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DETECTION OF FOOT-AND -MOUTH DISEASE VIRUS SEROTYPE 'O' IN MILK SAMPLES OF CATTLE USING RT-PCR AND DETECTION OF ANTIBODY AGAINST NON-STRUCTURAL PROTEIN USING DIVA ELISA

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Abstract–The present study was conducted to detect foot-and-mouth disease virus type 'O' in milk samples of FMD infected cattle and also to distinguish naturally infected from vaccinated cattle by use of DIVA ELISA. A total of 30 milk samples were collected from FMD infected cattle to carry out the study and serum samples were also collected from the same herd of animals. RNA extraction was done by Trizol method from milk samples. All the milk samples were found to be positive by RT-PCR for the presence of Foot and mouth disease virus type O. Detection of antibody against non-strucructural protein using r3AB3 kit showed 100% positivity rate, which indicates the animals were naturally infected by Foot and mouth disease virus type O.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-footed domestic and wild animals with a very high economic importance (Knight-Jones and Rushton, 2013). The FMD virus has seven serotypes-O, A, C, Asia 1 and Southern African Territories (SAT)1, SAT 2 and SAT 3 (Robson *et al.*,1977). The FMD virus has a single-stranded, positive sense RNA genome of approximately 8.4 Kb and it comes under the genus Aphtho virus and the family Picornaviridae (Grubman and Baxt, 2004). Clinically, FMDV infected animals show high rise of temperature, salivation with the formation of vesicles on the oral mucosa, nose, udder, and feet (Kitching, 2002; Alexandersen *et al*., 2003). FMD causes a heavy economic loss to the farmers every year by lowering the productivity of animals. Therefore, it is very much necessary to diagnose the disease rapidly and to control it. Many diagnostic techniques have been applied in different laboratories such as virus isolation (VI) in sensitive cell cultures and antigen detection ELISA (Ferris and Dawson, 1988). Recently, many PCR based techniques by amplification of FMDV viral genome, have been found to play a major role in rapid detection of FMD in animals, particularly automated rRT PCR (Reid *et al*., 2003).Reverse Transcription PCR can be used to detect FMDV in different biological samples such as tissues, fluids, blood, serum, milk and also this technique is useful for detection of the disease in infected animals before development of symptoms or also detection of the disease, when virus isolation may be negative (Callahan *et al*., 2002; King *et al*., 2006).

Milk is a non-invasive type of sample and also easy to collect from farms for detection of both FMDV and FMDV-specific antibodies (Burrows *et al*., 1971; Armstrong, 1997). In animals, the virus is reported to be secreted in milk for many days, after

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infection (Terbruggen, 1932; Sutmoller and Casas 2003). It is also reported that the FMD virus survives for 6 days at 18ºC and for 15 days at 4ºC in raw milk samples (Terbruggen 1932). The virus can be detected in milk 1–4 days before development of vesicles (Burrows *et al*. 1971, Blackwell *et al*. 1982). Therefore, milk can be considered as one of the important source of sample for detection of FMDV. The FMDV genome contains one open reading frame (ORF), which encodes a single polypeptide which is post-translationally modified into four structural proteins (VP1–4) and eight non-structural proteins (Lpro, 2A, 2B, 2C, 3A, 3B, 3Cpro, and 3D) (Belsham,1993). DIVA (Differentiation of infected from vaccinated animals) strategy based on non-structural protein (NSP) helps in detection of FMD virus in animals and also helps in discrimination of vaccinated from naturally infected populations (Biswal *et al.*, 2015). Recombinant non-structural proteins such as 2B, 2C, 3A, 3AB, 3B, 3ABC and 3D have been used in DIVA-ELISAs by many researchers (Clavijo *et al*., 2004b; Brocchi *et al*., 2006). Some of the NSPs like 2C, 3A, 3ABC or its derivatives such as 3AB3 based DIVA-ELISA have been found to be the most reliable indicators of FMD infection and also determine whether the infection is due to vaccination or natural infection (De Diego *et al*., 1997; Mackay *et al.*, 1998). Some researchers reported detection of anti NSP against 3ABC up to 395 days post infection in both cattle and sheep (Sorensen *et al*., 1998).

MATERIALS AND METHODS

Experimental samples

A total of 30 raw milk samples (10 ml) were collected from suspected FMDV infected cattle for detection of FMD virus. The milk samples were transported to laboratory by maintaining cold chain and stored at 4^0C. For performing DIVA-ELISA, serum samples (30) were collected from raw blood samples from the infected animals and stored at -20^0C.

A live virus O vaccine strain maintained at the Project Directorate on Foot and Mouth Disease, Indian Veterinary Research Institute (IVRI) Campus, Mukteswar, Uttarakhand was used as control in this study.

RNA extraction

RNA extraction from milk samples was carried out using Trizol method. Milk samples were centrifuged at 1500g for 15 min at 4^0C to separate the milk fat. After centrifugation, milk fat was removed carefully from the tube. A volume of 250 µl sample was taken in a 1.5 ml microcentrifuge tube and 750 µl of Trizol reagent was added. The mixture was vortexed properly and 200 µl of chloroform was added and vortexed vigorously, followed by incubation of the tube at room temperature for 15 min. After incubation, the tube was centrifuged at 12,000g for 15 min at 4 ºC and the aquous phase was transferred to a fresh microcentrifuge tube. An equal volume of isopropanol was added and mixed properly and the tube was then incubated at room temperature for 10 min for precipitation of the RNA. After incubation, the tube was centrifuged again at 12,000g for 10 min at 4 ºC. Then the supernatant was removed carefully and the RNA pellet was washed with 70% alcohol. The pellet was air dried and dissolved in 20 µl of nuclease free water by incubating at 55 ºC for 10 min. The extracted RNA was stored at -80 ºC.

cDNA synthesis

For cDNA synthesis, 3 µl (0.5-1 µg) of RNA was taken as template in a PCR tube. To the template, 0.5 µl random hexamer (0.2µg/ µl) and 3 µl of nuclease free water was added The reaction mixture was incubated at 70 °C for 5 min and then at 25 °C for 10 min in a thermal cycler. After incubation, the tube was immediately cooled to 4 ºC by placing in crushed ice followed by the addition of remaining reagents of the reaction mixture. To the above reaction mixture, 2 µl of 5X RT buffer, 0.5 µl RNase inhibitor (40 units/µl), 0.5 µl 10 mM dNTP mix and 0.5 µl RevertAid™ M-MuLV reverse transcriptase (200 units/ml) enzyme were added. The reaction mixture was mixed properly and incubated using the condition-25°C for 5 mins, 42°C for 1 hour and 70°C for 15 mins in a single cycle. cDNA was stored in -20^0C for further use.

Detection of FMD virus type O using RT PCR

For detection of FMD virus, PCR was performed using cDNAs with the primer sequence shown in Table 1. The primer sequence specific for detection of FMD O virus was designed in the laboratory using Primer Blast software. A total volume of 25 µl reaction mixture was prepared as follows: 12.5 µl of 2X Dream Taq master mix, 1 µl of each forward primer (10 pmol/µl) and reverse primer (10 pmol/ µl), 2 µl cDNA and the volume was make upto 25 µl with nuclease free water. The PCR was performed with the thermal cycling condition as follows: 95^0C

for 5 min, 1 cycle; 95 °C for 30 sec, 57 °C for 1min, 72°C for 30 sec, 35 cycles followed by 72 °C for 5 min, 1 cycle.

Agarose gel electrophoresis

The PCR amplified products were subjected to agarose gel electrophoresis (1.5%) for confirmation of the products. A product size of 81 bp was obtained after gel electrophoresis (Figure 1).

NSP based DIVA-ELISA using 3AB3 ELISA KIT

Presence of antibody against non-structural protein of foot and mouth disease virus was determined by using recombinant 3AB3 based indirect ELISA kit with above 90% sensitivity and 98% specificity for DIVA (Differentiation of Infected from Vaccinated Animal).

Recombinant 3AB3 based indirect EISA kit designed, developed and evaluated at Central Laboratory, Project Directorate on FMD, Mukteswar was used to detect the presence of antibody against non-structural protein (NSP) of FMDV (Mohapatra *et al*., 2011).

Freeze dried recombinant protein was dissolved in the vial with 1 ml of coating buffer. After reconstitution, the vial should be used for coating immediately without storing. The freeze dried vials of positive (one month post infection serum) and negative (one month post vaccination serum) control serum were reconstituted with distilled water and stored at -20 °C after reconstitution in single use aliquots. The contents of freeze dried vial carrying *Escherichia coli* lysate was dissolved in 70µl of PBS and stored at -20^{0}C after use. 96 -well polystyrene (Nunc Maxisorp). Immuno plate was coated with the diluted recombinant protein @ 50µl per well (~40 ng of purified recombinant protein per well). The plate was gently tapped from all sides and incubated at 4 °C for overnight. Next day plate was removed from refrigerator and thawed them at 37 °C for 15 min. The test and the supplied negative and positive control sera were diluted @ 1:20 in diluents buffer in a low protein binding Perspex plate. Only serum from bovine host is compatible with this test. A total volume of 220 µl of diluted serum was prepared so that 100 µl of the mixture can be transferred to the coated ELISA plate in duplicate. For background controls, only 100 µl of diluents buffer was dispended without any serum. Three continuous washings (no hold time) were given with wash buffer. 100 µl of serum and diluent buffer mixture from Perspex plate was transferred to the ELISA plate in duplicate wells. The plate was incubated for 1 hour at 37^{0}C and plates were tapped gently from all sides at every 15 min intervals. Three washings were given at 3 min soak period each. Anti- bovine –HRP conjugate diluted in the diluents buffer (1:2000) was dispensed @ 50 µl per well. The plate was incubated for 1 hour at 37^{0}C and tapped gently from all sides at every 15 min intervals. Three washings were given of 5 min soak period each. Freshly prepared substrate solution was added @ 50 µl per well and incubated for 15 min at 37 °C without shaking. The colour reaction was stopped by adding 1M H_2SO_4 @ 50 µl per well. Optical density values were measured at wave length of 492 nm (Reference 620 nm).The ELISA test results were expressed as the percent positivity (PP) value, which were calculated by dividing the OD of the test serum by that of the positive control serum and multiplying that value with 100. The test result was considered as NSP positive, if PP value was more than 40%; and for less than 40%, it was considered as negative for the test.

RESULTS AND DISCUSSION

Foot and mouth disease is considered as one of the most contagious diseases of cloven hoofed animals and it is very necessary to diagnose the disease rapidly, so that it cannot spread to other animals. Milk samples are easy to collect from animals for diagnosis of FMD and for surveillance in dairy cattle (Saeed *et al*. 2011). RT-PCR is one of the most widely used techniques for diagnosis of FMD with high sensitivity rate and speed (Shaw *et al*., 2004; King *et al*., 2006).

In the present study, all the 30 cDNA synthesized from RNA extracted from milk were subjected to RT PCR. All the 30(100%) cDNA were found to be positive for presence of FMD virus type O (Figure 1). In conformity to the present study, Ranjan *et al.*

Table 1. Primers Used for RT- PCR for detection of FMD 'O' serotype

Primer	Sequences	Annealing temperature	Product size	Reference
FMD O serotype	F: 5′-CAACAAAACACGGACCCGAC-3′ R: 5′-CAGTTCTGATAGCCTTCACTCCA-3′	57	81 bp	Designed

(2016) detected FMD virus in all the 15 (12 clinically infected and 3 asymptomatic) milk samples using mPCR and RT LAMP. They also reported presence of FMDV in individual milk and pooled milk samples m-PCR till 37 and 14 dpm (days post manifestations) by m-PCR and till 37 and 21 dpm by RT-LAMP, respectively. Present study was in agreement with the findings of Armson *et al.* (2018), who conducted a study to detect FMD virus in milk and serum samples of experimental infected cattle to evaluate two Real time RT-PCR (rRT-PCR) methods with two different chemistries. Four cattle were experimentally infected through direct contact with two infected cattle and milk and serum samples were tested for detection of FMDV. The virus was detected in all the four milk samples by both the Real time RT -PCR methods till 28 days post contact and the virus was detected 18 days longer in milk than the serum samples. The virus was also detected with both rRT-PCR methods in milk samples collected during the UK outbreak in 2007. Another study conducted by Armson *et al*., (2020) detected FMDV in 42(5.7%) out of 732 pooled milk samples collected from a large scale dairy farm in Saudi Arabia by Real time RT-PCR. These findings were not in agreement with the present study.

All the 30 serum samples were screened for presence of antibody against non structural protein (NSP) using recombinant 3AB3 ELISA kit. All the serum samples (100%) were found to be positive for antibody against NSP. In a study conducted in Haryana (Bora *et al*., 2014), where 4200 serum samples collected from 21 districts of the state were tested for detection of antibody against NSP and

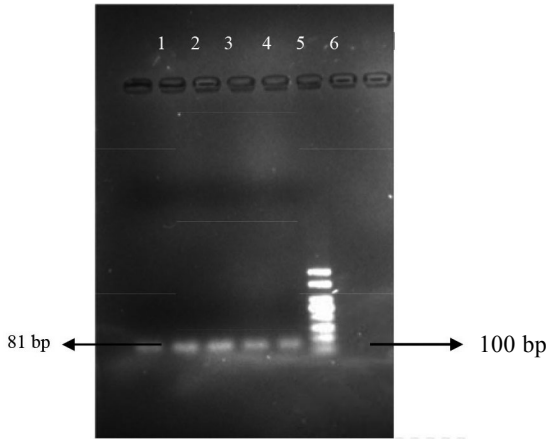

Fig. 1. Representative photograph showing amplified products (81 bp) of FMD serotype O (Lane 2-5), Lane 1: Control, Lane 6: 50 bp ladder

only 76 (1.809%) serum samples were found to be positive for anti 3AB3antibodies. These findings were not in conformity with the present study. Singh *et al.,* (2015) screened a total of 10,422 serum samples comprising of 5,038 samples from buffalo and 5,384 samples from cattle for presence of anti3AB3 NSP antibodies, collected on the day of vaccination during XVI phase of vaccination under FMD control programme (FMD-CP) from 65 districts of Uttar Pradesh. A positivity rate of 18.62% (1,941/10,422) serum samples were found for presence of anti-3AB3 NSP antibodies with 19.71% (1,061/5,384) for cattle and 17.47% (880/5,038) for buffalo serum samples, which is not in conformity with the present findings. Kumar *et al*., (2007) conducted a study in Haryana, where serum samples were collected from vaccinated cattle and buffaloes under FMD control programme (FMD-CP). A total of 3048 serum samples were collected prior to vaccination (1484) and before fourth phase of vaccination (1564) from randomly selected cattle and buffaloes and screened for presence of antibody against NSP using Baculovirus-expressed FMDV NSP 3A. A positivity rate of 31.94% and 18.40%, were found for presence of anti NSP antibodies against 3A for pre first phase and pre fourth phase of vaccination respectively. Whereas 35.2% of cattle and 14.81% of buffaloes were found to be positive for presence of anti 3A NSP antibodies. These results were not very close conformity to the present findings.

CONCLUSION

Detection of FMD virus from milk samples may be easier method as milk is a non invasive sample compared to other invasive techniques. RT-PCR can be used to detect the virus from milk samples at low cost compared to Real-time RT-PCR techniques. Detection of antibodies against NSP using r3AB3 ELISA kit can be very useful technique to differentiate vaccinated and naturally infected animals.

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Conflict of Interest

The author declares no conflict of interests regarding the publication of this paper.

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