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Novel pan-lineage VP1 specific degenerate primers for precise genetic characterization of serotype O foot and mouth disease virus circulating in India

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ABSTRACT

Analysis of the VP1 gene sequence of the foot and mouth disease virus (FMDV) is critical to understanding viral evolution and disease epidemiology. A standard set of primers have been used for the detection and sequence analysis of the VP1 gene of FMDV directly from suspected clinical samples with limited success. The study validated VP1-specific degenerate primer-based reverse transcription polymerase chain reaction (RT-PCR) for the qualitative detection and sequencing of serotype O FMDV lineages circulating in India. The novel degenerate primer-based RT-PCR amplifying the VP1 gene can circumvent the genetic heterogeneity observed in viruses after cell culture adaptation and facilitate precise viral gene sequence analysis from clinical samples.

Keywords: Picornaviridae; Aphthovirus molecular diagnosis; disease epidemiology; gene sequencing

INTRODUCTION

Foot-and-mouth disease (FMD) is an acute vesicular disease of domestic and wild ungulate species of animals. Although high mortality due to FMD is restricted to young animals, the direct loss of animal productivity, indirect loss due to FMD control strategies, and poor access to trade lead to severe economic devastation [1]. The disease is caused by the foot-and-mouth disease virus (FMDV) within the genus *Aphthovirus* of the family *Picornaviridae*. The disease is endemic in India. Among the seven distinct serotypes identified (O, A, C, Asia1, and SAT1–3], three serotypes (O, A, and Asia1) are currently prevalent in the country.

Regarding the FMD epidemiology, serotype O has been the leading research subject and has the widest geographical distribution [2]. In India, serotype O has been responsible for most FMD outbreaks reported across the states [3-6]. The geographical distribution, antigenic and

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Conflicts of Interest

The authors declare no conflicts of interest.

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genetic characterization of serotype O FMDV field isolates from India have been described [3-6]. Globally, FMD serotype O viruses were classically divided into 11 topotypes, and all the serotype O isolates reported thus far in India belonged to the ME-SA topotype [2,7].

Various lineages of FMDV serotype O under the ME-SA topotype exhibit a distinct pattern of evolution and geographical distribution [8]. In India, multiple lineages of FMDV serotype O, i.e., A, B, PanAsiaI, Ind2001, and Ind2018, have dominated the epidemic landscape at various periods [3-6]. Furthermore, co-circulation and gradual replacement of one lineage with another group of serotype O viruses have been documented in various studies [4-6].

In an endemic disease setting, confirmatory laboratory diagnosis of every suspected FMD case is vital for the effective and quick implementation of the disease control program. Conventionally, the serotype detection of suspected samples for FMD is done by multiplex polymerase chain reaction (mPCR) [9] in India. FMDV-positive samples were processed further for cell culture isolation and reverse transcription polymerase chain reaction (RT-PCR) sequencing of the VP1/P1 gene as per standard protocol [10]. The surface-exposed capsid protein VP1 is critical for the antigenic and phylogenetic characterization of FMDV [11].

Despite being a fast and specific diagnostic tool, RT-PCR is used with routine virus isolation and serotype-differentiating enzyme-linked immunosorbent assay protocols for detecting FMDV. RT-PCR has the specific advantage of sequencing the genome from original tissue material over cell culture virus isolation, wherein a genetic mutation is possible due to adaptation in cell culture passage [12]. Hence, RT-PCR assay is equally important for determining the genome sequence from a suspected clinical sample and for the positive samples where virus isolation is disconfirming.

A standard set of primers are used to amplify the VP1/P1 region from the viral strain of the respective FMDV serotype [10]. For serotype O, a universal set of primers, ARS4 and NK61, has been used worldwide for VP1 sequence analysis. Originally, ARS4 and NK61 were advocated for the theoretical detection of FMDV serotype O through RT-PCR, followed by sequence determination using various sequencing primers (1C244, 1C272, 1C283, and NK72) [10]. Although the primer pair ARS4–NK61 is highly specific to serotype O, it has low detection sensitivity (Dsn= ~50%) in clinical samples [13,14]. In addition, the ARS4–NK61 primer pair has been used in the authors' laboratory to amplify FMDV serotype O virus isolates from tissue samples with moderate success. Thus, the present study was designed to improve the qualitative detection and sequencing of the VP1 gene of serotype O FMDV from clinically suspected samples by degenerate primer-based RT-PCR.

MATERIALS AND METHODS

The study was designed according to the guidelines for the Standard for the Reporting of Diagnostic Accuracy Studies as per STARD 2015 guidelines [15].

Virus strains, clinical samples, and cell lines

The FMDV vaccine virus strains (for serotype O, A, and Asia1) and field clinical epithelial samples (n = 245) (**Supplementary Table 1**) were obtained from the repository of ICAR-Directorate of Foot and Mouth Disease, Mukteshwar. The BHK-21 cells were maintained in

Glasgow MEM (Thermo Fisher Scientific, USA), supplemented with tryptose phosphate, sodium hydrogen carbonate, and 10% fetal bovine serum.

Design of primers

The coding sequences of the VP1 gene of viruses from different lineages of serotype O FMDV available in the institute database were aligned to determine the consensus region. The degenerate oligonucleotide primers, forward *KSP21* (5′-CCGCGGCCGCACCACCTCCACAGGTGAGTCWG-3′), and reverse *KSP24* (5′-CTCGAGCTGYTTCACAGGTGCCACAATC-3′) spanning complete coding sequence (639 bp) were custom synthesized accommodating nucleotide variations at N′ and C′ terminal of VP1 gene across lineages.

KSP21- KSP24 RT-PCR assay setup

The degenerate primer RT-PCR was optimized using cell culture supernatant of serotype O FMDV strains. The reverse primer (*KSP24*) was used to synthesize complementary DNA (cDNA) from the total RNA extracted from samples using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™). The amplification was done using HotStarTaq™ DNA Polymerase (Qiagen, Germany) in 25 μL reaction volume (**Supplementary Table 1**). The time-thermal profile was optimized to 95°C for 15 min, 35 cycles of denaturation (95°C for 30 sec), annealing (50°C for 30 sec), and extension (72°C for 30 sec) followed by a single cycle of final extension (72°C for 10 min). Appropriate controls were placed for each amplification reaction.

Sensitivity and specificity of the assay

The analytical specificity (Asp) of the degenerate primers was determined using a pool of positive samples used in the laboratory as internal quality controls from cell culture supernatant samples of healthy BHK-21 cell culture, serotype O, A, and Asia1 FMDV isolates. The analytical sensitivity (Asn) for custom degenerate primers RT-PCR to detect serotype O was determined against a 10-fold serially titrated virus in BHK-21 cells. The diagnostic sensitivity (Dsn) and specificity (Dsp) of KSP21–KSP24-based RT-PCR was evaluated using archival serotype O FMDV mPCR positive and negative clinical epithelial suspensions (**Supplementary Table 1**).

VP1 sequencing and analysis

Sanger sequencing of representative VP1 amplicons from two recent serotype O FMDV outbreaks was performed using the KSP21–KSP24 primer pair. Phylogenetic analysis of these VP1 sequences was conducted using MEGA11.

RESULTS

Validation Degenerate primer-based RT-PCR for serotype O FMDV

The degenerate primer-based RT-PCR for serotype O FMDV did not return false positive results (Asp = 100%) for healthy cell culture, serotype A and serotype Asia1 isolates using serotype O specific degenerate primers (**Fig. 1A**). The analytical sensitivity (Asn) for custom degenerate primers RT-PCR to detect the serotype O FMDV were determined to be 199 $TCID_{50}$/ ml of 10-fold serially titrated virus in BHK-21 cells (**Fig. 1B**).

The clinical epithelial suspension samples (n = 245) from the major sites of FMDV amplification in the host, namely vesicular fluid, tongue epithelium, teat erosions, and

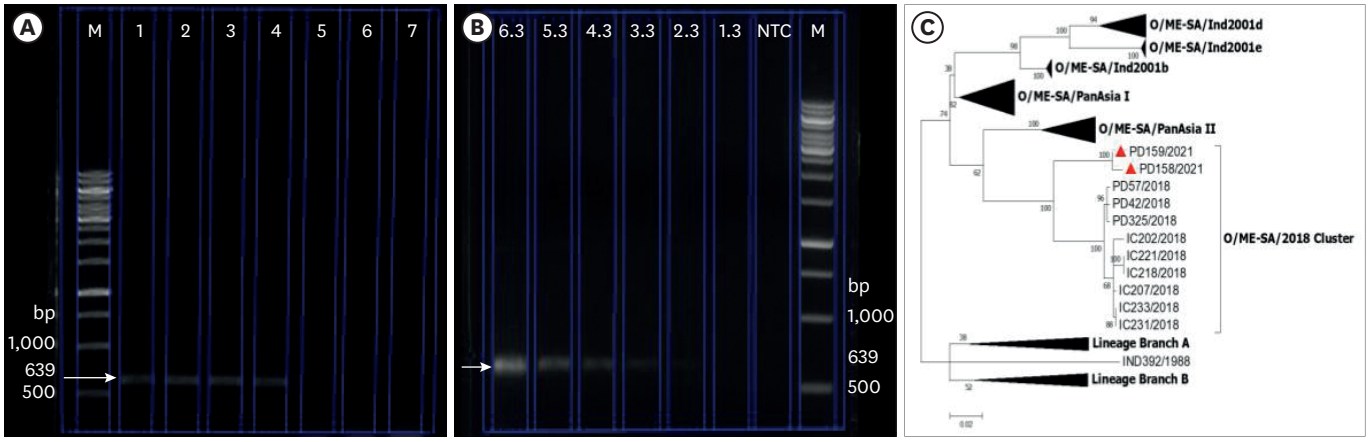

Fig. 1. Validation of VP1 gene degenerate primer-based reverse transcription polymerase chain reaction for serotype O FMDV. (A) Agarose Gel Electrophoresis showing lineage independent specific amplification of complete VP1 gene (639 bp) for the representative strain of serotype O FMDV lineage B, Ind2001, PanAsia, 2018 in lane 1, 2, 3, 4 respectively. Lane 5 and lane 6 serotype A and serotype Asia1 FMDV, Lane 7: NTC, Lane M: molecular weight marker 500 bp. (B) Agarose Gel Electrophoresis showing detection limits of degenerate VP1 primer. Numerical at each lane indicates infectious titer ($TCID_{50}$/mL) of the serotype O FMDV dilutions used for RNA extraction. Lane M: molecular weight marker 500 bp. (C) Maximum Likelihood phylogenetic tree showing different lineages of serotype O reported in India. The filled in the red triangle indicates isolates sequenced in this study.
FMDV, foot and mouth disease virus.

Table 1. Comparative diagnostic sensitivity and specificity of degenerate VP1 primer-based RT-PCR for serotype O FMDV

Sample type	Serotype O FMDV RT-PCR Assay				
	Serotyping mPCR [9]	KSP21-KSP24 primer		Dsn (%)	Dsp (%)
		(+)	(−)		
Epithelial suspension	(+/−)	105/33	0/107	76.08	100

RT-PCR, reverse transcription polymerase chain reaction; FMDV, foot and mouth disease virus; mPCR, multiplex polymerase chain reaction; Dsn, diagnostic sensitivity, Dsp, diagnostic specificity.

foot lesions, were examined. The KSP21–KSP24-based RT-PCR was determined to have the percent diagnostic sensitivity (Dsn) and diagnostic specificity (Dsp) of 76.083 and 100, respectively (**Table 1**).

Phylogenetic analysis

The complete VP1 sequence (639 bp) from two recent serotype O FMDV filed virus isolates were curated and deposited in NCBI under accession numbers (ON792382 and ON792383). The VP1 phylogenetic analysis placed these viruses appropriately under serotype O/ME-SA/ lineage 2018 (**Fig. 1C**).

DISCUSSION

Historically, serotype O FMDV strains were responsible for the highest number of disease outbreaks in India [3-6]. Despite the presence of limited antigenic heterogeneity within serotype O FMDV, a higher amount of genetic diversity was seen in viral nucleic acids can further classify these viral strains into numerous distinct lineages under each topotype [8]. The continuous turnover of serotype O FMDV strains has been documented regarding the disappearance, emergence, and re-emergence of lineages. Thus, the lineage-independent identification of serotype O FMDV becomes vital for an endemic country like India.

The RT-PCR and VP1 gene sequence analysis for FMDV combined to evaluate the relationship among virus strains and to trace the source of infection and the possibility of preventing further outbreaks [13]. The novel strategy of pan-lineage amplification and sequencing of

the VP1 gene of serotype O FMDV circulating in India directly from clinical samples using a single set of degenerate oligonucleotide primers is validated in this study. A primer set was designed to accommodate minimum accumulative degeneracy of many sequences and reduce the nonspecific PCR amplification of undesired DNA fragments. The assay could successfully amplify the complete VP1 gene (639 bp) of serotype O FMDV from clinical samples, which is critical in the rapid molecular epidemiological investigation of disease outbreaks [10,13]. The novel degenerate primer set presents an alternative and more sensitive approach to currently used primer sets for VP1 region amplification and sequence analysis of serotype O FDMV and could exclude the use of different primer sets for each step of RT-PCR (ARS4–NK61) and sequencing (1C244, 1C272, 1C283-, and NK72) [10]. The degenerate primer set was more sensitive than the ARS4–NK61 pair to detect viruses in suspected epithelial tissue samples. The primer pair is specific to serotype O and has no cross-reactivity to serotype A and Asia1 FMD viruses. The assay is useful for molecular epidemiological studies in endemic countries, such as India, to characterize the complete VP1 gene of various circulating serotype O FMDV lineages from suspected field samples without going for cell culture isolation of the virus. Similarly, the strategy can be used for precise genetic characterization of other serotypes and lineages of FMDV in different parts of the globe.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Determination of sensitivity and specificity of the test

Click here to view

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