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Molecular detection and seroprevalence of foot and mouth disease in goats of Assam, India

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Abstract
The present study was undertaken to detect the presence of FMDV in the goats of Assam. LPBE used to derive estimates of location wise incidence of FMDV. In addition, the study also involved the assessment of specific gene targets for detection of PPRV and FMDV by RT-PCR from the clinical samples. To detect the various serotypes of FMD by LPBE 382 serum samples were collected from different groups of goat from 15 districts of Assam. The titre $\geq$ 1.8 was considered to be protective titre in case of vaccinated animals. While in case of infected animals, titre $\geq$ 1.8 may indicate infection by particular type. Out of the total 382 serum samples screened for antibody titer against FMDV serotypes, LPBE titre of $\geq$ 1.8 was observed in 120, 82, 23 serum samples against serotype O, A, and Asia-1 of FMD virus, respectively. The multiplex PCR was used for detection of FMDV serotypes 'O', 'A' and 'Asia 1'. Freeze dried internal positive control (Type O: 249 bp; Type A: 376bp; Type Asia 1: 537 bp) were used as a reference of the test. All the samples were found positive for FMD virus serotype 'O'. Finally to confirm the FMDV RT PCR is done by using O type specific primer which showed prevalence of 'O' serotype in Assam.

Keywords: FMD; LPBE; RT-PCR

Introduction
Assam is a state of India in the north-eastern region. Located south of the eastern between $89^0 5' 96^0 1'$ East Longitude and $24^0 3' 27^0 58'$ North Latitude Himalayas, Assam comprises the Brahmaputra Valley and the Barak river valleys along with the Karbi Anglong and the North Cachar Hills with an area of 30,285 square miles (78,438 km²). Assam connected with international borders country of Bhutan in the northwest and with Bangladesh to the southwest. With the "Tropical Monsoon Rainforest Climate", Assam is temperate (summer max. at 95–100 °F or 35–38 °C and winter min. at 43–46 °F or 6–8 °C) and experiences heavy rainfall and high humidity. Assam is one of the richest biodiversity zones in the world and consists of tropical rainforests, deciduous forests, riverine grasslands and numerous wetland ecosystems.

Goats, one of the mainstays of subsistence farming for mainly the landless farmers and small size holders, provide a dependable source of income to most of the rural population who are below the poverty line and it contributes largely to the livelihood of the livestock-keeping households of low and medium-input farmers. As per the FAOSTAT, 2008, the population of goat in the world was approximately 861.9 million with India holding the second position with 125.7 million goats. In Assam alone, there are approximately 43, 76,150 population of goats [18th Livestock Census Data (2007-08)]. However, optimum productivity is hampered by various epidemics in goats due to infectious diseases which cause mortality rates of 50-80% in naïve populations (Abu Elzein *et al.*, 2004) [1].

Foot-and-mouth disease (FMD) is a highly contagious disease affecting *artiodactylae*, mostly cattle, swine, sheep, goats and many species of wild ungulates (Brooksby, 1982) [5]. It is recognized as a significant epidemic disease threatening the cattle industry since sixteen century and till date it is a major global animal health problem. FMD generally involves mortality rates below 5%, but even so it is considered the most important disease of farm animals since it causes huge losses in terms of livestock productivity and trade. Although FMDV rarely causes death in adult animals, the virus can cause severe lesion in the myocardium of young animals, leading to high mortality rates (Domingo *et al.*, 1990 [11];

Woobury *et al*., 1995; Doel *et al*., 1996) [35, 10]. The main constraints in controlling this disease and why it is considered as the most dreaded viral disease are its high contagiousness, wide geographical distribution, broad host range, ability to establish carrier status, antigenic diversity leading to poor cross immunity and relatively short duration of immunity. The *Office of International des Epizooties* (OIE) ranks the disease first in its list A diseases and the virus as Risk Group 4 of transboundary importance (OIE, 2008a and 2009) [24]. Poor surveillance and diagnostic facilities as well as inadequate control programs are major problems in control of this disease in the country. FMD is still a leading cause of loss of livestock economy in India. Outbreaks are still being reported from time to time round the year (Tamilselvan *et al*., 2009) [34]. Besides causing direct losses to livestock economy it also causes indirect losses in terms of severe trade restrictions, impact of which may be higher than direct losses. Although, the disease has been controlled successfully in many parts of the world by regular vaccination of susceptible animals and slaughtering of infected animals, no country has been considered safe, because of the highly contagious nature and rapid spread of the infection (Bruner and Gillespie, 1973) [6]. In India, FMD control is implemented by regular vaccination and for effective control, outbreaks should be detected at an early stage and persistent infections should also be recognized to prevent further transmittance. These can be achieved when vaccination is regular and effective and when diagnostic tools available are of accurate and sensitive at the same time rapid. Till date not much work has been done on sero-surveillance of FMD in goats of Assam. With the above views in mind, the present work was undertaken to evaluate the prevalence of FMD viral antibodies in the goat population of Assam and also to detect the presence of the viral nucleic acid by PCR.

Study area and sample collection

To ascertain the seroprevalence among goats in Assam, the serum samples were collected from different parts representing the whole province. Serum samples from goats were collected randomly from fifteen districts of Assam (Fig. 1). From each of the animals, blood samples were collected by jugular vein-puncture in vacutainer, separated the serum and transferred to small sterile screw capped plastic vials (Tarsons) labeled properly and stored at -20 ^{0}C without addition of any preservative till further use. Sera samples were collected from four groups of animals namely infected vaccinated, infected unvaccinated, vaccinated apparently healthy and uninfected unvaccinated from different districts of Assam (Table. 1).

Tissue samples comprising of tongue/feet epithelium collected from FMD infected goats were taken from the repository of Regional Research Center, All India Coordinated Research Project on FMD, Khanapara, Guwahati, which were collected during the period of February 2013 to January 2014 from different parts of Assam. The infected tissue materials were properly preserved in glycerol phosphate buffer and stored at -20 ^{0}C till further use.

Liquid phase blocking ELISA (LPBE)

The Liquid phase blocking ELISA was performed to determine the antibody titre of serum samples against FMD virus serotype O, A, Asia-1. Two-fold dilutions of serum samples were tested to estimate serotype-specific SP-Ab titer using the in-house LPB ELISA kit (ICAR-DFMD, Mukteswar) as per the procedure described earlier (Ranabijuli *et al*., 2010) [28]. The results were expressed as percentage reactivity for each serum dilution as follows:

$$\text{Percnt reactivity} = \frac{\text{Mean OD of each test serum dilution}}{\text{Mean OD of antigen control}} \text{X } 100$$

The antibody titers were expressed as logarithm of reciprocal of serum dilutions giving 50% of the absorbance recorded in the antigen control wells. The samples showing log10 titer of ≥1.5 in case of cattle and ≥1.2 in sheep or goat against one or multiple FMD virus serotypes (O, A and Asia 1) were considered as positive for infection or vaccination.

RNA extraction and quantitation

The tissue samples that were preserved in chilled condition at -20 ^{0}C and used for preparing 10% tissue suspension. The aliquots were used for viral RNA extraction. RNA extraction was done using Trizol Reagent as per standard protocol. RNA was quantified by spectrophotometric analysis.

RT PCR for detection of PPR viral nucleic acid

Complementary DNA (cDNA) was synthesised with 2 µg of the quantified RNA using 1 µl 10mM dNTP mix, 1 µl NK61 primer (5'GACATGTCCTCCTGCATCTG3), 0.5 µl RNase inhibitor, 5.5µl MQ water. The mixture was heated to 70 ^{0}C for 10 min and snap chill on ice for 5 min. After spinning down, and add previously prepared master mix containing 4 µl 5X RT buffer, 2 µl dTT (0.1M) incubate 25 ^{0}C for 15 mins snap freeze and added RT (200 U/µl). The PCR tubes were then placed in a thermal cycler and reverse transcription was done at 42 ^{0}C for 1 h followed by 35 PCR cycles followed by 72 ^{0}C for 10 mins and store at -20 ^{0}C for further process. PCR amplification was carried out by using a set of FMD specific forward (F-5'CGGGTGACTGAACTGCTTTA3') and reverse (5'GACATGTCCTCCTGCATCTG3') primers to amplify 249 bp product. PCR was carried out in thermocycler (Flexigene Cambridge, UK) in 25 µl of total volume containing 40 ng of each primer, 200 µMdNTP mix, 1.5 mM MgCl2, 2.5 mM 109 buffer, 50–100 ng cDNA template and 1 U Taq DNA polymerase. The PCR cycling parameters were optimized as follows; initial denaturation at 95 ^{0}C for 15 min, followed by 95 ^{0}C for 30 s, 58 ^{0}C for 30 s, 72 ^{0}C for 30 s for 35 cycles and final extension at 72 ^{0}C for 10 min. The PCR products were separated by horizontal submarine agarose gel (1.5%, free from DNAse and RNAse) electrophoresis in 1X TBE buffer at 80 V for 60 min and visualised using a gel documentation system (Gel Logic 100, KODAK).

Multiplexed PCR for FMD typing

Ready to use multiplex PCR kit for detection of FMDV along with the user manual was obtained from ICAR Directorate on Foot and Mouth Disease, Mukteswar, IVRI Campus, Uttarakhand. The kit comprised of ready to use mPCR lyophilized master mix vial which was reconstituted with 100 µl of nuclease free water. Positive and negative RNAs controls were also reconstituted with 50 µl of nuclease free water. The contents were mixed by pulse vortexing and 12.5 µl of RT-PCR mixture was pipetted out in 0.2 ml nuclease free vial. After that 1.5µl of extracted RNA,positive and negative controls were added in respective tubes. Mixed by pulse vortexing and briefly spin down to settle down the contents and placed in the thermo-cycler.

Statistical analysis

The statistical analysis of the generated experimental data was

done as per standard procedure (Snedecor and Cochran, 1994) [32].

Results

Detection of FMDV antibody using LPBE

All the 382 serum samples of different groups of goat collected from 15 districts of Assam were screened by LPBE against various serotypes of FMD *viz.* Type O, A and Asia l. LPBE was performed as per the method described by the ICAR Directorate of FMD, IVRI campus, Mukteswar, Nainital, Uttarakhand. The antibody tire was expressed in $^{\log_{10}}$ scale. The antibody levels $\geq$ 1.8 $\log_{10}$ titre was considered to be protective titre in case of vaccinated animals. While in case of infected animals, antibody levels $\geq$ 1.8 $\log_{10}$ titre may indicate infection by a particular serotype and antibody level below 1.8 $\log_{10}$ titre was considered sero-negative (Table. 2).

Out of the total 382 serum samples screened for antibody titer against FMDV serotypes, LPBE titre of $\geq$ 1.8 $\log_{10}$ titre was observed in 120(31.41%), 82(21.46%) and 23(6.02%) sera samples against serotype O, A, Asia-1 of FMD virus, respectively. The remaining sera samples were found to be sero-negative.

District wise LPBE titre

All Sera samples were collected from four groups of animals namely infected vaccinated, infected unvaccinated, vaccinated apparently healthy and uninfected unvaccinated from different districts of Assam. From Kamrup district, 100 numbers of samples were collected, out of which, 49,42 and 12 numbers of samples showed antibody titre $\geq$1.8 $\log_{10}$ against sero type O,A and Asia 1 respectively. Out of the 2 samples of vaccinated infected group, both the samples showed high antibody titre (1.8 $\log^{10}$ -2.1 $\log^{10}$) against type O but only one sample showed high antibody LPBE titre ($\geq$1.8 $\log^{10}$) against type A and type Asia 1. High titre was also observed in majority of the samples of vaccinated apparently healthy animals against serotype type O (27) and A (24). However, only 10 sample showed antibody titre ($\geq$1.8$\log_{10}$) against type Asia 1.Out of the 4 infected unvaccinated serum samples tested, high LPBE titre ($\geq$1.8$\log_{10}$) was observed against type O(3) and A(2). None of the samples showed antibody titre against serotype Asia 1 in this group. Out of the 57 serum samples collected from uninfected unvaccinated group, majority of samples exhibited a low level of antibody titre (<1.8) against serotype O (40), A (42) and Asia 1 (56). In case of Darrang district sera samples were collected from two groups i.e. infected unvaccinated and uninfected unvaccinated animals. In the former group, out of 2 samples, LPBE titre $\geq$ 1.8$\log_{10}$ was observed against FMDV serotype O but none of the samples showed antibody titre against serotype A and Asia 1. In the latter group, out of 23 samples, antibody titre $\geq$ 1.8$\log_{10}$ was seen in only 6 and 2 samples against serotypes O and A respectively.

In the Nalbari district out of 25 samples, 7 samples were collected from vaccinated apparently healthy group of animals and 5 samples showed high titre ($\geq$1.8 $\log_{10}$) against serotype O followed by 4 against type A and only 1 against type Asia -1. In case of infected- unvaccinated group, none of the serum samples could show antibody titre ($\geq$1.8 $\log_{10}$) except serotype O. In uninfected unvaccinated samples of the same district, the high titre could be detected only in 3 against serotype O and one against serotype A out of the 16 serum samples collected. In Bongaigaon district, out of the 20 serum samples collected from uninfected unvaccinated animals only two samples showed antibody titre $\geq$ 1.8$\log_{10}$ against FMDV serotype O. The serum samples collected from 5 vaccinated animals from Cachar district showed high titre against type O (4),type A (3) and type Asia 1(2) and out of the 10 serum samples collected from uninfected unvaccinated samples, high titre could be detected only in 2 against serotype O and one against serotype Asia1. From Jorhat district 25 samples were collected from two groups.From the 10 samples collected from vaccinated apparently healthy group of animals, high titre against serotype O, A and Asia 1 could be detected in 6, 6 and 2 of those samples, respectively and out of the 15 serum samples collected from uninfected unvaccinated animals of the same district, only two samples showed high titre (>1.8) against serotype O. All the uninfected unvaccinated serum samples of goat without any definite history of vaccination or infection in Karbi anglong district only 5 samples were found to have a high titre (>1.8$\log_{10}$) against serotype O. Rest of the samples were found to have low level of LPBE titre (<1.8) against all the serotypes of FMDV during the present study. A low level of LPBE titre (<1.8) was also observed in all the serum samples collected from uninfected unvaccinated animals from Golaghat district, except one sample exhibited high titre against type O. In case of Karimganj district out of the 30 serum samples and in Hailakandi district out of 12 samples, collected from uninfected unvaccinated animals, none of the samples showed high titre ($\geq$ 1.8$\log_{10}$) against FMDV serotype O,A and Asia 1. Among the 25 samples collected from uninfected unvaccinated animals of Barpeta district, high level of LPBE titre (>1.8) was observed against type O in 5 samples and only one against type A. In case of Lakhimpur district of Assam 15 samples were collected from uninfected unvaccinated animals,out of which 4 and 2 samples showed high level of LPBE titre (>1.8$\log_{10}$) against type O and type A. Out of the 15 serum samples collected from uninfected unvaccinated animals in Dhubri district, only 5 and 3 samples showed high titre (>1.8$\log_{10}$) against serotype O and serotype A. Very few numbers of samples (4) exhibited high LPBE titre (>1.8 $\log_{10}$) against FMDV serotype O and only two samples could show high titre against type A out of the 20 serum samples tested from uninfected unvaccinated animals in Dhemaji district. Not a single serum sample of that group of animals exhibited high LPBE titre against serotype Asia1. From Mongoldoi district, out of 20 samples, 11 samples were collected from vaccinated apparently healthy group of animals and majority of the samples (9) showed high antobody titre against serotype O and type A. However, against serotype Asia -1 only 5 exhibited high titre. In case of infected- unvaccinated group, all the serum samples could not show LPBE titre equal or more than 1.8 except serotype O. In 8 samples collected from uninfected unvaccinated animals of the same district, the high titre could be detected only in 3 against serotype O and two against serotype A.

Detection of FMD virus nucleic acid by Multiplex PCR

The multiplex PCR components were tested for their accuracy for detection of FMDV serotypes 'O', 'A' and 'Asia 1'. Samples were subjected to the multiplex PCR. The amplicons were confirmed in 1.5% agarose gel by electrophoresis. The specific products size used are 249bp for serotype 'O', 376bp for serotype 'A' and 573bp for serotype 'Asia1'. (Fig. 2). All the samples were found positive for FMD virus serotype 'O'. The amplicons corresponded to 249bp i.e Serotype O was found to be prevalent in all the collected FMDV samples.

Detection of FMDV nucleic acid by RT-PCR using type O specific primers

2.5 µl of the cDNA was utilized for amplification by PCR using type O specific primers. Five µl of the PCR amplified product was run on 1.5% agarose gel in 1X TBE buffer at 80V for 60 minutes. All the samples were found to be positive for serotype O showing the targeted amplification at 249bp (Fig. 3).

Discussion

The current work was designed to determine the seroprevalence of FMD in goats of Assam, India. Liquid Phase Blocking ELISA was employed for detection of FMD viral antibodies. LPBE is a serotype specific, highly sensitive prescribed test which detects antibodies elicited by both vaccination and infection. The test is known to be appropriate for confirming previous or on-going infection in non-vaccinated animals as well as for monitoring the immunity conferred by vaccination (Hamblin *et al*., 1986; Bronsvoort *et al*., 2008) [15, 4]. The LPBE has a sensitivity of 100% and specificity of 95% (Hamblin *et al*., 1986) [15].

FMD is difficult to diagnose in small ruminants as infected animals do not always show typical clinical signs or as the cardinal signs mimic other diseases (Ganter *et al*., 2001) [12]. The difficulty in making a clinical diagnosis should encourage the development of more rapid screening tests to assist in future control programmes (Patil *et al*., 2002) [26].

In this study, 382 serum samples of different groups of goat were collected from 15 districts of Assam, and were screened by LPBE against various serotypes of FMD *viz.* Type O, A and Asia l. LPBE was performed as per the method described by the ICAR Directorate on FMD, IVRI campus, Mukteswar, Nainital, Uttarakhand. The antibody titre was expressed in $^{\log_{10}}$ scale. The titre ≥ 1.8 was considered to be protective titre in case of vaccinated animals. While in case of infected animals, titre ≥ 1.8 may indicate infection by particular type. Out of the total 382 serum samples screened for antibody titer against FMDV serotypes, LPBE titre of ≥ 1.8 $\log_{10}$ titre was observed in 120(31.41%), 82(21.46%) and 23(6.02%) sera samples against serotype O, A, Asia-1 of FMD virus, respectively. The remaining serum samples were found to have a low LPBE titre (<1.8).

Majority of the samples, 31.41 percent in that group showed high LPBE titre (≥ 1.8 in $^{\log_{10}}$) against O, while 21.46 percent of samples against serotype A and only 6.02 percent against the serotype Asia-1 were found. The high percentage of titre (≥ 1.8 in $^{\log_{10}}$) against O and A might be attributed due to vaccination or infection status of goat from where the samples were collected. Martinez and Quintero (1996) could also observe presence of high LPBE titre against serotype O and A in the infected groups of animals. The present observation corroborates with the findings of Devi *et al* (2010) [9].

During the present study, it was observed that all the samples were found positive for FMD virus serotype 'O'. The amplicons corresponded to 249bp i.e Serotype O was found to be prevalent in all the collected FMDV samples. The present observation was in agreement with the finding of Giridharan *et al*., (2005) [13, 14], who could detect FMDV in 126 ES out of 142 ES (88.73%) of which 75 (52.81%) were typed as serotype 'O'. They also reported that mPCR worked more efficiently.

RT-PCR of several FMDV genome segments have been described (Meyer *et al*., 1991; Laor *et al*., 1992; Rodriguez *et al*., 1992; Amaral-Doe1 *et al*., 1993; Hofner *et al*., 1993; Leister *et al*., 1993; Stram *et al*., 1993; Donn *et al*., 1994; Prato Murphy *et al*., 1994; Rodriguez *et al*., 1994) [20, 21, 7, 30, 3, 16, 18, 33, 27, 29, 31]. Polymerase chain reaction was the most widely used nucleic-acid-based diagnostic techniques since its invention (Mullis *et al*., 1987; Callens *et al*., 1998) [22, 7]. With the development of RT-PCR to amplify RNA targets many workers have assessed the usefulness of it as a reliable tool for FMD diagnosis (Meyer *et al*., 1991; Laor *et al*., 1992; Hofner *et al*. 1993; Rodriguez *et al*., 1994) [20, 21, 17, 16, 29, 31] and in parallel with conventional assays (Marquardt *et al*., 1995; Callens *et al*., 1998; Callens *et al*., 1999) [19, 7, 8]. A particularly high sensitivity was reported by RT-PCR ELISA (Callens *et al*., 1998; Alexandersen *et al*., 2000) [7]. In this study all the samples (comprising of feet and tongue epithelium), from a total of 5 goats and the vaccine strain (positive control) IND R_2/75 against O serotype were processed for RNA extraction using Trizol method. The cDNA was prepared using NK 61 primer and for PCR, FMD virus type O specific primer was used. The reference vaccine virus as well as all the samples (100.00%), produced approximately 249 bp amplicon.

In conclusion, the results demonstrate that the prevalence of foot and mouth disease is awfully high in the sampled goat population. Seroprevalence of FMD was significantly associated with sampling locales. Highest seroprevalence was observed in western part followed by Central and western parts of Assam. Regular vaccination of FMD is recommended in goats in the studied areas as goats are usually ignored for vaccination. The present study is the first of its kind which demonstrates that FMD is highly prevalent in goats of Assam. Therefore, a broader study is required to find out the countrywide prevalence of FMD in goats.

Fig 1: Geographical location of different districts of Assam for sample collection

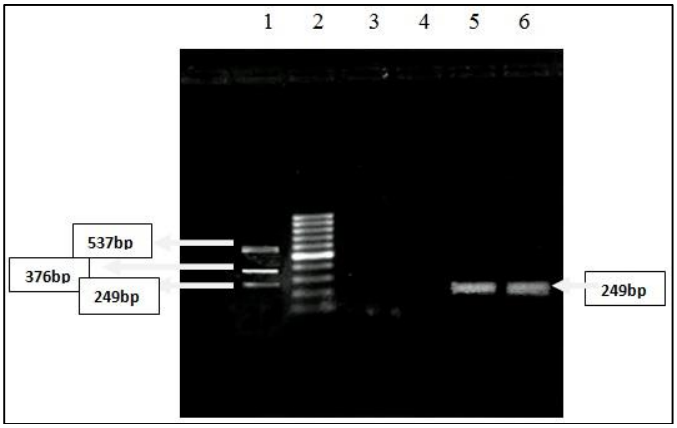

Fig 2: Multiplex PCR for FMDV detection. Lane No. 1: Freeze dried internal positive control (Type O:249 bp; Type A:376bp, Type Asia 1:537bp.); Lane No. 2: DNA marker of 100bp (Fermentas); Lane No. 3: Freeze dried internal negative control; Lane No.4: Test sample negative for FMD; Lane No.5 & 6: Sample positive for serotype O (249 bp).

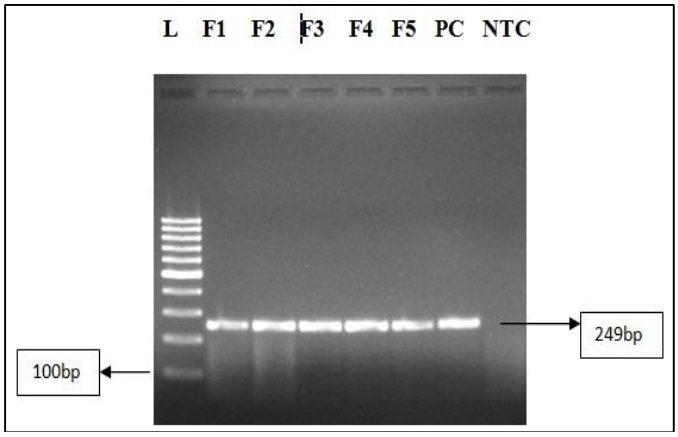

Fig 3: Representative gel photograph showing amplification of serotype O of FMDV. Standard (L) 100bp (Fermentas) ladder was used for checking serotype O amplification by agarose gel electrophoresis (F1, F2, F3, F4, F5= infected samples, PC= Positive control, NTC=Negative control).

Table 1: Details of samples collected from different districts of Assam.

Sl no.	Place of sample collection	No. of serum samples collected
1	Kamrup	39(V)+61(UV)
2	Bongaigaon	20(UV)
3	Barpeta	25(UV)
4	Nalbari	7(V)+18 (UV)
5	Dhubri	15(UV)
6	Cachar	5(V)+20(UV)
7	Darrang	25(UV)
8	Karbianglong	17(UV)
9	Karimganj	20(UV)
10	Dhemaji	20(UV)
11	Mongoldoi	11(V)+9(UV)
12	Jorhat	10(V)+15(UV)
13	Hailakandi	12(UV)
14	Golaghat	18(UV)
15	Lakhimpur	15(UV)
Total		382

Table 2: LPBE Titre (< 1.8 and ≥ 1.8 LOG^{10} scale) against different serotypes of FMD virus in different groups of goats.

Source of samples	No of Samples	No. of samples showing LPBE titer (log^{10} scale) against FMD serotype					
		O		A		Asia 1	
		< 1.8	≥ 1.8	< 1.8	≥ 1.8	< 1.8	≥ 1.8
Infected Vaccinated	2	0(0.00)	2(100.00)	1(50.00)	1(50.00)	1(50.00)	1(50.00)
Infected Unvaccinated	9	1(11.11)	8(88.88)	7(77.77)	2(22.22)	9(100.00)	0(0.00)
Vaccinated apparently healthy	70	19(27.14)	51(72.85)	24(34.28)	46(65.71)	50(71.42)	20(28.57)
Uninfected unvaccinated	301	242(80.39)	59(19.60)	268(89.03)	33(10.96)	299(99.33)	2(0.66)
Total	382	262(68.58)	120(31.41)	300(78.53)	82(21.46)	359(93.97)	23(6.02)

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Conflicts of Interest
The authors declare there is no conflict of interest.

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