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Use of nucleic acid 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 m-PCR 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

Foot-and-mouth disease (FMD) is an important trans-boundary animal viral infection affecting even-toed commercially important domestic animals such as cattle, sheep, goats, swine and water buffalo (Alexandersen et al. 2003). FMD virus (FMDV) belongs to genus *Aphthovirus* and family Picornaviridae. Out of 7 distinct serotypes in the world, O, A, C, and Asia-1 occur in the country and serotype O is most commonly prevalent (Ranjan *et al*. 2014).

After FMDV infection, the same is not cleared at a given point of time. The virus continues to be secreted in milk for many days (Terbruggen 1932, Sutmoller and Casas 2003). This virus has been reported to survive in raw milk for 6 days at 18ºC and for 15 days at 4ºC (Terbruggen 1932). During the 1967–1968 epizootic in England, the virus was detected in some milk bulk tanks and tankers at least 33 hours before clinical signs were reported in the affected herds (Hedger and Dawson 1970). Virus was found in milk 1–4 days before vesicles developed (Burrows *et al*. 1971, Blackwell *et al*. 1982). Milk collected from a clinically normal animal during an outbreak contains up to $10^{6.6}$ $TCID_{50}$/ml (Donaldson *et al*. 1982). The spillage of infected milk acts as a source of infection (Dawson 1970).

FMD causes considerable economic losses in livestock productivity, and were estimated at round ₹ 23,000 crore per annum (The Hindu, IST Bengaluru, January 12, 2015). It has been demonstrated already that virolactia precedes the development of clinical signs in experimentally infected animals (Blackwell and Hyde 1976, Blackwell *et al*. 1982, Burrows 1968). Automated real-time RT-PCR (rRT-PCR) is a valuable diagnostic tool for the laboratory detection of FMDV in vesicular epithelial tissue (Reid *et al*. 2003, Shaw *et al*. 2004), in serum, nasal swabs and oesophageal-pharyngeal scrapping (Zhang and Alexandersen 2003), in semen (Sharma *et al*. 2012), in milk (Reid *et al*. 2006) and in tongue epithelium (Ranjan *et al*. 2014). There is a paucity of literature dealing with the detection of FMDV secreted in milk after natural infection. The routine diagnosis of FMDV in milk is difficult following standard protocol due to the presence of inhibitory factors. The sensitive antigen trapping ELISA, although is a highly sensitive method for detection and serotyping of FMDV in the clinical samples, also was not effective in the spiked milk. Presence of non-specific PCR inhibitors such as lactoferrin, peroxidase and zinc residues hampers the action of polymerase enzyme

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reducing the sensitivity of the assay (Cohen *et al.* 1997). Hence, the present study was undertaken for the detection of FMD virus in milk in natural infection using nucleic acid recognition (NAR) methods.

MATERIALS AND METHODS

Animals: The present study was carried out at the experimental cattle herd, maintained at 29⁰28'N and 79⁰39'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.

FMD 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 was virus 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

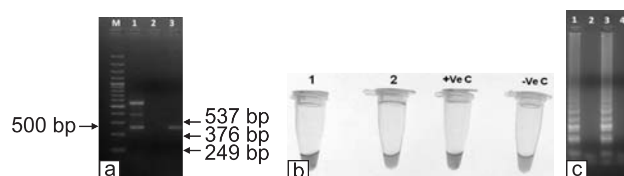

Fig. 1. Agarose gel electrophoresis. (a) multiplex PCR (mPCR) products showing presence of distinct bands of test sample in column 3–249 bp; negative control-column 2; positive control of O-249 bp, A-376 bp and Asia1–537 bp in column- 1; M: 100 bp molecular weight marker. (b) Tube 1 and 2 indicating positive and negative test sample in RT-LAMP test; +Ve and –Ve tube showing positive and negative control of RT-LAMP indicated by sky blue and purple colour, respectively. (c) Column 1 and 2 indicating positive and negative test sample in RT-LAMP; while column 3 and 4 showing positive and negative control of RT-LAMP, respectively.

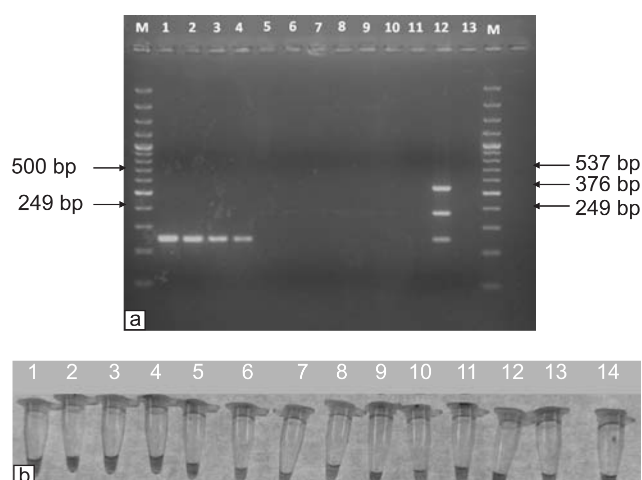

Fig. 2. Analytical sensitivity of uninfected negative whole milk spiked milk with $10^{5.7}$ $TCID_{50}$/ml FMD virus (IND R2/1975) diluted in decimal series (10^{-1} to 10^{-10}). (a) 1% gel electrophoresis of mPCR product in which column 1–11 indicates dilution of $10^{5.7}$ $TCID_{50}$/ml FMD virus from 10^0 to 10^{-10} while column 12 shows positive control of FMD virus of vaccine strain of serotype O, A and Asia 1 at 249 bp, 376 bp and 537 bp, respectively and column 13 shows negative control. (b) Showing change in colour of RT-LAMP product after completion of reaction in which tube 1–11 indicates dilution of $10^{5.7}$ $TCID_{50}$/ml FMD virus from 10^0 to 10^{-10} while tube 12 and 13 indicate positive FMD virus of vaccine strain of serotype O and negative control.

analytical ssensitivity of NAR methods, the detection limit of mPCR and RT-LAMP assay was $10^{2.7}$ and $10^{1.7}$ TCID50/ml, respectively (Fig. 2a,b). In previous study, detection limits of mPCR (Giridharand *et al.* 2005) and RT-LAMP (Ranjan *et al.* 2014) for FMDV serotype O were 158 and 4.2×10^{-4} $TCID_{50}$/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

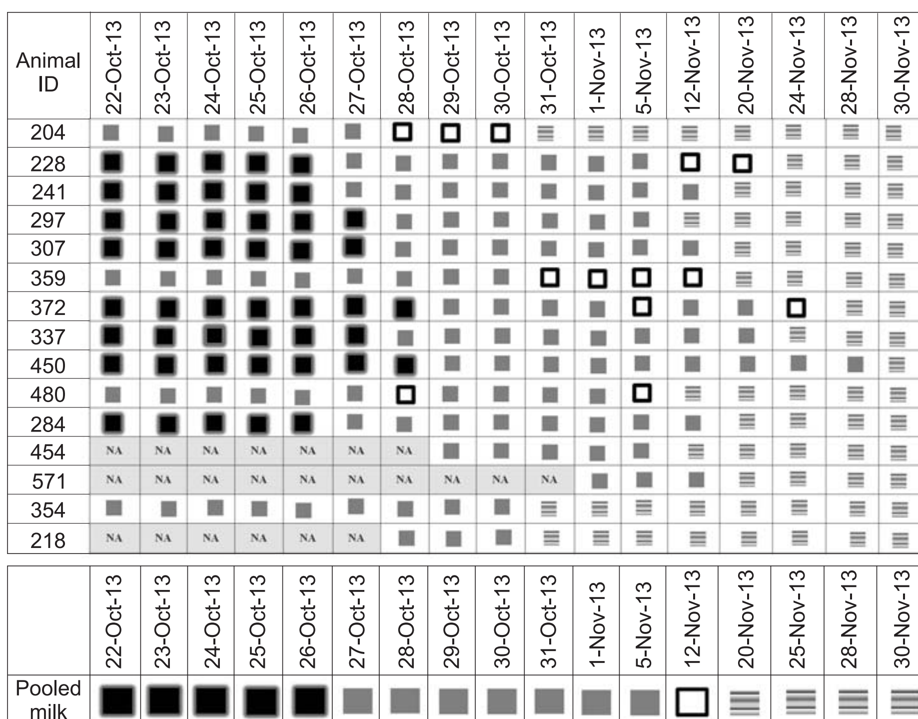

Fig 3. Demonstration of foot-and-mouth disease virus infection of dairy cows by virus /genome positive in milk samples monitored by virus isolation ■, multiplex PCR and RT-LAMP ■, RT-LAMP □, milk samples become negative by all test ≡, respectively. Upper figure showe result in milk sample collected from 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

Alexandersen S, Zhang Z, Donaldson A I and Garland A J M. 2003. The pathogenesis and diagnosis of foot-and-mouth disease. *Journal of Comparative Pathology* **129**: 1–36.

Bhattacharya S, Pattnaik B and Venkataramanan R. 1996. Development and application of sandwich enzyme linked immune sorbent assay (ELISA) for type identification of foot-and-mouth disease virus in direct field materials. *Indian Journal of Animal Sciences* **66**: 1–9.

Bisht P, Mohapatra J K, Subramaniam S, Das B, Pande V, Biswal J K, Sharma G K, Rout M, Ranjan R, Dash B B, Sanyal A and Pattnaik B. 2014. Efficient rescue of foot-and-mouth disease virus in cultured cells transfected with RNA extracted from clinical samples. *Journal of Virological Methods* **196**: 65–70.

Blackwell J H and Hyde J L. 1976. Effect of heat on foot-and-mouth disease virus (FMDV) in the components of milk from FMDV-infected cows. *The Journal of Hygiene* **77**: 77–83.

Blackwell J H, McKercher P D, Kosikowski F V, Carmichael L E and Gorewit R C. 1982. Concentration of foot-and-mouth disease virus in milk of cows infected under simulated field conditions. *Journal of Dairy Science* **65**: 1624–31.

Burrows R, Mann J A, Greig A, Chapman W G and Goodridge D. 1971. The growth and persistence of foot-and-mouth disease virus in the bovine mammary gland. *Journal of Hygiene (Lond)* **69**: 307–21.

Burrows R. 1968. Excretion of foot-and-mouth disease virus prior to the development of lesions. *Veterinary Record* **82**: 387–88.

Cohen M S, Hoffman I F, Royce R A, Kazembe P, Dyer Jr Daly, C C, Zimba D, Vernazza P L, Maida M, Fiscus S A and Eron J J Jr. 1997. Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. *Lancet* **349**: 1868–73.

Dawson P S. 1970. The involvement of milk in the spread of foot-and-mouth disease: an epidemiological study. *Veterinary Record* **87**: 543–48.

Donaldson A I, Gloster J, Harvey L D J, Deans D H. 1982. Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth disease outbreaks in Brittany, Jersey and the Isle of Wight in 1981. *Veterinary Record* **110**: 53–57.

Drew T W, Yapp F, Paton D J. 1999. The detection of bovine viral diarrhea virus in bulk milk samples by the use of a single-tube RT-PCR. *Veterinary Microbiology* **64**: 145–54.

Giridharan P, Hemadri D, Tosh C, Sanyal A and Bandyopadhyay SK. 2005. Development and evaluation of a multiplex PCR for differentiation of foot-and-mouth disease virus strains native to India. *Journal of Virological Methods* **126**: 1–111.

Heath G S, King D P, Turner J L E, Wakeley P R and Banks M. 2003. Use of an internal standard in a TaqMan1 nested reverse transcription polymerase chain reaction for the detection of bovine viral diarrhea virus. *Veterinary Microbiology* **96**: 357–66.

Hedger R S, Dawson P S. 1970. Foot-and-mouth disease virus in milk: an epidemiological study. *Veterinary Record* **87**: 186–88.

Hyslop N S. 1970. The epizootiology and epidemiology of foot and mouth disease. *Advances in Veterinary Science and Comparative Medicine* **14**: 261–307.

Ranjan R, Kangayan M, Subramaniam S, Mohapatra J K, Biswal J K, Sharma G K, Sanyal A, Pattnaik B. 2014. Development and evaluation of a one step Reverse Transcription-Loop Mediated Isothermal Amplification Assay (RT-LAMP) for rapid detection of foot and mouth disease virus in India. *Virus Disease* **25**(3): 358–64.

Reid S M, Grierson S S, Ferris N P, Hutchings G H and Alexandersen S. 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *Journal of Virological Methods* **107**: 129–39.

Reid S M, Parida S, King D P, Hutchings G H, Shaw A E, Ferris N P, Zhang Z, Hillerton J E and Paton D J. 2006. Utility of automated real-time RT-PCR for the detection of foot-and-mouth disease virus excreted in milk. *Veterinary Research* **37**: 121– 32.

Saeed A, Khan Q M, Waheed U, Arshad M, Asif M and Farooq M. 2001. RT-PCR evaluation for identification and sequence analysis of foot-and-mouth disease serotype O from 2006 to 2007 in Punjab, Pakistan. *Comparative Immunology, Microbiology and Infectious Diseases* **34**: 95– 101.

Samuel A R and Knowles N J. 2001. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *Journal of General Virology* **82**: 609–21.

Sharma G K, Subramaniam S, De A, Das B, Dash B B, Sanyal A, Misra A K and Pattnaik B. 2012. Detection of foot-and-mouth disease virus in semen of infected cattle bulls. *Indian Journal of Animal Sciences* **82** (12): 1472– 76.

Shaw A E, Reid S M, King D P, Hutchings G H and Ferris N P. 2004. Enhanced laboratory diagnosis of foot-and-mouth disease by real-time polymerase chain reaction. *Revue Scientifique et Technique (International Office of Epizootics)* **23**: 1003– 09.

Sutmoller P and Casas O R. 2003. The risks posed by the importation of animals vaccinated against foot and mouth disease and products derived from vaccinated animals: a review. *Revue Scientifique et Technique (International Office of Epizootics)* **22**: 823–35.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731–39.

Terbruggen F. 1932. Ueber die Haltbarkeit des Maul- und Klauenseuchevirus in Milch und Molkereiprodukten. I. Mitteilung. *Dtsch Tieraerztl Wochenschr* **40**: 129–34.

The Hindu. FMD caused loss of Rs 23,000 cr. in one year. The Hindu. IST Bengaluru, January 12, 2015.

Zhang Z and Alexandersen S. 2003. Detection of carrier cattle and sheep persistently infected with foot-and-mouth disease virus by a rapid realtime RT-PCR assay. *Journal of Virological Methods* **111**: 95–100.