Research Article

Detection, Isolation, and Molecular Characterization of Foot-and-Mouth Disease Virus from the 2022–2023 West Java Outbreak

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Abstract | Foot-and-mouth disease (FMD) is an acute and highly contagious viral infection that affects various cloven-hoofed animals. In 2022, FMD "re-emerged" in Indonesia, with outbreaks reported in several provinces, including West Java. This resurgence has raised concerns about the management and control of the disease, especially given the virus's complex characteristics. FMD virus exhibits distinct characteristics based on serotypes. This study aims to identify clinical symptoms of FMD, isolate and characterize the FMD virus causing the 2022-2023 outbreak in West Java. This study collected 31 samples from Sumedang and Sukabumi districts in West Java Province, which were affected by FMD outbreaks between 2022-2023. Clinical examinations were conducted on animals suspected of being infected with the FMD virus the 31 samples comprised 20 cattle, 9 buffaloes, and 2 goats. The study method used was molecular detection with RT-qPCR, isolation, and identification with RT-PCR followed by sequencing. The observed clinical symptoms included fever, excessive salivation and nasal discharge, vesicles in the mouth, lameness, and leg lesions. The RT-qPCR result of 30 out of 31 samples (97%) tested positive for FMD virus. Of the 30 samples that were positive for FMD, 8 were identified as serotype O, with an RT-PCR amplicon size of 1135 bp. FMD virus was successfully cultured in BHK-21 cells, producing a cytopathic effect (CPE) within 24–72 hours. The clinical symptoms of FMD observed in cattle, buffalo, and goats in West Java Province are consistent with those previously reported. FMD virus was detected in 97% of animals showing clinical signs in this study.

Keywords | Foot-and-mouth disease (FMD), Identification, Isolation, Molecular detection, Outbreaks, Viral infection

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INTRODUCTION

Foot-and-mouth disease (FMD) is an acute, highly contagious viral infection that predominantly affects cloven-hoofed livestock (Freimanis *et al*., 2016). Additionally, approximately 70 wildlife species are susceptible to the disease (Thomson *et al*., 2003). Clinical manifestations are characterized by the development of vesicles and erosions on the oral mucosa (including the mouth, tongue, and gums), nostrils, teats, and the coronary bands (Alexandersen *et al*., 2003).

Foot-and-mouth disease (FMD) causes substantial economic losses, both direct and indirect. Direct economic losses include reduced production, livestock mortality, and delayed marketing. Conversely, indirect losses arise from control measures, vaccination costs, and restrictions on both domestic and international trade (Knight-Jones and Ruston, 2013). These trade barriers extend beyond live animals to encompass all animal-derived products (Jamal and Belsham, 2013). In Southeast Asia and China, the predominant viral strains of FMD belong to serotype O, topotype ME-SA, and lineage Ind-2001 (Jamal and Belsham, 2018). Consequently, the May 2022 outbreak led the World Organisation for Animal Health (WOAH) to suspend Indonesia's FMD-free status (WOAH, 2022).

West Java Province was identified as one of the affected areas during the FMD outbreak (MoA, 2022). By November 2022, the disease prevalence in West Java had reached 35.02% (BNPB, 2022). While control and preventive measures such as vaccination have been implemented to sustain the local livestock industry, there remains a paucity of data regarding the specific clinical characteristics of the outbreak in West Java and detection results across various susceptible species. Therefore, this study aims to identify clinical symptoms of FMD in several livestocks species, isolate, and characterize the FMD virus causing the 2022–2023 outbreak in West Java.

MATERIALS AND METHODS

Study area

This study was conducted from November 2022 to December 2023. Sample collection was carried out at cattle, buffalo, and goat farms in Sumedang and Sukabumi Regencies, West Java Province. Samples were tested at the Virology Laboratory of the Research Center for Veterinary Science, Research Organization for Health, National Research and Innovation Agency (BRIN).

Sample collection

The sampling used a purposive sampling method and targeted, based on passive surveillance reports of suspected outbreaks. Specimens were obtained from susceptible livestock (cattle, buffalo, and goats) exhibiting clinical signs of FMD following a clinical examination. A clinical examination of FMD-susceptible animals (cattle, buffaloes, and goats) was conducted through a systematic physical inspection to identify pathognomonic clinical signs. Inspection and observation of body posture, the presence of excessive salivation, and lameness. Restraint to examine in more detail the oral mucosa (tongue, gums, dental pads), nostrils, nail gaps (interdigital spaces), and teats to detect the presence of vesicles, erosions, or ulcerations (Arzt *et al.* 2011). This procedure must include a rectal temperature measurement to detect infection (>40°C). Mucosal swabs were collected from the nasal and oral cavities, which were then immediately immersed in tubes containing non-inactivated viral transport medium (VTM). All samples were maintained in a cooler box at 2–6 °C and subsequently stored at -80 °C before further analysis.

RNA extraction

Mucosal swab samples in VTM were homogenized using a vortex mixer. Viral RNA was extracted using the Viral Nucleic Acid Extraction Kit II (Geneaid®) following the manufacturer's instructions and standard laboratory procedures. The extracted RNA was stored at -80 °C before further analysis.

Detection of foot-and-mouth disease virus (FMDV) using RT-qPCR

Detection of FMDV RNA from mucosal swabs using RT-qPCR (Oleksiewicz *et al.*, 2001). The assay utilized specific primers targeting the 3D gene to generate a 107 bp amplicon. The primer sequences used were: forward (GenBank AF189157) 5'-ACT GGG TTT TAC AAA CCT GTG A-3' and reverse 5'-GCG AGT CCT GCC ACG GA-3' (Callahan *et al.*, 2002). Amplification was carried out using the EXPRESS One-Step Superscript™ RT-qPCR Kit (Invitrogen™). The thermal cycling conditions consisted of reverse transcription at 50 °C for 30 min, followed by initial denaturation at 95 °C for 15 min. Subsequent amplification involved 50 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 1 min. Results were interpreted based on the cycle threshold (Ct) value; amplification curves with a Ct value < 40 were considered positive for FMDV, while samples with a Ct value > 40 were considered negative (WOAH, 2021).

Virus isolation

The positive mucosal swabs of FMDV by RT-qPCR were subjected to trials of virus isolation with the blind passage method. The samples were centrifuged at 1,500 ×g for 15 min at 4 °C. The supernatant was collected and transferred into 1.5 ml microcentrifuge tubes. Confluent monolayers of BHK-21 cells in T25 tissue culture flasks were inoculated with 0.5 ml of the supernatant and incubated for 30 min. Following this adsorption period, 5 ml of Roswell Park Memorial Institute (RPMI) medium was added, and the cultures were incubated at 37 °C with 5% CO (Tesfaye *et al.*, 2020). Incubation continued until cytopathic effects (CPE) were observed, and confirmation of isolates with CPE using the RT-qPCR procedure testing. The virus was subsequently harvested by centrifugation at 3,000 ×g for 20 min and stored in sterile cryogenic vials at -80 °C.

Molecular characterization

Samples confirmed positive by RT-qPCR and viral isolates

obtained from BHK-21 cell culture were subjected to conventional RT-PCR. This step served as a preparatory phase for sequencing. The complete VP1 coding region was amplified using specific primer sets, forward (O-1C272) 5'-TBGCRGGNCTYGCCCAGTACTAC-3' and reverse (EUR-2B52) 5'-GACATGTCCTCCTGCATCTGGTTGAT-3' (Knowles *et al.*, 2016). The reaction master mix was prepared in a PCR hood by combining 8 µl of nuclease-free water, 2.5 µl of forward primer (4 pmol/µl), 5 µl of reverse primer (4 pmol/µl), and components of the QIAGEN OneStep RT-PCR Kit (Qiagen), which included 5 µl of 5× buffer (containing 12.5 mM MgCl2), 1 µl of dNTP mix, and 1 µl of enzyme mix. Finally, 2.5 µl of viral RNA template was added to the reaction, and amplification was performed in a thermal cycler. The cycling conditions consisted of reverse transcription at 50 °C for 30 min and initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation (95 °C for 1 min), annealing (60 °C for 1 min) for the FMDV serotype O, and extension (72 °C for 2 min). The process concluded with a final extension at 72 °C for 5 min. PCR products were visualized on a 1.5% agarose-Tris-borate-EDTA (TBE) gel containing 1× GelRed nucleic acid stain (Biotium), using a DNA size marker (GeneRuler 100 bp DNA Ladder Plus).

Analysis of conventional PCR products that are FMDV positive is then followed by sequencing. Sequencing is performed using the Sanger Sequencing method with BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The total volume of 10 µl consisted of 2 µl of 5× sequencing buffer mixed with 0.5 µl of BigDye® Terminator v3.1 and 3 µl of additional primer forward (O-CRH2F) 5'-GAYTACGCSTACACSGCGTC-3' and reverse (NK-72) 5'-GAAGGGCCCAGGGTTGGACTC-3'. The PCR sequencing reaction was performed in duplicate for each primer in a 0.2 ml PCR plate. Thermal cycling: cycler temperature 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Sequencing results were analyzed by counting the number of nucleotide base differences from the total sequence obtained using the VP1 capsid protein sequencing protocol or the 1D gene (Knowles *et al.*, 2016).

Data analysis

Data were analyzed descriptively and presented in tables and figures using Microsoft Excel. Sequencing results were analyzed using BioEdit 7.2 software (Hall, 2011). Sequence similarity to GenBank databases was assessed using NCBI's BLAST tool. Phylogenetic relationships were inferred using MEGA XI software (Tamura *et al.*, 2021). Nucleotide homology of the VP1 protein was analyzed between field samples, BHK-21 culture isolates, and serial passage samples.

RESULT AND DISCUSSION

Clinical symptoms of foot-and-mouth disease

A total of 31 samples were obtained in this study from two districts in West Java Province that experienced FMD outbreaks in 2022-2023, at Sumedang and Sukabumi Districts. Clinical symptoms of FMD in cattle and buffalo included high fever up to 40°C upon infection and excessive salivation Figure 1. Vesicles were found on the tongue, oral cavity, gums, snout, and between the hooves. Vesicles can also be found on the teats of lactating cows (Kitching, 2002).

Figure 1: Clinical signs of FMD in cattle: (A) Excessive salivation and (B) Vesicles on the gum. Clinical signs of FMD in buffalo: (C) vesicles on the gums of buffalo. Clinical signs of FMD in goats: (D) vesicles on the gums (personal documentation).

Clinical symptoms of FMD in sheep and goats, such as vesicles, were found in the mouth, between the hooves, and on the teats (Kitching and Hughes, 2002). The incidence of clinical symptoms in cattle and buffalo was higher than in sheep and goats. This was confirmed in this study, in which very few FMD-suspected goats showed clinical symptoms.

Clinical symptoms found during examination before sampling were influenced by the level of infection and the incubation period of the FMDV. In this study, goats infected with FMDV had milder symptoms, although only 2 goats exhibited clinical symptoms. Based on the data collected in this study, fever, excessive salivation, nasal discharge, and vesicles in the mouth were the most commonly found clinical signs. The results of clinical examinations of 31 animals, consisting of 20 cattle, 9 buffaloes, and 2 goats, with clinical symptoms included fever (52%), excessive salivation or secretion (45%), vesicles in the mouth (42%), lameness (23%), and lesions on the feet (16%) as shown in Figure 2. We observed that

lameness (23%) was indeed more prevalent than visible foot lesions (16%). This discrepancy is likely due to the prodromal phase of inflammation supported by physical palpation findings heat or pain response. In FMD, acute coronitis (inflammation of the coronary band) often causes significant pain and lameness before the rupture of vesicles or the appearance of ulcerative lesions. Therefore, several animals were scored as 'lame' due to pain sensitivity, even though gross lesions had not yet fully developed.

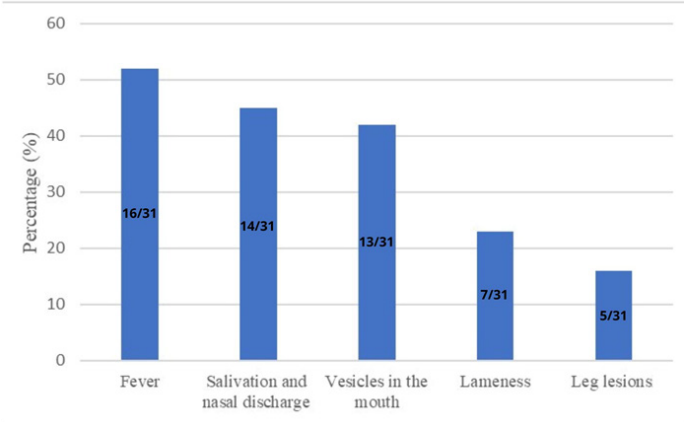

Figure 2: Clinical symptoms of FMD found in cattle, buffalo, and goats. The most commonly found clinical symptoms are fever, excessive salivation, and the presence of vesicles in the mouth. The observed clinical symptoms included fever (52%), excessive salivation and nasal discharge (45%), vesicles in the mouth (42%), lameness (23%), and leg lesions (16%).

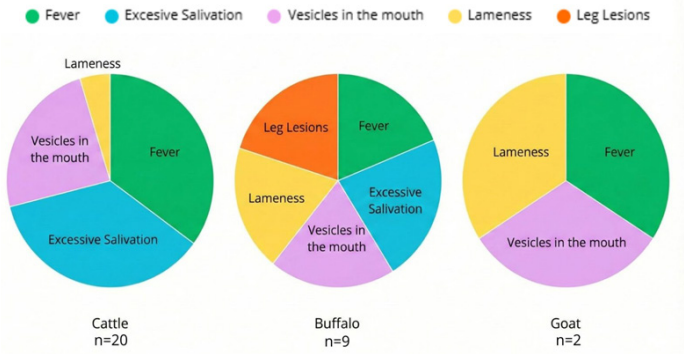

Figure 3: Differences in the clinical manifestations of foot-and-mouth disease in cattle, buffalo, and goats. Frequency of observed clinical signs in animals sampled (n=31). Differences in clinical symptoms found in cattle (fever, excessive salivation, and vesicles in the mouth), buffalo (lesions on the feet), and goats (fever, vesicles in the mouth, and lameness).

The clinical manifestations of FMD in ruminants show pathognomonic variations between species that are crucial for accurate diagnosis in the field Figure 3. In cattle, symptoms are found acutely as a primary indicator (sentinel) characterized by high fever, excessive salivation and nasal secretions (foamy), and large, obvious vesicles on the tongue and mouth. In buffalo, although systemic symptoms are similar, the severity of the lesions is often more dominant and persistent in the leg area (interdigital) than in the mouth area. In goats, clinical symptoms tend to be subclinical (silent spreader), where hypersalivation is rarely found, and oral lesions are very small (the size of a needle) and heal quickly, so the focus of diagnosis in goats must be shifted to detecting lameness. Yoon *et al.* (2012) stated that the most frequently observed clinical symptoms in FMD epidemics that are specific to cloven-hoofed animal species are excessive salivation, vesiculation, and ulceration. Differences in clinical symptoms due to inter-species variations were also found, but lesions on the feet and oral vesicles can be used as early predictors of FMD disease (Ismail *et al.*, 2023).

Detection of foot-and-mouth disease virus (FMDV)

Molecular detection of FMDV using RT-qPCR on 31 (n=31) mucosal swab samples (mouth, nose, and oropharynx) taken from animals with clinical symptoms of FMD. 30 samples from cattle, buffalo, and goats were successfully detected as positive for FMDV with the 3D protein target, and 1 sample showed a negative result for FMDV from a buffalo sample in Sukabumi. The curve formed during amplification was negative for FMD virus with a Ct value >40. RT-qPCR can accurately detect viral RNA in samples obtained from animals infected with FMD virus. Viral RNA can even be detected in mouth and nose samples 24 to 96 hours before the onset of clinical symptoms (Callahan *et al.*, 2002).

RT-qPCR with the 3D gene target is highly sensitive for detecting FMDV in cattle, buffalo, and goats because the 3D gene is part of the virus's RNA polymerase, which is highly conserved and essential for viral replication, and rarely undergoes significant mutations. RT-qPCR can detect very low levels of viral RNA, even in samples with minimal viral load or in the early stages of infection, due to its high sensitivity and exponential amplification capability. Infection levels can also be monitored through RT-qPCR results. Speed and efficiency are also advantages of detection using this method, making it suitable for surveillance and epidemiological monitoring (Paton *et al.*, 2009).

Cattle, buffalo, and goats are some of the species susceptible to FMDV. Early detection using RT-qPCR with the 3D target gene is crucial to prevent the spread of this disease. By accurately and quickly detecting the virus, preventive measures such as vaccination and biosecurity can be implemented to control outbreaks. For WOAH (OIE) reporting, this finding supports a strategy of 'presumptive positive' reporting. Given the high correlation between symptoms and viral presence, surveillance should pivot to immediate containment based on clinical sign and rapid

detection, but still waiting for laboratory confirmation.

Virus isolation

A total of 10 samples that showed positive results for FMDV with Ct values <20 were selected for virus isolation in BHK-21 cells Table 1. The presence of a cytopathic effect (CPE) indicated the presence of FMDV growth in BHK-21 cells. CPE in the form of cell rounding, cell enlargement, granulation, and detachment of BHK-21 cells from the surface of the culture flask. BHK-21 cells initially exhibit a fibroblast-like morphology and are densely arranged to form a confluent monolayer on the culture surface Figure 4. FMDV infection in BHK-21 cell cultures triggers morphological alterations, initiated by cytoplasmic retraction due to cytoskeletal disruption. The BHK-21 cells subsequently undergo cell rounding and exhibit increased membrane refractility, indicating the occurrence of CPE during the active replication phase. Progression of the infection results in loss of cell adhesion to the substrate surface (cell detachment) and nuclear condensation (pyknosis) at advanced stages. This degenerative process culminates in total cell lysis, characterized by massive disintegration of the monolayer integrity and the accumulation of cellular debris at the final stage.

Table 1: Results of FMDV isolation attempts in BHK-21 cells for ten selected samples with low Ct values.

No	Sample name	Ct value	intepretation	CPE P3*
1	SS1/Cattle/SMD/2022	19,42	Positive (+)	No (-)
2	SS2/Cattle/SMD/2022	19,34	Positive (+)	No (-)
3	SK2/Cattle /SKB/2022	18,11	Positive (+)	No (-)
4	KS1/Buffalo/SMD/2022	18,20	Positive (+)	No (-)
5	S4/Cattle/SKB/2023	18,56	Positive (+)	Yes (+++)
6	S5/Cattle/SKB/2023	17,28	Positive (+)	Yes (+++)
7	S9/Cattle/SKB/2023	16,74	Positive (+)	Yes (+++)
8	S10/Cattle/SKB/2023	18,08	Positive (+)	Yes (+++)
9	S11/Cattle/SKB/2023	15,93	Positive (+)	No (-)
10	S12/Cattle/SKB/2023	16,39	Positive (+)	Yes (+++)

*P3: Passage 3; (+++): extensive CPE.

A total of 5 samples showed FMDV growth. The cytopathic effect causes severe cell damage and eventual cell death within 24-72 hours after infection. Negative virus growth results indicate the absence of CPE, thus causing no morphological changes in BHK-21 cells (El-Ansary *et al*., 2023).

These observations align with previous findings establishing that BHK-21 cells are highly permissive to FMDV replication, particularly for samples collected during the acute viremic phase of infection (Ranjan *et al*., 2016; Arzt *et al*., 2017; WOAH, 2021). The rapid onset of CPE from a high viral titer in the mucosal swab samples confirming that the circulating strain in West Java indicates high replicative fitness in the BHK-21 cells. The true virulence require controlled animal challenge studies to determained.

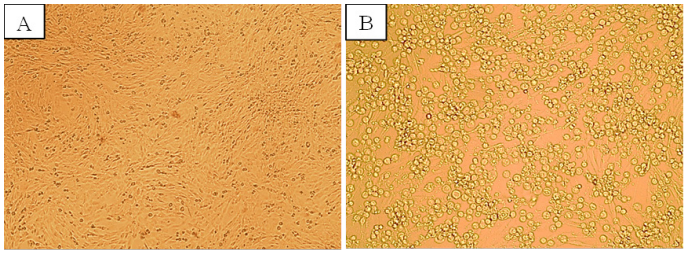

Figure 4: Cytopathic effects on BHK-21 cells due to FMDV growth. (A) Normal BHK-21 cells, (B) cytopathic effects on BHK-21 cells after 72 hours of incubation. Magnification 100x.

BHK-21 cells remain a gold standard for FMDV isolation due to their high sensitivity and ability to support rapid viral replication, specifically for Serotype O, which is predominant in the Southeast Asian region. The mechanism of CPE observed in this study is attributed to the virus's interaction with cellular integrin receptors, which facilitates viral entry and subsequent hijacking of the host cellular machinery, resulting in cell lysis (Jackson *et al*., 2002; Freimanis *et al*., 2016).

Molecular characterization

RT-PCR testing 31 samples were chosen for this test with the target protein VP1, which is a structural protein. A total of 8 samples were detected with FMDV serotype O. The results can be seen in the formation of electrophoresis bands corresponding to the length of the target gene, Figure 5 with an amplicon size of 1135 bp.

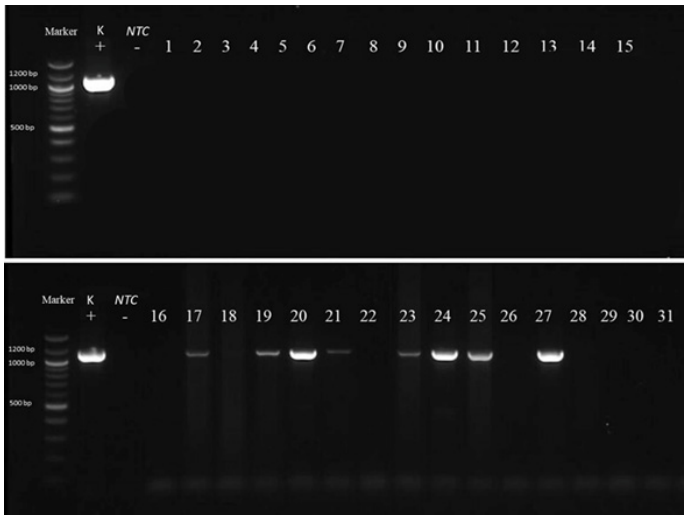

Figure 5: Visualization of RT-PCR products for determining FMDV serotypes. FMDV serotype O positive samples are indicated by a single band at a length of 1135 bp, namely samples numbered 17, 19, 20, 21, 23, 24, 25, and 27. K+ = positive control. NTC = Non template control. Marker = 100 bp.

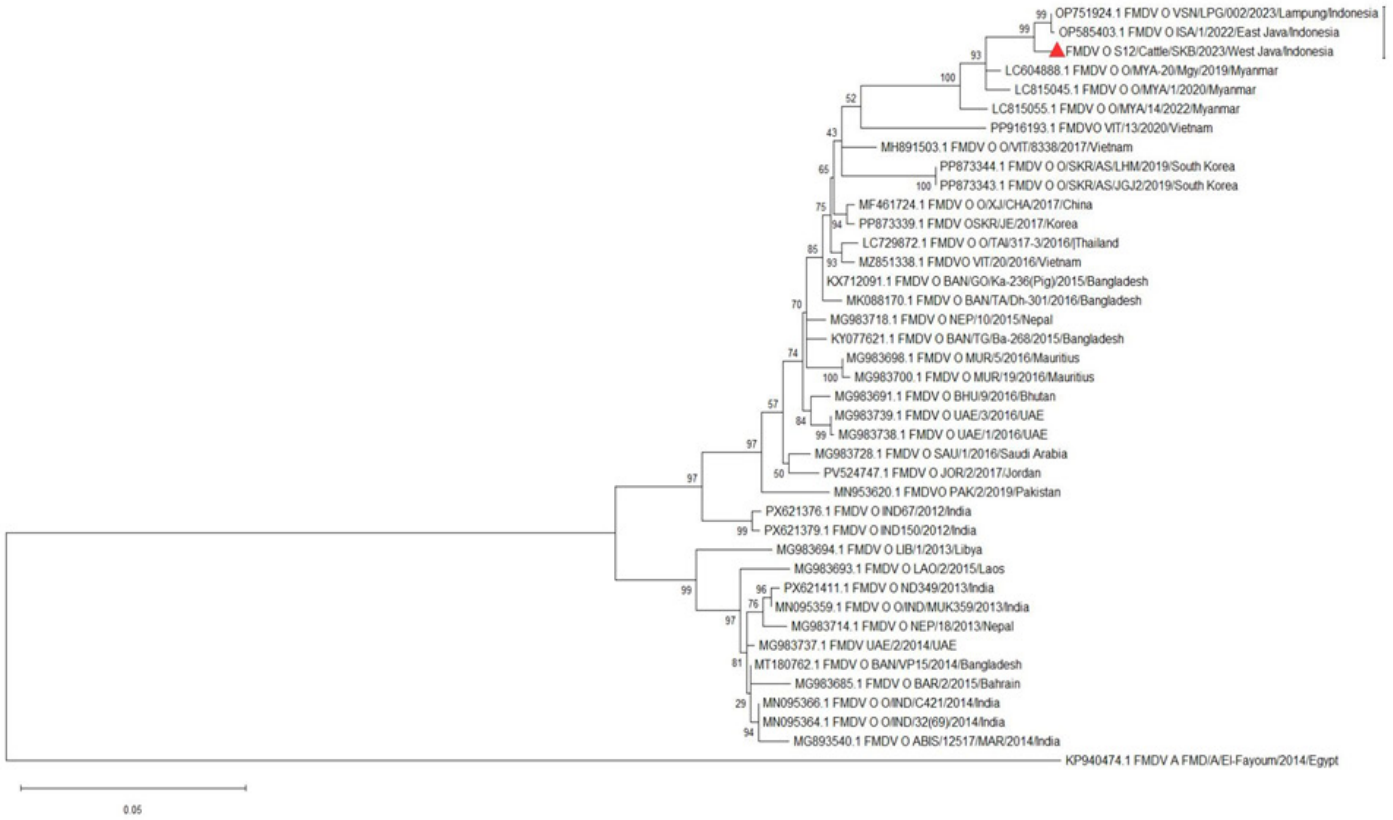

Figure 6: Phylogenetic tree of FMDV serotype O based on VP1 nucleotide sequences. The West Java isolate (S12/Cattle/SKB/2023) is highlighted in red triangle symbol ( ). Phylogenetic analysis was performed using the maximum likelihood method, bootstrap value 1000, and Kimura-2 parameter model. Bootstrap values >70% are shown at nodes. The scale bar indicates nucleotide substitutions per site.

Samples that were not detected as serotype O in this test were likely due to differences in sensitivity and detection limits between the two methods. RT-qPCR is inherently much more sensitive than RT-PCR, so RT-PCR cannot detect the virus if its concentration is below the detection limit (Bao *et al*., 2008). The sensitivity of RT-qPCR can be 10 to 1,000 times higher because it can detect DNA amplification products in real-time every cycle using fluorescent probes, enabling it to detect very low amounts of viral genetic material (viral load). Conventional RT-PCR only detects the final product after all cycles are complete through gel electrophoresis visualization.

The next factor that can also cause RT-PCR results to be undetectable is the genetic variability of the FMDV in the VP1 target region and the potential for primer mismatch. The gene encoding the VP1 protein was chosen because it is an important target for serotyping, but this gene is also one of the most variable parts of the FMDV genome. RNA viruses such as FMD have a high mutation rate. If there are mutations or sequence variations at the site where conventional RT-PCR primers bind in the 23 samples, the amplification process will not occur or will be highly inefficient. The type of primers used in this study for some samples was not suitable; testing with other primer pairs for serotype O, which has various options, or serotype-specific primers is necessary (Knowles *et al*., 2016).

The phylogenetic analysis placed the West Java isolate (S12/Cattle/SKB/2023) into a distinct cluster within Serotype O (Figure 6), sharing >98% nucleotide homology with strains from the 2022 East Java outbreak (GenBank Acc. No. OP585403; Zainuddin *et al*., 2023) and the 2023 Lampung case (GenBank Acc. No. OP751924). This high degree of genetic conservation suggests the possibility of a single introduction event followed by extensive local transmission. A whole genome analysis can provide important information in order to confirm a single introduction event in East Java, Lampung, and West Java Province on FMD outbreak.

These findings reflect established transboundary disease patterns in archipelagic nations, where inter-island transport facilitates transmission. Phylogenetically, the West Java strain clusters closely with lineages circulating in the Indian subcontinent and Southeast Asia, including Myanmar, Thailand, Vietnam, and Malaysia. This underscores Indonesia's susceptibility to regional transboundary animal disease (TAD) dynamics. Therefore, strengthening border biosecurity and regional collaboration is essential to monitor viral evolution and prevent the reintroduction of variants via informal trade

and human mobility (Jamal and Belsham, 2013; Knight-Jones *et al*., 2016).

Rapid antigen tests or clinical diagnosis should trigger immediate zoning measures for immediate respond and RT-qPCR must follow within 24–48 hours to confirm the diagnosis. If negative, restrictions are lifted to minimize economic disruption. Active RT-qPCR surveillance is mandatory for sheep/goat populations in outbreak zones, regardless of clinical appearance.

CONCLUSION

In conclusion, the clinical symptoms of FMD found in cattle, buffalo, and goats in West Java Province are still the same as the clinical symptoms of FMD that have been reported. The FMDV was successfully detected in 97% of animals showing clinical signs of FMD in this study. Serotype O was still found in several positive samples of the FMDV from West Java and was successfully grown in BHK-21 cells. The confirmation of Serotype O provides immediate scientific validation for the current government vaccination program. It confirms that the deployment of monovalent Serotype O vaccines remains the correct strategy.

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NOVELTY STATEMENT

The study is the first time a detection and molecular characterization of FMDV in West Java Province. The first study conducted to isolate FMDV in West Java Province.

AUTHOR'S CONTRIBUTION

MHN, NLPIM, HN, and IWTW contributed equally to this work. They conceptualized the study, conducted the research, analyzed the data, and finalized the manuscript. RI, RSA: Authors analyzed the data, and NLPID: Reviewed the manuscript. All authors read, reviewed, and approved the final content of the manuscript and agreed to the conditions outlined in the copyright assignment form.

Ethical approval

Ethical approval for this study was obtained from the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences, Number: 144/KEH/SKE/XII/2023.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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