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ORIGINAL PAPER

One-step SYBR green-based real-time RT-PCR assay for detection of foot-and-mouth disease virus circulating in India

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Abstract

Rapid, sensitive, and reliable laboratory detection of foot-and-mouth disease virus (FMDV) infection is essential for containing and controlling virus infection in any geographical area. In this report a SYBR green-based $3D^{pol}$-specific one-step real-time RT-PCR (rRT-PCR) assay was developed for the pan-serotype detection of FMDV in India. The detection limit of the SYBR green-based rRT-PCR was 10^{-2} $TCID_{50}$/50 µl, which is 10 times more sensitive than the traditional agarose gel electrophoresis-based RT-multiplex PCR (RT-mPCR). The standard curve exhibited a linear range across 8-log_{10} units of viral RNA dilution. The reproducibility and specificity of this assay were reasonably high suggesting that the $3D^{pol}$-specific SYBR green rRT-PCR could detect FMDV genome specifically and with little run-to-run variation. The new $3D^{pol}$-specific SYBR green rRT-PCR assay was evaluated alongside the established RT-mPCR using the archived FMDV isolates and clinical field samples from suspected FMD outbreaks. A perfect concordance was observed between the new rRT-PCR and the traditional RT-mPCR on viral RNA in the archived FMDV cell culture isolates tested. Furthermore, 73% of FMDV-suspected clinical samples were detected positive through the $3D^{pol}$-specific SYBR green rRT-PCR, while the detection rate through the traditional RT-mPCR was 57%. Therefore, the SYBR green-based $3D^{pol}$-specific one-step rRT-PCR could be considered as a valuable assay with higher diagnostic sensitivity to complement the routine assays that are being used for FMD virus diagnosis in India.

Keywords FMDV · SYBR green · Real-time PCR · Diagnosis

Introduction

Foot-and-mouth disease (FMD) is a highly contagious vesicular, viral disease of both wild and domesticated cloven-footed animals. Although the disease causes a low mortality, it can affect a large number of livestock in a short span of time, leading to the loss of livestock product and productivity in the disease-endemic countries of Asia and Africa [1]. Approximately, three-fourth of world's livestock population is concentrated in the FMD-endemic countries; therefore, the disease poses a serious threat to food and nutritional security [2]. FMD is caused by FMD virus (FMDV), a single-stranded positive-sense RNA virus, in the genus *Aphthovirus* within the family *Picornaviridae* [3]. The virus exists as seven immunologically and genetically distinct serotypes: O, A, C, Asia1, SAT (Southern African Territories)-1, -2, and -3. Each serotype of FMDV contains multiple variants (topotypes/genotypes) which are often restricted to specific geographical locations [4]. In India, FMDV serotypes O, A, and Asia1 are circulating with a predominance of serotype O [5].

FMD is characterized by fever, formation of vesicles and epithelial erosions in the tongue, oral cavity, feet, coronary band, and mammary gland [6]. However, these clinical signs may not distinguish FMD from other vesicular and look-alike diseases, such as vesicular stomatitis, swine vesicular diseases, Seneca valley virus infection, bovine viral diarrhea, and lumpy skin disease. Therefore, laboratory confirmation of any suspected case of FMD is essential. Laboratory diagnosis of FMD can be performed either by detecting the virus and/or any of its component such as viral antigen/genome,

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or by detecting the presence of virus-specific antibodies in the serum or mucosal samples. Although, FMDV isolation in cell culture from suspected clinical samples is considered to be gold standard for FMD diagnosis, the method is tedious and requires high-containment laboratory facility [7]. However, detection of FMDV genome by real-time reverse transcription polymerase chain reaction technology (rRT-PCR) has several advantages over the traditional method of virus detection [8]. The World Organization for Animal Health (OIE) approved the use of real-time RT-PCR for pan-serotypic detection of FMDV in the clinical samples [9, 10]. However, the OIE-approved assays are based on the TaqMan technology, which depends upon the primers and probe targeting a single-conserved region of FMDV genome. Therefore, TaqMan-based assays may produce false-negative results if any one of the probe/primer binding regions shows nucleotide variation in the event of the emergence of new variants of FMDV [11, 12]. To overcome this bottleneck, SYBR green-based rRT-PCR may be used in conjunction with TaqMan-based assay for improved diagnosis of FMDV. Furthermore, in comparison to TaqMan assay, SYBR green-based rRT-PCR is advantageous due to the relative low cost and simplified primer design [12].

In India, an agarose gel electrophoresis-based reverse transcription multiplex PCR (RT-mPCR) targeting the VP1-coding region has been developed and is in use to detect and differentiate FMDV serotypes for more than a decade now [13]. However, the traditional RT-mPCR is time consuming and vulnerable to false-positive result due to the carry-over of PCR amplicons [14, 15]. Therefore, the RT-mPCR assay may not be suitable for routine testing of large number of clinical samples from FMD outbreaks in India. Compared to the conventional RT-PCR, rRT-PCR has many advantages, such as shorter detection time, simplicity, lower carry-over contamination rate, and higher sensitivity of detection [8]. However, there has been no report on the application of SYBR green-based rRT-PCR for detection and quantification of FMDV RNA on large number of samples in India. Consequently, the aim of the current study was to develop and evaluate a rapid, specific, and sensitive SYBR green-based rRT-PCR assay targeting the 3D-polymerase region ($3D^{pol}$) of FMDV for detection and quantification of viral RNA in various FMD-suspected clinical samples and to compare its performance with the in-use traditional RT-mPCR.

Materials and methods

Clinical samples and virus isolates

FMDV serotypes O, A, and Asia1 isolates available in the virus repository as infected cell culture supernatant ($n = 121$) and 10% (w/v) tongue/feet epithelial suspension of clinical samples in PBS ($n = 690$) received from field outbreaks was used in this study. In addition, saliva and nasal swabs collected from apparently healthy and FMDV-antibody free animals were used as negative controls in this study.

RNA extraction

Total nucleic acid from the clinical samples was extracted using QIAamp Viral RNA Mini Kit (Qiagen) as per the manufacturer's instructions. The extracted viral RNA was stored at – 80 °C until further use.

Design of oligonucleotide primers

The design of oligonucleotide primers was based on nucleotide sequences encoding the 3D-polymerase region of FMDV serotypes O, A, and Asia1. The nucleotide sequences were retrieved from both the GenBank (http://www.ncbi.nlm.nih.gov/) and the local sequence database of FMDV maintained at ICAR-Directorate of FMD, Muteswar, India. The 3D-polymerase coding sequences were aligned using BioEdit [16] and primers were designed from the conserved sequences using the PCR primer design software Primer3. The primers were verified for their thermodynamics properties, secondary structures, and potential primer–dimer formation using OligoAnalyzer software [17]. Subsequently, the specificity of the primer sequences was determined using the BLASTn searches at GenBank for short and near-exact matches. While determining the specificity of primers through BLASTn searches, 3D-polymerase sequences of other FMDV serotypes (Serotypes SAT1-3) and viral genome sequences of other FMDV look-alike diseases were also considered. Accordingly, the sequence of the selected primer pair was 3D-F-5′-AGACACTATGAGGGAGTT GAGCT-3′ and 3D-R-5′-AGTGTCTTTTGAGGAAAG TGACA-3′ with a calculated amplicon size of 200 bp.

SYBR green-based one-step rRT-PCR

During the optimization of one-step 3D-polymerase-specific rRT-PCR protocol, several combinations of experiments were conducted to set up the selection of rRT-PCR kit, reagent concentration, and the thermal cycling parameters (data not shown). Real-time one-step RT-PCR was carried out using Luna® Universal One-Step RT-qPCR Kit (NEB) with a final reaction volume of 20 µl containing 10 µl of Luna Universal One-step reaction mix (2×), 1 µl of Luna WarmStart® RT-enzyme mix, 0.8 µl each of 10 µM forward (3D-F) and reverse (3D-R) primer, 4 µl of extracted RNA from clinical samples, and 3.4 µl of nuclease-free water. The optimized thermal cycling conditions were as follows: 1 cycle of 55 °C for 10 min (for reverse transcription), 1 cycle of 95 °C for 1 min (for initial denaturation), and 40

cycles at 95 °C for 10 s and 60 °C for 30 s. The fluorescence was measured at the end of each amplification cycle. A final melting curve analysis at 65–95 °C, plate read/0.5 °C, and hold 5 s was performed to determine the amplicon specificity. All the real-time one-step RT-PCR were conducted using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). The detection limit and amplification efficiency of the SYBR Green rRT-PCR assay were estimated on the basis of viral RNA extracted from tenfold serial dilution series of a representative sample each from FMDV serotypes O, A, and Asia1. The obtained Ct values were plotted against the viral RNA dilutions to construct the standard curve. The amplification efficiency of the assay is calculated as per the formula, $E\% = 10^{-1/\text{slope}} \times 100$.

Analytical specificity and reproducibility

To determine the analytical specificity of the assay, total RNA extracted from saliva and nasal swab samples ($n = 20$) from apparently healthy animals were tested in duplicate.

To determine the repeatability of the SYBR green rRT-PCR assay, intra-assay and inter-assay variation tests were performed using five different tenfold dilution series of RNA extracted from representative FMDV sample. The test for intra-assay variation was performed in triplicate within a single plate, while the test for inter-assay variation was conducted on three different days. The mean, standard deviation (SD), and co-efficient of variation (CV) of Ct values for both the intra-assay and inter-assay tests were calculated separately.

Conventional RT-mPCR

The conventional agarose gel electrophoresis-based one-step RT-mPCR assay for amplification of targeted VP1 region of FMDV serotypes O, A, and Asia1 was conducted as per the methods described earlier [13]. In this assay, the reverse transcription and PCR amplification were performed in a single tube using One*Taq*® one-step RT-PCR kit (NEB). The total reaction volume was 25 µl and the reaction mixture contained 12.5 µl of OneTaq one-step reaction mix (2×), 1 µl of OneTaq one-step enzyme mix, 10 pmol of FMDV-specific NK61 reverse primer, and 10 pmol each of the forward primers DHP13, DHP15, and DHP9 for FMDV serotypes O, A, and Asia1, respectively, 3 µl of extracted RNA from clinical samples, and 7.5 µl nuclease-free water. The thermal cycling program consisted of 48 °C for 20 min, 94 °C for 1 min, and 40 cycles 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 30 s, followed by a final extension of 68 °C for 5 min. The PCR was conducted using Veriti™ Thermal Cycler (Applied Biosystems). The amplified products were resolved on 2% agarose gel and visualized by ethidium bromide.

Results

Analytical sensitivity of SYBR green one-step rRT-PCR

To determine the analytical sensitivity of the $3D^{pol}$-specific SYBR-green rRT-PCR assay at $TCID_{50}$ level, total RNA extracted from tenfold serial dilutions of FMDV serotypes O, A, and Asia1 having an infectivity titer of 10^5 $TCID_{50}$/50 µl (titer determined by Spearman–Karber method) was used. The assay was conducted in duplicate and $3D^{pol}$-specific positive amplification signals with specific melt curve temperature (83.0 ± 0.5ºC) was determined until about 10^{-2} $TCID_{50}$ sample dilutions for FMDV serotypes O, A, and Asia 1 (Fig. 1). Therefore, SYBR green-based one-step rRT-PCR assay was able to detect the total RNA in the samples up to 10^{-2} $TCID_{50}$ dilution, with the corresponding threshold cycle (C_t value) of 32.5 (Fig. 1A). However, the detection limit of FMDV RNA through the agarose gel-based RT-multiplex PCR was up to 10^{-1} $TCID_{50}$ dilution (Fig. 1C). Hence, the new SYBR green-based $3D^{pol}$-specific rRT-PCR is 10 times more sensitive as compared to the multiplex PCR.

Further, the standard curves were generated by plotting the Ct values against the viral RNA dilutions series. The standard curves exhibited a linear range within the RNA dilutions 10^5 to 10^{-2} $TCID_{50}$ (Fig. 2). The amplification efficiencies ($10^{-1/\text{slope}}$) was about 2 for the three-tested serotypes of FMDV with an R^2 value of 0.99 in the standard curve (Fig. 2), thereby indicating the doubling of PCR amplicon after each reaction cycle.

Specificity and reproducibility of SYBR green one-step rRT-PCR

The specificity of the $3D^{pol}$-specific SYBR green rRT-PCR assay was determined by testing saliva and nasal samples from apparently healthy animals. In addition, FMDV serotype O vaccine strain (O IND R2/1975) and nuclease-free water were used as positive and negative controls, respectively. In the assay only FMDV serotype O RNA was detected as a single melt-peak and no peak was detected for RNA extracted from samples of healthy animals and nuclease-free water (Fig. 3).

The reproducibility of the optimized assay was determined by testing five tenfold serial dilutions of FMDV RNA in triplicate by inter- and intra-assay comparison. The inter-assay SD and CV ranged from 0.65 to 1.27 and 2.4% to 4.7%, respectively, while the intra-assay SD and CV ranged from 0.19 to 0.51 and 0.73% to 3.3%, respectively (Table 1). The small CV values indicate a more

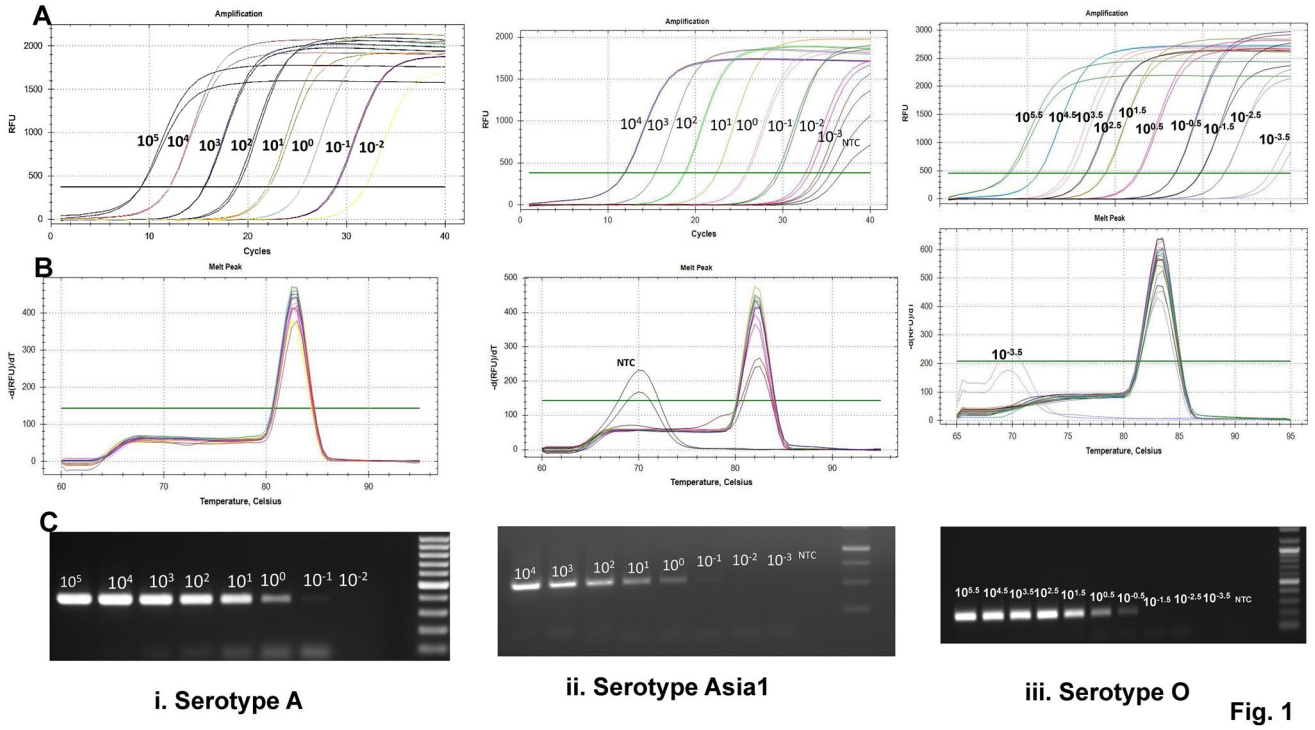

Fig. 1 Detection of tenfold serial dilutions of FMDV serotypes Asia1, A, and O RNA using both the one-step SYBR green-based 3D^{pol}-specific real-time RT-PCR and agarose gel-based RT-multiplex PCR. **A** SYBR-based 3D^{pol}-specific real-time RT-PCR amplification plots for FMDV serotypes A (i), Asia1 (ii), and O (iii). **B** SYBR-based 3D^{pol}-specific real-time RT-PCR melt curves for FMDV serotypes A (i), Asia1 (ii), and O (iii). **C** Agarose gel-based RT-multiplex PCR for FMDV serotypes Asia1 (i), A (ii), and O

reliable and consistent measurement, since CV value of < 5% is deemed acceptable [18].

Sensitivity of 3D^{pol}-specific SYBR green rRT-PCR assay during co-infection of different FMD serotypes

To determine the sensitivity of the 3D^{pol}-specific SYBR green rRT-PCR assay during the FMDV serotypes co-infection, different serotypes (O + A, A + Asia1, or O + Asia1) were mixed at different concentrations and the viral RNA was extracted and analyzed by the current assay. The C_t-value determined from the mixed serotypes samples was compared with the C_t-value from samples containing single FMDV serotype. As depicted in Table 2, viral co-infection has no significant effect on the sensitivity of detection of viral genome in the 3D^{pol}-specific SYBR green rRT-PCR assay as because the primer binding sites are conserved pan-serotypes.

Comparative evaluation of 3D^{pol}-specific SYBR green one-step rRT-PCR assay and in-use RT-mPCR assay

In order to evaluate the suitability of the new SYBR green-based rRT-PCR assay for the detection of FMDV genome in the field samples, RNA extracted from cell culture supernatant infected with FMDV serotypes O, A, and Asia1 ($n = 121$) were analyzed by both traditional RT-mPCR and SYBR green rRT-PCR. The strains were isolated from field outbreaks in India over the last 20 years. The results from the comparative analyses showed a 100% concordance between the traditional RT-mPCR and 3D-polymerase-specific SYBR green rRT-PCR with respect to the detection of FMDV genome (Supplementary Table 1).

Application of 3D^{pol}-specific SYBR green one-step rRT-PCR assay to field samples

FMDV-suspected clinical samples ($n = 690$) from different geographical regions of India were analyzed for the detection of FMDV genome by 3D^{pol}-specific SYBR green RT-rPCR. The same set of samples were also tested by traditional RT-mPCR (Table 3). The results from the comparative analyses showed that 397 samples (detection rate = 57%) were found positive by traditional RT-mPCR; however, 507 number of samples (detection rate = 73%) were found positive by the new SYBR green one-step rRT-PCR assay at the cut-off Ct value of 32.5. These additional samples were confirmed to be positive by subsequent agarose gel electrophoresis of 3D^{pol} rRT-PCR product showing an amplicon size of about 200 bp. In

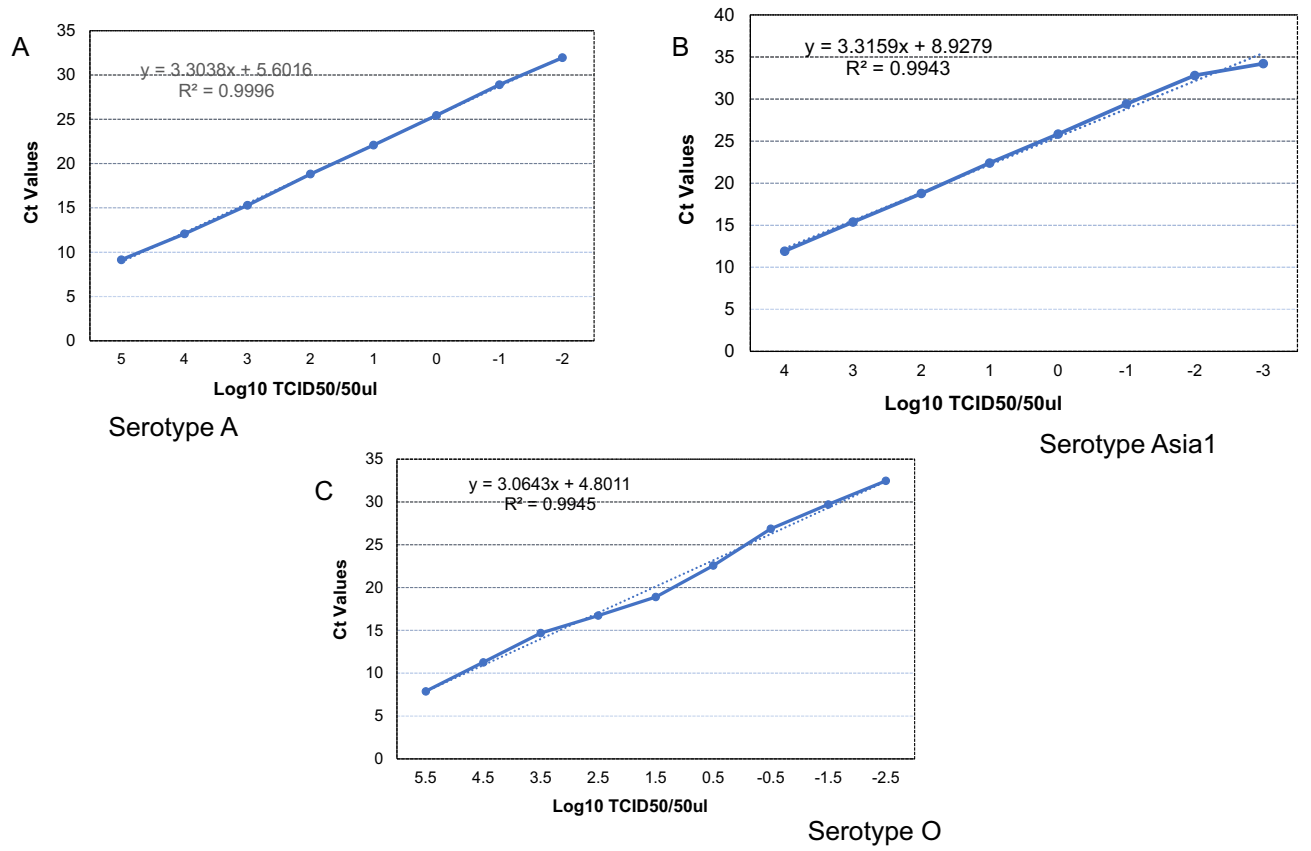

Fig. 2 Standard curves generated from the amplification of tenfold serial dilutions of FMDV serotypes A, Asia1, and O RNA using the SYBR green-based $3D^{pol}$-specific one-step real-time RT-PCR. The standard curves were linear for all the tested serial dilutions with a R^2 value of 0.99

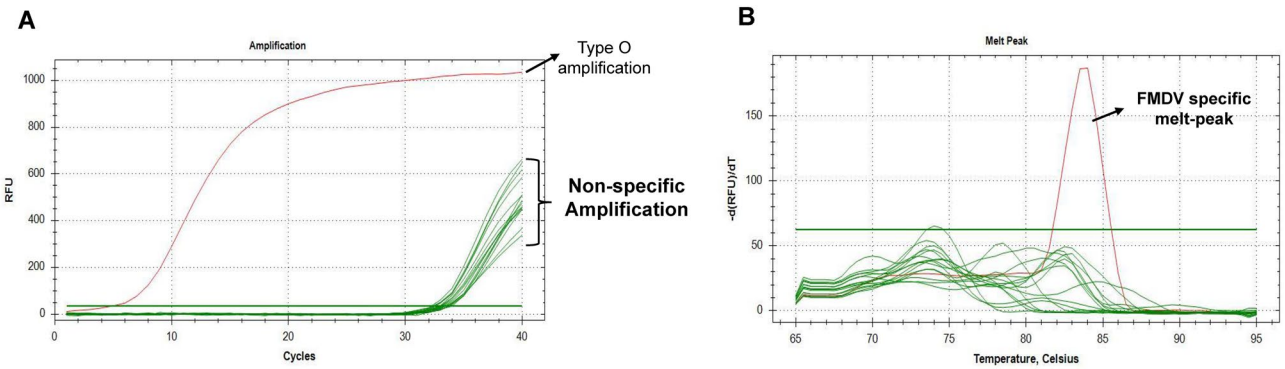

Fig. 3 Specificity of the SYBR green-based $3D^{pol}$-specific one-step real-time RT-PCR assay. No specific melt curve was obtained for the RNA extracted from saliva and nasal samples obtained from the FMDV naïve animal, whereas amplification curve with low C_t value and specific melt curve was determined for the RNA obtained from FMDV serotype O

addition, the specificity of the amplified PCR-product was further confirmed upon sequencing (ABI 3500xL automatic DNA sequencer). These data indicated that SYBR green-based one-step rRT-PCR can detect FMDV RNA in the clinical samples with higher sensitivity than conventional RT-mPCR.

Furthermore, SYBR green-based rRT-PCR was also used for detection of FMDV genome in the blood samples collected sequentially from FMDV-infected cows ($n = 12$) in a natural FMDV outbreak [19]. As illustrated in Fig. 4, the traditional RT-mPCR can detect viral RNA in the blood samples of all the 12 infected animals until 3-day

Table 1 Repeatability of inter- and intra-assay with different FMDV RNA dilutions by SYBR green-based $3D^{pol}$-specific one-step RT-PCR

Variations	FMDV RNA dilutions	C_t values					
		1	2	3	Mean	SD	CV%
Inter-assay	10^{-1}	15.27	14.23	13.98	14.49	0.68	4.72
	10^{-2}	19.89	21.22	20.47	20.53	0.67	3.25
	10^{-3}	23.52	24.82	22.67	23.67	1.08	4.57
	10^{-4}	26.67	25.76	27.02	26.48	0.65	2.46
	10^{-5}	30.58	31.28	29.1	30.32	1.11	3.67
Intra-assay	10^{-1}	15.3	15.82	14.8	15.31	0.51	3.33
	10^{-2}	19.29	19.67	20.21	19.72	0.46	2.34
	10^{-3}	24.52	24.02	24.76	24.43	0.38	1.55
	10^{-4}	27.12	26.74	27.02	26.96	0.20	0.73
	10^{-5}	30.12	30.55	30.2	30.29	0.23	0.76

Table 2 Detection of FMDV genome by $3D^{pol}$-specific one-step SYBR green rRT-PCR during FMDV serotypes co-infection

Experiments	FMD virus	C_t-value	FMDV infection status (either single or co-infection)
1	Serotype O (10^3 $TCID_{50}$)	17.17	Single
2	Serotype A (10^3 $TCID_{50}$)	16.14	Single
3	Serotype Asia1 (10^3 $TCID_{50}$)	19.47	Single
4	Serotype O (10^{-2} $TCID_{50}$)	32.27	Single
5	Serotype A (10^{-2} $TCID_{50}$)	33.21	Single
6	Serotype Asia1 (10^{-2} $TCID_{50}$)	32.12	Single
7	Serotypes O (10^3 $TCID_{50}$) + A (10^{-2} $TCID_{50}$)	19.00	Co-infection
8	Serotypes A (10^3 $TCID_{50}$) + O (10^{-2} $TCID_{50}$)	18.38	Co-infection
9	Serotypes Asia1 (10^3 $TCID_{50}$) + A (10^{-2} $TCID_{50}$)	21.81	Co-infection
10	Serotypes A (10^3 $TCID_{50}$) + Asia1 (10^{-2} $TCID_{50}$)	18.27	Co-infection
11	Serotypes O (10^{-2} $TCID_{50}$) + A (10^{-2} $TCID_{50}$)	33.62	Co-infection
12	Serotypes O (10^{-2} $TCID_{50}$) + Asia1 (10^{-2} $TCID_{50}$)	34.48	Co-infection

post-manifestation of clinical symptoms; however, all the infected animals were consistently found positive until 4-day post-manifestation of clinical samples by SYBR green-based rRT-PCR. In addition, a greater number of animals were found positive for FMDV genome for longer duration in the SYBR green rRT-PCR as compared to the traditional RT-mPCR assay (Fig. 4).

Discussion

Availability of rapid, sensitive, and accurate diagnostic assay is essential for the effective surveillance and control of FMD in endemic country, like India. Although virus isolation is considered as the gold-standard method for the confirmatory diagnosis of FMD, the method lacks sensitivity in case of the clinical samples with low viral load [20, 21]. Furthermore, successful virus isolation depends on the quality of field samples to a greater extent. FMDV serotyping by antigen-captured ELISA can be considered an alternative to virus isolation method [22]. However, antigen-ELISA requires the access to standard-biological reagents. For these reasons, real-time RT-PCR (rRT-PCR) assays have been considered as practical tool for the diagnosis of FMD [8]. The World Organization for Animal Health (OIE) has approved the use of FMDV 5′-UTR and 3D-polymerase-specific TaqMan rRT-PCR assays for the diagnosis of FMD. However, since the TaqMan assay relies on 5′–3′ fluorophore and quencher-labeled oligonucleotide probe, it is not economical for routine diagnosis of large number of suspected clinical samples. As an alternative, SYBR green-based rRT-PCR coupled to melting curve analysis can be applied directly for the diagnosis of FMD, without the need to design and synthesize fluorescent-tagged probes. Furthermore, SYBR green-based assay is less expensive than the TaqMan assay. In this study, a rapid, simple to operate, and sensitive SYBR green-based one-step rRT-PCR assay targeting the 3D-polymerase region of FMDV was developed and evaluated for its accuracy and sensitivity in detecting FMDV genome in the clinical samples.

Table 3 Comparative diagnosis of FMDV-suspected clinical samples by both traditional RT-multiplex PCR and SYBR green-based 3D^{pol}-specific one-step RT-PCR

SL no	Name of the state	No. of samples[a] tested	RT-mPCR result		3D^{pol}-SYBR green RT-qPCR	
			Positive	Negative	Positive	Negative
1	Odisha	14	5	9	10	4
2	Madhya Pradesh	27	16	11	18	9
3	Telangana	21	3	18	3	18
4	West Bengal	39	26	13	30	9
5	Maharashtra	124	36	88	70	54
6	Karnataka	295	200	95	242	53
7	Bihar	21	13	8	17	4
8	Andaman & Nicobar Islands	1	1	0	1	0
9	Goa	8	5	3	6	2
10	Tamil Nadu	55	26	29	38	17
11	Rajasthan	24	8	16	14	10
12	Chhattisgarh	6	6	0	6	0
13	Assam	15	15	0	15	0
14	Punjab	20	20	0	20	0
15	Himachal Pradesh	2	1	1	1	1
16	Haryana	12	10	2	10	2
17	Gujarat	6	6	0	6	0
	Total	690	397	293	507	183

[a]10% PBS suspension of tongue/foot epithelium

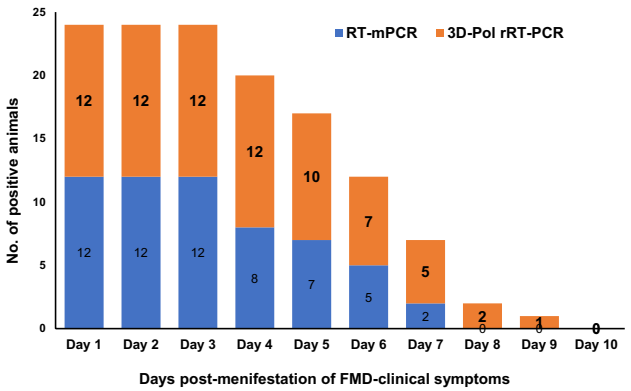

Fig. 4 Kinetics of FMDV genome detection in the blood samples collected from cattle ($n=12$) after the manifestation of FMD clinical symptoms. RNA extracted from the blood samples was tested by both agarose gel-based RT-multiplex PCR and SYBR green-based 3D^{pol}-specific one-step real-time RT-PCR assay

Owing to the highly conserved nature of FMDV 3D-polymerase, we designed the primer to amplify about 200 bases of FMDV 3D-polymerase. The 3D^{pol}-specific SYBR green rRT-PCR assay was optimized to achieve maximum efficiency of amplification. In our assay, the efficiency ($10^{-1/slope}$) of amplification was ($10^{0.33}$) approximately 2, thereby indicating the doubling of expected product after each thermal cycle. The assay amplifies 200-bp fragment of FMDV 3D-polymerase with a T_m of 83.0 ± 0.5. The limit of detection of FMDV RNA was 10^{-2} dilution, at the same time the analytical sensitivity of conventional agarose gel-based RT-mPCR was found to be 10^{-1} dilution. Therefore, the sensitivity of SYBR green-based 3D^{pol}-specific rRT-PCR was 10 times higher than that of the conventional RT-mPCR. Through standard curve analysis the linearity in the curve was observed over a wide range of total viral RNA (8-$\log_{10}$ orders) concentration; therefore, the assay can be expected to accurately detect over a wide range of viral RNA concentrations in the clinical samples. Furthermore, the low co-efficient of variation as determined from the inter- and intra-assay comparison indicated that the new SYBR green-based rRT-PCR assay is repeatable and reproducible with low variation.

Since, RT-PCR assay relies on oligonucleotide primers and/or probes that target the conserved region of viral genome, they are susceptible to produce false-negative result in the event of genetic variations within the complementary target regions [12]. Therefore, to determine the diagnostic specificity and robustness of the new SYBR green-based rRT-PCR assay, the viral RNA extracted from different lineages of FMDV serotypes O, A, and Asia1 which have been isolated in the country during the last 20 years were analyzed by the new assay. The results from this analysis suggested that a perfect concordance was observed for the detection of viral genome between the 3D^{pol}-specific rRT-PCR and the conventional RT-mPCR. Therefore, the new rRT-PCR assay can be used for detection of viral genome in the event of

emergence and re-emergence of various lineages of FMDV serotypes in India. Furthermore, since the oligonucleotide primer designed for the 3D-polymerase-based rRT-PCR is pan-serotypic in nature, it may also detect any future FMDV incursion of strains exotic to India.

To compare the efficacy of SYBR green rRT-PCR assay over the traditional RT-mPCR, sequential blood samples obtained from cattle affected with FMDV during a natural FMD outbreak were tested. From the comparative analyses, SYBR green rRT-PCR assay proved to be more sensitive than conventional RT-mPCR. Evaluation of diagnostic performance on the clinical samples ($n=690$) revealed that viral RNA from considerably a greater number of FMDV-suspected samples ($n=507$) could be detected in the $3D^{pol}$-specific rRT-PCR as compared to the agarose gel-based RT-mPCR ($n=397$). Altogether, these data indicate that the new $3D^{pol}$-specific SYBR green rRT-PCR is more sensitive than the conventional RT-mPCR for early detection and surveillance of FMDV infection in India. The new SYBR green rRT-PCR displays additional advantages over traditional RT-mPCR, such as increased laboratory throughput, simultaneous detection of several samples, and ability to quantify the viral load in the suspected clinical samples. In addition, the assay gives results within 3–4 h and the one-step reaction is performed in a closed tube; therefore, the assay is less prone to carry-over contamination [23].

During an FMD outbreak in the disease-endemic country, the identification of serotypes of the causative virus strain is important for disease control through vaccination and for tracing the source of outbreak. Since the current $3D^{pol}$-specific rRT-PCR is serotype independent, it may be argued that the new assay would not be useful in the disease surveillance and control measures. However, since in India trivalent FMD vaccine is being used for the control of the disease and historically only Eurasian FMDV serotypes are prevalent in the country, and the identification of the serotypes of the causative FMDV strain bears less significance in the context of enforcing immediate measures to contain the spread of the disease. Moreover, owing to the high sensitivity and rapid nature of the new rRT-PCR assay, the 3D-polymerase-based SYBR green rRT-PCR could be used as a rapid screening assay in the country-wide FMD surveillance. Since the assay can quantify the viral load in the clinical samples, the $3D^{pol}$-specific rRT-PCR could be useful in FMDV pathogenesis and transmission studies.

In conclusion, a SYBR green-based real-time one-step RT-PCR targeting the 3D-polymerase coding region of FMDV for detection and quantification of FMDV RNA was developed in this study. The new assay was proven to be efficient, specific, reproducible, and more sensitive than the conventional RT-multiplex PCR in the detection of FMDV RNA extracted from a range of samples, such as tongue epithelium, saliva, nasal swabs, blood samples, and cell culture isolates. Since effective disease management relies on accurate diagnostic assays to confirm the viral infection early in the course of infection, the new SYBR green-based rRT-PCR assay would be a useful test if applied in the FMD surveillance activities.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11262-021-01884-3.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Animal ethics No experiment was conducted on animals.

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