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Detection of Foot and Mouth Disease Virus in Salted Raw Cowhide from Malaysia in Tanjung Priok Port, Indonesia

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Abstract

Animal products such as raw salted cowhide are thought to have the potential to transmit the foot and mouth disease (FMD) virus from the infected zone. Indonesia imports raw salted cowhide from Malaysia, so it has the potential to transmit FMD to Indonesia which enters through Tanjung Priok Port. This study aimed to investigate the presence of the FMD virus in raw salted cowhide from Malaysia. The number of samples was collected from each container of raw salted cowhide imported through Tanjung Priok Port during August–December 2022. A total of 21 samples were obtained from 21 bulk containers containing raw salted cowhide. Real time q Polymerase Chain Reaction (RT-qPCR) was used to investigate foot and mouth disease virus in samples. The RT-qPCR screening test on 21 samples reported that salted raw cowhide was free from the FMD virus. Continuous monitoring and surveillance protocols for salted rawhide imported from non-free countries need to be carried out at other points of entry.

Keywords: foot and mouth disease, cowhide, surveillance

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INTRODUCTION

Beef is one of the animal proteins consumed by the public. Meanwhile, cowhide is used as a craft material in the form of bags, shoes, wallets and gloves through a drying and salting process. Animal products such as salted raw cowhide have the potential to carry the foot and mouth disease (FMD) virus from areas infected with FMD.

Foot and mouth disease (FMD) is a highly contagious animal disease in animals with even-numbered hooves (cloven hoops) such as cattle, buffalo, sheep, goats, pigs, deer, camels and elephants (Garcia *et al*., 2022) . FMD outbreaks in endemic areas cause a decrease in fertility in dairy cattle (Chaters *et al*., 2018; Purnama *et al*., 2019). There are seven types of FMD virus, namely: A, O, C, Asia, and South African Territory (SAT) 1, 2, and 3. Each type of FMD virus is further divided into several subtypes and strains. Indonesia has one type of FMD virus, namely type O virus (Rohma *et al*., 2022). The World Organization for Animal Health (WOAH) states that FMD is not transmitted to humans and is not a public health risk (WOAH, 2023).

Animals infected with the FMD virus will show typical clinical symptoms in the form of fever, blisters or lesions on the tongue and lips, in the mouth, nipples and all over the paws (Buetre *et al*., 2013). FMD is known as an airborne disease, namely a disease that spreads through the air. Airborne transmission of the FMD virus has been reported to spread up to 50 km on land (Gloster *et al*., 2005), and more than 200 km in water (Gloster and Burgin, 2007), and the virus remains infective for up to 7 days in the air at room temperature (Kristensen *et al*., 2021). FMD transmission occurs through direct or indirect contact with transmission rates reaching 90–100% and very high economic losses (Nason, 2022). Losses due to the FMD epidemic occurred in England in 2001 with an estimated loss of more than 9 billion USD (Rp. 119.9 trillion) (Jones, 2014). FMD cases in other countries, including Taiwan in 1977 caused a loss of 6.617 billion USD, Uruguay in 2001 lost 0.08 billion USD, Japan in 2010 lost 0.55 billion USD and South Korea in 2010–2011 experienced losses of up to 2.8 billion USD (Rushton and Knight, 2015). The total annual impact due to FMD is estimated at 6.5

billion USD to 21 billion USD (Naipospos and Suseno, 2017).

After 32 years of being declared free from FMD, Indonesia has again been hit by an FMD outbreak. The determination of areas affected by the FMD outbreak is contained in the Decree of the Minister of Agriculture Number 652/KPTS/PK.300/M/08/2022 concerning Determination of Foot and Mouth Disease Outbreak Areas which covers 24 provinces in Indonesia (Ministry of Agriculture, 2022). The Indonesian government re-designated FMD as a strategic infectious animal disease in the Decree of the Minister of Agriculture of the Republic of Indonesia No. 121/KPTS/PK.320/M/03/2023 which already exists in regions or regions in Indonesia so that it becomes a priority for controlling and overcoming animal diseases (Ministry of Agriculture, 2023). The costs used by the Indonesian government in handling FMD for 100 years (1887–1986) reached 1.66 billion USD (Khudori, 2022).

The spread of FMD between farms or between regions generally occurs through traffic or transportation of infected livestock, products from infected livestock, and carrier animals (Prihatin *et al*., 2023; Oktanella *et al*., 2023). Trade in livestock products is the biggest risk factor in the cross-border spread of FMD in Southeast Asia, involving complex and rapidly changing market chains that connect producers with consumers (Blacksell *et al*., 2019). The FMD outbreak in Malaysia shows that the main factor in the spread of FMD is animal movement, which is 66% of the cases (Ramanoon *et al*., 2013).

Malaysia is one of Indonesia's neighboring countries with a status that is not completely free from FMD and there are still several zones that are not yet free from FMD (Silitonga, 2016). Based on a report from WOAH, from January to June 2019, there were still reports of FMD in Malaysia attacking livestock including cattle, buffalo, sheep, goats and camels (WOAH, 2020). Products of animal origin from Malaysia in the form of salted raw cowhide destined for the Garut and Cikarang areas are still imported by Indonesia via the Tanjung Priok Port in order to meet the needs of the leather tanning industry for making bags, shoes, gloves, wallets and other finished products. The import of salted raw cowhide in 2020 was 931.42 tons, in 2021 it was 1,935,909 tons and in January 2022 it was 72.3 tons. The large amount of raw, salted cowhide entering Indonesia from Malaysia, which is not yet free from FMD, has the potential to bring the FMD virus to Indonesia and could hamper the FMD control program.

This study aimed to investigate the presence of the FMD virus in salted raw cowhide from Malaysia entering through Tanjung Priok Port. It was expected that the results of this study can be used as material for consideration in drafting regulations regarding the import of salted raw cowhide from Malaysia and to control the handling of FMD that has entered Indonesia.

MATERIALS AND METHODS

Ethical Approval

This study did not require ethical approval because there was no treatment of animals.

Study Period and Location

The study was carried out from August 2022 to January 2023. Sample collection was carried out at the Tanjung Priok Agricultural Quarantine Center (BBKP). Sample testing was carried out at the Agricultural Quarantine Standard Testing Laboratory (BBUSKP). Data processing was carried out at the Veterinary Public Health and Epidemiology Division, School of Veterinary Medicine and Biomedicine, IPB University (SKHB IPB).

Samples

Based on 2022 data from the Tanjung Priok Agricultural Quarantine Center, the frequency of imports of salted raw cowhide from Malaysia at Tanjung Priok Port in the period August–December 2022 was 21 containers. The data was presented in Table 1.

The sampling method for each container used a simple random method, with lesion criteria in a random area. Samples were collected from each import container of salted raw cowhide. The sample size was collected randomly in each

container with cowhide samples in the form of pieces on the edge of the cowhide amounting ± 250–500 g. Sampling was carried out using one of the techniques available to the sampling officer in accordance with Indonesian National Standard Number 19-0428-1998 concerning Methods for Sampling Solids, namely by taking samples randomly at 5 points in each container and forming the letter "X" (right corner top left, bottom and middle) in the form of pieces of raw cowhide salted on the edges (BSN, 1998). Evaluation was carried out using 5:1 pooling, which means 5 samples were combined and tested in one test for one container.

Evaluation

The materials used were QIAamp Viral RNA Mini Kit (Qiagen, Germany) for sample extraction, PBS for elution/rinsing cowhide samples, AgPath-ID One-Step RT-PCR Kit (Thermo Fisher, USA) absolute ethanol mastermix, primers and probes. referring to Callahan *et al*. (2002), with the following sequence: 3D Forward Primer 5'-ACT-GGG-TTT-TAC-AAA-CCT-GTG-A-3' (positive strand), 3D Reverse Primer 5'-GCG-AGT-CCT-GCC-ACG-GA-3' (negative strand), and 3D Probe Primer 5'-FAM-TCC-TTT-GCA-CGC-CGT-GGG-AC-TAMRA-3', real-time 3D synthetic positive control of FMD virus derived from 3D primary sequences (Dinana *et al*., 2023).

Sample testing was carried out using the Real Time q Polymerase Chain Reaction (RT-qPCR) method. The first step of testing was to extract viral RNA from each salted raw cowhide sample with the QIAamp Viral RNA Mini Kit (Qiagen, Germany). The extraction process was carried out according to the kit manual. Rinsing or elution of the sample is carried out before the extraction process, to remove salts which act as inhibitors by weighing 5 g of cowhide in 50 mL of phosphate buffer saline (PBS) and homogenizing. The rinsed cowhide was weighed as much as 25 mg in a 2 mL microtube and 1 stainless steel bead was added. A total of 300 µL of phosphate buffer saline (PBS) was added to the microtube, then placed in the tissue lyser adapter set, then tissue lyser II was operated for 2 minutes at 25 Hz. The next step, centrifuged at 14,000 g for 2 minutes, and 140 µL of the supernatant formed was taken as "starting material".

The resulting supernatant was put into a 2 mL microtube as much as 140 µL, 560 µL of AVL buffer and 5.6 µL of carrier RNA were added, homogenized and incubated for 10 minutes at room temperature. A total of 560 µL of absolute ethanol was added, then homogenized again for 15 seconds, then a quick spin to reduce the liquid in the tube cover. The next step, 630 µL of solution was transferred into a mini spin column tube, then centrifuged at 8,000 rpm for 1 minute. The liquid was discarded and the mini spin column collection tube was replaced with a new one and the previous steps were repeated with the remaining solution. The mini spin column collection tube was replaced with a new one and 500 µL of AW 1 buffer was added, centrifuged at 8,000 rpm for 1 minute. In the next step, the mini spin column collection tube was replaced with a new one, then 500 µL of AW 2 was added, centrifuged at 14,000 rpm for 3 minutes, then the liquid was discarded and centrifuged again for 1 minute to dry the membrane. After that, the mini spin column collection tube was replaced with a new 1.5 mL Eppendorf tube and 60 µL of AVE buffer was added, then incubated for 1 minute at room temperature, then centrifuged at 8,000 rpm for 1 minute and the extraction results were stored in a freezer -20°C.

The second step is the PCR amplification process. The amplification process begins by adding the PCR master mix using the AgPath-ID One-Step RT-PCR Kit (Thermo, United States). Every real-time PCR test is always accompanied by a positive control, negative control and non-template control (NTC). Master mix ingredients in 1 reaction with 20 µL reaction formula consisting of RNAse-free water 1.5 µL H2O, 12.5 µL 2× RT-PCR buffer, 2 µL 3D Forward primer [20 µM] with a concentration of 1.6 µL, 2 µL 3D primer Reverse [20 µM] with a concentration of 1.6 µL, 1.5 µL probe primer [10 µM] with a concentration of 0.6 µL, and 0.5 µL 25× RT-PCR enzyme mix. These materials were put into a 1.5 mL PCR tube, then homogenized. The resulting master mix solution was distributed as much as 20

µL into PCR microtubes for each sample. For each sample to be identified, 20 µL of master mix solution is required plus 5 µL of RNA template so that the total solution is 25 µL. In the positive control, a template from a synthetic positive control preparation was used, while in the negative control 5 µL of RNAse-free water was added as a template.

In the third step, the PCR tube was placed into the Rotor-Gene Q thermal cycler PCR amplification machine (Qiagen, Germany). The amplification program in the real-time RT-PCR method is reverse transcription at 50ºC for 2 minutes, RT inactivation/initial denaturation at 95ºC for 10 minutes, and amplification at 95ºC for 15 seconds and 60ºC for 1 minute (annealing/extension/data acquisition, FAM-TAMRA probe reading) with 50 cycles. The final step, analysis of the test results was carried out using the Rotor-Gene Q Series Software 2.3.1.49 (Qiagen, Germany). Interpretation is seen based on the CT (cycle threshold) value produced on the CT amplification plot. A positive result is indicated if the test sample has a CT value < 40, while a negative result is indicated by a CT value > 50, and if there is a CT value between 40-50, then the test is repeated. If the results of the retest still give the same results then it is concluded with dubius and the sample is taken again.

Data Analysis

The data obtained were analyzed descriptively to describe the presence of the FMD virus from raw cowhide salted at Tanjung Priok Port. Test results are presented in the form of table and figure.

RESULTS AND DISCUSSION

RT-qPCR testing on 21 samples of raw salted cowhide imported from Malaysia via Tanjung Priok Port showed negative results for the FMD virus. The test results can be seen in Table 1 for the period August–December 2022. The results of this study show that no FMD virus was detected in the raw salted cowhide samples examined using the RT-qPCR method.

The test results of salted raw cowhide samples are simply displayed graphically in Figure 1 using the Rotor-Gene Q Series Software 2.3.1.49 (Qiagen, Germany).

Based on the sample test results, Figure 1 explains that negative results occurred in all 21 samples of raw salted cowhide, negative control (NC) and non-template control (NTC) tests. Non-template control functions as an indicator for investigators whether the mastermix kit used has had contamination or no contamination. Positive results only occurred in positive control 1 which intersected with the threshold line at CT-value 22.36 and positive control 2 intersected with the threshold line at CT-value 36.08. The test results in Table 1 showed that the synthetic positive control 3D real-time FMD virus derived from the 3D primer sequence used was in accordance with the standard, because it was indicated by the CT-value with positive results, namely < 40. There was no NC that intersects the auto- threshold line (0.028) so that the sample tested was reported to be negative and the negative control did not contain contamination. The results revealed to be positive if the CT-value was < 40 and the results reported to be negative as indicated by the CT-value > 50. This means that in 50 replications of PCR amplification there was no FMD virus genetic material in raw salted cowhide sample.

The explanation regarding the negative results in 21 samples of raw salted cowhide tested using RT-qPCR was possible because the cowhide samples came from zones that had undergone perfect salting treatment according to World Organization for Animal Health (WOAH) regulations, the raw salted cowhide came from a free zone. FMD which has been required through a Veterinary Health Certificate, and/or salted raw cowhide comes from farms that are not infected with FMD and the test samples taken are scattered, so it was possible that the FMD virus was not expressed in samples.

The characteristics of the FMD virus are that it survives in the environment or nature depending on the situation and conditions of temperature and acidity level, and is more stable and infective if the virus is in the skin layer, mucous fluid and protected from exposure to sunlight or at

relatively low temperatures in the environment, as well as other conditions (MacLachlan *et al*., 2017). Other characteristics of the FMD virus include being able to survive longer in cold temperatures, being susceptible to acidic and alkaline pH (pH 6–9), but under certain conditions, it can maintain its infectivity in the environment for a long time if organic material is present, and is easily inactivated at drying temperatures >56°C (USDA, 2020). Therefore, in this study, the samples taken were fresh salted raw leather with conditions of humidity in the leather container that were quite high, so it was possible that the FMD virus could still be detected in laboratory tests.

Tabel 1. Imported salted raw cowhide and RT-qPCR evaluation during August–December 2022

Month	Number of samples (n)	Test results
August	1	Negative
September	11	Negative
October	1	Negative
November	4	Negative
December	4	Negative

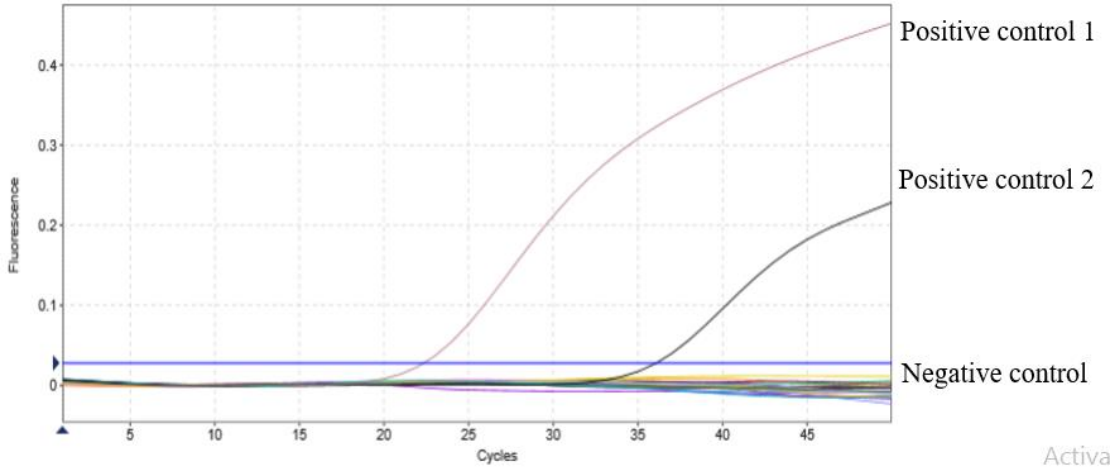

Figure 1. RT-qPCR amplification plot on test samples.

The FMD virus will die with the use of disinfectants containing sodium carbonate, so it is proper to use for decontamination (Haskell, 2014). Based on study by Hong *et al*. (2015), it was reported that it only took a maximum of 5 minutes to obtain a titer reduction of 4 logs for type O virus with sodium carbonate in a concentration of 4% at a temperature of 4°C. Based on the study above, it shows that the sodium carbonate concentration level, temperature and time used greatly influence the reduction of FMD virus titers (Majid *et al*., 2023).

The FMD virus on the skin can still be detected for up to 32 days or more, depending on humidity and drying and storage temperature. Leather that was salted (salted rawhide) and stored at 4°C still had detectable FMD virus on day 352, while skin dried at 20°C with a relative humidity of 40%, still had detectable FMD virus after 42 days (USDA, 2020). Raw salted cowhide from Malaysia entering through the Tanjung Priok Port uses bulk container transportation without refrigeration, which causes the temperature inside the raw salted cowhide container to be higher and more humid, so it is possible that the FMD virus is still present.

Every import of salted cowhide on quarantine documents must require a Veterinary Health Certificate from the country of origin. The official veterinarian who signed the Veterinary Health Letter stated that the salted raw cowhide product had met the requirements, including that the raw salted ruminant hide came from a country that was free from FMD outbreaks in ruminants,

had gone through a salting process using sea salt containing 2% of sodium carbonate for a minimum of 28 days and have been processed in accordance with applicable standard procedures, come from livestock that have passed ante- and post-mortem examinations, shipped in clean and protected containers, and have been inspected by the authorized party on the day of shipment and declared healthy, safe, and industrially viable (WOAH, 2023).

The negative test results were also possible due to the immersion process in salt water which is able to clean viruses and other microorganisms. If the soaking and salting process is not carried out perfectly with the right time and concentration, it will not be able to deactivate the FMD virus in salted raw cowhide. This is explained based on the WOAH rule which states that the FMD virus can be inactivated by 2% of sodium hydroxide, 4% of sodium carbonate, 0.2% of citric acid, 2% of acetic acid, 3% of sodium hypochlorite, 1% of sodium chloride, and chlorine dioxide (WOAH, 2021).

The high protein content of leather makes it very susceptible to rot and bacterial degradation, so it is important that it is properly preserved for transportation and storage before tanning. Preservation using the salting method is the most widely used method because it is easy, cost effective and the quality of the leather is better (Wu *et al*., 2017). There are two ways to limit or control microbial attacks, namely by killing microorganisms which is called bactericidal or creating conditions that are unfavorable for microorganisms to reproduce, which is called bacteriostatic (Septiyani *et al*., 2023). The bacteriostatic method uses salt, generally sodium chloride. The salt-based leather preservation method uses soaking in a 95% saturated salt solution or 40%–50% sodium chloride based on the weight of the raw hide (Sarker *et al*., 2018).

Preserving leather using salt is done by destroying active bacteria, preventing bacterial activity, or preventing bacterial contamination. Skin treatment by providing a mixture of compounds containing 5% of sodium chloride and 1% of sodium hexafluorosilicate can prevent rot for 14 days when the storage temperature is 22ºC. The use of 15% sodium chloride allows preservation of the skin for more than 28 days. Preservation using salt causes a decrease in skin water content by 6% and soaking with salt improves impregnation conditions (Valeika *et al*., 2017). Leather preservation in the leather soaking process using a technical table salt solution measuring 1–2 mm or the size of rice grains with a content of ±90–95% with a concentration of 20–24°Be, as well as adding a disinfectant or skin poison in the form of sodium arsenate or cortymol G or antimucin SP and soaked for 24 hours (Juliyarsi *et al*., 2019). This may be the reason why the FMD virus was not detected in this study, due to the effects of immersion in NaCl and disinfectant, thus the results reported negative for the FMD virus.

CONCLUSION

Screening tests using the RT-PCR method on 21 samples of raw salted cowhide imported from Malaysia collected at Tanjung Priok Port during the arrival period from August–December 2022 did not reveal the presence of the FMD virus. It is necessary to carry out continuous monitoring and surveillance of salted shells imported from non-free countries over a longer period and other places of entry.

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AUTHORS' CONTRIBUTIONS

CB: Conceptualization and drafted the manuscript. DAS, CB, and DWL: Performed sample evaluation. CB and DWL: Prepared table and figure. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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