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Optimization of fluorescent antibody techniques for demonstration of foot-and-mouth disease virus in bovine tongue epithelium and dorsal soft palate

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ABSTRACT

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Nucleic acid detection and virus isolation (VI) techniques are preferred for many applications in diagnostic virology, but these techniques are not enough to provide microscopic localization virus or viral antigen at cellular or at tissue level. Though the anti-FMDV fluorescent antibody technique (FAT) has been used as a tool for investigation of viral pathogenesis, they could also be useful as an ancillary diagnostic tool, particularly when necroptic tissues might be the only available samples. In this study FAT was optimised for the detection of FMDV structural proteins in frozen tissues of bovine origin. Tongue epithelium (TE) and dorsal soft palates (DSP) collected from FMDV suspected animals and later confirmed for FMDV positive by RT-LAMP and its serotype by mPCR were used. TE and DSP of apparently healthy bovine collected from slaughter house used as negative control, which was previously confirmed negative for FMDV by RT-LAMP and mPCR. Fluorescent antibody labelling of FMDV was performed in conjunction with labelling of cell markers like pancytokeratin/β-tubulin. Epithelial cells were identified with anti-pancytokeratin for DSP while β-tubulin for TE. As expected, FMDV antigen was predominantly colocalized in vesicle along with β-tubulin/pancytokeratin. Tissues (TE & DSP) from the FMDV positive animals incubated with isotype control antibodies have no corresponding FMDV specific signal. The FAT established in the current study could identify virus-positive cells within the TE and DSP. As a research tool, this technique could allow the precise localization of FMDV during various stages of infection.

Keywords: Dorsal soft palate; foot-and-mouth disease virus; fluorescent antibody technique; tongue epithelium.

INTRODUCTION

Foot-and-mouth disease (FMD) is a most contagious and economically devastating viral disease of domestic cloven-hoofed animals and more than 70 wild species including elephant[1,2]. The disease is caused by FMD virus (FMDV), genus *Aphthovirus,* family *Picornaviridae* and characterised by pyrexia, lameness and vesicular lesions of the tongue, feet and teats etc.[3,4]. FMDV exists in seven serotypes (O, A, C, Asia1, SAT-1, -2, -3)[5], which are not uniformly distributed across the globe. Formation of vesicles in the tongue, gum, coronary band, interdigital skin and teats of affected animal are most important macroscopic lesions of FMD4. The histological characteristics of FMD vesicles have been extensively described in veterinary literatures[6,7]. Never the less, the histogenesis of cells involved in the morphogenesis of vesiculation has not been examined thoroughly in naturally infected animals.

The nucleic acid detection and virus isolation (VI) are preferred for many diagnostic virology applications owing to their relative ease of standardization and high throughput; however, these techniques are inadequate for providing information regarding microscopic viral localization to specific anatomic tissues regions or individual cells types. When such data are required, researchers may utilize immunohistochemistry (IHC), fluorescent antibody technique (FAT), and in-situ hybridization (ISH) methods. Though, anti-FMDV FAT has been used as a tool for investigation of pathogenesis, they could also be effective ancillary diagnostic procedure, particularly when necroptic tissues might be the only available samples. Precise diagnosis of FMD is of utmost importance because of the severe economic impact associated with detection of the disease. Furthermore, definitive diagnosis is rarely made with a single modality of testing. Rapidity of FMD diagnosis is crucial as small delays in detection can lead to rapid geographic dissemination through movement of animals and aerosolization of infectious droplets. FAT has been used worldwide to demonstrate the localization FMDV antigen in different tissues[7]. However, there is a paucity of literature dealing with localization of FMDV antigens by FAT in domestic species in India. In the present study, through multi-channel FAT, for the first time we have optimized and demonstrated localization of FMD viral antigen within tongue and dorsal soft palate obtained from bovine.

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MATERIALS AND METHODS

Animals and tissue sample collection

Tongue epithelium (TE) was collected from bovine during acute phase of FMDV infection and dorsal soft palates (DSP) were collected from calf died due to FMD viral infection which was subsequently confirmed positive for FMDV by RT-LAMP and its serotype by mPCR. TE and DSP of apparently healthy bovine collected from slaughter house used as negative control, which was previously confirmed negative for FMDV by RT-LAMP and mPCR. Clinical signs exhibited by FMDV-infected animal were pyrexia, erosive stomatitis, off -feed and profuse salivation while animal for negative control were apparently healthy and found negative in NAR methods. Tissue samples were collected in duplicate and stored in 50% (v/v) buffered glycerin for virus isolation (VI) and FMD viral genome detection and in OCT embedding medium for pathological studies.

Nucleic acid detection

The tissue samples in 50% glycerol phosphate buffer were used to prepare a 10% (w/v) suspension in phosphate-buffered saline (pH: 7.4). Viral RNA was extracted from this suspension using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instruction. The extracted RNA was quantified by NanoDrop™ 1000 spectrophotometer. The cDNA synthesis was performed with Thermoscript ™ Reverse Transcriptase (Invitrogen, USA) enzyme and specific RT primers for FMDV, NK61[8] at 55°C for 2 hr. RNA extracted from the above suspension was also used for the detection of FMDV by RT-LAMP[9] and it serotype was confirmed by multiplex PCR[10] using the prepared cDNA. Further, RT-LAMP and mPCR amplified products were resolved on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Histological studies

Tissue samples collected in OCT sectioned (5μm) on positively charge slide [slides were cleaned by immersing either in 4% Dextran (neutral) in pre-warmed distilled water (DW) for 30 min, then slides were rinsed thoroughly in DW, followed by acetone treatment for five minutes each, and air dried. Slides were then immersed in 2% (v/v) 3-Aminopropyl-triethoxysilane (Sigma Chemicals, USA) in acetone for 30 minutes and finally, slides were rinsed in acetone for 5 minutes, washed in DW; air dried and stored in dust free condition for further use] by cryostat (Leica 1800 UV cryostat). Cryosections were fixed in acetone at -20°C for 10 minutes, air dried at room temperature and stored at -80°C until use.

Cryosections were processed for routine histopathological examination (haematoxylin and eosin stain, H&E) in TE and DSP as per method described earlier[11]. Multilevel FAT was performed on cryosections. Antibodies used in the current study and their specificities are described in Table 1.

All primary and secondary antibodies were optimized and validated previously by serial dilution using sections of bovine tongue epithelium and DSP from known FMD animals (Table 1). The negative control samples were comprised of duplicate sections with the primary antibody substituted with a species-and isotype-matched antibody, derived from normal sera for polyclonal antibodies or purified immunoglobulins for monoclonal antibodies. Slides were removed from -80°C and kept it at room temperature for 10 minute and wash in TBS three times for 5 min each. Sections were washed with 0.025% Triton X-100 in TBS two times for 5 minutes each. Blocking of non–specific antigen binding sites with sufficient amount (100-150 μl) of blocking buffer (5% of each normal goat, horse and pig serum in 1% BSA in TBS) for 2 hr at room temperature followed by washing in TBS (3x5 min each). Slide were drained carefully and wiped around the sections and multi-label FAT was performed on cryosections. All primary antibodies were collectively diluted in blocking buffer and simultaneously 100-150μl were added to the each section and incubated at 4°C for overnight. Next day slide were removed carefully and rinse in 0.025% Triton X-100 in TBS (2x5min each) with gentle agitation. Mouse mAbs used to label cell markers were anti-pancytokeratin. Goat anti-mouse IgG1, isotype-specific, fluorescently labelled secondary antibodies were diluted collectively in blocking buffer and applied to slides for 60 min at room temperature. For most stains, virus was detected with Alexa Fluor (AF)- 594/rodamine (R), and cell markers were labelled with AF-488/ FITC. For double stains, nuclei (DNA) were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and the cover slip was placed with mounting media. Slides were evaluated with a wide-field epi-fluorescence microscope (Leica, DM2500), and images were captured with a digital camera (Leica, DFC450C). Individual images were captured in each detection channel and subsequently adjusted for brightness and contrast, and then merged using commercial software (LabX, Leica). Isotype controls were included with each experiment and were used to establish camera sensitivity settings. Tissues from apparently healthy animal incubated with anti-FMDV antibodies were also included as negative controls.

RESULTS

All tongue epithelium (TE) and dorsal soft palate (DSP) collected from bovine during active phase of infection or dead animals showed clinical signs of FMD likes lameness, stomatitis, feed refusal, pyrexia and subsequently diagnosed as FMDV infection by RT-LAMP and its serotype by mPCR (Fig.1) in laboratory, where as the TE and DSP samples from apparently healthy bovine

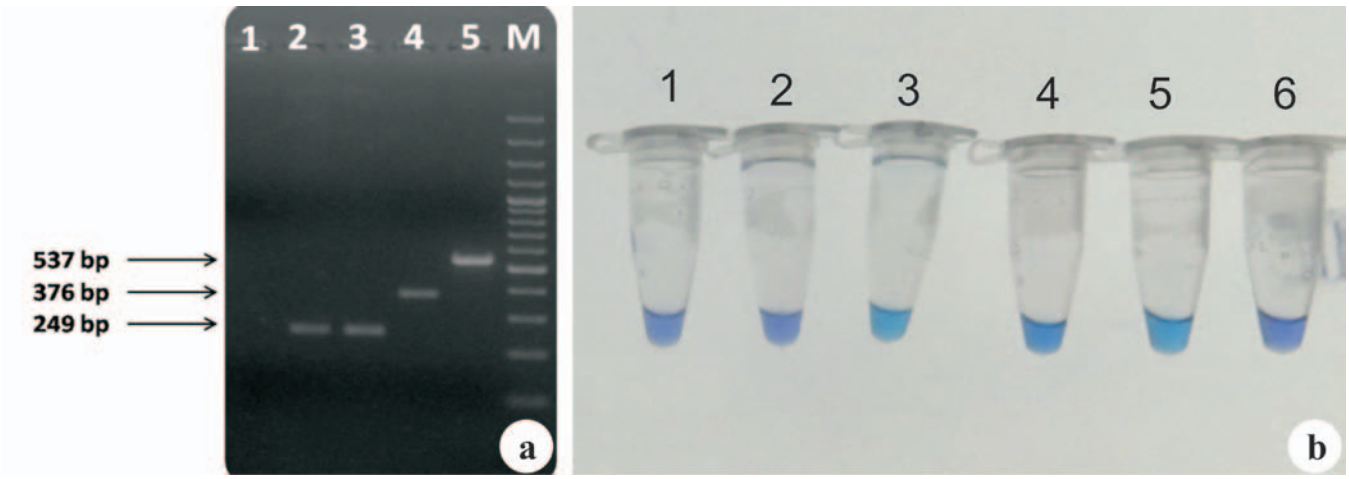


Fig.1. Agarose gel electrophoresis: **(a)** multiplex PCR (mPCR) products showing presence of distinct bands in test sample in column 2- 249 bp; Negative Control- column 1; Positive control of O- 249 bp, A- 376 bp and Asia1- 537 bp in column- 3, 4 and 5; M: 100 bp molecular weight marker. **(b)** tube 1 and 2 negative test samples while tube 3 and 4 positive test samples; tube 5 and 6 showing positive and negative control of RT-LAMP indicating by sky blue and purple colour, respectively.

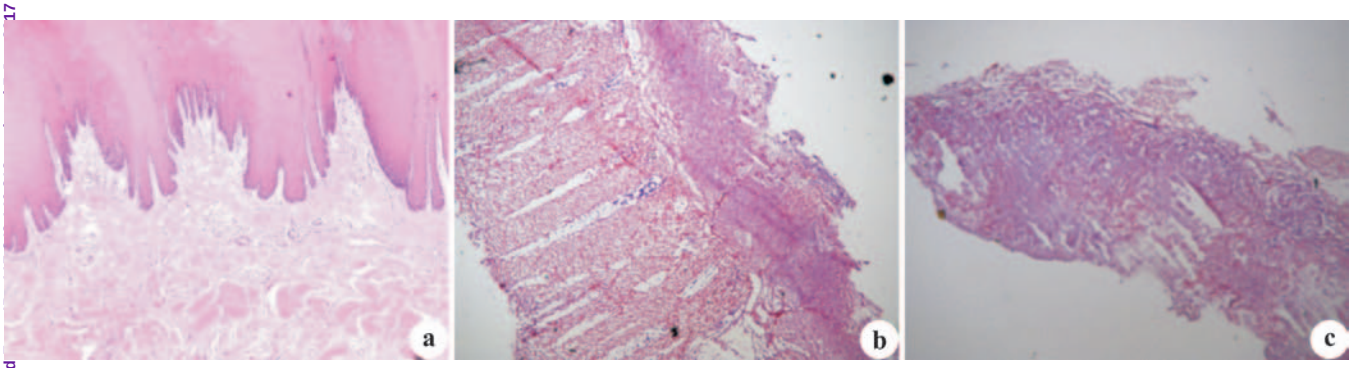
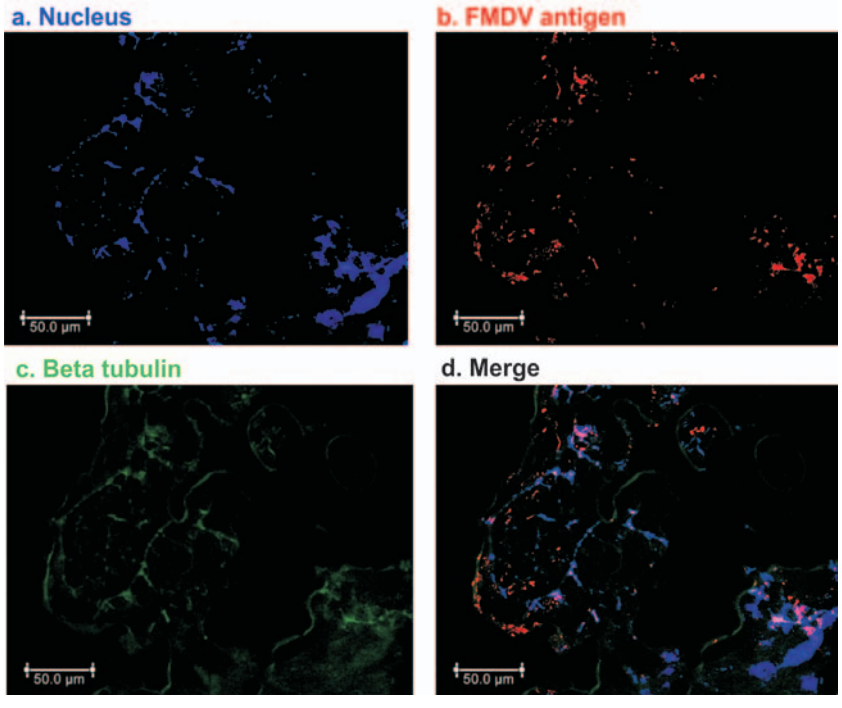


Fig.2. Light microscopy of tongue, **(a)** tongue collected from apparently healthy bovine showing no significant change at microscopic level, **(b)** and **(c)** increased cytoplasmic eosinophilic staining of the cells in the stratum spinosum along with mixed mononuclear cells infiltration was noticed in vesicular lesions. H&E x100.



population from slaughter house diagnosed negative by NAR methods.

Genome detection in TE and DSP

All TE collected from FMD infected animals during acute phase of infection and DSP collected from dead animals during necropsy found positive for FMDV serotype O by mPCR (Fig. 1).

Histologic and immunofluorescence characterisation of FMDV antigen

Necrotic cellular debris along with fibrin with mixed mononuclear cells

Fig.3. Immunofluorescence of tongue (positive control): **(a)** nuclei- blue, **(b)** FMDV capsid protein- red, **(c)** β-tubulin-green, and **(d)** co-localization of FMDV antigen with β-tubulin within the vesicle. Primary antibody- FMDV guinea pig (GP) polyclonal antibody, β-tubulin rabbit polyclonal antibody. Secondary antibody- goat anti GP- R and goat anti rabbit IgG-FITC. Bar= 50µm.

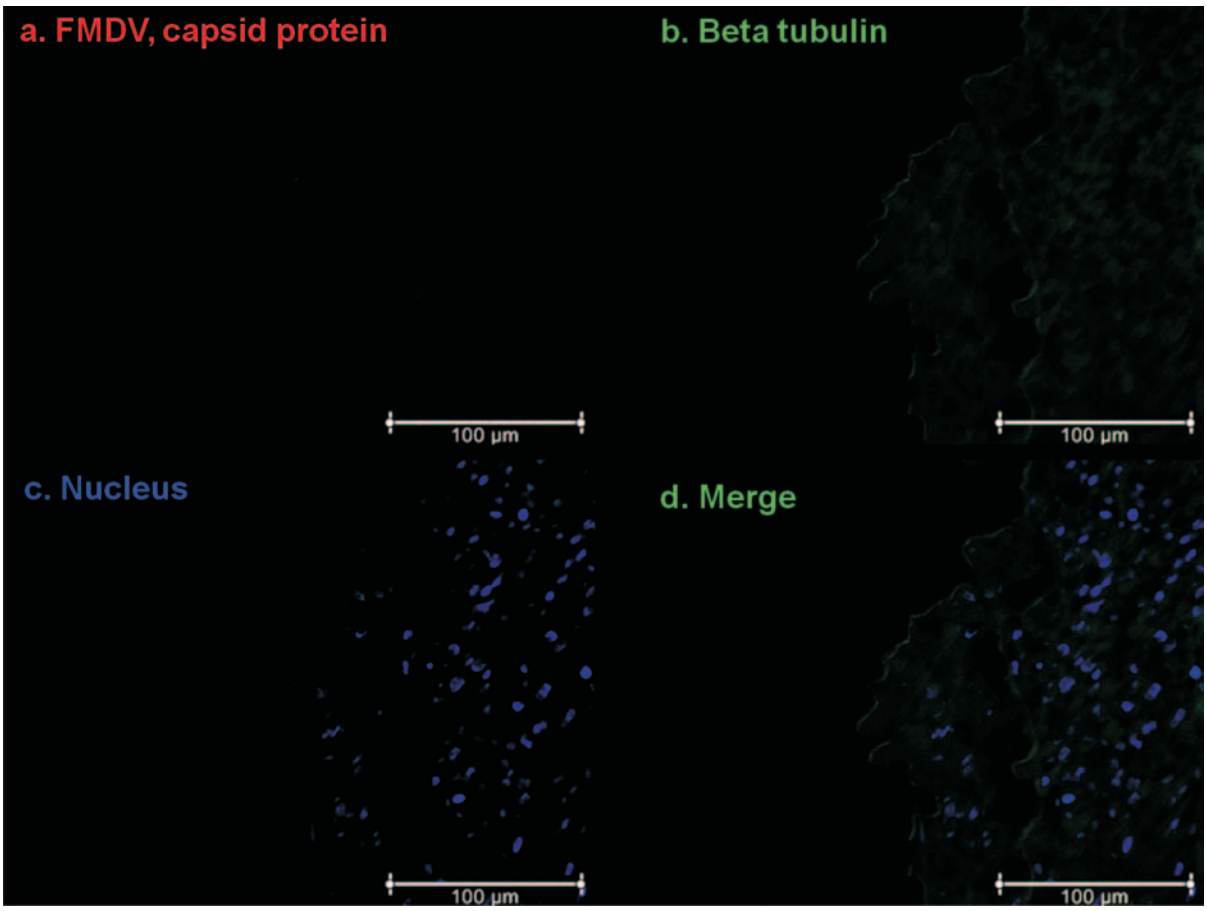


Fig.4. Immunofluorescence of tongue, FMDV carrier animal, FMDV capsid protein- red, β-tubulin-green, nuclei- blue. **(a)** negative for FMDV antigen, **(b)** positive for β-tubulin. Primary antibody- FMDV guinea pig (GP) polyclonal antibody, β-tubulin rabbit polyclonal antibody. Secondary antibody- goat anti GP- R and goat anti rabbit IgG-FITC. Bar= 100µm.

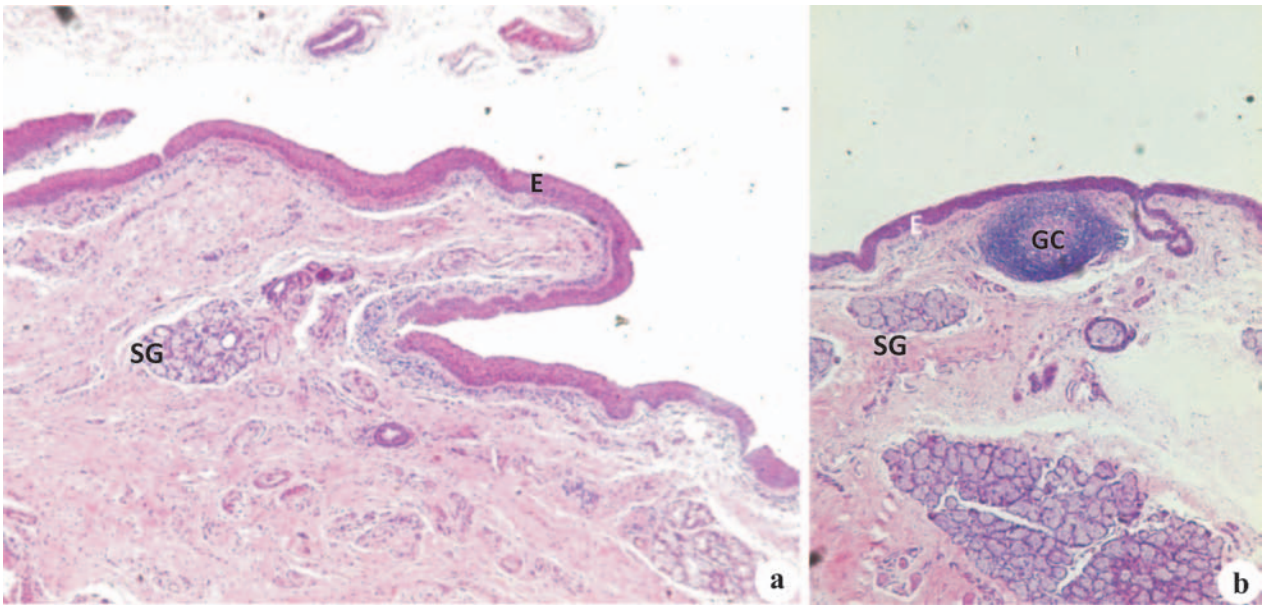


Fig.5. Light microscopy of soft palate, **(a, b)** Sections of the DSP showing, salivary glands (SG) and germinal centres (GC) located within the connective tissue of the lamina propria below the respiratory epithelium (E). The germinal centres were orientated with the light zone towards the apical surface. H&E x100.

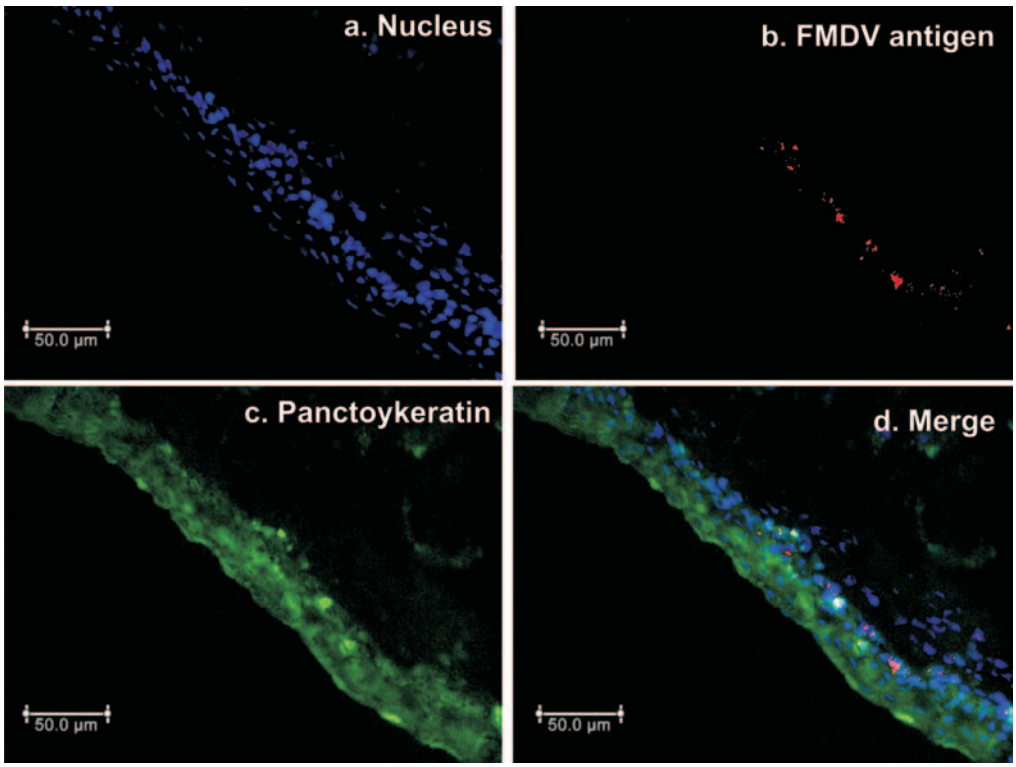


Fig.6. Immunofluorescence of DSP 2 (FMDV positive animal)- **(a)** nuclei- blue, **(b)** FMDV capsid protein- red, **(c)** Pan cytokeratin-green, and **(d)** co-localization of FMDV antigen with pan cytokeratin within in the vesicle. Primary antibody- FMDV guinea pig (GP) polyclonal antibody, pancytokeratin rabbit polyclonal antibody. Secondary antibody- goat anti GP- R and goat anti rabbit IgG-FITC. Bar= 50 µm.

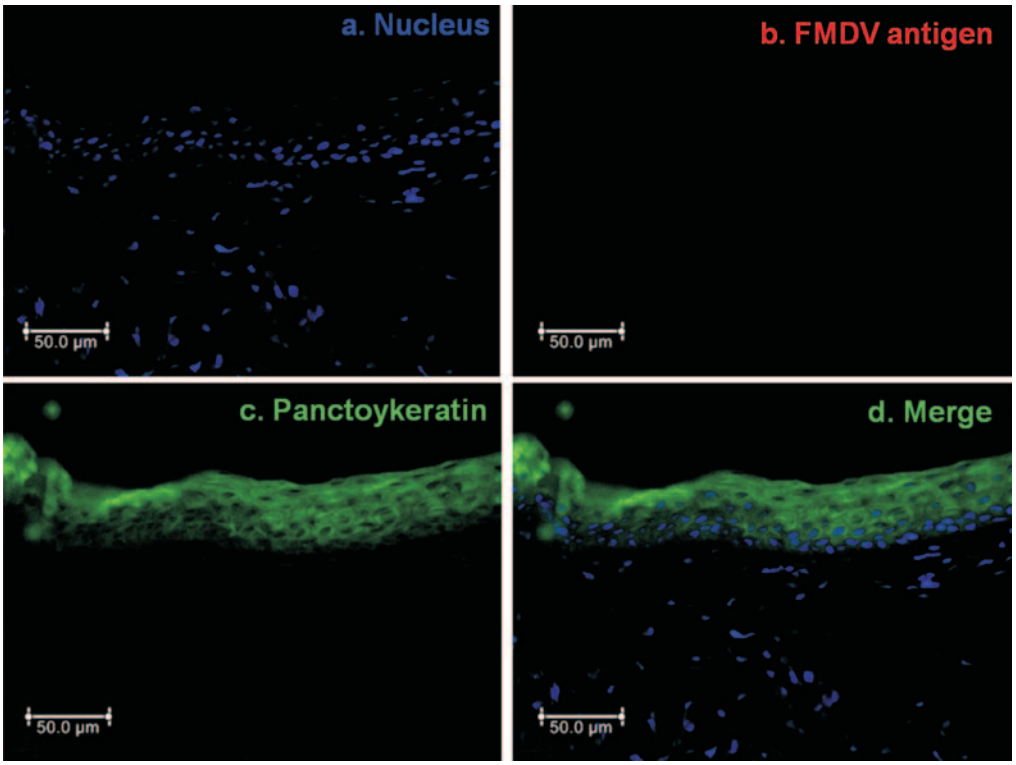


Fig.7. Immunofluorescence of dorsal soft palate (FMDV negative animal): **(a)** nuclei- blue, **(b)** FMDV capsid protein- red, no signal was observed, **(c)** pancytokeratin-green, and **(d)** no co-localization of FMDV antigen with pan cytokeratin within in the vesicle. Primary antibody- FMDV rabbit polyclonal antibody, pancytokeratin mouse monoclonal antibody. Secondary antibody- goat anti rabbit IgG (H+L) Alexa fluor-594 and goat anti mouse IgG (H+L) Alexa fluor-488. Bar= 50 µm.

Table 1. Primary and secondary antibodies used for fluorescent antibody technique.

Sr. No.	Types of Antibodies	Dilution used	Reference	Source
1	Polyclonal FMDV guinea pig antibody	1:2000	Biswal *et al*.[12]	DFMD, Mukteshwar
2	Polyclonal FMDV rabbit antibody	1:2000	Biswal *et al*.[12]	DFMD, Mukteshwar
3	Normal guinea pig and rabbit serum	1:2000	Ranjan[13]	DFMD, Mukteshwar
4	SC-2043- Normal goat serum	5%	Ranjan[13]	Santa Cruz
5	RM1239-Horse serum	5%	Ranjan[13]	HiMedia
6	RM10415- Pig serum	5%	Ranjan[13]	HiMedia
7	SC-9104-β Tubulin (H-235) antibody rabbit IgG	1:50	Ranjan[13]	Santa Cruz
8	sc-81714- Pancytokeratin antibody (AE1/AE3) anti mouse	1:50	Ranjan[13]	Santa Cruz
9	A11037-Alexa fluor 594 goat anti-rabbit IgG(H+L)	1:100	Ranjan[13]	Invitrogen
10	A11029-Alexa fluor 488 goat-anti mouse IgG(H+L)	1:100	Ranjan[13]	Invitrogen
11	SC-2012-Goat anti-rabbit IgG FITC	1:100	Ranjan[13]	Santa Cruz
12	SC-2441- Goat anti-guinea pig IgG FITC	1:100	Ranjan[13]	Santa Cruz
13	sc-2091- Goat anti-rabbit IgG-R	1:100	Ranjan[13]	Santa Cruz
14	sc-2442- Goat anti-guinea pig IgG-R	1:200	Ranjan[13]	Santa Cruz

infiltration was noticed in vesicular lesions (Fig. 2). Fluorescence antibody labelling of FMDV was performed in conjunction with labelling of cell markers like pancytokeratin/β-tubulin. Epithelial cells were identified with anti-pancytokeratin in DSP while β-tubulin in tongue epithelium. As expected, FMDV antigen predominantly colocalized in vesicle with β-tubulin/ pancytokeratin (Fig. 3, 6). Tissues from the FMDV positive animals incubated with isotype control antibodies did not show corresponding FMDV specific signals in both the tissue (Fig. 4, 7). Immunofluorescence detection of FMDV antigens was typically limited to regions of morphologic vesiculation as observed in basal cells of tongue (Fig. 4).

No significant macroscopic changes were observed in dorsal soft palate (Fig. 5). In the soft palate, FMD viral antigen was detected within the basal layer of the epithelium in individual cells or in small clusters (Fig. 6).

DISCUSSION

In modern era, research institute and diagnostic laboratories have various options for demonstration of FMDV in animal's tissues[14-17]. Application of this technique used as a simple, single method to detect different serotype of FMDV in bovine tissues.

FA techniques in the current study identify virus-positive cells within the dermal papillae. Examination using a single, virus-only label visualization system might have led to misidentification of these cells as dermis-derived. Furthermore, the detection of microvesicles surrounding dermal papillae distant from developing vesicles suggests that a single vesicle is often formed through multiple transfer events of virus from blood to epithelium. Capsid antigen positivity in dermal cytokeratin-negative cells supports the notion that a population of non-epithelial cells becomes infected as the lesions progress. In addition, since these cells are identified near mature lesions, it is not possible to discern whether the FMDV antigens contained within these cells or came from the vasculature or the adjacent vesicle.

In the characterization of FMD vesicles, authors commonly describe affected regions using nomenclature that is appropriate for typical epithelia with uniformly lamellar stratum layer like basal layer, spinosum layer, granulosum and corneum layer. However, the typical FMD lesion sites (interdigital skin, coronary band, tongue, and snout) are composed of epithelia that are anatomically complicated by the interdigitation of long dermal papillae with epithelial pegs. Vesicular cavities contained variable quantities of fibrin and necrotic cellular debris has also been reported earlier[11].

The pharyngeal epithelium is supposed to be a primary site for FMDV replication during acute[14-16,18] and persistent stages of infection[17]. In present study, no significant macroscopic changes were observed in DSP in histopathology. In some tissues from animal, notably the dorsal soft palate, clusters of positively staining cells were occasionally discernible in the upper stratum spinosum in the absence of any histologically apparent lesion[19]. Presumably, the source of this staining may be draining of virus from secondary replication in the tongue and soft palate[19]. In the current study, epithelia and subjacent mucosa within the dorsal soft palate (DSP) from FMDV infected animals was used to investigate possible sites of virus replication and this has been also shown earlier[20]. Viral RNA was also detected earlier[21]. Microscopic localization of FMDV RNA in the basal layers of the epithelium of the dorsal soft palate (DSP) and pharynx has been demonstrated by in situ hybridization[17,22]. Previous literatures on tissue-specific

localization of FMDV persistence within the dorsal soft palate and dorsal pharynx implicated as the most frequent sites in cattle post viraemic[23,24]. Microscopic studies utilizing immunohistochemistry in bovine tissues have provided further support for the importance of pharyngeal tissue and soft plate, in the acutely infected animals[15,20,25]. Additionally, a study in sheep showed that the tonsil and soft palate were the replication sites of FMDV after 10 day post infection (dpi)[24,26].

In the current study, tongue epithelium and dorsal soft palate were found positive for viral RNA suggesting that virus-host interaction in these animals favored replication at these sites. Previous record also explained about presence of viral RNA in numerous tissues up to two days after cessation of viremia, but in pharyngeal tissues duration of detection of viral RNA up to 72 dpi[27,28]. Previous studies have demonstrated that the dorsal soft palate is a site of unique FMDV-host tropism in adult cattle during the early stages of FMD pathogenesis[15,25,29]. In present study, FMDV localized in sub-epithelium region of dorsal soft palate and this was differs from earlier findings[25]. Localization of FMDV antigen within the superficial epithelium of the nasopharynx of cattle has been demonstrated earlier[25,27] but FMDV RNA localization in the basal layers of the epithelium of the dorsal soft palate and pharynx recorded by earlier worker[17,22].

The FA techniques described in the current study could facilitate various aspects of FMDV investigation. With more thorough examination of sensitivity and specificity, rapid and effective diagnostic tests could easily be validated, which could readily be utilized by laboratories lacking resources for diagnosis through PCR or VI. As research tools, these techniques have allowed precise localization of FMDV in various stages of infection, which will be described in additional manuscripts forthcoming from the authors' laboratory.

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