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Comparing Typing Methods for Foot-and-Mouth Disease Virus Serotypes: Sandwich ELISA, Multiplex PCR, RT-LAMP and SYBR Green Real-time PCR

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ABSTRACT

Background: Foot and Mouth Disease (FMD) poses a significant economic threat to the cattle population in Assam, with recurrent outbreaks occurring annually.

Methods: This study aimed to compare different diagnostic techniques for the molecular detection and serotyping of Foot and Mouth Disease Virus (FMDV) outbreaks in the region. Epithelial tissue samples (n=29) were collected following standard procedures. SYBR Green real-time PCR targeting the 3D gene, sandwich enzyme-linked immunosorbent assay (S-ELISA), multiplex PCR (mPCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) were utilized for detection and serotyping.

Result: All 29 tissue samples tested positive for FMDV using SYBR Green real-time PCR, mPCR and RT-LAMP, with 100% congruence in serotyping (20 serotype O, 9 serotype A). In contrast, S-ELISA detected only 86.21% of samples as positive (17 serotype O, 8 serotype A). Statistical analysis revealed significant differences between the sensitivity of S-ELISA and molecular techniques ($p = 2.91 \times 10^{-7}$). The findings underscore the superior sensitivity of SYBR Green real-time PCR, mPCR and RT-LAMP, highlighting their potential for enhancing FMDV surveillance and control efforts in Assam.

Key words: Foot and mouth disease virus, Multiplex PCR, Reverse transcription loop-mediated isothermal amplification, Sandwich ELISA, SYBR Green real-time PCR.

INTRODUCTION

Foot and mouth disease (FMD) is a transboundary and highly contagious disease affecting mammals, characterized by fever, loss of appetite, depression, lameness, and the appearance of vesicles on the feet and in or around the mouth, resulting in severe economic losses in susceptible cloven-hoofed animals, including domestic and wild ruminants and pigs [World Organisation for Animal Health (OIE)] 2012; Hegde *et al*., 2021; Rout *et al*., 2016; Rout *et al*., 2017). In India, FMD is endemic, with outbreaks reported yearly, with varying prevalence rates across different regions (Tamilselvan *et al*., 2009; Subramaniam *et al*., 2013).

Among the seven serotypes of FMD virus (FMDV), serotypes O, A, C and Asia-1 have been recorded in India, with serotype C not reported since 1995 and the last global outbreak recorded in Ethiopia in 2005 (Rweyemamu *et al*., 2008). Between 2011 and 2020, FMDV serotype O was the most prevalent, responsible for approximately 92% of all recorded outbreaks in India. This was followed by serotype Asia1, which accounted for about 5% of outbreaks and serotype A, which comprised 3% of outbreaks (Subramaniam *et al*., 2022).

While virus isolation is the gold standard for FMD diagnosis, it is slow and cumbersome. Serological techniques such as sandwich-ELISA (S-ELISA), Liquid-Phase Blocking ELISA (LPBE) and indirect ELISA (DIVA) offer alternatives (Longjam *et al*., 2011). Various nucleic acid-based molecular diagnostic techniques, including reverse transcription-polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA) and reverse transcription loop-mediated isothermal amplification (RT-LAMP), have been developed for rapid and specific detection of FMDV RNA (Biswal *et al*., 2012). Additionally, nucleotide sequencing, microarray, recombinant antigen-based detection, biosensors, phage display and pen-side tests such as lateral flow and Immunostrip tests are available for field detection (Longjam *et al*., 2011).

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RT-LAMP, an isothermal auto-cycling and strand displacement DNA synthesis method, holds the potential for field deployment in FMD diagnosis as a point-of-care test (Dukes *et al*., 2006; Ranjan *et al*., 2014). Real-time reverse transcription polymerase chain reaction (rRT-PCR) has become a widely used method in molecular diagnostics and research, offering sensitive, specific, and swift detection and quantification of viral RNAs, essential for diseases like FMD (Hoffman *et al*., 2009). Multiplex PCR, which co-amplifies different sets of primers targeting different FMDV serotypes, enables simultaneous diagnosis, saving time and reagents (Giridharan *et al*., 2005).

Given the array of diagnostic options, this study aims to conduct a comparative analysis of molecular detection and typing of FMDV in Assam using different diagnostic techniques.

MATERIALS AND METHODS

Collection of Samples

The research work has been carried out with high biosafety and biosecurity standards in the BSL-3 laboratory, DBT-Advanced Animal Disease Diagnosis and Management Consortium (ADMaC), Guwahati center, Department of Veterinary Microbiology, Assam Agricultural University, Khanapara-781022, Assam, India. Epithelial tissue samples (n=29) were collected from un-ruptured or freshly ruptured vesicles on the tongue, gums, and feet of suspected FMD-infected animals during the period 2018-2021. Samples were placed into scintillation vials containing a transport medium composed of 50% glycerol and 0.04M phosphate buffer with antibiotics (penicillin, myostatin, neomycin and polymyxin) adjusted to pH 7.4. The antigen preparation was conducted following standard procedures (World Organisation for Animal Health [OIE], 2012).

Serotyping of FMDV by S-ELISA and PCR

Serotyping of the circulating FMD virus serotypes was conducted using Sandwich ELISA (S-ELISA), multiplex PCR (mPCR) and reverse transcription-loop-mediated isothermal amplification (RT-LAMP). For the standardization of different methods, live virus O, A and Asia-1 vaccine strains maintained at the Central FMD Virus Typing Laboratory, Directorate on Foot-and-Mouth Disease (DFMD), Indian Veterinary Research Institute (IVRI) campus, Mukteswar, India, were used as positive controls in this study.

Sandwich ELISA (S-ELISA)

The S-ELISA was performed according to bench protocols using reagents standardized at the Central FMD Virus Typing Laboratory, Directorate on Foot-and-Mouth Disease (DFMD), Indian Veterinary Research Institute (IVRI) campus, Mukteswar, India. Type-specific anti-146S FMDV sera, raised in rabbits and guinea pigs, were used as coating and tracing sera respectively, in accordance with ICAR-DFMD recommendations, Mukteswar, India. Rabbit anti-guinea pig IgG-HRP conjugate was utilized in the assay.

Reverse transcription of RNA (RT-PCR) for cDNA synthesis

Genomic RNA extraction was performed using the QIAamp Viral RNA kit (Qiagen, Cat No. 52904) according to the manufacturer's protocol. Extracted RNA was quantified and cDNA synthesis was performed immediately using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. EP0441). Thermal cycling conditions and reagent concentrations were based on manufacturer protocols and previous studies (Notomi *et al*., 2000; Mori *et al*., 2001).

Multiplex polymerase chain reaction (mPCR)

The multiplex polymerase chain reaction (mPCR) is a widely used technique in molecular biology, known for its ability to amplify multiple templates within a single reaction, thereby conserving both time and reagents. The primer sets utilized for the mPCR study were chosen based on the recommendations provided by Giridharan *et al*., (2005). These primer sets consist of a universal primer (reverse sense) and serotype-specific primers (genome sense) for each of the FMDV Serotypes O, A and Asia 1.

The components of the mPCR reaction mixture included 12.5 µl of 2X PCR Master Mix, along with specific amounts of forward primers (Fwd primer1, Fwd primer2 and Fwd primer3), a reverse primer (universal primer), nuclease-free water (NFW) and cDNA. To achieve a total volume of 25 µl, 5 µl of cDNA was added to the mixture. The list of primers used in reverse transcriptase multiplex polymerase chain reaction (RT-mPCR) is provided in Table 1. Positive controls included live virus O/A and Asia-1 vaccine strains from the Central FMD Virus Typing Laboratory at IVRI, Mukteswar, India, while negative controls were Non-Template Controls (NTC). The thermal cycling conditions for mPCR involved an initial denaturation step at 95°C for 5 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, repeated for 30 cycles. A final extension step at 72°C for 10 minutes was performed to complete the reaction. Subsequently, agarose gel electrophoresis was conducted to analyze the mPCR product.

This method allows for the simultaneous amplification and detection of multiple FMDV serotypes in a single

Table 1: Primers used for amplification of serotype-specific FMDV by RT-mPCR.

Serotypes of FMDV	Primers	Sequences (5′ –3′)
O	Fwd primer1/DHP15	CAACGGGACGARCAAGTACTC
A	Fwd primer2/DHP13	GTGACTGAACTGCTTTACCGCAT
ASIA-I	Fwd primer3/DHP9	GACCTGGAGGTYGCGCTTGT
Universal	Reverse primer/NK61	GACATGTCCTCCTGCATCTG

reaction, providing a cost-effective and efficient approach for serotyping analysis (Giridharan *et al*., 2005).

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

Reverse transcription Loop-Mediated Isothermal Amplification (RT-LAMP) is a cutting-edge diagnostic technique that utilizes autocycling and strand displacement DNA synthesis to amplify viral cDNA under isothermal conditions, requiring minimal reagents and equipment, such as a water bath (Notomi *et al*., 2000). This method offers an advantage as positive RT-LAMP reactions cause the solution to become cloudy due to the formation of magnesium pyrophosphate, eliminating the need for gel electrophoresis for DNA product visualization (Mori *et al*., 2001).

In this study, RT-LAMP (Madhanmohan *et al*., 2013) was employed for the detection and serotyping of FMDV from epithelial tissue samples. The RT-LAMP reaction was conducted in a Loopamp real-time turbidimeter (LA-500; Eiken Chemical, Tokyo, Japan). Each sample was tested against three FMD RT-LAMP primers targeting serotypes O, A and Asia-1, with positive and negative controls included in each run to prevent cross-contamination.

The components of the RT-LAMP reaction mixture consisted of Reaction Buffer (10x), Bst Polymerase, $MgSO_4$, Betaine, dNTP, HBN and various primers, along with nuclease-free water and cDNA, resulting in a total volume of 20 µl. The thermal cycling conditions for RT-LAMP involved initial denaturation of cDNA, isothermal amplification and inactivation.

During amplification, the progress could be monitored in real-time on the turbidimeter screen. After completion of the cycle, RT-LAMP products were analyzed based on colour development with positive samples turning violet while negative samples retained their original blue colour. Additionally, positive samples could be identified based on the turbidity of the RT-LAMP product.

SYBR Green real-time PCR targeting 3D gene for detection of FMDV

The study utilized SYBR Green real-time PCR targeting the 3D gene for rapid detection of Foot-and-Mouth Disease Virus (FMDV). Primer sets by Callahan *et al*. (2002), endorsed by the World Organisation for Animal Health (formerly OIE) in 2012, were employed. cDNA from suspected tissue samples was used, with samples run in duplicate alongside appropriate controls. The Applied Biosystems (ABI) StepOnePlus Real-Time PCR System was utilized. The components of the SYBR Green real-time PCR reaction mixture were as follows: 2X PCR Master Mix (SYBR Green) 5 µl, 3D forward primer (10 pmol) 0.2 µl, 3D reverse primer (10 pmol) 0.2 µl, cDNA 2 µl and nuclease-free water (NFW) 2.6 µl, resulting in a total volume of 10 µl.

Thermal cycling conditions for the SYBR Green real-time PCR targeting the FMDV-specific 3D gene were as follows: holding at 50°C for 2 min, initial denaturation at 95°C for 10 min; denaturation, annealing and extension at 95°C for 15 sec and 60°C for 1 min, respectively, for 40 cycles. Subsequently, a melt curve analysis (ramp rate @ 0.3°C/sec) was performed at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

RESULTS AND DISCUSSION

Molecular detection and typing of FMD virus

FMD is highly contagious among cloven-hoofed animals, both domestic and wild. Effective control of FMD relies on the availability of sensitive, specific and rapid diagnostic tools. Diagnosis traditionally involves virus isolation, considered the gold standard despite its slow and labour-intensive nature. Alternatively, detection of FMD viral antigen or nucleic acid in clinical samples has become the modern, commonly employed method due to its rapidity, sensitivity, and specificity (WOAH, 2009; Longjam *et al*., 2011). Both serological and molecular biology-based methods are utilized globally and domestically for FMD diagnosis (Muthukrishnan *et al*., 2008). In our study, we employed a combination of tests for the molecular detection and typing of FMDV (Table 2).

Sandwich ELISA (S-ELISA)

In the present study, all the epithelial samples (n=29) collected from FMD-infected animals showing clinical signs and lesions of FMD were subjected to S-ELISA after

Table 2: Results of S-ELISA, mPCR, RT-LAMP and 3D gene SYBR green Real-time PCR on tissue samples for detection and typing of FMDV.

Diagnostic tests	No. of tissue samples tested	No. of tissue samples positive	Serotypes		
			O	A	Asia1
S-ELISA (Central FMD Virus Typing Laboratory, Directorate on Foot-and-Mouth Disease (DFMD), Indian Veterinary Research Institute (IVRI) campus, Mukteswar, India)	29	25 (86.21%)	17	8	0
mPCR (Giridharan *et al*., 2005)	29	29 (100%)	20	9	0
RT-LAMP(Madhanmohan *et al*., 2005)	29	29 (100%)	20	9	0
3D gene SYBR green real-time PCR (Callahan *et al*., 2002)	29	29 (100%)	-	-	-

the preparation of antigen. Out of all the tissue samples (n=29) collected from the FMD suspected animals, only 25 (86.21%) samples were found to be positive for FMDV in S-ELISA, where 17 were Serotype O and 8 were Serotype A positive (Fig 1). However, 4 numbers of tissue samples were found to be negative for FMDV.

ELISA is the preferred procedure for the detection of FMD viral antigen and identification of viral serotype (Ferris and Donaldson, 1992; Roeder and Smith, 1987; WOAH, 2009). According to the WOAH (2012), it is the recommended test for the detection and typing of viral antigens from tissue samples. In our study, the FMDV serotype could be identified in 25 (86.21%) epithelial tissue samples collected from clinically FMD-infected animals by S-ELISA. In the case of clinically affected animals, lesion materials were the richest source of FMD virus and the sample of choice for diagnosis by S-ELISA. In fact, S-ELISA is being used for antigen detection using the material from the lesions though because of its low sensitivity currently mPCR is being used (Longjam *et al*., 2011).

Multiplex PCR

Analysis of various primer sets designed for universal and serotype-specific diagnosis of FMDV, targeting all seven serotypes, revealed that no single primer set could independently diagnose the disease or type of the virus. Consequently, multiplex PCR, incorporating multiple primer sets, has been developed to enhance the diagnostic sensitivity of conventional RT-PCR (Bao *et al*., 2008; Giridharan *et al*., 2005).

In this study, multiplex PCR (Giridharan *et al*., 2005) was utilized for confirmatory diagnosis and typing of FMDV from tissue samples. All 29 (100%) tissue samples from clinically FMD-infected cattle tested positive for FMDV. The serotype distribution among the 29 tissue samples was as follows: 20 were positive for Serotype O and 9 were positive for Serotype A. Fig 2 shows multiplex PCR identifying FMDV serotype “O” with a 249 bp product size in all positive samples, while Fig 3 shows the identification of FMDV serotype “A” with a 376 bp product size in all positive samples. No Asia-1 serotype was detected.

Giridharan *et al*. (2005), using the multiplex PCR they developed, reported 100% efficiency on cell culture samples, with both ELISA and mPCR performing well. However, mPCR exhibited superior efficiency over ELISA on clinical samples, accurately identifying serotypes and detecting dual infections in some cases. Their study demonstrated that mPCR was sensitive, specific and reliable, capable of serotyping ELISA-negative samples. Similarly, in our study, mPCR detected FMDV in 100% of tissue samples from clinically FMD-infected cattle, while S-ELISA only detected 86.21% of positive tissue samples. Other studies have also demonstrated the effectiveness of mPCR. For instance, a one-step multiplex RT-PCR adapted for detecting and typing FMDV serotypes O, A and Asia-1 in Vietnam showed high sensitivity and specificity (Le *et al*., 2011). Additionally, using mPCR and RT-LAMP assays for detecting FMDV excreted in cow milk, the detection limits were found to be 10^2.7 and 10^1.7 TCID50/ml, respectively (Ranjan *et al*., 2016). A multiplex RT-PCR developed for the simultaneous detection and differentiation of Turkish FMDV serotypes A and O from clinical samples demonstrated diagnostic sensitivity and specificity of 95% and 84%, respectively, compared to ELISA (Sareyyüpoğlu and Burgu, 2017). Thus, multiplex PCR emerges as a rapid and highly reliable molecular technique for detecting and typing FMDV from clinical samples.

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

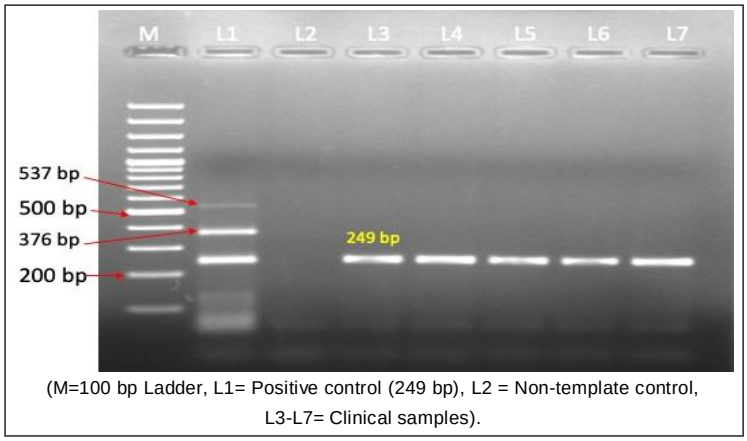
The LAMP reaction, completed in less than an hour using a standard water bath or heating block, allows for results visualization with the naked eye due to visible colour development. This simplicity, rapidity and potential for visual interpretation without instrumentation make the technique an attractive alternative for use in field conditions, especially in endemic countries (Dukes *et al*., 2006).

Although RT-LAMP assay has not been extensively evaluated for replacing or supplementing current molecular



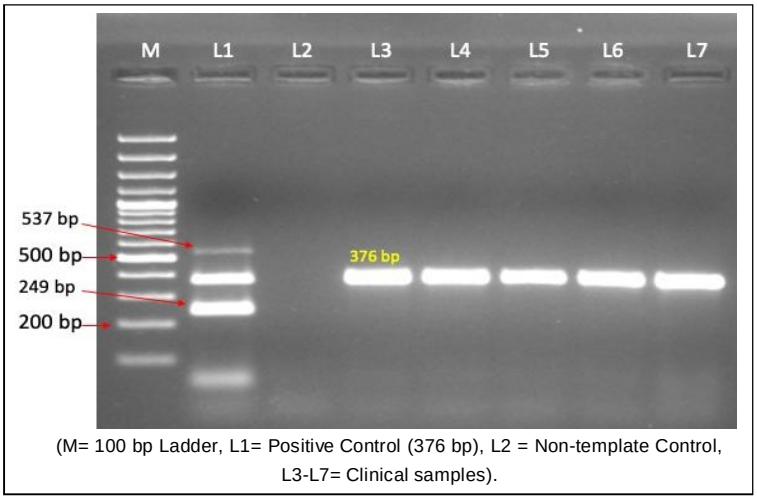
Rows: T1-T5 = tissue samples, (-) = background control, (+) = Positive control; Column 1-3(O) = serotype O, column 5-7(A) = serotype A, column 9-11(X) = serotype Asia 1.

Fig 1: Typing of foot and mouth disease virus by sandwich ELISA.



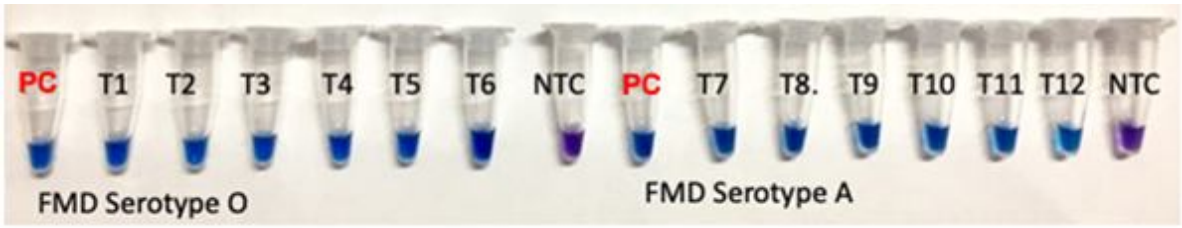
(M=100 bp Ladder, L1= Positive control (249 bp), L2 = Non-template control, L3-L7= Clinical samples).

Fig 2: Multiplex PCR products for FMDV positive (Serotype O= 249 bp) field samples in 1.5% Agarose Gel electrophoresis.



(M= 100 bp Ladder, L1= Positive Control (376 bp), L2 = Non-template Control, L3-L7= Clinical samples).

Fig 3: Multiplex PCR products for FMDV positive (Serotype A= 376 bp) field samples in 1.5% Agarose Gel electrophoresis.



(PC= Positive control and NTC= Negative control).

Fig 4: Results of RT-LAMP for FMDV, Serotype O and A, in tissue (T1-T12) samples.

diagnostic techniques, in this study, alongside mPCR, RT-LAMP (Madhanmohan *et al*., 2013) was utilized for confirmatory diagnosis and typing of FMDV from tissue samples using serotype-specific primers. All tissue samples (100%, n=29) from clinically FMD-infected cattle tested positive for FMDV. Serotype distribution among the 29 tissue samples revealed 20 positive for Serotype O and 9 positive for Serotype A (Fig 4-5). No Asia-1 serotype was detected. RT-LAMP and mPCR exhibited the same sensitivity (100%) in detecting FMDV from acutely infected animals.

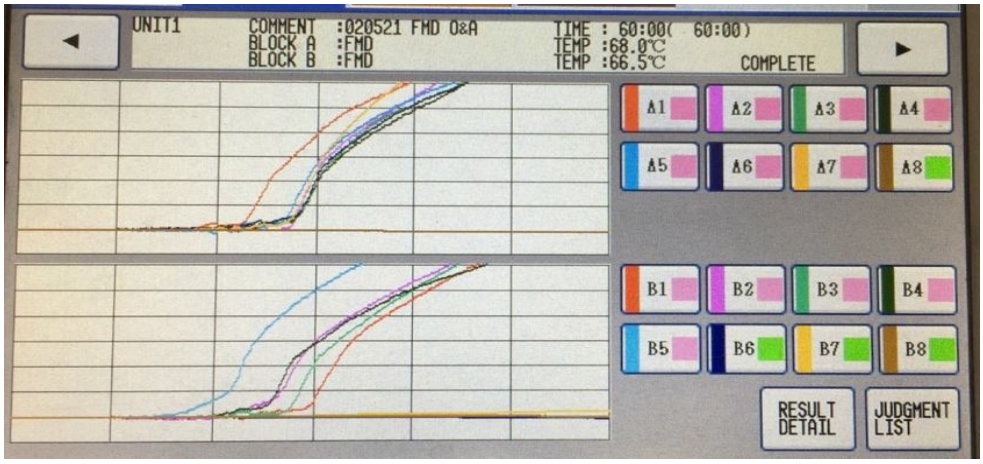
Madhanmohan *et al*. (2013) developed a one-step real-time RT-LAMP assay for rapid detection and serotyping of Indian FMDV, finding it to be 10^3-10^5 fold more sensitive compared to RT-PCR, with a detection limit ranging from 10^{-3} to 10^{-5} $TCID_{50}$ of FMDV of all three serotypes. The RT-LAMP assay detected 100% of clinical samples of the three serotypes, similar to qRT-PCR, while RT-PCR detected 69.7%, 58.1% and 60.0% of type O, A and Asia-1 samples, respectively. This suggests that RT-LAMP had the same sensitivity as real-time RT-PCR, without cross-reactivity within the three serotypes of FMDV and FMDV negative samples. Similarly, in our study, RT-LAMP detected FMDV in 100% of tissue samples from clinically FMD-infected cattle. An RT-LAMP assay based on the 3D

polymerase gene for specific and rapid detection of FMDV was found to be more sensitive than routinely used multiplex PCR. RT-LAMP with HNB dye was identified as a simple, specific and sensitive assay for rapid diagnosis of FMDV infection, with the potential for field deployment and rapid FMDV surveillance in India (Ranjan *et al*., 2014). With its cost-effectiveness, sensitivity, rapidity and simplicity, RT-LAMP holds promise as a point-of-care diagnostic in field conditions for diagnosing and typing FMDV.

SYBR Green real-time PCR targeting 3D gene

SYBR Green real-time PCR targeting the 3D gene is a modern diagnostic technique that eliminates the need for post-PCR gel electrophoresis by directly monitoring signal amplification during target cDNA/DNA amplification. In this study, SYBR Green real-time PCR targeting the 3D gene, which is specific to FMDV of all serotypes was employed for confirmatory diagnosis of FMD in tissue samples. All 29 tissue samples from clinically FMD-infected cattle tested positive for FMDV (Fig 6) indicating the high sensitivity of real-time PCR for FMDV detection.

Callahan *et al*. (2002) evaluated a portable real-time RT-PCR assay designed to detect all seven viral serotypes of FMDV from various sample types and found it to have 100% specificity. The assay demonstrated equal or greater sensitivity compared to viral culture in samples from experimentally infected animals and detected viral RNA in the mouth and nose before the onset of clinical disease in some cases. Similarly, in our study, real-time PCR detected FMDV in 100% of tissue samples from clinically FMD-infected cattle. Real-time PCR assays are now commonly used as routine tests for FMD diagnosis and virus quantification in many reference laboratories worldwide and in developed countries. However, these assays do not differentiate between FMDV serotypes, although they target highly conserved regions across all seven serotypes (King *et al*., 2006; Jamal and Belsham, 2013).



(Included Positive control, Positive sample and Negative control).

Fig 5: Results of RT-LAMP of tissue sample targeting FMDV, Serotype O (Block A) and Serotype A (Block B) in a real-time turbidimeter.

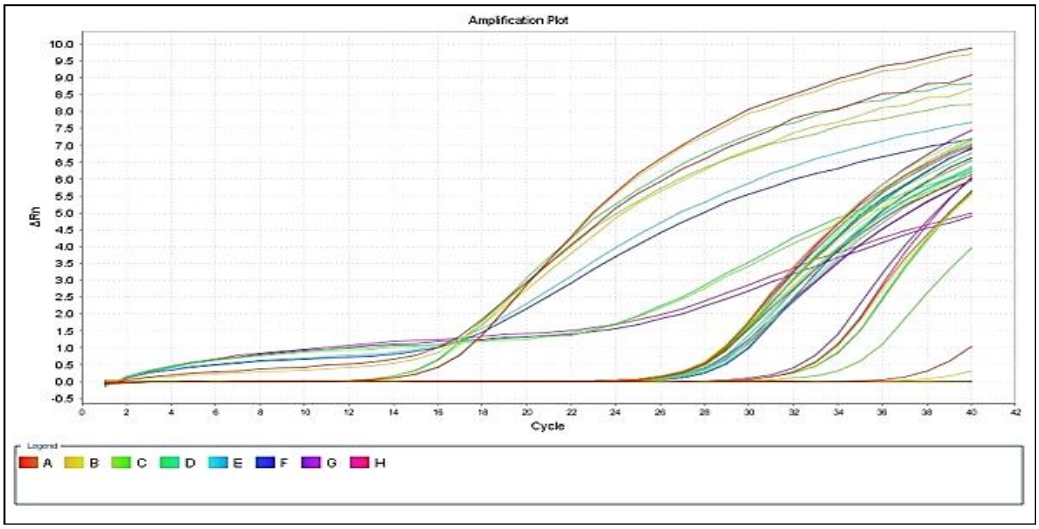


Fig 6: Amplification plot of SYBR green real-time PCR targeting 3D gene of FMDV.

Comparison of the diagnostic techniques used and their statistical analysis

In the present study, four diagnostic techniques were employed for the detection and typing of foot-and-mouth disease virus (FMDV) including SYBR Green real-time PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP), multiplex PCR (mPCR) and sandwich enzyme-linked immunosorbent assay (S-ELISA) kit. Madhanmohan *et al*. (2013) reported that real-time PCR and RT-LAMP demonstrated similar sensitivity, whereas mPCR was less sensitive compared to both real-time PCR and RT-LAMP. Upon comparison of the diagnostic tests on clinical samples, real-time PCR proved to be the most rapid and highly sensitive in detecting FMDV, followed by RT-LAMP, mPCR and S-ELISA.

In the present study, FMDV serotype identification rates were as follows: 86.21% with S-ELISA and 100% with mPCR, RT-LAMP and SYBR Green real-time PCR. A binomial test for P-value, considering real-time PCR as the gold standard, showed a significant difference for ELISA (P-value approximately 2.91×10^{-7}) but no significant difference for mPCR, RT-LAMP and SYBR Green real-time PCR (P-value 1.000) indicating their equal sensitivity in detecting FMDV in tissue samples from acutely infected animals.

All samples positive for FMDV by S-ELISA were also positive by mPCR, RT-LAMP and SYBR Green real-time PCR, suggesting the higher sensitivity of these molecular techniques compared to S-ELISA in detecting FMDV in tissue samples. The lower virus detection rate by S-ELISA could be attributed to environmental factors affecting antigen degradation, while molecular techniques were able to amplify intact RNA even from degraded samples. The concentration of viral load in samples may also influence the positivity rate between ELISA and molecular techniques.

CONCLUSION

Circulation of FMDV Serotypes O and A was observed in Assam between 2018 and 2021. Molecular detection and serotyping of FMDV are crucial for effective FMD control programs due to their rapid and high sensitivity. RT-LAMP emerges as a promising pen-side test for swift detection and serotyping of FMDV. In conclusion, the combination of different diagnostic techniques enhances diagnostic accuracy, with molecular techniques being more rapid and reliable for diagnosing FMD from epithelial tissue samples.

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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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