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Research Article

LITTER LOSS IS AN INDICATION OF CANNIBALISM IN FOOT-AND-MOUTH DISEASE VIRUS INOCULATED BALB/C MICE

Rajeev Ranjan[1]*, Jitendra K Biswal[1], Saurabh Chawla[2], Jajati K Mohapatra[1]

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ABSTRACT: BALB/c mice are commonly used in biomedical research globally. Despite a high mortality, due to infanticide and/or cannibalism, little is known about the way pups die and may have an impact on 4 'R's (replacement, reduction, refinement, rehabilitation) and research. This report provides about litter loss, which could be an indication of cannibalism in foot-and-mouth disease (FMD) virus inoculated laboratory BALB/c mice. Animals used in this experiment were BALB/c strain suckling pups in two groups (Gr). In Gr-I, 100 µl of FMD virus ($10^{6.3}$ $TCID_{50}$/ml) was inoculated intraperitoneally (IP) in 3-days-old suckling mice, while Gr-II was kept as the control without any inoculation. In Gr-I, on 4th day post partum, six pups were found dead, one pup was missing, and one pup was partially cannibalized. However, in Gr-II, neither infanticide nor cannibalism was observed. Lungs and kidney tissues were collected from partially cannibalized pups and tested positive for FMD virus (FMDV) serotype 'O'. The FMDV infection associated death of suckling mice could be confirmed by pathological findings also. Infanticide is rare in mice, but cannibalism of dead pups by adult mice is often observed, and this finding is supported by current observations. In the present study, pups were not killed by the dam, but they died due to inoculation of FMDV, and which has been confirmed by the detection of the FMDV genome, virus isolation, and other pathological findings suggesting FMDV infection. In line with earlier reports, a female mouse eating dead pups has also been observed in this study.

Keywords: BALB/c mice, Cannibalism, Foot-and-mouth disease virus, Litter loss, Suckling mice.

INTRODUCTION

Foot and mouth disease (FMD) is a contagious and infectious disease that affects cloven-hooved animals such as cattle, buffalo, goats, sheep, and pigs, as well as over 70 wild species [1]. It is caused by FMD virus (FMDV), which belongs to the genus *Aphthovirus*, a positive sense single-stranded RNA virus. Clinically FMD has fever, lameness, foamy salivation, and vesicular lesions on the tongue, muzzle, feet, and teats [2, 3]. In general, cows, buffaloes, pigs, and other animals have been widely used to study the pathogenesis of FMD virus (FMDV), but laboratory animals such as mice, rats, guinea pigs, and chickens have been used as experimental animals but have not been considered as targeted species [4]. According to previous report, mice have been used to study many viral diseases for treatment and vaccination [5, 6].

C57BL/6J and BALB/c mice strains are commonly used in biomedical research. Pre-weaning mice mortality is a major concern in animal facilities around the world [7], and many times dams may mutilate or cannibalise their pups after birth due to multiple reasons. Earlier reports stated that cannibalism behaviour is primarily observed in litters with malformed or defective pups [8], but this has not been experimentally demonstrated.

Despite a high mortality rate, little is known about how pups die, which may have an impact on the four 'R's (replacement, reduction, refinement, and rehabilitation) by increasing the number of pups needed for research or an indirect increase in number of breeding pairs needed

[1]*ICAR-National Institute on Foot and Mouth Disease, International Centre for Foot and Mouth Disease, Arugul, Bhubaneswar-752050, Odisha, India.*
[2]*National Institute of Science Education and Research (NISER) Jatni, Bhubaneswar-752050, Odisha, India*
**Corresponding author, e-mail: Rajeev.Ranjan1@icar.org.in*

to get those numbers. There are numerous factors that can influence the incidence of cannibalism in different species, including pigs, rabbits, hedgehogs, hamsters, ground squirrels, rats, and mice. Some strains of rat and mice are more prone to cannibalism which includes most used strains such as C57BL/6 and BALB/c, which may eat up substantial part of their litters [9]. In some of the studies related to loss of litter from one day to the next, or the discovery of some of them dead or partially eaten, and it was assumed that the dam had killed them [10, 11]. Cannibalism causes litter loss in C57BL/6 and BALB/c mice, which can routinely lose more than 30% and 20% of their litters, respectively [12], and it can even reach 50% [13]. It has also been observed in some of the genetically modified mice strains (GEM) [14]. After parturition, offspring are blind, immobile, hairless, and unable to suckle, and they are completely dependent on their parents, particularly the dam, during the first stage of their lives (up to 12-14 days post parturition). During this time, brown adipose tissue (BAT) in suckling mice can generate heat through non-shivering thermogenesis [15, 16].

The most common belief among laboratory mouse caregivers is that dams frequently kill their offspring (infanticide) or eat up the pups (cannibalism). Cannibalism is thought to be common in rodents, but actual cases of infanticide are uncommon [9]. Dams selectively cannibalise malformed, abnormal, defective, and nonviable offspring [8, 17]. It is thought that the dam has some discriminatory ability in identifying such offspring using her senses (tactile, visual, olfactory). Significant numbers of suckling mice dying for unknown reasons are a concern for animal welfare, and identifying the major causes of perinatal mortality in a particular strain is critical for developing preventive strategies for mouse breeding or experimental research. In current report, litter loss has been reported in FMDV inoculated laboratory BALB/c mice, which could be an indication of cannibalism.

MATERIALS AND METHODS

Animals and housing

The necessary numbers of animals (mice and their offspring) were used solely for the current study from IAEC-ICFMD protocol no. P07-02/2022 dated 28.01.2022. All animals were kept in a suitable environment, and experiments were carried out in accordance with committee for the purpose of control and supervision of experiments on animals (CCSEA) guidelines. The animals used in this experiment were adult BALB/c strain mice with their suckling offspring divided into two groups and observed throughout the experiment. The dam and her suckling offspring were housed in a cage together and kept at a temperature of 19-24^{o}C, relative humidity of 50-65%, and a photoperiod of 12 hours. All the animals were given free access to standard food and RO water.

Monitoring of animals

The laboratory attendant kept a close eye on the animals. The lab attendant recorded the time of parturition, and the number of pups delivered, and then kept a close eye on them daily.

Virus inoculation

In group I, 100 µl of FMDV containing a dose of $10^{6.3}$ $TCID_{50}$/ml was administered intraperitoneally (IP) in 3 days old suckling pups, while Gr-II served as a control with no inoculation.

Post mortem inspection

Fresh dead puppies were examined within 4 hrs of being collected. External and internal examinations were performed in accordance with the necropsy protocol. Lung morphology was assessed, and lung float tests were performed alongside liver tissues, which served as a control. Lung and kidney tissues were taken in pairs. The first set of tissue was collected in viral transport medium, 50% buffered glycerin for FMD virus isolation and genome detection, while the second set was stored in optimal cutting temperature (OCT) compound for microscopic examination.

Microscopic examination

Tissue samples viz. lungs were collected in OCT from cannibalized and dead suckling mice (administered with FMDV) and store at -80^{o}C until further processing. Histopathology was performed on tissue samples [18].

Nucleic acid extraction and FMD viral genome detection

PBS 10% was made from tissue kept in 50% buffered glycerin for virus isolation (VI) and nucleic acid extraction for genome detection. Total RNA was extracted from suspension made from lung and kidney tissues as per the manufacturer instruction (QIA amp total RNA mini kit, QIAGEN, Hilden, Germany). Total RNA extracted from lung and kidney tissues were subjected for detection FMD viral genome by RT-mPCR [19] and 5'UTR RT-qPCR [20] with minor modification. In brief, the mPCR mix includes FMDV serotypes O, A, and Asia 1, serotype-specific forward

and one FMDV-specific reverse primer and amplicon size (249 bp, 376 bp and 537 bp specific for serotype O, A, and Asia 1, respectively) were observed. RT-mPCR amplified products were electrophoretically separated and stained with ethidium bromide on a 2 % agarose gel. The 5'UTR RT-qPCR assay was performed in a one-step format using the AgPath-IDTM One-step RT-PCR reagents (Applied Biosystems). 5 µl of Extracted RNA from the tissue sample was amplified in a reaction mixture consisting of 12.5 µl 2 X RT-PCR buffers, 1 µl of 25 X RT-PCR enzyme mixes, 400 nM each of 5'UTR forward and reverse primers, and 120 nM of 5'UTR-probe. The final volume of the reaction mixture was adjusted up to 25 µl with nuclease-free water. The probe targeting the 5'UTR was labelled with 5'-FAM and 3'-TAMARA (Eurofins Scientific). The parameters for RT-qPCR reaction condition start with reverse transcription (RT) step at 45°C for 10 minutes, a RT-inactivation step at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 sec, and annealing/ extension at 60°C for 45 sec. The RT-qPCR assay was performed with CFX96 Touch Real-Time PCR detection system (Bio-Rad).

Virus isolation by chemical transfection

Total RNA was used for FMD virus rescue using the BHK-21 cell line via chemical transfection [21]. In brief, 1g of extracted total RNA was mixed with 2 µl lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) diluted in OPTI-MEM®I (Gibco, Life Technologies, NY, USA) and incubated at room temperature for 20 minutes. These mixtures were transferred to monolayer BHK-21 cells in 24-well plates with 200µl of GMEM overlaid and incubated for 4 hrs. After 4 hrs, 700 µl of GMEM was added and incubated at 37°C for 48 hrs. The entire contents of the wells were then harvested and stored at -80°C. A 200µl harvest aliquot was passed through BHK-21 cells to amplify the virus rescued by chemical transfection for future use.

RESULTS AND DISCUSSION

BALB/c mice are widely used in FMD research for pathogenesis, pathophysiology, vaccinal response,

Fig. 1. Mouse observation. [Observational behaviour of dam and its pups in standard management practicein cage showing healthy appearance of suckling mice and their mouth manipulation by mother].

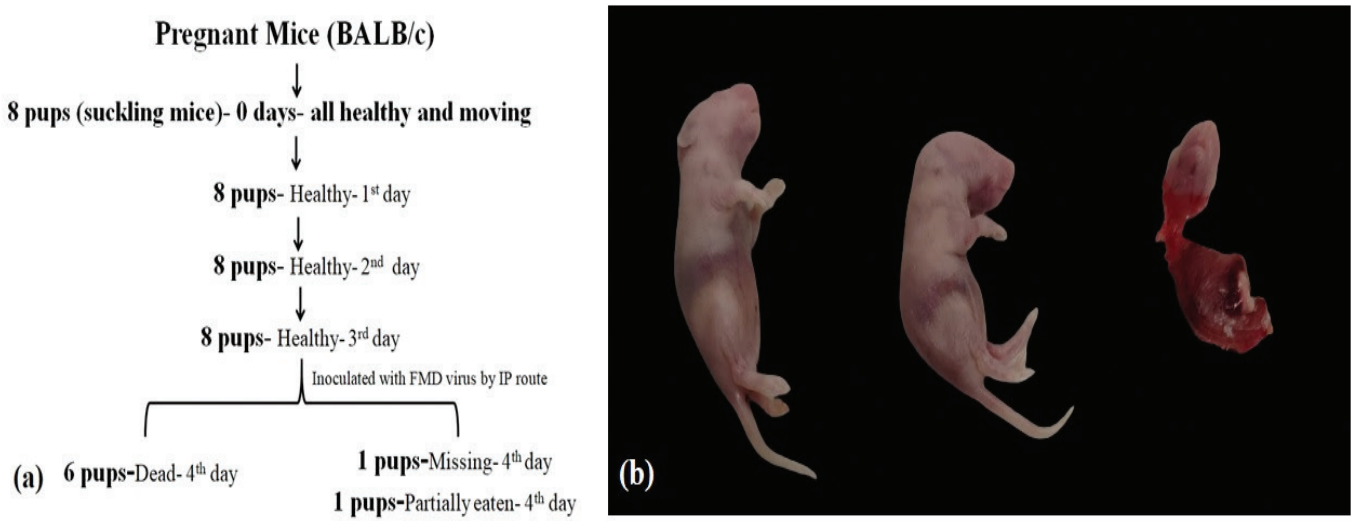

Fig. 2. Observational procedures of dam and its pups. [(a) all the pups were healthy and moving until the third day after birth during the daily cage check process. (b) Dead pups after inoculation of foot-and-mouth disease virus without showing any visible lesion (first two) and last pups were partially eaten by dam (cannibalised pup)].

and to reduce research costs, among other things [22]. However, there is little information available during the experimental process about litter loss, which may affect the outcome and the 4 'R's of laboratory animals. As a result, the current study provides data on litter loss due to cannibalism in FMDV inoculated laboratory BALB/c suckling pups. Cannibalism in mice was thought to be caused by poor infant care, malnutrition, and vices, but the current study found otherwise.

In the current study, all pups were healthy and active after birth, and the dam did interact with both healthy moving and still pups, as well as do mouth and paw manipulation up to 3 days post parturition (Fig. 1 and 2a). In Gr-I, FMDV inoculated group, six pups were found dead on the fourth day (one day after FMDV inoculation), one pup was missing, and one pup was partially eaten/cannibalized (Fig. 2b) by their mother. However, no infanticide or cannibalism

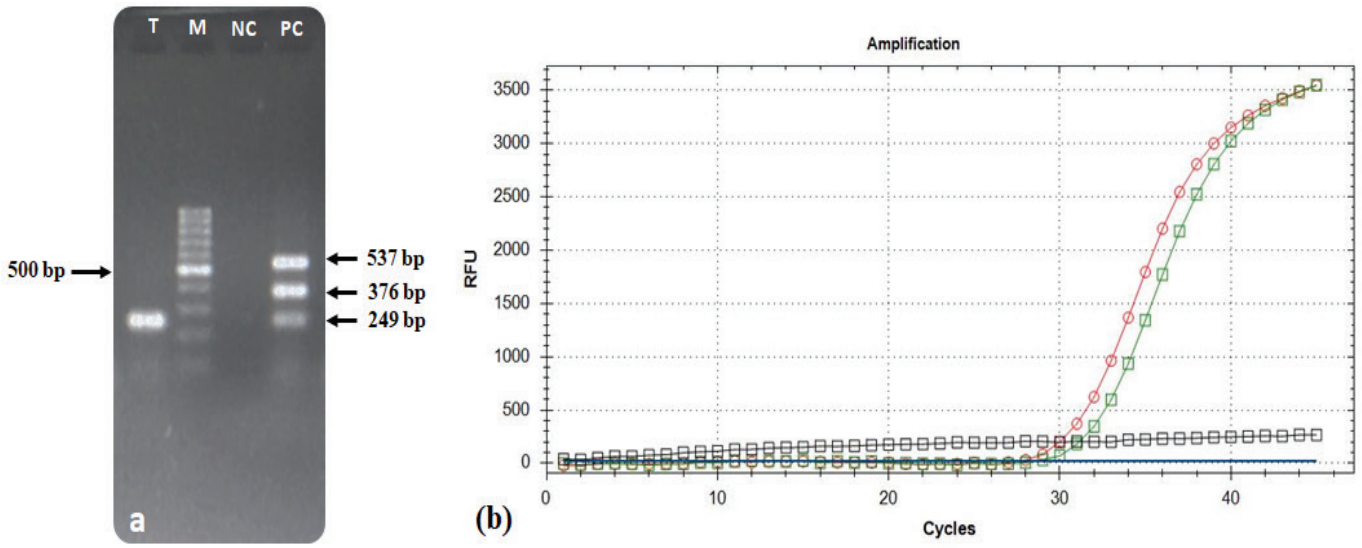

Fig. 3. Molecular diagnosis of FMD virus infection by detection of FMD virus specific genome. [(a) agarose gel electrophoresis of reverse transcriptase -multiplex PCR (RT-mPCR) products showing presence of distinct bands of test sample, 100 bp molecular weight marker, negative samples, and positive samples in column 1 (T), column 2 (M), column 3 (NC) and column 4 (PC), respectively. Column 4 showing positive control for FMDV serotype 'O', 'A' and 'Asia 1' with band of 249 bp, 376 bp and 537 bp, respectively. (b) Showing threshold cycle value in quantitative reverse transcription-polymerase change reaction (RT-qPCR) amplification curve of foot-and-mouth disease serotype 'O' virus in lungs (red colour) and kidney (green colour) tissues and not template control (black colour) samples].

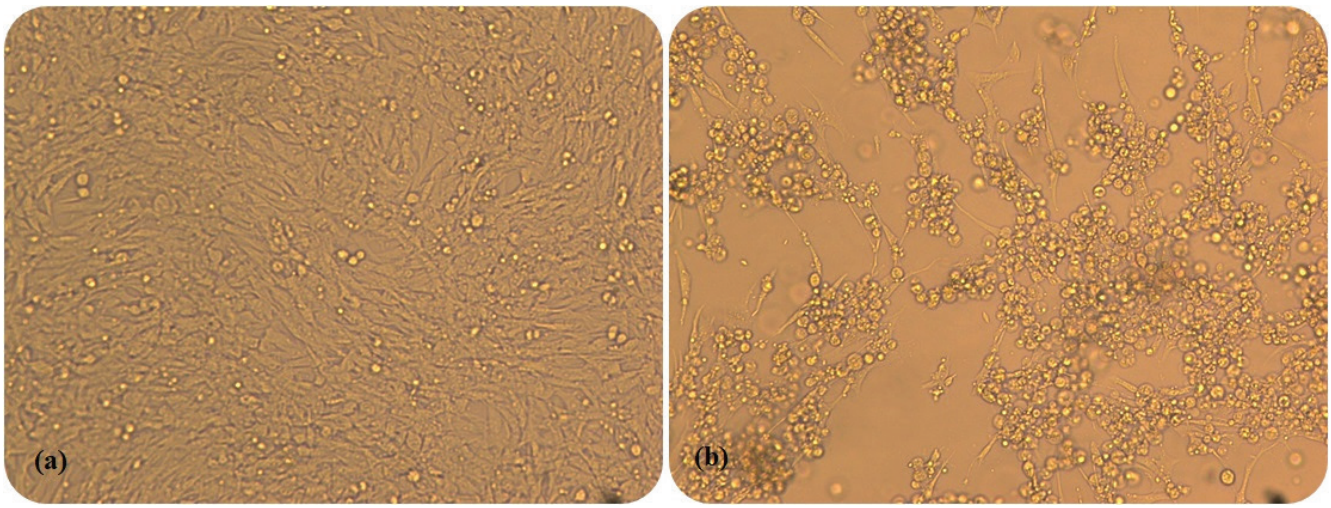

Fig. 4. Virus isolation from lungs tissues. [a) normal healthy BHK-21 cell line, b) cytopathic effect of FMD virus on BHK-21 cells line revealed cell swelling, rounding, and detachment of affected cells from the surface on which they cultured].

was observed in the Gr-II and all pups survived and were healthy. In experimental conditions, disease pathogenesis in natural hosts is determined by species and FMDV strain; similarly, FMDV pathogenesis in mice (adult or suckling) is determined by mouse strain and FMDV strain [2]. The disease incubation period during experimental conditions is 24 hours [23], which was also observed in the present study. A postmortem examination of dead puppies reveals milk inside the stomach.

Lung and kidney tissues were collected from partially cannibalized pups for genome detection and virus isolation. Both the tissues were found positive for FMD virus serotype 'O' by RT-mPCR (Fig. 3a) and it was also confirmed by probe-based RT-qPCR (Fig. 3b). Similarly, FMD virus was rescued on BHK-21 cells from lungs tissues, and the cytopathic effect of FMD virus on BHK-21 cells, such as cell swelling, rounding, and detachment of affected cells from the surface on which they cultured, was observed (Fig. 4a & 4b). This confirms the presence of FMDV, and death of pups could be due to FMDV not by other means.

Gross morphology of lungs of the affected mice appeared edematous, congested, and with rounded edges, whereas the kidneys appeared swollen and congested (Fig. 5a & 5b).

The presence of marked degeneration, emphysema, congestion, and eosinophilic appearance due to edematous fluid in alveoli and interstitial space with infiltration of mononuclear inflammatory cells in alveolar epithelium was revealed by microscopic examination of the lungs. Necrosis caused bronchioles to dilate and fill with tissue debris (Fig. 6a & 6b).

Kidneys showed congestion in the cortical and medullary regions, as well as degenerative and necrotic changes in the tubules, swelling of the glomerulus, and infiltration of mononuclear cells in both the cortical and medullary regions (Fig. 7).

Congestion, interstitial pneumonia, and cellular infiltration in the lungs are observed in large animals due to FMDV infection [3], and it has also been observed in mice due to FMDV infection [24]. In the current study, sucking pups inoculated with FMDV showed similar results. Microscopic changes in the alveoli, interstitial septum, bronchi of the lungs, and dysfunction of vital organs such as the kidney cause cardiac dysfunction in suckling mice, which may be the cause of death.

Suckling pups can die from malnutrition, starvation, or hypothermia because they are so reliant on their food [25]. In the current study, suckling pups did not die from malnutrition or starvation, as evidenced by the presence of milk in the stomachs of all 06 dead suckling pups, indicating adequate suckling from respective dam.

Sucking pups were lying idle or not moving for several hours before the female began eating, but they did not begin eating immediately after they stopped moving [9]. Infanticide is exceedingly rare in mice [9], and cannibalism is also observed in animals during food scarcity [26, 27], but food scarcity is not the case in the current study, as previously stated. Periparturient behaviors, i.e. infanticidal dams, were not observed in

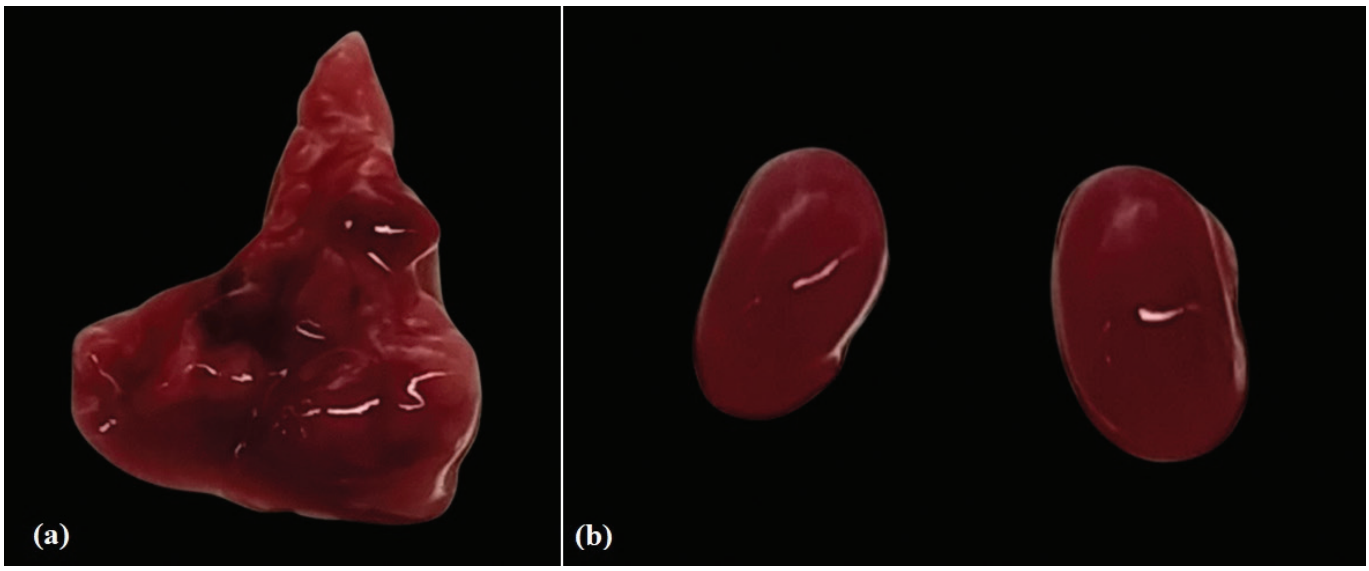

Fig. 5. Gross pathology of lungs and kidneys. [Showing gross changes in lungs and kidneys. (a) lungs appear edematous, congested, and having rounded edges, and (b) kidney was swollen and congested].

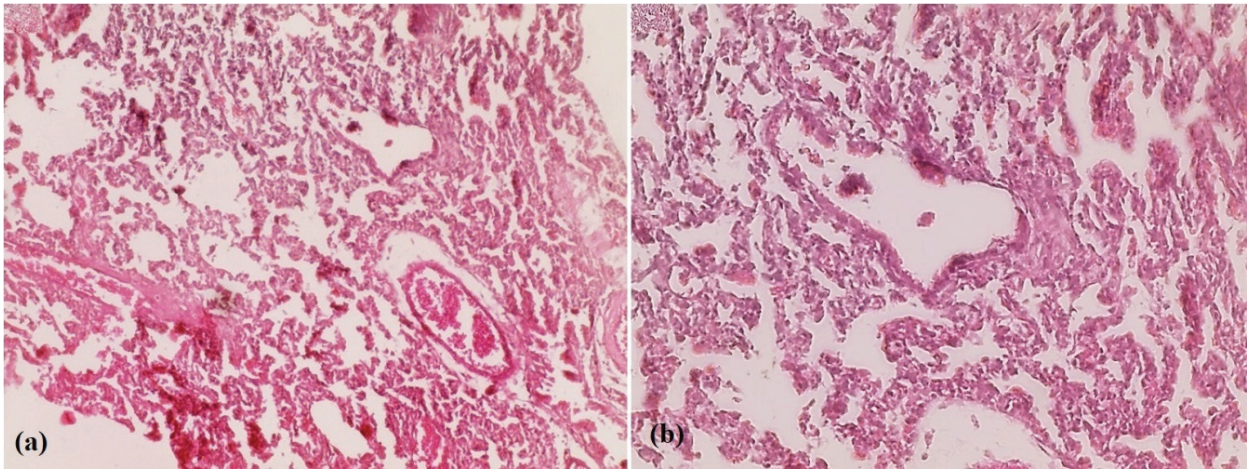

Fig. 6. Microphotograph of lungs. [Microscopic examination of lungs revealed presence of marked degeneration, congestion, eosinophilic appearance due to edematous fluid in alveoli and interstitial space with infiltration of mononuclear inflammatory cells in alveolar epithelium. Emphysema and dilated interalveolar space were observed. Bronchioles were dilated and filled with tissues debris due to necrosis (Stain: HE, Magnification: (a) 100X, (b) 400 X)].

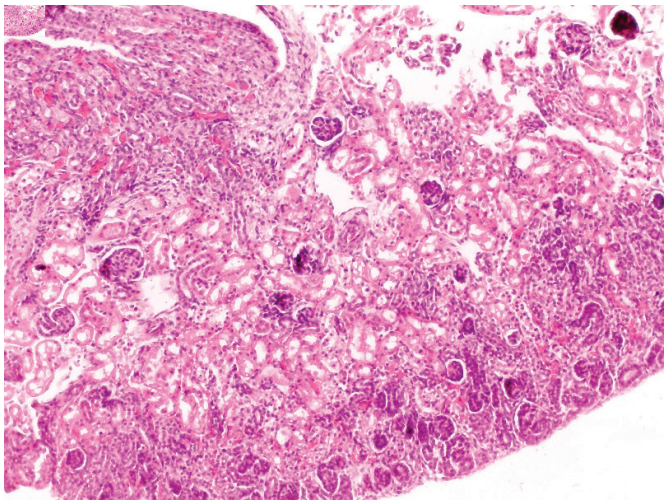

Fig. 7. **Microphotograph of kidneys.** [Microscopic examination of kidneys revealed presence of congestion in cortical and medullary region, degeneration and necrotic in tubules, glomerulus of kidney became swollen, and infiltration of mononuclear cells in both cortical and medullary region (Stain: HE, Magnification: 200X)].

group II, contrary to previous research [28]. However, for the exact behavioral observation, continuous video recording could have been better option.

Maternal cannibalism has also been linked to non-adoptive behavior [29], which was observed in the current study, and this non-adoptive behavior could be attributed to the FMD virus's effect on pups. Infanticide is uncommon in mice, but cannibalism of dead pups by adult mice is common [9, 30, 31], and the current findings support this finding. In the current study, suckling pups were not killed by the dam, but died because of FMD virus replication, which was confirmed by the presence of FMD virus serotype 'O' infection by RT-mPCR and RT-qPCR. Pathological findings (macroscopic and microscopic changes) observed in tissues collected from dead suckling mice have also confirmed FMD virus infection-related death. However, more study needs to be conducted in future with visual evidence to establish the litter loss by cannibalism of dead pups only not by infanticide.

CONCLUSION

In this study, a female mouse was observed eating dead sucking pups, which is consistent with previous reports. Cannibalism is normally exceedingly rare, and it varies from species to species. According to the author's knowledge, this is the first report of litter loss due to cannibalism in laboratory BALB/c mice administered with foot-and-mouth disease virus. Our findings suggest that infanticide is not a common cause of death in BALB/c mice, but cannibalism of dead mice is. As a result, litter loss can be avoided if the cause of death is identified. More laboratory experiments, as well as real-time recordings, are required to confirm the rates of cannibalism of dead pups (because of FMD virus inoculation) by adult female mice.

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ETHICAL STATEMENT

The small house animal facility is registered with the Committee for the purpose of Control and Supervision of Experiments on Animals (CCSEA) with facility registration number: 2018/GO/R-S/RNRc-L/18/CPCSEA. The animals used in this study were sourced from breeding colonies of healthy mice housed in research laboratories. There were no animals created specifically for this study. All experiments were carried out with the approval of the Institute Animal Ethics Committee (IAEC) ICFMD protocol no. P07-02/2022 dated 28.01.2022.

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