Abnormal Music Recognition Methods (m-PCR and RT-LAMP) for the Detection of Foot-and-Mouth Disease Virus Excreted in Cow Milk

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ABSTRACT

The present study was undertaken to see the excretion of FMD virus in milk during and after the subset of FMD outbreak. Fore-milk (50 ml) was sampled from 12 clinically infected and 3 asymptomatic cows in the morning. Analytical sensitivity of NAR methods was estimated using uninfected negative milk sample spiked with 10\(^{5.7}\) TCID\(_{50}\)/ml FMD serotype O virus (IND R2/1975) in 10 fold serial dilution. Detection limit of mPCR and RT-LAMP assay was 10\(^{2.7}\) and 10\(^{1.7}\) TCID\(_{50}\)/ml, respectively. 15 individual and pooled cows’ milk samples infected with FMD virus were processed for virus isolation (VI) and detection till 37 days post clinical manifestation (dpm). Virus isolation from individual and pooled milk from infected cow was positive till 6 and 4 dpm, respectively. Individual milk and pooled milk samples were found positive by mPCR till 37 and 14 dpm, respectively, but by RT-LAMP till 37 and 21 dpm, respectively. In case of asymptomatic cows, viral genome was detected 2–5 days before appearance of disease in other animals. Milk virus isolate had 100% nucleotide identity at VP1 coding region. mPCR and RT LAMP assays has potential to detect FMD virus in milk and help to prevent the spread of FMD virus from one place to another place.

Key words: Cow milk, FMD virus, Nucleic acid recognition method, Virus isolation
Materials and Methods

Animals: The present study was carried out at the experimental cattle herd, maintained at 29°28’N and 79°39’E in the Kumaon ranges of Himalaya (7,500 feet above mean sea level), where 40 crossbred cows were reared for milk purpose, practicing regular biannual vaccinations against FMDV infection. An FMD outbreak was reported in 2013. Twelve clinically infected and 3 in-contact healthy (asymptomatic) cows were selected for the present study.

Sampling: Milk samples (50 ml fore-milk) were collected from infected cow to detect the FMD by NAR methods. The sample was taken in 2 separate tubes during milking from each cow in the morning on 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, 29, 33, 37 and 39 days post clinical manifestation (dpm). First individual tubes were used for virus isolation (VI)/viral genome detection and 2nd tubes were pooled for VI/viral genome detection.

FMDV virus isolate: National FMD virus repository isolate of serotype O (IND R2/1975) was revived in BHK–21 cell monolayer and TCID_{50} was determined using Reed-Muench method.

Analytical sensitivity/optimization of NAR methods: Analytical sensitivity was carried out on spiked milk samples with 10^{5.7} TCID_{50}/ml FMD virus (IND R2/1975) diluted uninfected whole milk in decimal series (10^{-1} to 10^{-10}) and used for detection of viral genome by NAR methods viz. mPCR and RT-LAMP.

Viral RNA extraction and cDNA preparation: Before RNA extraction, whole milk was passed through Qia shredder and supernatant was used for viral RNA extraction from spiked, individual and pooled samples. Viral RNA was extracted from this suspension using QIAamp viral RNA mini kit as per the manufacturer’s instructions. The extracted RNA was further purified by treating with DNaseI and quantified by spectrophotometer using the OD_{260}/OD_{280} ratio. Total RNA preparations were stored at -80°C. The cDNA synthesis was performed with reverse transcriptase enzyme and specific RT primers at 55°C for 2 h.

Detection of FMDV in milk: The extracted viral RNA was used for the detection of FMDV by RT-LAMP (Ranjan et al., 2014) and serotype O of FMDV in milk and cell cultures was confirmed by antigen detection by ELISA (Bhattacharya et al., 1996) and mPCR (Giridharan et al., 2005). mPCR and RT-LAMP amplified products were resolved on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Chemical transfection and sequencing of FMDV: FMD virus was rescued from individual and pooled milk samples in the BHK–21 cells by the chemical transfection of viral RNA (Bisht et al., 2014). The serotype specific mPCR amplified products were gel purified using gel extraction kit and subjected to nucleotide sequencing of VP1 coding region of FMDV using primer ARS4 and NK61 (Samuel and Knowles, 2001). Agarose gel purified VP1 amplicon was sequenced on ABI 3130 automated DNA sequencer by big dye terminator v3.1 cycle sequencing kit.

Results and Discussion

Milk is an easily available ideal sample for laboratory diagnosis of FMD and also for the surveillance in dairy herds (Saeed et al., 2011). It is also used to monitor individual animals in other viral diseases like bovine viral diarrhoea (Drew et al., 1999, Heath et al., 2003).

FMDV and its serotypes were confirmed by using RT-LAMP (Fig. 1a,b,c) and mPCR (Fig. 1a), respectively. In
analytical sensitivity of NAR methods, the detection limit of mPCR and RT-LAMP assay was $10^{-7}$ and $10^{-17}$ TCID50/ml, respectively (Fig. 2a, b). In previous study, detection limits of mPCR (Giridharan et al. 2005) and RT-LAMP (Ranjan et al. 2014) for FMDV serotype O were 158 and $4.2 \times 10^{-4}$ TCID50/ml, respectively, reflecting the low sensitivity and this could be due to presence of PCR inhibitors (Cohen et al. 1997). It was also reported that the sensitivity of RT-LAMP assay was higher than that of mPCR assay (Ranjan et al., 2014). These results were found repeatable and uniform for all the 3 serotypes (FMDV serotype O, A and Asia1) tested.

FMDV was rescued in individual and pooled milk samples from infected cows till 6 and 4 dpm, respectively by the chemical transfection method. Presence of FMDV in milk could be due to its replication in the epithelial cells of the mammary gland, resulting in high viral titers in milk (Hyslop 1970). Individual milk samples were found positive for viral genome by mPCR and RT-LAMP till 37 dpm while the pooled milk samples were positive till 14 dpm by mPCR and 21 dpm by RT-LAMP. In individual milk sample, mPCR detected only 1 sample while RT-LAMP detected 2 samples on 33 dpm (Fig 3). RT-LAMP was thus more sensitive than mPCR as already reported (Ranjan et al. 2014). In earlier report, FMDV was excreted in milk up to 23 day post infection (Reid et al. 2006) but in this study, duration of FMDV excreted in milk was up to 37 dpm. However, all cows at the farm became negative for FMDV in milk samples by 39 dpm. In asymptomatic cows (218, 354 and 571), viral genome was detected 2–5 days before appearance of clinical symptoms in line with the findings of earlier workers (Burrows et al. 1971, Blackwell et al. 1982). In this study, 2 cows (218 and 571) were asymptomatic and delivered calves during the outbreak. The colostrums from these 2 cows were positive for FMDV. These findings indicated that infected milk could be a source of infection in areas where it may be distributed knowingly or otherwise.

Both mPCR and RT-LAMP amplified products were resolved on 2% agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 2a, b). In the infected BHK-21 cells, cytopathic effects characteristic of those due to FMDV replication were observed within 16–18 hours post infection at the second passage onwards. The presence of infectious serotype O virus in the BHK-21 cell culture supernatant was also confirmed by the serotype specific ELISA and multiplex PCR (data not shown). Nucleotide sequencing of amplified product (partial 1D region of FMDV), further confirmed the specific amplification by mPCR. Processed sequences were aligned with reference 1D sequences of FMDV serotype O, A and Asia 1 retrieved from NCBI database using the Clustal W algorithm accessible in the MEGA5 software (Tamura et al. 2011). Milk virus isolate had 100% nucleotide identity at VP1 coding region with contemporary virus isolates isolated from domestic animals in nearby areas which clearly indicated that FMDV isolated from milk was from the same outbreak.

Our findings indicated that the duration of FMDV secretion in milk may play an important role in transmission of FMDV in disease free area. Multiplex and RT-LAMP

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Fig 3. Demonstration of foot-and-mouth disease virus infection of dairy cows by virus/isolate positive in milk samples monitored by virus isolation , multiplex PCR and RT-LAMP , RT-LAMP , milk samples become negative by all test , respectively. Upper figure showe result in milk sample collected from individual cow and lower figure show pooled milk sample results from all fifteen cows.
assays has potential to detect FMD virus in milk and help to prevent the spread of FMD virus from one place to another place. However, further studies are required to ascertain the duration of virus secretions in milk of infected, recovered and subclinical animals in endemic states of the country.

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REFERENCES


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