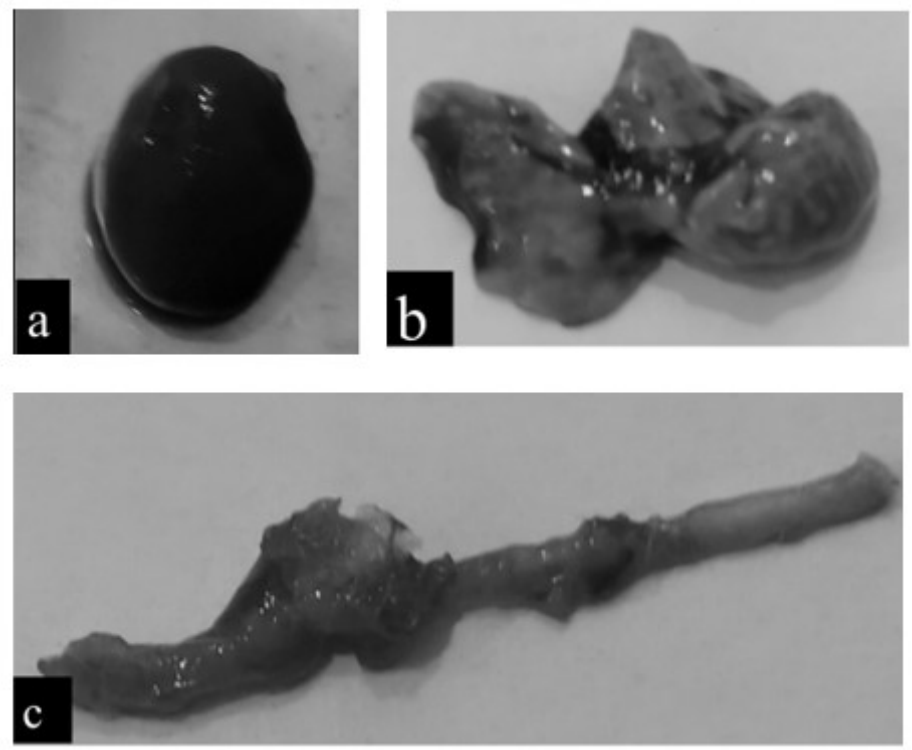

Figure 4. Hemorrhagic heart (a), lungs (b) and trachea (c) of mice at 3rd day post challenge.

Table 3. Histopathological lesions scoring in heart of mice.

Days post infection	Lesion scoring			P-value
	Congestion/ hemorrhage	Inflammation	Necrosis	
1	2	1	0	
2	3	1	0	
Up to 4th	3	2	2	**0.001**
Up to 14th	2	1	2	
Up to 28th	1	0	0	

Confidence interval 95%.

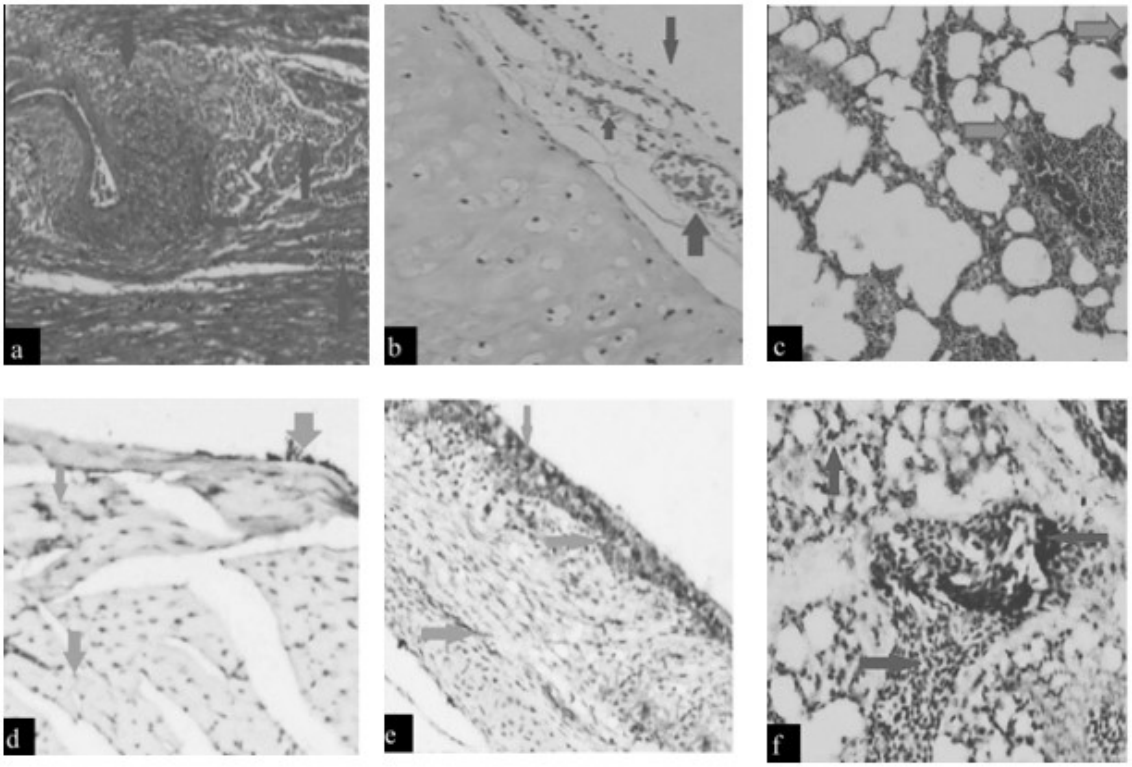

Figure 5. a: photomicrographs of heart longitudinal section, arrows showed congestion at 3rd day post infection; b: longitudinal section of trachea, arrowhead showed congestion, deciliated epithelium and polmorphonuclear leukocytes presence in mucosa at 3rd day post challenge; c: longitudinal section of lungs showed emphysema, congestion, inflammation and bronchial edema at 3rd day post infection (H& E Stain 10x10X); d: photomicrographs with arrow heads showed antigen spread in the myocardial fibers of heart; e: viral load in the sloughed up epithelium, mucosa and submucosa of trachea; f: arrow heads showed antigen load in the bronchioles and alveolar walls of lungs (IHC Stain, 10x10X).

Table 4. Histopathological lesions scoring in trachea of mice.

Days Post Infection	Lesions Scoring			P-value
	Congestion/ hemorrhage	Deciliation of epithelium	Inflammation	
1	1	1	1	
2	2	2	2	
Up to 4th	2	3	2	**0.001**
Up to 14th	1	0	0	
Up to 28th	0	0	0	

Confidence interval 95%.

in few animals. (Table 5). Lesion scoring was done as 0, 1, 2, 3 based on the severity of different histopathological lesions observed according to the ordinal method described by Gibson-Corley et al. (2013) (Table 6).

3.6. Immunohistochemical observations of FMD virus in tissues

After two days of experimental challenge, antigen spread was observed in the cardiac muscles and tracheal epithelium. After three days, antigen diffused rapidly in the heart, trachea and lungs. The lumen of the bronchioles and alveoli of the lungs were affected by the viral load. (Figure 5e, 5f and 5g, respectively). From 14 days onwards till the end of the study, virus presence was not identified in these tissues.

3.7. Statistical results

The lesions scoring was analyzed statistically by Non-parametric, Mann-Whitney test and the results were found significant with P-value<0.05 (Cruz et al., 2011).

Table 5. Histopathological lesions scoring in lungs of mice.

Days post infection	Lesions scoring			P-value
	Congestion/ hemorrhage	Interstitial pneumonia	Inflammation/ necrosis	
1	2	0	1	
2	2	2	2	
Up to 4th	3	2	3	**0.002**
Up to 14th	2	1	1	
Up to 28th	1	0	0	

Confidence interval 95%.

Table 6. Gross lesion scoring.

No change 0	<25%
1	26–50%
2	51–75%
3	76–100%

3.8. Ethical handling of the animals

For ethical handling, restraining and scarifying the animals, standard protocols were followed and permission was granted by the ORIC Division, University of Veterinary and Animal Science, Lahore.

4. Discussion

FMD is one of the significant devastating livestock diseases and is liable for heavy monetary losses to the cattle industry. A lot of research work has been conducted on this problem worldwide including Pakistan. The primary research areas were to explore the serotypes of FMDV emphasizing its phylogeny. This work aimed to study the virus-host relationship in the experimental animals leading towards a thought whether the Swiss albino mice could replace the target species for further investigation towards the pathogenesis of the disease, its prophylaxis in vaccine development, vaccine safety and potency testing. The mice model used in this project showed that tissue tropism of FMD virus in mice had no remarkable difference from large animals. The cost of a research project would also reduce to meet budget complications, time and labor (Lee et al., 2016).

For the current study, the suspected cattle's tongue epithelia and feet tissues were collected from high prevalence areas of district Kasur and Lahore, Punjab province, Pakistan. The tongue epithelium was successfully adapted on the BHK-21 cell line as compared to feet tissues. These results were in accordance with Shahiduzzaman et al. (2016) who claimed that epithelial tissues were more accessible to adapt on cell lines for FMD virus isolation than feet tissues. The confirmation of the isolated virus was done through RT-PCR. The phylogenetic analysis of FMD virus showed lineage "Ind-2001", sub-lineage "e" with topotype "ME-SA," which was recently documented in Pakistan by Hicks et al. (2020) and Jamal et al. (2021). In addition to previously reported Pan-Asia II (Abubakar et al., 2022), the emergence of a new lineage of FMD in different areas of Pakistan suggested that regular surveillance, control of animals movement and effective vaccination should be part and parcel for the different eradication plans continuing in the country. After determining the biological titer and mice infectious dose$_{50}$, the virus was injected intraperitoneally (IP) in 2-weeks old Swiss albino mice.

According to Arzt et al. (2011), disease pathogenesis in adult mice depends on the mouse strain and FMDV strain just like the natural host. Experimentally, the disease incubation period is 24 hours (OIE, 2018) and the same was observed in the current study. Salguero et al. (2005) observed lesions on the lips of mice after 24 hours of infection and similar findings were observed in the present results. The erosive epithelium on the lips and vesicles on the footpads of mice were similar to those reported in large animals by Islam et al. (2017) but after that, the mice showed a recovery stage as the death rate declined. In the first 48 hours, out of 45 experimental animals, seven died and after 72 hours, five mice died. However, in the 2nd and 3rd weeks, deaths of five animals were observed and in the last week, the other animals remained alive. The reason behind fewer deaths with increasing time period might be the age factor. These observations were in line with the previous studies of Stenfeldt et al. (2016) that the animals' immunity increased against the disease with age.

The gross pathological changes in the myocardium of young calves and death was often attributed to myocarditis in addition to the rare manifestation of FMDV-associated deaths in adults known as 'malignant FMD, characterized by lesions and degeneration of the myocardium (Hussain et al., 2020). Similar gross lesions were observed in mice in the 1st week of the study period and myocardial necrosis was observed in histopathology on 3rd dpi. These changes are age-related as $\alpha v\beta 3$ integrin receptors in cardiac muscles respond to age-related diseases. FMD virus receptors in cardiac muscles are vulnerable in young age. In large animals, gross and microscopic lung lesions showed severe congestion, interstitial pneumonia and cellular infiltration (Ranjan et al., 2016). Identical observations were recorded during this study in mice. In the current study, histopathological changes seen in the interlobular septa and interstitium of lungs in mice attributed that

the death in mice could be due to cardiac failure and pulmonary dysfunction. Similarly, all the vital organs like the spleen, kidneys, liver, and pancreas are disrupted due to cardiac changes.

To the best of our knowledge, this is the first study of Immunohistochemical changes in vital organs of mice due to FMDV so no relevant data was available to compare the results. In a research trial, Arzt et al. (2011) claimed that during infection of FMD, antigen resided firstly in the respiratory organs of the cattle. The current data also suggested that the FMDV was mainly present in mice's lungs and tracheal tissues. This study would also be helpful in the future for immunohistochemical studies in mice related to disease pathogenesis.

5. Conclusion

The molecular characterization of foot-and-mouth disease virus in this study showed the newly introduced lineage of FMDV in Pakistan. Vaccine matching studies should be conducted routinely by the concerned authorities to eradicate the disease effectively. For vaccine safety and potency trials, laboratory animal species should be used as cost-effective replacements for large animals. The data generated from this study was strong enough to understand pathogenies of disease in laboratory animals. This cost-effective study also opens a gateway to further analyze the disease mechanism in other laboratory experimental animals like guinea pigs and rabbits. This experimental laboratory model can be further used to study different factors related to age resistance in FMD. Furthermore, in laboratory animals role of the pancreas and different hormones can be studied for diabetes and panting in large animals after recovery from FMD.

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