



VIRUS PERSISTENCE AND ANTIBODIES OF FOOT AND MOUTH DISEASE IN DAIRY CATTLE IN BOYOLALI DISTRICT, CENTRAL JAVA

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Summary

Susilo, J., F. P. C. Santoso, A. Budiyanto, E. M. N. Setyawan & M. H. Wibowo, 2026. Virus persistence and antibodies of Foot and Mouth Disease in dairy cattle in Boyolali District, Central Java. *Bulg. J. Vet. Med.*, **29**, No 2, 267–279.

Foot and Mouth Disease (FMD) is a contagious disease of ruminants that causes significant economic losses for livestock farmers. It is caused by viruses from the *Picornaviridae* family, order Picornavirales, and the *Aphthovirus* genus. The FMD virus and the antibodies produced can be detected using laboratory tests. This research aims to conduct molecular testing on cattle suspected of FMD infection, detect post-infection virus shedding and antibodies. A total of forty dairy cows that had not been vaccinated against FMD from the smallholder farmers in Jemowo village, Banyusri, Taman Sari sub-district, Boyolali district were used in this research. Detection of viral ribonucleic acid (RNA) was carried out by real time Reverse Transcriptase-Polymerase Chain Reaction (rRT-PCR) using oropharyngeal fluid (OPF) samples taken using a probang cup. Testing for non-structural protein (NSP) and structural protein (SP) serotype O antibodies by the enzyme linked immunosorbent assay (ELISA) technique was performed on serum samples collected from the jugular vein. Detection of FMD virus RNA, NSP and SP antibodies was carried out twice each, namely 1 month and 6 months after FMD infection. The results showed that FMD virus RNA was detected 1 month and 6 months after infection of 39/40 (97.5%) and 3/40 (7.5%) respectively. The NSP test results at 1 month and 6 months post-infection were 37/40 (92.5%) and 35/40 (87.5%), and the SP results were 36/40 (90%) and 40/40 (100%). This research concluded that the cows were infected with FMD serotype O. The persistence of the FMD virus six months after outbreak was 7.5%, the persistence of NSP and SP antibodies was 87.5% and 100% respectively.

Key words: Foot and Mouth Disease, non-structural protein, oropharyngeal fluid, rRT-PCR, structural protein

INTRODUCTION

Foot and Mouth Disease (FMD) is a highly contagious and acute disease in livestock, leading to significant economic losses (Sissay, 2017). This disease is caused by the FMD virus, a member of the genus *Aphovirus* from the *Picornaviridae* family (MacLachlan & Dubovi, 2017). The main currently circulating serotypes of the FMD virus are serotypes A, O, C, Asia 1, South African Territories (SAT) 1, SAT 2, and SAT 3. The FMD virus has a capsid consisting of 4 structural proteins (VP1, VP2, VP3 and VP4) and 8 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D). These viral proteins have an important role in the life cycle of the FMD virus in infected cells (Clavijo *et al.* 2004; Gao *et al.* 2016). FMD infection produces antibodies against structural (SP) and non-structural (NSP) proteins. The vaccinated animals have an immune response against SP only, if the vaccine seed originated from purified virus (Elnekave *et al.*, 2016). Antibodies to NSP are an indicator of FMD virus replication, active infection, history of infection or viral proliferation (Robiolo *et al.*, 2006; Gao *et al.*, 2016).

The incubation period for FMD is 2–14 days. The mortality rate is 1–5% in adult livestock and more than 20% in young livestock (WOAH, 2022b). Clinical signs of animals infected with FMD are anorexia, fever, excessive salivation, and formation of vesicular blisters on the nose, muzzle, tongue, teats, feet, and other hairless skin parts (Knipe & Howely, 2001; El Damaty *et al.*, 2021; Soltan *et al.*, 2022), accompanied by lameness (Azeem *et al.*, 2020), and decreased milk production (Adjid, 2020; Sudarsono, 2022). Other studies report hypersalivation as the most common symptom, followed by lesions on

the feet and mouth, lameness, loss of appetite, depression, decreased milk production, lesions on the nipples and death (Nyaguthii *et al.* 2019; Urge *et al.* 2020).

Foot-and-mouth disease (FMD) negatively impacts livestock productivity through reduced milk production, increased mortality, low fertility rate and mastitis incidence, leading to higher culling rates and economic losses from control measures and trade restrictions (Knight-Jones & Rushton, 2013; Casey *et al.*, 2014; Jemberu *et al.*, 2014; Lyons *et al.*, 2015; Forbes *et al.*, 2016; Dos Santos *et al.*, 2017; Feng *et al.*, 2017; Paton, 2018; Truong, 2018).

FMD can be detected using virological and serological tests. Reverse transcription-PCR (rRT-PCR) is the confirmatory test for diagnosing the FMD virus because of its high specificity and sensitivity (WOAH, 2022a). Singh *et al.* (2020) stated that PCR is a simple, fast, sensitive, reliable, reproducible test and additional confirmation method for identification of FMD virus. Animals that recover from FMD infection can become disease carriers and have the potential to cause new outbreaks (Parthiban *et al.*, 2015). Previous research on OPF fluid samples specifically on nasopharyngeal mucosal epithelial cells using a probang cup can be used to detect the persistence of the FMD virus (Sutmoller & Gaggero, 1965). rRT-PCR can be used to amplify FMD virus genome fragments in diagnostic materials including epithelium, milk samples, serum and OPF (WOAH, 2022a). Further research has proven that the pharyngeal epithelium is a site for viral persistence (Parthiban *et al.*, 2015; Stenfeldt *et al.*, 2016).

The enzyme-linked immunosorbent assay (ELISA) is a widely used method for detecting FMD virus antigens and identifying serotypes (WOAH, 2022a). It is easy to perform by many laboratories, fast, and highly specific (Hussain *et al.*, 2019; Wang *et al.*, 2019). Additionally, ELISA-based serodiagnostics targeting the non-structural protein (NSP) 3ABC have been developed for differentiating infected from vaccinated animals (DIVA) and are recommended by WOAH (Fukai *et al.*, 2018; Gelkop *et al.*, 2018; Abd Hatem *et al.*, 2022; Keck, 2022). The presence of NSP antibodies indicates prior infection, suggesting the animal may have a role in disease persistence (Singh *et al.*, 2020).

The Ministry of Agriculture of the Republic of Indonesia includes FMD as a type of Strategic Infectious Animal Disease (Penyakit Hewan Menular Strategis/PHMS) that does not yet exist in Indonesia based on Minister of Agriculture Decree No. 4026/Kpts/OT.140/4/ 2013. Indonesia was declared free from FMD in 1986. In 2022, foot-and-mouth disease re-emerged in Indonesia, spreading to 22 provinces and causing significant economic losses. By November 2022, the outbreak affected 54.7 million livestock, with 570,137 reported cases, a morbidity rate of 1.04%, a mortality rate of 0.02%, and 12,650 animals culled (Anonymous, 2022). Molecular characterisation study of FMD in Indonesia confirmed infection in field cases and identified the circulating virus as serotype O, topotype ME-SA, lineage Ind-2001e, based on VP1 gene analysis, revealing high genetic similarity (99–100%) with viruses from the early 2022 outbreak (Sulistyaningrum *et al.*, 2024).

The research was conducted on dairy farms owned by smallholder farmers in

Boyolali Regency, Central Java because this district is a dairy farming center and its population is affected by the FMD outbreak in Indonesia. This research aims to confirm the presence of the FMD virus using the rRT-PCR method for 1 month and to detect latent infection 6 months after FMD infection of dairy cows. In addition, the study also aims to detect NSP and SP antibodies at 1 month and 6 months after the onset of clinical symptoms.

MATERIALS AND METHODS

Ethical approval

All research procedures follow the ethical aspects of animal welfare as stated in the Ethics Committee of the Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia No. 71/EC-FKH/Int./2023.

Samples origin

This research used 40 female dairy cows aged 2–10 years belonging to smallholder farmers in Jemowo village, Tamansari, Boyolali Regency, Central Java. A total of 40 cows were selected from 242 cows that still showed clinical symptoms of FMD. Cattle were kept using a traditional system, where animals are tethered in a fixed position, and fed concentrate pellets and forage. All cattle used in this study had a history of clinical FMD symptoms and had never been vaccinated against FMD. The research was conducted from October 2022 to March 2023. RNA virus detection was performed on OPF samples using rRT-PCR, while rRT-PCR for FMD and ELISA were performed at the Lampung Veterinary Center.

Oropharyngeal fluid and serum sampling

Collection of oropharyngeal fluid (OPF) samples using a probang cup was carried out twice, namely at 1 and 6 months after FMD infection by inserting a sterile probang into the oropharyngeal cavity to obtain fluid. OPF samples were taken by inserting the probang cup over the tongue into the oropharyngeal area and then passing it forcefully backwards and forwards 5–10 times between the first part of the esophagus and the back of the esophagus of the pharynx. The aim was to collect oropharyngeal fluid and especially superficial epithelial cells from this area, including the proximal part of the esophagus, the pharyngeal wall, tonsillar crypts, and the surface of the soft palate. The oropharyngeal fluid was added to 2 mL viral transport media containing 0.08M phosphate buffer of 0.01% bovine serum albumin, 0.002% phenol red, antibiotics (1000 units/mL penicillin, 100 units/mL mycostatin, 100 units/mL neomycin, and 50 units/mL polymyxin), with pH 7.2 (Kitching & Donaldson, 1987). (WOAH, 2022a).

Blood samples of 2 mL were collected twice, namely at 1 and 6 months after being infected with FMD from the jugular vein using a venoject needle with a vacuum tube without anticoagulant. Blood samples were centrifuged at 3000–4000 rpm for 10–15 min to separate serum from pellets. Serum was stored in a 1.5 mL microtube at a temperature of 4–8°C. Serum was inactivated by incubating at 58 °C for 30 min and then stored at –20 to –28°C for further analysis.

FMD virus extraction

RNA extraction from the 40 OPF samples was performed using an automatic machine with automatic pipetting pro-

grammes for the rRT-PCR. Automatic extraction uses the OptiPure kit with 96 wells in 8 columns containing lysis buffer, washing buffer, magnetic beads, and elution buffer. The sample extraction was then carried out according to the automatic extraction procedure using the OptiPure Viral Auto Plate (M665A46) on the TANBead Maelstrom 4800.

FMD virus VP-1 gene amplification using rRT-PCR

Amplification was carried out on the structural protein of the VP-1 gene for rRT-PCR of FMD (Knowles *et al.*, 2016). Reagents for amplification of deoxyribonucleic acid (DNA) are inserted into wells or microplates using the AgPath-IDTM One-step RT-PCR Kit (Cat.no. 56907371). The total volume of the reaction mix in the PCR tube was 20 µL consisting of 3.8 µL nuclease free water (NFW), 10 µL 2× RT-PCRBBuffer mix, 0.8 µL RT-PCREnzyme Mix, 0.8 µL forward primer, 0.8 µL of reverse primer, 1 µL each with a concentration of 20 pmol and 1 µL probe with a concentration of 5 pmol and 3 µL of extracted RNA template. FMD RT-PCR testing used Forward primer: ACTGG-GTTTT-ACAAA-CCT GT-GA; Reverse primer: GCGAG-TCCTG-CCACG-GA and probe: TCCTT-TGCAC-GCCGT-GGGAC (Callahan *et al.*, 2002). The amplification programme on the ABI 7500 Real time PCR Thermo Cyclor machine was Reverse Transcription at 45 °C for 10 min for 1 cycle; pre denaturation (95 °C for 10 min) for 1 cycle; denaturation (95 °C for 15 s), and annealing (60 °C for 45 s). The results were visible on the monitor screen in the form of curve and Cycle Threshold (CT) value numbers. A CT value < 40 was positive, CT values of 40–45 – indeterminate and CT values > 45 were negative.

Detection of NSP and SP of O serotype using ELISA

The NSP and serotype O-specific antibodies were examined in bovine serum samples collected 1 and 6 months post-FMD infection using ELISA. NSP-specific antibody detection was using the ID Screen® FMD NSP Competition commercial kit from ID. Vet, Batch no: FMDNSPC ver 0914 EN which was conducted based on the work instructions in the kit. Non-structural protein from the FMD virus as an antigen coated by detecting 3 ABC NSP antibodies. The presence of NSP antibodies in serum was determined by the S/N% value of the OD of each sample. NSP antibody was interpreted as seropositive if the S/N% value was $\leq 50\%$ and seronegative if $S/N\% > 50\%$. The ELISA NSP results were presented in the S/N ratio range table.

$$S/N\% = 100 \times (OD_{\text{sample}} / OD_{\text{NC}})$$

SP serotype O specific antibody detection uses the ID Screen® FMD Type O Competition ELISA kit from ID. Vet, Batch no: FMDOC ver 0314 GB which has been inactivated as an antigen coated to detect SP serotype O antibodies based on the work instructions in the kit. The presence of SP serotype O antibodies in serum was determined by the S/N% value of the OD of each sample. Seropositivity for SP serotype O antibodies was assumed if the S/N% value was ≤ 35 , equivocal (dubius): if the value was $35\% < S/N\% \leq 45\%$ and seronegative if $S/N\% > 45\%$. Then, the sample was read and recorded with an ELISA reader at a wavelength of 450 nm. The ELISA SP results were presented in the S/N ratio range table.

$$S.N\% = 100 \times [(OD_{\text{sample}} - OD_{\text{PC}}) / (OD_{\text{NC}} - OD_{\text{PC}})]$$

RESULTS

Clinical findings

The results of observations in the dairy cow population in this study were cows that had a history of clinical symptoms suspected of being FMD. Clinical symptoms that were still visible 1 month after FMD infection from 242 animals showed anorexia 119/242 (49.2%), hypersalivation 57/242 (23.6%), fever 9/242 (3.7%), lesions on the mouth or tongue 10/242 (4.1%), leg lesions 7/242 (2.9%), lameness 8/242 (3.3%), abscess 5/242 (2.1%) and mastitis 5/242 (2.1%) (Fig. 1).

VP-1 Gene Amplification of FMD virus

The rRT-PCR results from OPF samples of 40 dairy cows 1 month after showing clinical symptoms of FMD showed that 39 cows were detected positive for FMD (97.5%). FMD viral shedding was also still detected in 7.5% (3/40) 6 months post infection using the same testing techniques (Table 1). The rRT PCR results of gene amplification are shown on Fig. 2. The results were visible in the form of a curve and the CT value < 40 was positive, CT value 40–45 was indeterminate and CT value > 45 is negative.

NSP and SP ELISA results

The results of the ELISA NSP with Cut-off = 50% of 40 dairy cow serum samples showed that one month after the outbreak, 37/40 (92.5%) FMD seropositive cattle were detected and 35/40 (87.5%) were detected 6 months after the outbreak (Table 2). ELISA SP results with Cut-off S/N 35–45% detected 36/40 (90%) FMD seropositive one month after the outbreak, and 40/40 (100%) six months after the outbreak (Table 3).



Fig. 1. Clinical symptoms of FMD were seen in the dairy cows in this study: hypersalivation (a), fever (b), lesions in the oral cavity and upper palate (c), very severe tongue erosion (d), lesions and vesicles on the legs (e), interdigital lesions (f).

Table 1. rRT-PCR Results for 40 dairy cows 1 month and 6 months after FMD

Time after FMD infection	rRT-PCR results				Total
	<20	20-30	>30	Negative	
1 month	14	21	4	1	40
6 months	0	0	3	37	40
Positive control (+)	1				
Negative control (-)				1	

DISCUSSION

The results of observations of clinical symptoms in this study, which were seen 1 month after FMD infection, included hypersalivation (Fig. 1A), fever (Fig. 1B), lesions in the oral cavity (Fig. 1C), tongue lesions (Fig. 1D), lesions on the legs (Fig. 1E and 1F), anorexia, lameness, abscess,

and mastitis. This is in accordance with the study of Azeem *et al.* (2020), that the clinical symptoms caused by FMD include anorexia, lesions in the mouth area, feet, rashes, hypersalivation, decreased milk production, fever, weight loss and abortion. Ismail *et al.* (2023) also stated that clinical symptoms of FMD-infected dairy cows included hypersalivation, anorexia,

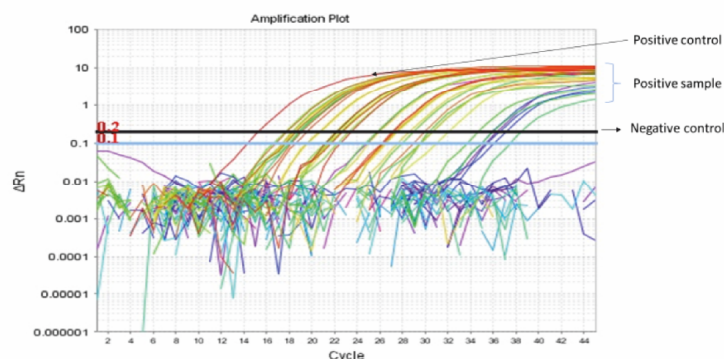


Fig. 2. The rRT-PCR results for FMD first month after the sampling of 40 cows showing clinical symptoms of FMD.

Table 2. Results of ELISA NSP Testing on 40 dairy cows 1 month and 6 months after FMD

S/N Cut-Off (%)	Number of samples		Interpretation of result
	1 month after FMD	6 months after FMD	
0–10	29	20	Positive
11–20	6	8	Positive
21–30	1	5	Positive
31–40	1	2	Positive
41–50	0	0	Positive
51–60	0	0	Negative
61–70	0	0	Negative
71–80	3	0	Negative
81–90	0	4	Negative
91–100	0	0	Negative
> 100	0	1	Negative
Total	40	40	

lameness, leg lesions, fever, and rupture of oral vesicle lesions. Other researchers reported that clinical symptoms of FMD included hypersalivation (77.3%) followed by lesions on the feet (53.6%), mouth lesions (52.7%), lameness 39.1%, anorexia 30.9%, depression 15.9%, decreased milk production 7.2%, nipple lesions 3.9%, and mortality of the adult cows of 0.5% (Nyaguthii *et al.* 2019). Urge *et al.* (2020) reported the following

symptoms of FMD in dairy cows from the Adea Berga district, central Oromia: hypersalivation (39%) was the most common symptom, followed by vesicles in the oral and interdigital cavities (22.6%), lameness (7.5%), and weakness (7.5%).

The results of examining oropharyngeal samples using the rRT-PCR 1 month and 6 months after FMD infection were 97.5% (39/40) and 7.5% (3/40), respectively, positive for FMD virus detection

Table 3. Results of ELISA Structural Protein Testing on 40 dairy cows 1 month and 6 months after FMD

S/N Cutt-Off (%)	Number of samples		Interpretation of result
	1 month after FMD	6 months after FMD	
0–10	31	32	Positive
11–20	3	7	Positive
21–30	2	1	Positive
31–40	0	0	Positive
41–50	0	0	Negative
51–60	0	0	Negative
61–70	0	0	Negative
71–80	0	0	Negative
81–90	0	0	Negative
91–100	2	0	Negative
> 100	2	0	Negative
Total	40	40	

(Table 1). FMD outbreaks often lead to a high proportion of virus carriers within the affected population. This is partly due to the practice of not culling infected animals (Hayer *et al.*, 2017; Bertram *et al.*, 2018b). Several studies were conducted regarding the detection of cattle carrying the FMD virus using OPF. In Vietnam an overall prevalence of 2.4% in Asian cattle and buffalo (de Carvalho Ferreira *et al.*, 2017), with a duration of 2 months to 2 years after the outbreak was shown. In another study, 4 out of 10 infected cows were still positive for FMD virus in OPF 32 months after the outbreak (Bertram *et al.*, 2018a). In another longitudinal investigation, the prevalence of FMD virus carriage 12 months after the outbreak was 0.7% (Bronsvort *et al.*, 2016). Longitudinal studies in India and Vietnam demonstrated FMD persistence 32% – 51% with a duration of 12 months after the outbreak (Bertram *et al.*, 2020). The decrease in the prevalence of disease carriers over time ranged from 0.03 to 0.1% per month, with a duration of 6 to 24 months, but also oc-

curred over 32 months in some studies (Stenfeldt & Arzt, 2020).

Previous research states that FMD carrier status will occur up to 9 months (Bronsvort *et al.*, 2016) and 12 months (Hayer *et al.*, 2017). Variations in carrier status duration are influenced by factors such as livestock management (breed, nutrition, movement, vaccine composition and frequency), host characteristics (age, sex, immune status), the causal virus strain (virulence, carrier prevalence), and the FMD virus itself, which is highly contagious through direct and indirect contact as well as through viral aerosols in the cage environment (Isha *et al.*, 2017). Differences in RT-PCR based analysis methodology compared to virus isolation and culture will influence the research conclusions (Bronsvort *et al.*, 2016).

The results of examining serum samples for ELISA antibodies about NSP 1 month and 6 months after FMD infection showed rates of 92.5% (37/40) and 87.5% (35/40), respectively (Table 2). Research examining the duration of anti-NSP anti-

bodies after natural FMD virus infection in animals is still limited (Elnekave *et al.*, 2016; Hayer *et al.*, 2017). Hayer *et al.* (2017), stated that throughout the post-outbreak study period, more than 75% of animals were NSP seropositive, with percent positivity (PP) more than 40. Clinically infected animals were 100% seropositive at 7.5 months post-outbreak, and 83% were seropositive at 12 months post-outbreak. In contrast, 87% and 69% of subclinically infected animals tested seropositive at least once at 7.5 and 12 months after the outbreak, respectively, respectively (Hayer *et al.*, 2017). The PP values in subclinical cattle were significantly lower than those in clinically affected animals (Hayer *et al.*, 2017; Elnekave *et al.*, 2015), reflecting the lack of viral replication during subclinical infection. However, a slight increase in the mean PP values of subclinical animals was observed 7.5 and 12 months post-outbreak, which may suggest ongoing immune response activity or delayed seroconversion. The persistence rates of FMD non-structural protein (NSP) antibodies in cattle slaughtered at abattoirs were reported to be high in Rawalpindi, India (44.55%), Ethiopia (48.1%), and Kenya (52.5%), indicating a high incidence of FMD exposure in these regions (Yousaf *et al.*, 2021; Megersa *et al.*, 2009; Kibore *et al.*, 2013).

In this study, the rates from examining serum samples for ELISA antibody SP antigen O at 1 month and 6 months after FMD infection were 90% (36/40) and 100% (40/40), respectively (Table 3). This shows that the dairy cows in this study were infected with the FMD virus serotype O. This result is in accordance with research conducted by Susila *et al.*, (2023) which stated that phylogenetic analysis based on the *VP1* coding sequence showed that all FMD viruses de-

tected during the disease outbreak in Indonesia in May 2022 (named ISA-22) were in the order O/ME-SA/Ind-2001e. Although all ISA-22 viruses are grouped under the Ind-2001e lineage, they form a distinct group separate from the Ind-2001e group from other Asian countries with an average nucleotide sequence similarity of 95.3%.

CONCLUSION

Based on the results of observing clinical symptoms, followed by rRT-PCR using oropharyngeal samples and ELISA testing of NSP and SP, the dairy cows in this study were infected with FMD virus serotype O. Persistence of the FMD virus was detected using the rRT-PCR test after 1 month (39/40; 97.5%) to 6 months (3/40; 7.5%). The persistence of NSP antibodies detected using ELISA of NSP was 37/40 (92.5%) at 1 month to 35/40 (87.5%) at 6 months. Persistence of SP antibodies detected using ELISA was 36/40 (90%) at 1 month to 40/40 (100%) at 6 months.

ACKNOWLEDGEMENTS

The authors are thankful to the Ministry of Agriculture Republic Indonesia for funding the research and publication support. We thank Hasan Abdullah Sanyata, DVM, Head of Lampung Investigation Centre, Enny Saswiyanti, DVM, M.Sc. for contribution in rRT-PCR assay and ELISA. In particular, the authors are grateful to the Head of Microbiology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta along with the laboratory technical assistant team, and Disease Investigation Center Lampung, for providing the necessary support during the study.

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Paper received 05.09.2024; accepted for publication 07.04.2025

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