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Detection of foot-and-mouth disease virus type O in recovered as well as healthy cattle to study carrier status in Assam

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Foot and mouth disease (FMD), one of the most contagious diseases of animals, affects different host species including wild animals. Asymptomatic FMD recovered animals may remain as carrier, which may be threat to other healthy animals. Hence, it is necessary to monitor the carrier status of the FMD recovered animals to effectively prevent further spread of the disease. Out of all the seven serotypes of FMD, O serotype is most commonly found in livestock. Therefore, in the present study, we chose to detect serotype 'O' in oropharyngeal fluid (OP) and to quantify cytokines, *viz*. IL-1α, IL-1β and IL-2. A total of 30 OP fluids and 30 blood samples were collected from 10 animals (1 in-contact healthy animal) for 3 months post infection. FMD O serotype could be detected in all the animals (100%). The RQ values were found to be 0.014 to 63.118 and 0.162 to 46.889 for IL-1α and IL-1β genes respectively, while insignificant RQ values were obtained for IL-2. In the second and third months, two animals showed down regulation for IL-1α gene, while IL-1β and IL-2 genes were down regulated in 7 animals and in all 10 animals, respectively for all the three months.

Keywords: Cytokines, Interleukins, Livestock, Oropharyngeal fluid

Foot-and-mouth disease (FMD) is a highly contagious viral infection which affects cloven-hoofed animals including cattle, pigs and sheep[1]The disease spreads rapidly to susceptible animals and causes heavy loss to farmers and agricultural industries in affected areas[2].The Foot-and-mouth disease virus (FMDV), the causative agent of FMD, comes under the family Picornaviridae, genus Aphthovirus and is a single stranded (ss) with positive sense RNA, sedimentation coefficient of 146S and a genome size of ~8.5 Kb[3].FMD can be considered as one of the most important limitation for international trade of animals and animal products[4].

There are seven immunologically distinct serotypes of FMD virus (FMDV), namely, O, A, C, Asia1, SAT1, SAT2 and SAT3[5], with more than 65 subtypes. FMD is being considered as an endemic in many parts of the world and in most of the South-Asian countries, outbreaks occurs due to serotypes O, A, and Asia-1[6,7]. Production losses caused by FMD include reduced milk production[8], which results in 80% reduction in milk yield in dairy herds[9]. A large amount of money is invested every year for vaccinating animals against FMD in the world. The overall economic loss, calculated based on the costs of a vaccine with its application even a decade ago, was estimated to be between 0.4 and 3 USD, with most likely cost of 1 USD[10]. The direct, visible production loss due to FMD outbreak ranges from 100 to 370 USD per case, with the most likely value 100 USD[11]. The total economic loss due to FMD inIndia ranges from 12,000 crore to 14,000 crore per year[12]. The controlling method adopted is either slaughter of diseased animals or maintaining proper vaccination. The clinical symptoms include development of vesicular lesions in areas covered by cornified stratified squamous epithelium such as the oral cavity and coronary bands[13], which causes excessive salivation, in appetence and lameness. Infected animals develop transient viraemia which last for about 2-4 days, which is effectively counteracted by the development of circulating anti-FMDV antibodies. The severity of the disease varies greatly between different viral strains and different host species, with development of more severe form in swine, followed by cattle with mild to moderate symptoms and sheep may remain asymptomatic till

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the occurrence of disease[2]. The mortality rates are higher in animals with lower age group with myocarditis in comparison to adult animals. Initially, FMDV replication occurs in nasopharynx or lungs[14], followed by viraemia, which spreads the infection to secondary site of replication leading to vesicle formation and lesions[15].

Although, the clinical symptoms appear till 14 days of infection, but the persistent infection in ruminants have been found to cause many complications as the animals may remain as carrier making very difficult to control the disease. Persistent FMDV infection is found to occur following clinical or subclinical infection in ruminants[16]. Carriers can be defined as an animal from which virus can be detected 28 days post infections[16]. The carrier animals may act as a potential threat of outbreaks for in-contact healthy animals and making it difficult to regain FMD free status[4]. Animals may remain as carrier for several months or years and may spread the disease to other animals of the herd. Hence, it is essential to identify those carriers to prevent further spread of the disease. Persistence of FMD virus has been reported from many host species including cattle, sheep, goats, Asian buffalo and various wild animals[17]. Real-Time PCR is one of the reliable techniques to detect persistent FMDV infections in terms of specificity and sensitivity[18]. It has previously been demonstrated that analysis of samples of oropharyngeal fluid using specific qRT-PCR is suitable for detection of FMDV carriers in experimentally infected animals[19]. Persistent infection is detected in oropharyngeal (OP) fluid collected using probang cup. Although the virus persist in OP fluid, the viral load becomes very low and RT-PCR may be useful in detecting the persistent infection. Moreover, RT-PCR is faster and more samples can be run in a single assay with absolute quantification of the virus, than the conventional RT-PCR[20].

Cytokines have been found to play role in host cell response during persistent infection of FMDV. There may be possibilities of involvement of cytokines during persistent infections, preventing infections and helping in clearance of the virus[16]. It is also reported that FMDV disrupts the early immune response by targeting innate immune system[21]. Majority of cytokines including TNF-α, IL-1, IL-2, IL-4, IL-5, IL-10, IL-12, type I interferons and IFN-γ play a vital role in natural and adaptive immunity by means of immuno-modulatory mechanism for clearance of viral infection. Interferon (IFN) type I, II and III have been demonstrated to diminish replication of FMDV both *in vitro* and *in vivo*[22]. Cytokines such as IP-10, IL-2, IL-4, IL-10, IL-12, IL-15 and IL-18 have been reported to affect replication of the virus through activation and maturation of dendritic cells and proliferation and activation of NK cell[23]. FMDV replication and transmission is reported to be controlled by cytokines like IL-1, IL-6, TNF-α, IL-10[24]. IL-1 is produced by activated macrophages and causes activation of T cells. Upon viral infection, macrophages attempting to destroy the virus particle by release of IL-1 into circulation. However, IL-2 is produced by Th1 and causes T cell proliferation, promotes growth of B cells, induces production of IFN-γ and IL-5 and regulates TNF-α expression. They also activate macrophages and enhance their cytotoxic activity resulting in destruction of the pathogen. In this context, here, we investigated FMD virus type O in recovered as well as healthy cattle to understand the carrier status and to determine possible factors of persistence of FMD virus type 'O' in cattle.

Materials and Methods

Source of samples

A total of 60 clinical materials collected from Instructional Livestock Farm, College of Veterinary Science, Khanapara, AAU, Guwahati, Assam (India) during the period of November 2012 to January 2013, were used in the present study. Samples were collected 7 months of post infection as the outbreak occurred in the month of May, 2012. The clinical materials comprised of oropharyngeal fluid (OP), i.e. probang samples collected by probang-cup (30) and blood (30) collected at an interval of one month from 10 animals and preserved in phosphate buffer and stored at 080°C, the 30 whole bloods were collected in EDTA vials and stored at 020°C. Necessary approval from the Institute's ethics committee was obtained. Approval No: 770/ac/CPCSEA.FVSc/AAU/IAEC/13-14/192 Dated10.2.2014

Reference virus

For standardization of different methods, 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 controls in this study.

Detection of FMD virus nucleic acid by Real-Time PCR

RNA isolation from OP fluid was done as recommended by the manufacturer of Trizol reagent

(Invitrogen). Reverse transcription of the viral RNA was performed using RevertAid™ M-MuLV Reverse Transcriptase {Cat.# EP0441; MBI Fermentas (Vilnius), Lithuania} and was obtained using NK61 as RT-primer(5'-GACATGTCCTCCTGCATCTG-3')[25]. Detection of FMD virus in op fluid was done by using Step One Plus Real-Time PCR (Applied Biosystem by Life Technology, Foster City, CA, USA). SYBR Green dye was used as the active chemistry and Rox was being used as passive dye. All samples were measured in triplicates. The primer sequence for Real-Time PCR was designed using Primer Blast software (Table 1). Reactions were performed in 20 µL volume reaction mixture and Non-Template Control (NTC) comprised of the following components listed in Table 2. The thermal cycling conditions were set using the PCR thermal-cycling conditions specified in the Table 3.

Optimization of assay

Initially, specificity of all assays were checked by conventional PCR using thermal cycler and product size of the PCR product was checked in agarose gel electrophoresis. If a single band was obtained then the optimization of Real-Time PCR were performed. The optimal primer concentration for the primer pair was determined by performing duplicate RT PCR assay at different primer concentrations over the range of 5-20 pmol/µL. From the fluorescence data and melting curve analysis, the optimal primer concentration was obtained.

Quantification of IL-1 and IL-2 genes by RT PCR in blood

RNA isolation was done from blood using Ribozol™ Reagent and Reverse transcription of the viral RNA was performed using RevertAid™ M-MuLV Reverse Transcriptase {Cat.# EP0441; MBI Fermentas (Vilnius), Lithuania}.

Real-Time PCR for cytokine quantification

SYBR Green based Real-Time PCR using Step One Plus, Real-Time amplification instrument (Applied Biosystem by Life Technology, Foster City, CA, USA) was used for cytokine quantification and the reaction mixture had components as listed in Table 2, and thermal cycling conditions for IL-1α, IL-1β, IL-2 and GAPDH as shown in Table 3

Table 1 — Primers used for Real-Time PCR for detection of FMD 'O' serotype and for quantification of cytokines

Primer	Sequences	Product size
FMD O serotype	Forward: 5'-CAACAAAACACGGACCCGAC-3' Reverse: 5'-CAGTTCTGATAGCCTTCACTCCA-3'	81 bp
IL-1α	Forward: 5'-TTGGGAGGACTGAGGCTACT-3' Reverse: 5'-ATTCCAACTGCTGTCATGGGT-3'	106 bp
IL-1β	Forward: 5'-AACGTCCTCCGACGAGTTTC-3' Reverse: 5'-GGAGAGCCTTCAGCACACAT-3'	116 bp
IL-2	Forward: 5'-GTGCCCAAGGTTAACGCTAC-3' Reverse: 5'-TGATCTCTCTGGGGTTCAGGT-3'	118 bp
GAPDH	Forward: 5'-TGTGCCAGCCGCATCC-3' Reverse: 5'-ATGGCGACGATGTCCACTTT-3'	118 bp

[Annealing temperature for FMD O serotype was 57.5 °C and for the rest 57 °C]

Table 3 — Thermal cycling conditions for Real-Time PCR of IL-1α, IL-1β, IL-2, GAPDH and FMD 'O' serotype

Step	Temperature(°C)	Duration	Cycles
Initial Denaturation	95	15 s	HOLD
Denaturation	95	10 min (IL-1α, IL-1β, IL-2 and GAPDH) 1 min (FMD O serotype)	30
Annealing/Extension	57 (IL-1α, IL-1β, IL-2 and GAPDH) 57.5 (FMD O serotype)	1 min (IL-1α, IL-1β, IL-2 and GAPDH) 45 sec (FMD O serotype)	
Melt curve stage	95	15 s	1
	60	1 min	
	95	15 s	

Table 2 — Components of Real-Time PCR for IL-1α, IL-1β, IL-2 and GAPDH and FMD serotype 'O'

Components	IL-1α		IL-1β		IL-2		GAPDH		FMD 'O' serotype	
	Non-Template Control (NTC)	Reaction mixture	Non-Template Control (NTC)	Reaction mixture	Non-Template Control (NTC)	Reaction mixture	Non-Template Control (NTC)	Reaction mixture	Non-Template Control (NTC)	Reaction mixture
Fast SYBR Green Master Mix (2X)	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL
Forward primer(10 pmol/µl for IL-1α, IL-1β, IL-2 & GAPDH) (7.7 pmol/ µl for FMD O)	0.25 µL	0.25 µL	0.2 µL	0.2 µL	0.2 µL	0.2 µL	0.25 µL	0.25 µL	0.2 µL	0.2 µL
Reverse primer (10 pmol/µl for IL-1α, IL-1β, IL-2 & GAPDH) (7.7 pmol/ µl for FMD O)	0.25 µL	0.25 µL	0.2 µL	0.2 µL	0.2 µL	0.2 µL	0.25 µL	0.25 µL	0.2 µL	0.2 µL
cDNA	-	1.5 µL	-	2.0 µL	-	3.5 µL	-	1.5 µL	-	3.5 µL
Nuclease free water	9.5 µL	8.0 µL	9.6 µL	7.6 µL	9.6 µL	6.1 µL	9.5 µL	8.0 µL	9.6 µL	6.1 µL
Total	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL	20.0 µL

Relative quantification of gene expression and data analysis

The relative quantification of target genes expression was calculated using $2^{-\Delta\Delta Ct}$. The threshold cycle (Ct) values were based on triplicate measurements and each experiment was repeated twice. The quantification values obtained for target genes in control were used for calibration and were arbitrarily set to 1 and 0 for linear and log graph types respectively. The data analysis was carried out by StepOne® software v 2.2.2.

The RT-PCR products obtained were subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide (10 mg/mL).

Results and Discussion

Detection of FMD virus nucleic acid by RT PCR

In the present study, all the 30 samples (OP fluid) were subjected to RT PCR and the virus was detected in all the samples. RNA concentration of the test samples was measured by nanodrop spectrophotometer which ranges 25-50 ng/µL. The results revealed that there was a negative correlation between the RNA concentration and the C_T value. This indicates that as the concentration of RNA increases, there is decrease in the C_T value of RT PCR.

The present analysis showed that all 30 OP fluids collected for three months were positive for FMD virus O serotype with mean C_T values ranging from 24.613 to 26.305, and standard deviation between triplicate wells ranging from 0.211 to 1.554 for first month, 23.291 to 26.035 and standard deviation between triplicate wells ranging from 0.19 to 1.88 for second month and 22.331 to 25.868 and standard deviation between triplicate wells ranging from 0.352 to 1.913 for third month, respectively. RT PCR amplification log, linear and melt curve obtained during detection of FMD virus type 'O' are shown in Figs. 1 and 2. RT-PCR amplicons of OP fluid showed a product size of 81 bp for FMD 'O' serotype in 1.5% agarose gel electrophoresis (Fig. 3).

A flurogenic Real-time RT PCR could detect FMD virus in 57 of the 59 (97%) epithelial suspension and 70 (99%) out of 71 cell culture fluid; out of the 20 oral swab samples used in the study, three samples were positive with real-time PCR and only one of these samples were positive by virus isolation[20].These findings were in close conformity to the present findings. Another group researchers reported that three samples out of two hundred and sixty oral swabs collected from area with outbreaks of FMD was positive by real-time PCR while only two of those samples were positive by virus isolation and ELISA[26]. This study was not in agreement with the present findings. Another study conducted to detect FMD virus serotype O by reverse transcription PCR in 18 samples comprising of tongue and feet epithelium and oral swab and 15 (84%) were found to be positive for O serotype[27]. These findings are in close conformity to the present findings. Carrier status was detected by screening of oral swab samples[1], where 7 out of 12 samples (58%) were positive, which has consistent with the results found previously in experimental infections with serotype O FMDV by many researchers[2,16,]. Presence of FMD O serotype in OP fluid indicates that all the animals have asymptomatic persistent infection. Persistent FMD serotype Asia 1 as detected from 12 (71%) out of 17 experimentally infected cattle by RT PCR from oesophageal pharyngeal fluid[28].

The above results are in agreement with that the RT-PCR is a highly sensitive and specific under laboratory and experimental conditions[29]. Correlation between RNA concentration and C_T value of RT-PCR showed that there was a negative correlation between them thus indicating that as the concentration

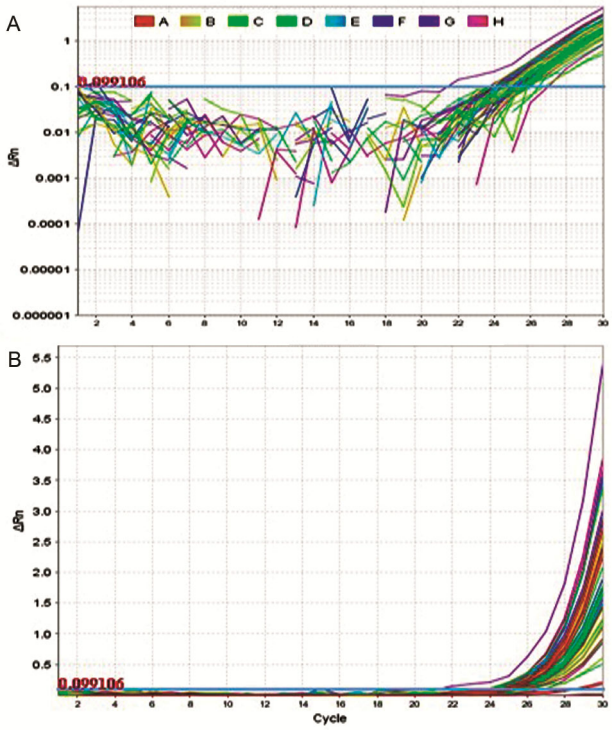

Fig. 1 — Representative Real-Time polymerase chain reaction amplification curves obtained during detection of FMD virus type 'O' (A) Log curve; and (B) Linear curve

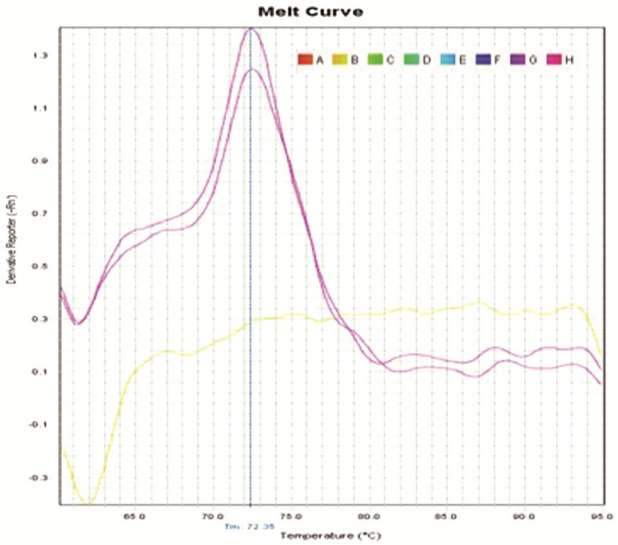

Fig. 2 — Melt curves for (A) GAPDH; (B) IL-1α (C) IL-1β; and (D) IL-2 genes

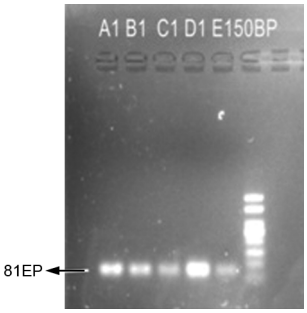

Fig. 3 — Real-Time PCR amplicons of OP fluid showing specific bands for FMD 'O' serotype in 1.5% agarose gel. [Lane 1: Control; Lanes 2-5: Amplified products of FMD positive samples; Lane 6: 50 bp ladder]

of RNA increases, the C_T value (threshold cycle) of RT-PCR decreases.

Quantification of cytokines associated with persistence by RT PCR

In the present study, values of relative quantification of cytokines (IL-1α, IL-1β and IL-2) obtained for target genes in the negative control was used for calibration and was arbitrarily set to 1 and relative quantification for all the genes were obtained for 10 animals in three months period. The RQ values for IL-1α gene during the three months of study were found in the range of 0.014 to 63.118. In the first month, IL-1α gene was not found to be down regulated in any animal, but 2 animals (no.4 and 5) showed down regulation of the gene in second month and third month. During the study period there was no acute infection or any inflammatory reaction which

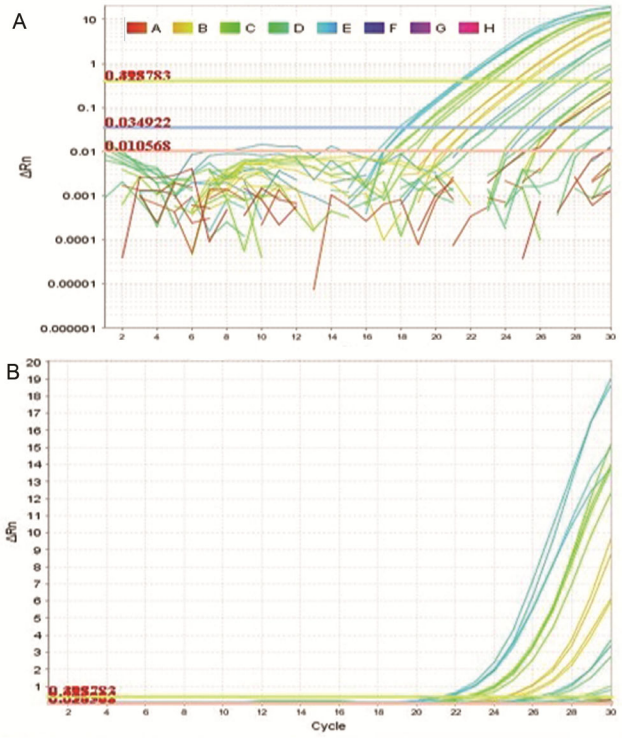

Fig. 4 — Representative Real-Time PCR amplification curves for relative quantification of genes IL-1α, IL-1β and IL-2 (A) Log curve; and (B) Linear curve

may be the reason for IL-1α down regulation. In contrast to the present findings, an increased expression of IL-1α gene during persistence was reported[19]. Log and linear curves for RT-PCR amplification for relative quantification of GAPDH, IL-1α, IL-1β and IL-2 genes were shown in Fig. 4 A and B. The melt curves for all the four genes (GAPDH, IL-1α, IL-1β and IL-2) are shown separately in supplementary Fig. S1B (*All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). The 1.5% agarose gel electrophoresis of RT PCR amplicons of GAPDH, IL-1α, IL-1β and IL-2 showed product size of 118,106,116 and 118 bp, respectively (Fig. 5).

IL-1β gene expression was studied in the present study to correlate with persistent FMDV infection. The RQ values were found in the range of 0.162 to 46.889 for IL-1β gene for three months. In case of IL-1β, a downregulation was observed in month 1 and month 3, whereas in month 2, an upregulation was observed. Out of 10 animals, 7 animals were found to be consistently showing down regulation of the gene in all the 3 months. Another study reported absence of IL-

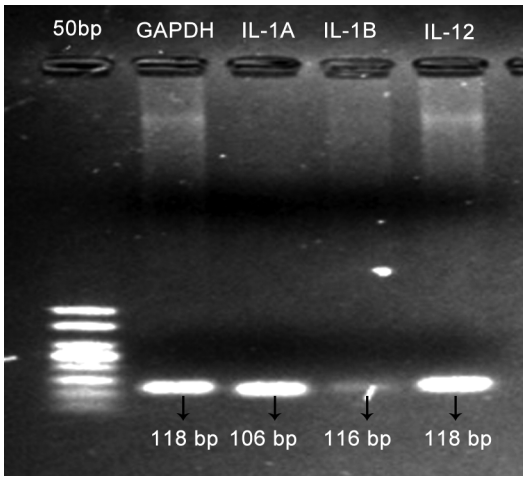

Fig. 5 — Real-time amplicons of GAPDH, IL-1α, IL-1β and IL-2 in 1.5% agarose gel electrophoresis. [Lane 1: 50 bp ladder; Lanes 2-5: Amplified product of GAPDH, IL-1α, IL-1β, IL-2 genes, respectively]

1β and TNF-α in the cells persistently infected with measles virus and hypothesized that this could be a consequence of the persistent infection inhibiting signal transduction pathways involved in the induction of the corresponding genes[14]. They also reported that persistently infected clones of astrocytoma cells continually expressed various amounts of IL-6 and TNF-β, but not IL-1β and TNF-α. IL-1β is produced by activated macrophages and responsible for inflammation, cellular activities including cell proliferation and differentiation. As, during the study period, acute infection had already subsided and no visible clinical signs were observed, so probably this resulted in downregulation of IL-1β gene.

In the present study, expression of IL-2 gene was also studied to establish any relationship with virus persistence. The RQ values for IL-2 gene were found to be consistently insignificant across 3 months of study. In all the10 animals, IL-2 gene was found to be downregulated for all the 3 months. However, there was a slight increase in mRNA expression of IL-2 during persistence reported by another group of researchers[20]. IL-2 is secreted by T cells responsible for T- & B- cell proliferation and NK cell activation and proliferation.

Conclusion

The present study provided preliminary data on carrier status of Foot-and-mouth disease in cattle in Assam. This study showed a high rate of persistent FMDV infection in cattle, as it showed 100% positivity in RT PCR. It suggests that RT PCR can be used as a useful technique for detection of carrier status, and thereby prevent the spread of the disease to in-contact healthy animals. Transmission of the disease from FMDV recovered (carrier) cattle to other in-contact healthy cattle highlights the importance of the study with early detection of carriers. The findings also indicated downregulation of all the three cytokine genes IL-1α, IL-1β and IL-2 with progression of recovery of the animals from the disease.

Conflicts of interest

Authors declare no competing interests.

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