



Detection of Persistence of Foot and Mouth Disease Virus in Local and Crossbred Cattle of Assam

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ABSTRACT

Background: This study aimed to investigate the persistence of Foot-and-Mouth Disease Virus (FMDV) in clinically recovered cattle following an outbreak, focusing on both local indigenous breeds (Lakhimi) and crossbred cattle (Holstein Friesian-cross) in Assam.

Methods: A total of 129 cattle (36 local and 93 crossbred), clinically recovered from FMD, were included in the study. Oropharyngeal fluid (OPF) samples (n=178) were collected at various intervals post-recovery. FMDV detection was carried out using three molecular diagnostic techniques: Multiplex PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and SYBR Green real-time PCR, all targeting the FMDV 3D gene.

Result: FMDV was detected in 38 samples (21.35%) by multiplex PCR, 47 samples (26.40%) by RT-LAMP and 49 samples (27.53%) by SYBR Green real-time PCR. While all three methods showed comparable diagnostic accuracy, real-time PCR proved to be the most sensitive, rapid, reliable and cost-effective technique. FMDV persistence was observed in 24.81% (32/129) of cattle up to the 1st month post-recovery, 11.63% (15/129) up to the 3rd month and 1.55% (2/129) up to the 6th month. Local non-vaccinated Lakhimi cattle exhibited higher rates of viral persistence for serotype O, with 33.33% (12/36), 22.22% (8/36) and 5.55% (2/36) showing persistence at the 1st, 3rd and 6th months post-recovery, respectively. In contrast, vaccinated Holstein Friesian-cross cattle, demonstrated lower persistence rates, with 19.23% for Serotype A and 22.39% for Serotype O at the 1st month and 10.45% for Serotype O at the 3rd month post-recovery. Importantly, no transmission of the virus to healthy in-contact animals was detected during the study period. Statistical analysis ($P=0.23$, Chi-square=5.57) revealed no significant difference in viral persistence between local and crossbred cattle, suggesting that factors beyond vaccination status might influence FMDV persistence. However, the findings underscore the critical role of vaccination in reducing viral persistence in recovered cattle.

Key words: Foot-and-mouth disease (FMD), Multiplex PCR, Oropharyngeal fluid, RT-LAMP, SYBR green real-time PCR, Viral persistence in cattle.

INTRODUCTION

Foot-and-Mouth Disease (FMD) is a persistent and recurrent disease of significant economic importance in India, with frequent outbreaks occurring despite extensive vaccination programs (Rout *et al.*, 2016; Baro *et al.*, 2018). The geographical distribution of FMD in India varies by region, with prevalence rates reported at 43% in the Eastern region, 31.5% in the Southern region, 11.6% in the North-eastern region, 5% in the Central region, 4.4% in the Western region and 4% in the Northern region (Barman *et al.*, 2020). Among circulating FMDV serotypes, serotype O is predominant (92%), followed by Asia1 (5%) and serotype A (3%) (Subramaniam *et al.*, 2022).

FMDV typically resolves systemically within 8-15 days post-infection in cattle (Juleff *et al.*, 2013). However, certain animals may become carriers, harboring the virus in their pharyngeal tissues for extended periods post-infection, particularly if viral persistence extends beyond 28 days (Salt, 1993). FMDV persistence can occur in naive, convalescent, or vaccinated animals, regardless of whether they experienced symptomatic or asymptomatic infections. Both primary and persistent infections are linked to the nasopharyngeal mucosa-associated lymphoid tissue (MALT), where viral capsid proteins and/or genome can be

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detected in the germinal centers and follicular dendritic cells of this tissue (Cortey *et al.*, 2019).

In both domestic and wild ruminants, FMDV persistence can result in intermittent viral shedding in oropharyngeal fluids, which may last for extended periods

(Stenfelt and Arzt, 2020). This phenomenon plays a critical role in FMD epidemiology, particularly at the interface between wildlife and domestic cattle (Jori *et al.*, 2016). Continuous viral replication in specialized cellular sites within carrier animals allows for the possibility of new outbreaks initiated by these carriers (Zhang *et al.*, 2004).

Despite its importance, few studies have investigated FMDV persistence in local or crossbred cattle, particularly in India. The carrier state in these animals is difficult to detect due to the absence of overt clinical signs, complicating the accurate assessment of prevalence and epidemiology in field conditions. Thus, labor-intensive sampling and the application of highly sensitive molecular diagnostic tools are required for confirmatory diagnosis. Key aspects of FMDV carriers, including the viral factors influencing persistence, emergence, competition, transmission and spread, remain poorly understood and warrant further study (GFRA Gap Analysis Report, 2022). Understanding the mechanisms and duration of FMDV persistence is crucial for developing effective and sustainable control strategies.

This study, therefore, aims to detect and characterize FMDV persistence in local and crossbred cattle (both vaccinated and non-vaccinated) in Assam, India. The findings will provide valuable insights for refining FMD control programs, ultimately contributing to the goal of FMD eradication

MATERIALS AND METHODS

Collection of samples

Oropharyngeal fluid (OPF) samples (n=178) were collected using the probang cup method from follow-up cases of FMD-infected (129) and in-contact healthy animals (n=12) during various outbreaks in Assam during 2018-2021. Sampling was conducted 28 days post-outbreak and repeated at 3-month intervals until viral clearance. Samples were collected in equal volumes of 0.08M phosphate-buffered saline containing 0.01% bovine serum albumin with added antibiotics (pH 7.6) (WOAH, 2012). Additionally, whole blood samples (n=24; 12 from healthy in-contact and 12 from FMD-recovered cattle) were collected in EDTA vials and stored at -20°C until processing.

Extraction of RNA

Genomic RNA extraction from OPF and whole blood samples was performed using the QIAamp RNA mini kit (Hilden, Germany, Cat. No. 52904) according to the recommended protocol.

Reverse transcription of extracted RNA for cDNA synthesis

For cDNA synthesis, 11 µl of extracted RNA from each sample was subjected to initial incubation at 65°C for 7 minutes after adding Random hexamer (0.2 µg/µl) 1µl. Subsequently, RevertAid™ M-MuLV Reverse Transcriptase (200 units/µl) 1µl, Ribolock RNase Inhibitor (40U/µl) 0.5 µl, 5X RT buffer 4 µl and 10 mM dNTP mix 2 µl were added to

a final volume of 20 µl. The reaction mixture underwent thermal cycling at 25°C for 5 minutes, 42°C for 1 hour and 72°C for 10 minutes, followed by storage at 4°C.

Multiplex polymerase chain reaction (mPCR)

Multiplex PCR was performed for simultaneous detection and serotyping of FMDV using specific primers for Serotypes O, A and Asia-1 (Giridharan *et al.*, 2005). The mPCR reaction mixture included Master Mix (2X) 12.5 µl, specific primers (10 pmol each) for FMDV serotypes and cDNA 5 µl in a total volume of 25 µl. Thermal cycling conditions involved initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

RT-LAMP was used for FMDV detection and serotyping (Madhanmohan *et al.*, 2013) using specific primers for Serotypes O, A and Asia-1, using Loopamp real-time turbidimeter (LA-500; Eiken Chemical, Tokyo, Japan). The RT-LAMP reaction mixture included Reaction Buffer (10x), Bst Polymerase, MgSO₄, Betaine, dNTPs and specific primers along with cDNA. The reaction underwent initial denaturation at 95°C for 5 minutes, followed by isothermal amplification at 63°C for 60 minutes and inactivation at 83°C for 2 minutes.

SYBR green real-time PCR (3D gene)

Real-time PCR targeting the FMDV 3D gene was performed using SYBR Green dye (Callahan *et al.*, 2002) on an Applied Biosystems StepOnePlus Real-Time PCR System. The real-time PCR reaction mixture included 2X PCR Master Mix (SYBR Green), specific primers for the 3D gene, cDNA and nuclease-free water. Thermal cycling conditions consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. A melt curve analysis was performed at the end of the reaction. All samples were analyzed in duplicate, with appropriate positive and negative controls included in each run to ensure accuracy and reliability of results.

RESULTS AND DISCUSSION

Multiplex PCR (mPCR)

In this study, multiplex PCR (mPCR) (Giridharan *et al.*, 2005) was utilized for the confirmatory diagnosis and typing of FMDV using OPF (n=178) and blood (n=24) samples. Among the follow-up cases of clinically FMD-recovered cattle, 38 OPF samples (21.35%) tested positive for FMDV by multiplex PCR (Table 1; Fig 1 and 2), indicating persistence, with 33 samples (18.54%) being Type O and 5 (2.81%) being Type A. However, OPF (n=12) and blood (n=12) samples from all healthy in-contact (control) and blood (n=12) from persistently infected cattle were negative

for FMDV. Therefore, this study reveals that investigation of the FMD virus in clinically FMD-recovered animals using OP fluid is suitable for detecting persistently infected animals.

Multiplex PCR (mPCR) is a sensitive, specific and reliable technique that can be used for serotyping ELISA-negative samples. Multiplex assays provide multiple advantage as they are used to detect several pathogens

and their serotypes at the same time (Deka *et al.*, 2024). Giridharan *et al.* (2005) demonstrated that both mPCR and ELISA performed well with 100% efficiency on cell culture samples, with mPCR showing better efficiency than ELISA on clinical samples. The development of one-step mRT-PCR for detection and typing of FMDV serotypes O, A and Asia 1 exhibited high sensitivity and specificity (Le *et al.*, 2011). For the detection of FMDV excreted in cow milk, the

Table 1: Detection of persistence of FMDV from OPF samples (n=178).

FMD serotypes	mPCR		RT-LAMP		SYBR green real-time PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
Type O	33 (18.54%)	145	42 (23.60%)	136	49 (27.53)	129
Type A	5 (2.81%)	173	5 (2.81%)	173		
Type Asia-1	-	178	-	178		
Total (n=178)	38 (21.35%)	140	47 (26.40%)	131	49 (27.53%)	129

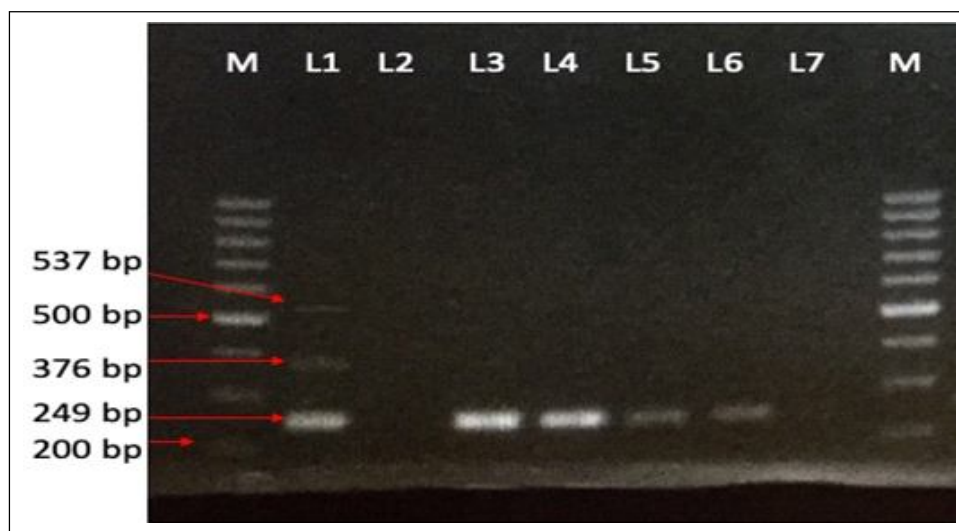


Fig 1: Multiplex PCR products for FMDV positive (Serotype O) OPF samples in 1.5% Agarose Gel electrophoresis (M=1 kb Ladder, L1=PC, L2=NC, L3-L7= OPF samples).

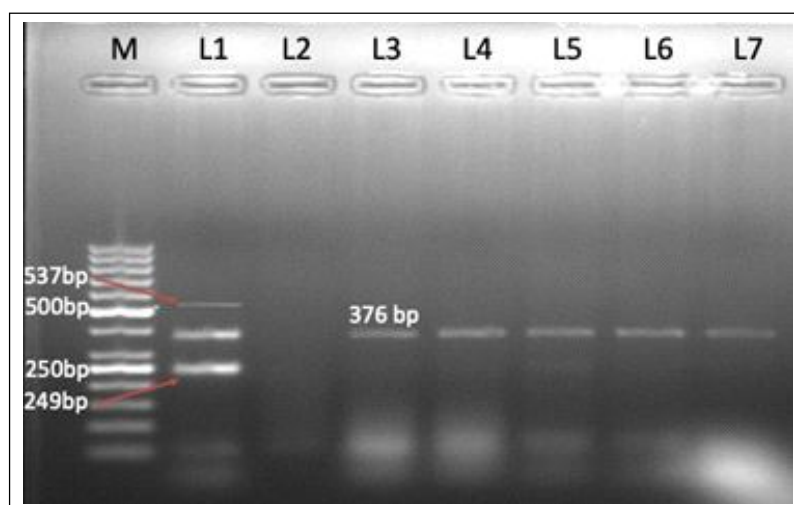


Fig 2: Multiplex PCR products for FMDV positive (Serotype A) OPF samples in 1.5% Agarose Gel electrophoresis (M=50 bp Ladder, L1=PC, L2 = NTC, L3-L7=OPF samples).

detection limit of mPCR was 102.7 TCID₅₀/ml (Ranjan *et al.*, 2016). Therefore, mPCR emerges as a rapid and reliable molecular technique for detecting and typing FMDV, particularly useful for identifying persistence of FMDV from OPF samples in clinically FMD-recovered cattle.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

RT-LAMP is a recently developed diagnostic technique that employs a simple isothermal amplification process, allowing rapid and highly sensitive detection of nucleic acids (Notomi *et al.*, 2000). In this study, RT-LAMP (Madhanmohan *et al.*, 2013) was utilized alongside multiplex PCR (mPCR) for confirmatory diagnosis and serotyping of FMDV from oropharyngeal fluid (OPF) samples (n=178) using serotype-specific primers. Among FMD-recovered cattle, 47 OPF samples (26.40%) tested positive for FMDV (Table 1; Fig 3 and 4), with 42 samples (23.60%) belonging to serotype O and 5 (2.81%) to serotype A, indicating viral persistence. Notably, all OPF (n=12) and blood (n=12) samples from healthy in-contact control animals, as well as blood samples (n=12) from clinically recovered but persistently infected cattle, were negative for FMDV.

The one-step RT-LAMP assay used in this study, designed for the rapid detection and serotyping of FMDV, was found to be 10³-10⁵ times more sensitive than conventional RT-PCR, with a detection limit ranging from 10⁻³ to 10⁻⁵ TCID₅₀ of FMDV across all three serotypes. It detected 100% of clinical samples for the three serotypes, comparable to qRT-PCR, whereas RT-PCR detected 69.7%, 58.1% and 60.0% of type O, A and Asia 1 samples,

respectively. This demonstrates that RT-LAMP has sensitivity comparable to real-time RT-PCR, with no cross-reactivity among the three FMDV serotypes or negative samples (Madhanmohan *et al.*, 2013).

RT-LAMP was able to detect serotype Asia 1 of FMDV within 45 minutes at 61°C (Chen *et al.*, 2011), making it faster than conventional methods. Compared to the real-time TaqMan assay, RT-LAMP was found to be not only sensitive and specific but also more rapid and cost-effective (Dukes *et al.*, 2006). An RT-LAMP assay based on the 3D polymerase gene demonstrated even greater sensitivity than routine mPCR (Ranjan *et al.*, 2016). The assay, enhanced with hydroxynaphthol blue (HNB) dye for colorimetric detection, offers a simple, specific and sensitive tool for rapid diagnosis of FMDV, making it a viable option for field deployment and surveillance in regions like India and other developing countries (Dukes *et al.*, 2006).

For FMDV detection in cow milk, the RT-LAMP assay exhibited a detection limit of 101.7 TCID₅₀/ml (Ranjan *et al.*, 2016), further showcasing its versatility. An RT-LAMP assay developed to detect serotype C of FMDV had 10-fold higher sensitivity than conventional RT-PCR (Ding *et al.*, 2014). A modified FMDV-specific RT-LAMP assay, combined with a lateral flow device (LFD) for dual-labeled product detection, achieved sensitivity equivalent to laboratory-based real-time RT-qPCR and showed 10,000-fold greater analytical sensitivity than the FMDV-specific antigen LFD used in the field (Waters *et al.*, 2014).

Lyophilized RT-LAMP and real-time PCR assays are also compatible with fluorescence detection for real-time amplification monitoring and endpoint detection can be achieved using molecular lateral flow devices (Howson

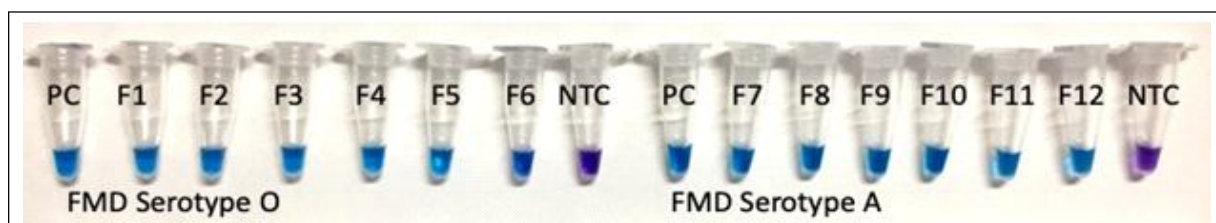


Fig 3: Results of RT-LAMP for FMDV Serotype O and A in OP fluid (F1-F12) samples (PC=Positive control and NTC=Negative control).

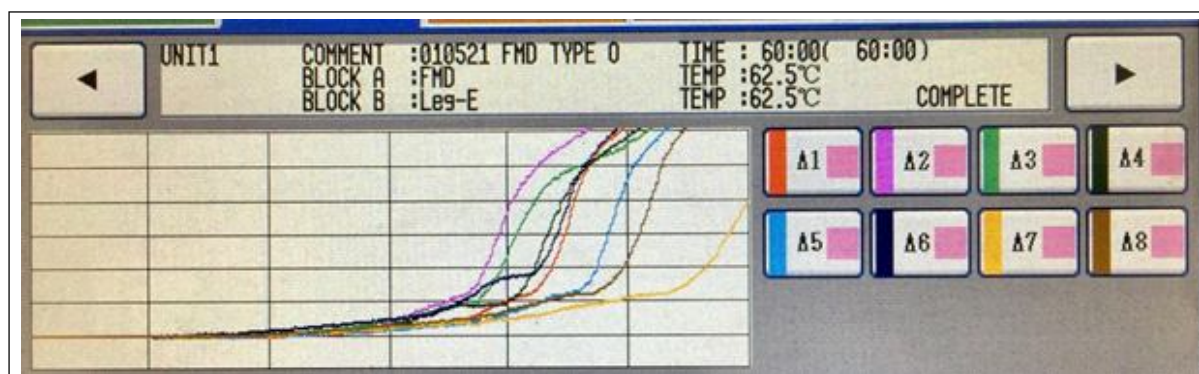


Fig 4: Image of RT-LAMP for FMDV Serotype O and A in OPF sample using a real-time turbidimeter (Included Positive control, Positive sample and Negative control).

et al., 2017). While RT-LAMP assays often target specific local epidemiological settings due to primer design constraints, pan-FMDV assays have also been developed (Lim *et al.*, 2020). Additionally, the need for specialized equipment can be minimized by using indicator chemistries that produce color changes visible to the naked eye, making it an ideal candidate for point-of-care diagnostics under field conditions (Zhang *et al.*, 2022).

In conclusion, RT-LAMP offers numerous advantages, including being inexpensive, sensitive, rapid and easy to use, making it a promising tool for the diagnosis and typing of FMDV, particularly in field settings and resource-limited environments.

SYBR green real-time PCR (3D gene)

Real-time PCR is a highly advanced, rapid and reliable diagnostic technique that allows for real-time monitoring of fluorescence during amplification, eliminating the need for post-PCR processing. In this study, we employed SYBR Green real-time PCR, targeting the FMDV 3D gene across all serotypes (Callahan *et al.*, 2002), for the confirmatory diagnosis of FMDV in oropharyngeal fluid (OPF) samples (n=178). Among the follow-up cases of FMD-recovered cattle, 49 OPF samples (27.53%) tested positive for FMDV (Table 1; Fig 5 and 6), indicating viral persistence. Conversely, OPF (n=12) and blood (n=12) samples from all healthy in-contact controls and blood (n=12) from clinically recovered but persistently infected cattle were negative for FMDV. Real-time PCR proved to be a highly sensitive method, especially for detecting viral persistence in OPF samples.

The high sensitivity of real-time RT-PCR enables the detection of FMDV even in non-conventional samples such

as OPF, pooled milk (Armson *et al.*, 2020), airborne particles, environmental swabs (Brown *et al.*, 2021) and meat juice (Yeo *et al.*, 2022). A real-time RT-PCR assay, designed to detect all seven FMDV serotypes from various sample types, demonstrated 100% specificity, with the ability to detect viral RNA even before the onset of clinical symptoms in some cases (Callahan *et al.*, 2002). Viral RNA levels typically peak between 30 to 53 hours post-infection (p.i.) and decline thereafter, becoming undetectable between 7 and 18 days p.i. In persistently infected animals, however, viral RNA remained detectable in OPF samples beyond 28 days p.i. (Stenfeldt *et al.*, 2013; Zhang *et al.*, 2004). This persistence in OPF samples plays a critical role in understanding the long-term presence of FMDV.

Studies using one-step RT-PCR have indicated that FMDV persistence is significantly higher in unvaccinated cattle compared to vaccinated animals (Thwiny, 2016). Real-time PCR assays are widely used in reference laboratories for routine FMD diagnosis and viral quantification, although they cannot distinguish between different FMDV serotypes (Jamal and Belsham, 2013).

When comparing multiplex PCR (mPCR), RT-LAMP and SYBR Green real-time PCR for FMDV detection, positive results were found in 21.35%, 26.40% and 27.53% of OPF samples, respectively, highlighting the superior sensitivity of real-time PCR over mPCR and RT-LAMP (Table 1). Real-time PCR was the most rapid and sensitive technique, identifying more cases of persistent FMDV infection compared to the other methods. However, combining these diagnostic techniques can enhance overall diagnostic accuracy, as observed in previous studies (Brahma *et al.*, 2024). A multi-assay approach can significantly improve

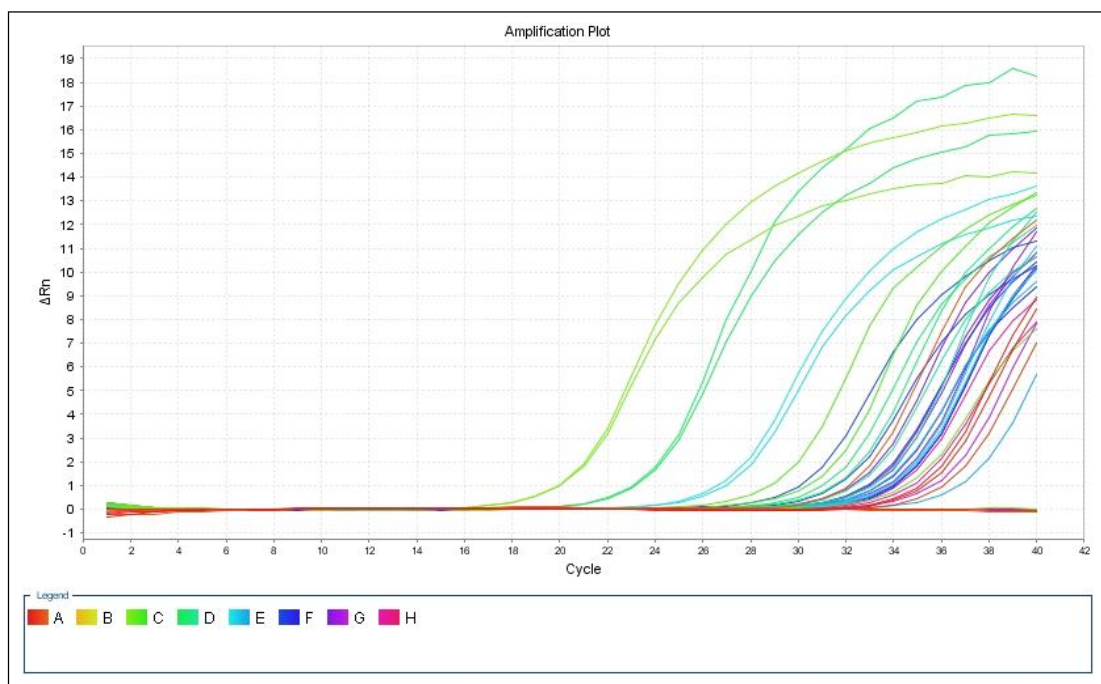


Fig 5: Amplification Plot of SYBR Green real-time PCR targeting 3D gene of FMDV.

diagnostic performance and resolution, particularly in challenging epidemiological scenarios (Foglia *et al.*, 2021).

Persistence of FMDV in local and crossbred cattle

Foot-and-mouth disease virus (FMDV) persistence has been observed across various species, including cattle, sheep, goats, Asian buffalo and wildlife species (Weaver *et al.*, 2013). The establishment of a carrier state does not seem to depend on specific viral genome determinants (Arzt *et al.*, 2019). Instead, factors such as species, disease incidence, vaccination coverage and immune status influence the prevalence of carriers within a population (Thwiny, 2016). Viral persistence may be modulated by factors like residual viral RNA replication, RNA degradation/clearance, or the characteristics of infected cells and their ability to induce cell death (Zhang *et al.*, 2004). Additionally, impaired cellular immune responses, inhibition of apoptotic pathways, elevated Th17 activity and upregulated aryl hydrocarbon signaling have been associated with persistence (Zhu *et al.*, 2022). While low levels of FMDV RNA may be detected in pharyngeal epithelial samples during early and persistent infection phases, higher viral levels are often present in pharyngeal secretions (Stenfeldt

and Belsham, 2012). Infectious FMDV can be isolated from milk and oropharyngeal fluid (OPF) samples from persistently infected animals (Buckle *et al.*, 2021), including those subclinically infected after vaccination (Navid *et al.*, 2018).

FMDV may persist for months or even years post-infection in a subclinical state in a significant proportion of animals (Stenfeldt and Arzt, 2020). In our study, persistence in cattle was mostly detected up to 3-4 months post-infection, with no persistence beyond 4 months in crossbred (vaccinated) cattle. However, 2 local (non-vaccinated) cattle (1.6%) exhibited persistent infection up to 6-7 months post-outbreak. No cases of persistence were detected at the 9-month mark. Overall persistence rates in cattle (n=129) were 24.81%, 11.26% and 1.6% at the 1st, 3rd and 6th months, respectively. Persistence rates in the 1st month varied by serotype and vaccination status: 22.39% in vaccinated serotype O, 19.23% in vaccinated serotype A and 33.33% in non-vaccinated serotype O. At the 3rd month, persistence was 10.45% in vaccinated serotype O and 22.22% in non-vaccinated serotype O. By the 6th month, only 5.55% of non-vaccinated serotype O animals remained persistently infected (Table 2). Statistical analysis revealed no

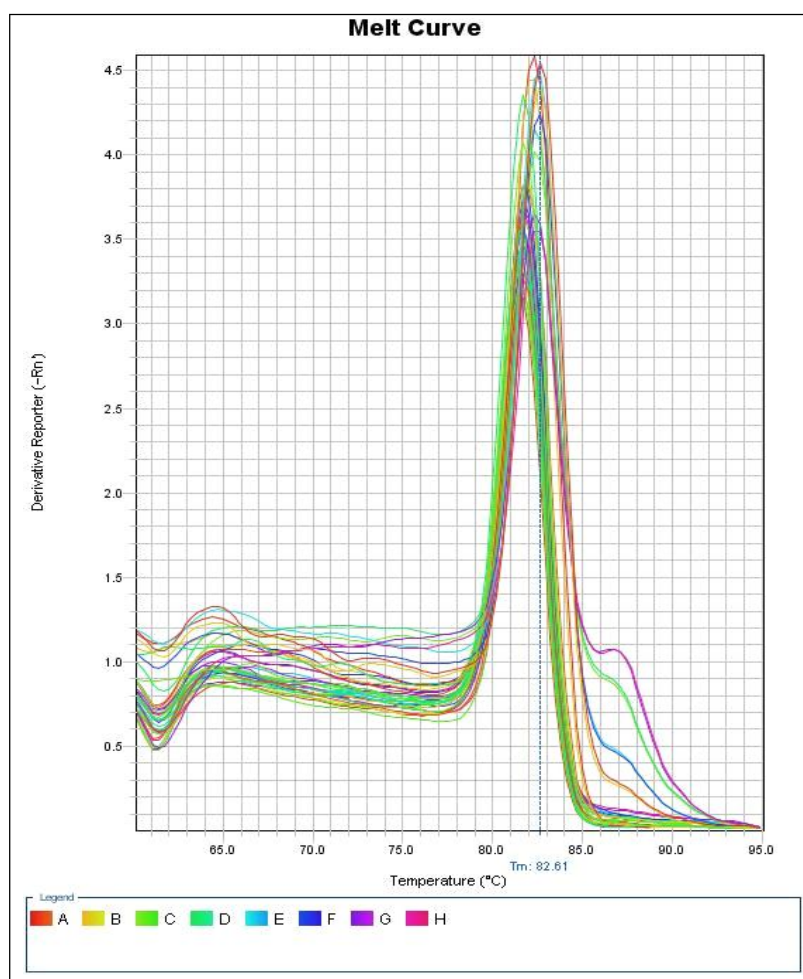


Fig 6: Melt Curve of SYBR Green real-time PCR targeting 3D gene of FMDV.

Table 2: Month-wise rate of persistence of FMDV in local (non-vaccinated) and crossbred (vaccinated) cattle.

Status	FMDV Serotype	Rate of Persistence			
		1 st month	3 rd month	6 th month	9 th month
Local lakhimi cattle (Non-vaccinated)	"O" (n=36)	12 (33.33%)	8 (22.22%)	2 (5.55%)	Nil
Crossbred cattle of Holstein friesian (Vaccinated)	"O" (n=67)	15 (22.39%)	7 (10.45%)	Nil	Nil
	"A" (n=26)	5 (19.23%)	Nil	Nil	Nil
Total no. of animals	129	32 (24.81%)	15 (11.63%)	2 (1.55%)	Nil

significant difference between local (non-vaccinated) and crossbred (vaccinated) cattle, with a p-value of 0.23 (>0.05) and a Chi-square value of 5.57.

Other studies similarly report varying rates of FMDV persistence in both vaccinated and non-vaccinated cattle. In Indian cattle, the average duration of persistence was reported to be 10-13 months based on OPF sampling and FMDV-specific RT-PCR, with no statistical difference in carrier state duration between clinically and subclinically infected animals (Hayer *et al.*, 2017a; Hayer *et al.*, 2017b). In abattoir samples, up to 11.1% of healthy buffalo OPF samples tested positive for FMDV using real-time PCR (Navid *et al.*, 2018). Approximately 50% of FMD-recovered ruminants develop persistent infections, regardless of vaccination status (Parida, 2009). The carrier state in cattle typically does not exceed 6 months, although a small percentage may remain carriers for up to 3 years (WOAH, 2012).

In experimental studies, FMDV Asia 1 infection via intradermal-lingual inoculation resulted in 65.2% to 71% of cattle developing persistent infections, with carriers exhibiting significantly higher neutralizing antibody titers than non-carriers (Mohan *et al.*, 2008). Even vaccinated cattle can become carriers (Zhang *et al.*, 2009), although increasing vaccine doses may reduce viral shedding and the frequency of carrier states (Parida *et al.*, 2006). Persistence rates of 62% and 67% have been reported in vaccinated and non-vaccinated cattle, respectively, with similar levels of viral RNA shedding during persistence (Stenfeldt *et al.*, 2016). One study reported 91% of vaccinated cattle remaining persistently infected up to or beyond 28 days post-challenge (Parida *et al.*, 2006). Unvaccinated cattle tend to shed higher viral loads for longer periods, whether they become carriers or not (Parthiban *et al.*, 2015).

The risk of transmission from FMDV carriers appears to be low (Arzt *et al.*, 2021; Bertram *et al.*, 2018; Bronsvoort *et al.*, 2016). In our study, no transmission to in-contact healthy animals (n=12) was detected. However, experimental studies have shown that naive cattle can develop clinical FMD after intranasopharyngeal inoculation with untreated OPF from persistently infected carriers (Stenfeldt and Arzt, 2020). Transmission from carriers is rare, as evidenced by few seropositive animals detected more than 12 months after an outbreak (Bronsvoort *et al.*, 2016). No FMDV infection or seroconversion was detected in three calves born to carrier animals (Bertram *et al.*, 2018). Nonetheless, persistently

infected carriers play a crucial role in FMDV ecology and are a significant factor in trade regulations and outbreak responses in disease-free regions (GFRA Gap Analysis Report, 2022).

Given the potential role of carriers in FMDV ecology, developing rapid and reliable diagnostic methods for early detection and addressing the carrier state remains critical for effective control and prevention strategies.

CONCLUSION

In conclusion, our study demonstrates that local cattle, particularly those unvaccinated, exhibited a higher prevalence and extended duration of FMDV persistence compared to crossbred cattle. This finding highlights the critical role of vaccination in reducing FMDV persistence in both local and crossbred cattle populations. Implementing comprehensive vaccination programs is essential to minimize viral persistence, thereby enhancing disease control and supporting broader efforts to prevent FMD outbreaks in susceptible cattle breeds.

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Animal welfare and ethics

Prior approval No.770/GO/Re/S/CPCSEA/FVSc/AAU/IAEC/18-19/665 Dated 28.12.2018 was taken under Institutional Animal Ethics Committee.

Conflict of interest

The authors declare that they have no conflict of interest.

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