Molecular Detection of Foot and Mouth Disease Virus (FMDV) from 2017 Outbreaks in Punjab by RT-PCR and RT-LAMP Assays

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Abstract

Foot and mouth disease is infectious, transboundary, disease of cloven hoofed domesticated ruminants. In this study, ELISA confirmed positive infected and field samples were analyzed through RT-PCR using universal primer set (IF/R and P1/P2) and RT-LAMP assays. A total of N=100 samples were collected from different livestock population areas in Punjab. Types of samples were serum, nasal secretions, tissue, saliva and swab samples. Out of N=100 infected samples, n=19 samples were serotyped into A, O and Asia 1 strains through antigen ELISA (Ag-ELISA) and these samples were further confirmed through RT-PCR and RT-LAMP assays. ELISA confirmed samples revealed 100% results with RT-PCR and RT-LAMP assays. Field samples revealed 33.33%, 27.16% and 37.03% with RT-PCR (IF/R and P1/P2) and RT-LAMP assays respectively. It was concluded that ELISA confirmed samples were successfully analyzed with RT-PCR and RT-LAMP assays. Both the assays successfully analyzed 2017 outbreaks of FMD in Punjab.

Keywords: Optimize; Outbreaks; RT-PCR; RT-LAMP

Introduction

Foot and mouth disease is contagious and transboundary disease of domesticated ruminants. More clinical signs and symptoms are observed in pigs than cattle and sheep. But, no clinical carrier state was observed in pigs [1-4]. FMD is caused by Foot and Mouth Disease virus (FMDV). Pakistan, Afghanistan, Iran, India and China are endemic countries for FMDV serotype A, O and Asia 1 and they have been facing a huge economic losses in livestock production annually [5,6]. For detection purpose, vesicular fluids are considered suitable samples but oropharyngeal fluid, nasal secretions, mouth swabs and serum are also used for detection purpose [7]. In a laboratory, FMD is diagnosed through antigen captured Enzyme Linked Immunosorbant Assay (ELISA) and virus isolation [8,9]. Reverse-transcription Polymerase Chain Reaction (RT-PCR) and Real-time PCR (RT-qPCR) are widely used for detection of virus in suspected samples [10]. FMDV detection by RT-PCR using universal primer sets is also a promising technique in the early detection of virus [11]. RT-qPCR has higher sensitivity than tests based on detection of antibodies that are raised against FMDV [12]. RT-qPCR also has an additional property to detect very small quantity of RNA; so it has much higher diagnostic sensitivity [13]. But, RT-qPCR is an expensive technique as it requires thermal cycler along with probes and commercially available kit of reagents to perform the reaction. So, there is a requirement of simple, fast and economically technique for the identification of FMD suspected samples. A novel technique has been recently established designated as loop-mediated isothermal amplification [14]. It allows rapid and easy detection of FMDV (cDNA). Its principle relies on the auto cycling strand displacement activity. Large fragment of Bst DNA polymerase forms DNA synthesis. It is more sensitive technique than Reverse Transcription Polymerase Chain Reaction (RT-PCR). This technique requires heating block such as water bath to provide temperature in a constant way to precede reaction [15]. There is no need of expensive equipment like thermal cycler for amplification of DNA [16-19]. For enzymatic activity of Bst DNA polymerase, it requires constant range of temperature i.e., 60-65°C [20]. Kasanga et al. had reported that RT-LAMP has higher sensitivity (30.2%) that RT-qPCR (17.3%) [4]. A similar study was also reported by Chen et al. by evaluating the performance of RT-LAMP and RT-PCR and their results showed that RT-LAMP has higher specificity [21]; as it can detect 10 copies of FMDV in a single reaction while RT-PCR can detect 100 copies per reaction. Farooq et al. had also performed RT-LAMP on serotyped A, O and Asia 1 strains [22]. Our study emphasized on 2017 outbreaks of FMDV infected samples and ELISA confirmed positive infected samples. This paper describes the optimization of RT-PCR and RT-LAMP with ELISA known infected samples and analysis of samples from field outbreaks in 2017 from different livestock populated areas of Punjab (Jhang, Gujranwala, Islamabad and Lahore) with RT-PCR and RT-LAMP techniques.

Materials and Methods

Sample collection

Samples were collected from different populated areas in Punjab from October 2016 to December 2017. Islamabad, Jhang, Lahore and Gujranwala were selected for sample collection. Type of samples were saliva (n=9), swab (n=28), tissue (n=33) and serum samples (n=30) from cow, buffalo and cattle. Saliva samples (n=9) were already known positive infected samples that were borrowed from Dr. Manzoor Hussain (National Project Director, FAO Pakistan).
RNA extraction and cDNA synthesis

RNA extraction was executed according to TRIzol® kit method (TRIzol® Reagent, Invitrogen) [11,23]. Extracted RNA was dissolved in 20 μl RNase free water (DEPC treated water) and then kept at -70°C till further use. cDNA were synthesized using RevertAid® First Strand cDNA synthesis Kit using oligo (dT)18 primer. RNA mix were prepared by adding RNA, oligo (dT)18 and nuclease free water. The above reagents were briefly centrifuged and incubated at 65°C for 5 minutes. Meanwhile, RT-mix was prepared by adding 5X reaction buffer, ribolockRNase Inhibitor (20 U/ul), 10 mM dNTPs mix, Revert Aid m-MuLV RT (200 U/ul) and incubated it at 42°C for 60 minutes. The reaction was terminated at 70°C. Then, tubes were stored at -20°C until to perform RT-PCR [23,24].

RT-PCR

RT-PCR was performed by using two universal primer sets (IF/IR and P1/P2). To perform PCR, following reagents were added in following order; master mix (before adding briefly centrifuge it), primer set (IF/IR or P1/P2), template cDNA and nuclease free water. The total PCR reaction volume was 50 ul. PCR profile was made on thermal cycler ((BIO RAD T100TM); annealing at 58°C (for P1/P2 and for IF/IR at 54°C) for 1 minute, elongation and final elongation at 72°C for 1.5 and 1 minutes respectively. Number of cycle were 30 and then finally hold at 4°C. Amplification was checked through Mini gel electrophoresis apparatus (Wealtec, USA) by making 2% gel in 1X TEA EDTA buffer. Results were visualized under gel documentation system (InGenius3) (Figures 1 and 2).

RT-LAMP

3 primer sets two outer (F3 and B3), Two inner (BIP and FIP) and additional loop primer set (Loop F and Loop R) was used in RT-LAMP assay [14]. This technique is easy to perform, economical and gives result in short time. LAMP reaction mixture was made by adding nuclease free water, cDNA, BIP and FIP (50 p mol), F3 and B3 (10 p mol), Loop F and Loop R (10 p mol), LAMP reaction buffer (10X), dNTPs (1.5 mM) and Bsm DNA polymerase (8U) per reaction. The total reaction volume was 25 ul. Then, incubated it 58°C in water bath for an hour. The positive reaction was checked by making 2% gel (agarose). The amplification was 174 bp. It was checked under gel documentation system (InGenius 3) (Figure 3).

Results

Out of N=100 samples, 81 samples were analyzed for RT-PCR and RT-LAMP assays. ELISA confirmed samples (n=9) gave 100% result
with both assays; RT-PCR and RT-LAMP. Field samples \( n=81 \), \( n=27 \) samples were positive with IF/IR (33.33%) showing amplification of 328 bp. \( n=22 \) samples were positive with P1/P2 (27.16%) primer set showing amplification of 216 bp. \( n=30 \) samples showed positive result with RT-LAMP assays (37.03%) (Table 1).

| Results                  | RT-PCR(IF/IR) | RT-PCR(P1/P2) | RT-LAMP |
|--------------------------|---------------|---------------|---------|
| ELISA confirmed samples  | 100%          | 100%          | 100%    |
| Field samples \( n=81 \) | 33.33%        | 27.16%        | 37.03%  |

Table 1: Comparison of number of positive samples detected through RT-PCR (IF/IR) and (P1/P2) and RT-LAMP assays.

**Discussion**

In our study, we checked ELISA confirmed positive FMDV infected samples and field samples collected from different livestock population areas of Punjab from 2017 outbreaks. As FMDV is RNA virus, the mutation rate is comparatively very high in population leading to emergence of new sub-serotypes. That is also a case of failure of existing FMD vaccine in field. So, continuous surveillance of FMD outbreaks in endemic regions through easy to perform and reliable diagnostic tests are emphasized. To get this goal, we had planned this study on improved diagnostic tests of RT-PCR and RT-LAMP to check the presence of FMD. ELISA and RT-PCR are routinely diagnostic tests in many laboratories. According to OIE, 2012 for detection purpose, vesicular fluids are considered suitable samples but oropharyngeal fluid, nasal secretions, mouth swabs and serum are also used for detection purpose. FMDV detection by RT-PCR using universal primer sets was also a promising technique in the early detection of virus [11]. RT-LAMP is a fast diagnostic test as there is no need to extract DNA/RNA and simply, it can be performed on crude sample. So, there is no issue of contamination like PCR. Similar study was conducted by Saeed et al. who had checked samples through Ag-ELISA, and further performed RT-PCR and RT-LAMP. In addition to this, they had also evaluated the cross reactivity with other viruses like NDV, IBV and PPRV and specificity.

But, our study focused on optimization of ELISA confirmed samples with RT-PCR and RT-LAMP assays and checked presence of FMDV in infected samples with above mentioned assays in 2017 outbreaks. No study was conducted before regarding the optimization of ELISA confirmed positive infected samples with RT-PCR using universal primer sets (IF/IR and P1/P2) and RT-LAMP assays in 2017. All ELISA confirmed positive infected samples were positive with both assays; RT-PCR and RT-LAMP showing 100% results while \( n=30 \) samples were positive with RT-LAMP (37.03%) followed by \( n=27 \) samples with RT-PCR (IF/IR) (33.33%) and \( n=22 \) samples were positive with P1/P2 primer set (27.16%).

Previous many studies have been designed for detection purpose of FMDV and further evaluating these techniques on the basis of their specificity and sensitivity. In our study, RT-PCR performing with F1 and F2 primers showed exactly the same results as performed by Barbour et al. [25]. Saeed et al. had also used the same primer sets F1/F2 and P1/P2 and reported same amplification 328 and 216 bp respectively [11]. Similar work was also done by Waheed et al. [26]. They had checked 156 samples, collected from different areas of Punjab, Pakistan and checked them through RT-PCR using P1/P2 primer set. Their results revealed that 23% samples were positive. Ali et al. used the same primer set 1F/1R as mentioned in our work and reported amplification of 328 bp [27]. Primers used in present study were already mentioned by Dukes et al. that were designed against 3D region of genome. But, they had reported results at 65°C [28]. However, we got results at 58°C. Monitoring and surveillance of suspected population can be performed through RT-PCR, ELISA and rRT-PCR. Successful control of FMD relies on accurately and rapidly diagnosis of FMD. FMD can be easily and accurately detected by using molecular techniques. As only a small quantity of suspected samples is required for detection purpose [11,29-33]. In a short way, it was concluded that RT-PCR and RT-LAMP was successfully optimized with field outbreaks and ELISA confirmed positive infected samples. Both the assays showed equal efficacy in detection of FMD. There is considerable need for molecular characterization of FMDV in endemic areas to access the current situation of FMDV. We suggest this for future that there is a need for sequencing of a variable region of genome i-e VP1 and further phylogenetic analysis to access the current prevalent situation of FMD in Pakistan for development of a good vaccine as, only control of FMD is a proper vaccination of animals [34].

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**Conflict of Interest**

The author affirms that there is no conflict of interest.

**Author’s Contribution**

UW, SE, FH participated in study design, experimental work and drafted the manuscript. MA revised the final manuscript. All authors accepted the final description of manuscript.

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