

The localisation of persistent foot and mouth disease virus in the epithelial cells of bovine soft palate and pharynx



Zhidong Zhang and Paul Kitching

Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey, GU24 0NF, UK

ABSTRACT

Following contact with foot and mouth disease virus (FMDV), cattle can become persistently infected regardless of the preexisting of immune status of the animal and whether it develops clinical disease. The cellular site(s) of FMDV persistence has not been determined. Using in situ hybridisation in combination with tyramide signal amplification (TSA), we provide the first direct evidence that the FMDV RNA is localized within the epithelial cells of the soft palate/pharynx during persistent infection, indicating that these cells remain persistently infected following contact with FMDV.

INTRODUCTION

Foot and mouth disease virus (FMDV) is a member of the family Picornaviridae, genus Aphthovirus which causes a highly contagious disease in cloven-hoofed animals characterised by the appearance of vesicles on the feet and mouth. Following contact with foot and mouth disease virus (FMDV), cattle can become persistently infected regardless of the preexisting of immune status of the animal and whether it develops clinical disease. The specific cells which become persistently infected with FMDV have not been determined although the sequential distribution of the virus through the body has been studied extensively (Burrows et al 1981, Murphy et al, 1999). In order to identify the cellular site(s) of FMDV persistence, in situ hybridisation was used in combination with tyramide signal amplification (TSA). The FMDV was localised within the epithelial cells of the soft palate/pharynx during persistent infection

MATERIALS AND METHODS

Samples Four cattle were infected in high containment animal accommodation with the FMDV C Oberbayern by intradermal inoculation with 107 TCID₅₀. Samples of soft palate and pharynx were collected at post-mortem from cattle killed at 7, 42, 72 and 82 days after infection. Tissues were immediately fixed in 10% formal-FIXX (Shandon, UK) for 16 hours, washed with PBS and embedded in paraffin wax. Negative controls included similar tissue sections from an uninfected animal processed identically to those taken from the FMDV infected cattle. Oropharyngeal fluid (OPF) was collected approximately weekly for the duration of the study using a probang cup. The infectivity of each OPF was determined by inoculation of monolayers of primary bovine thyroid (BTY) cells.

Oligo probe and labelling Oligonucleotide (oligo) sequences (Table 1) were chosen from the highly conserved regions of the FMDV non-structural genes and synthesized by Crunchem (Crunchem, UK). The oligos were labelled with biotin-11 dUTP (Boehringer-Mannheim) as described by Zhang and Kitching (2000).

Pretreatment of tissue samples Paraffin embedded samples of fixed tissues were cut into 4 mm thick sections, adhered onto superfrost microscope slides (BDH), then deparaffinised in xylene for 30 min at 37°C followed by two further xylene treatments of 10 min each at room temperature. Tissue sections were placed in 100% ethanol for 2 x 10 min and then transferred and rehydrated through 75, 50, 25% ethanol (5min each) to DEPC treated H₂O for 5 min. The tissue samples were incubated in 0.02N HCl for 10 min and then rinsed in PBS for 5 min. The slides were transferred to a humidified chamber and treated with 10 mg/ml proteinase (GIBCO) in PBS for 45 min at 37°C. The slides were washed twice with DEPC-treated PBS containing 100 mM glycine for 5 min each and then twice for 5 min each with DEPC-treated PBS. To acetylate sections, slides were washed twice with 0.1M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma) for 5 min each wash and then washed twice with DEPC-treated H₂O for 5 min each wash and air-dried.

Hybridisation and detection The sections were hybridised with 100 ml of In Situ Hyb buffer (Ambion, UK) containing a cocktail of oligo probes (100 ng/ml each probe) at 42°C overnight in a humidified chamber. After ISH, the sections were washed once in 2 x In Situ Wash Solution (Ambion, UK) for 4 minutes and then 1 x In Situ Wash Solution for 4 min at room temperature. The slides were washed in water for 2min and endogenous peroxidase was quenched by treatment with 3% H₂O₂ in PBS for 10 minutes. The slides were then washed in water for 2 min and buffer A (100mM Tris-HCl, pH7.5, 150mM NaCl) for 2 min before being attached to a disposable immunostaining chamber and then placed in Sequenza (Shandon). The sections were washed three times for 5 min each wash with TNT buffer (buffer A containing 0.05% tween-20) and then incubated in 300 ml of TNB (buffer A containing 0.5% blocking reagent (NEN)) for 30 min. 100 ml of streptavidin-HRP (NEN) at 1:100 dilution in TNB buffer was applied and the slides were incubated for 30 min at room temperature. The sections were then washed in TNT buffer three times for 5 min each wash and 100 ml of biotinyl tyramide (NEN, diluted 1:50 in amplification buffer) was added and the tissues were incubated for 10 min at room temperature. The sections were washed in TNT buffer three times for 5 min each wash and then incubated with ABC-alkaline phosphatase (Vector Laboratories) for 30 min at room temperature. The sections were incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories) in 100mM Tris-HCl, pH8.2 for 30 min until the colour appeared fully developed. The sections were counterstained in Vector Methyl green (Vector Laboratories) and mounted with Synthetic mountant (Shandon).

Immunocytochemistry Immunocytochemistry was performed on the tissue sections adjacent to each section found positive for persistent FMDV RNA by ISH. Sections were deparaffinized and rehydrated through graded alcohols, washed in PBS and blocked with serum corresponding to the species in which the primary antibody was raised. Mouse anti-human cytokeratin type II antibody (Serotec) was used at 20mg/ml for identification of epithelial cells. The primary antibody was detected with biotinylated anti-mouse and ABC-alkaline phosphatase (Vector Laboratories). The sections were stained with Vector Red alkaline phosphatase substrate (Vector Laboratories), counterstained in Vector Methyl green (Vector Laboratories) and mounted with Synthetic mountant (Shandon).

RESULTS

As shown in Figure 1, FMDV RNA was localized in the soft palate of persistently infected cattle. At 42 days after infection, sections of dorsal soft palate showed strong red chromagen in the cytoplasm of cells in the stratum germinativum of the epithelium (Fig.1A). Similarly, hybridization of the ventral soft palate showed ISH-positive cells also present in the cells of the epithelium (Fig.1D). Pretreatment with RNase abolished the signal in adjacent tissue sections (Fig 1B and 1E). The location and morphology of the ISH-positive cells suggested that these cells were epithelial cells. Immunocytochemistry with anti-cytokeratin antibody was carried out on adjacent tissue sections from the same FMDV infected cattle. Cytokeratin is continuously expressed on epithelial cells in vivo and staining with anti-cytokeratin antibody clearly showed the presence of the ISH positive cells in the same location as cytokeratin positive cells in the soft palate, strongly indicating that the ISH-positive cells are epithelial cells (Fig. 1C and 1F). In pharyngeal sections ISH-positive cells also found within the epithelium (Fig. 2A). No signal was seen in the pharyngeal tissue pretreated with RNase (Fig.2B) or from the uninfected animal (Fig.2C). In tissues from the soft palate collected at 72 days (Fig 3B) and 82 days (Fig.3C) post infection respectively, ISH-positive cells were also detected in the epithelial cells in the stratum germinativum of the epithelium (Fig.3).

CONCLUSION

FMDV RNA is localized within the epithelial cells of the soft palate/pharynx during persistent. The location and morphology of the ISH-positive cells strongly suggest that these cells are epithelial cells.

REFERENCES

- Burrows, R., Mann, J. A., Garland, A. J., Greig, M. A. & Goodridge, D. (1981). Pathogenesis of natural and simulated natural foot and mouth disease viruses strains (O1 campos and C3 Rescende). *Journal of General Virology*, **72**, 2821-2825.
- Murphy, P. M. L., Forsyth, M. A., Belsham, G. J., Salt, J. S (1999). Localization of foot-and-mouth disease virus RNA by in situ hybridization within bovine tissues. *Virus Research*, **62**, 67-76.
- Zhang, Z.D. and Kitching, P. (2000). A sensitive method for the detection of foot and mouth disease virus by in situ hybridisation using biotin-labelled oligodeoxynucleotides and tyramide signal amplification. *Journal of Virological Methods* (in press).

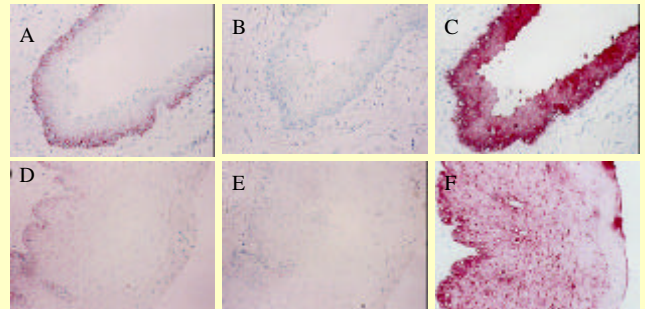


Figure1

ISH-TSA detection of FMDV RNA in persistently infected bovine soft palate tissues. Bovine soft palate tissues were collected at 42 days after infection with type C FMDV and fixed with 10% neutral buffered formalin. FMDV RNA was hybridised with a cocktail of biotin-labelled oligo probes. Detection was performed using biotin TSA. FMDV RNA was localised in the stratum germinativum of the epithelium of dorsal soft palate tissues (A) and ventral soft palate tissues (D). Pre-treatment with RNase A abolished the positive signals (B, E). Sections showed positive reaction for cytokeratin (C, F). Stained using Vector Red with Vector Methyl Green counterstain. (Original magnification x 200).

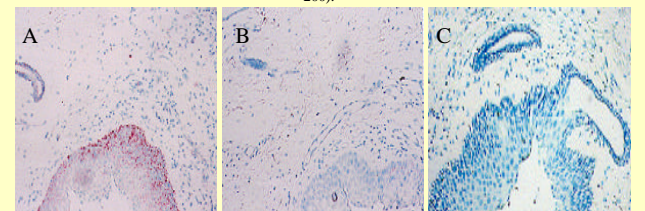


Figure 2

ISH-TSA detection of FMDV RNA in persistently infected bovine pharynx tissues. Bovine pharynx tissues were collected at 42 days after infection with FMDV C and fixed with 10% neutral buffered formalin. FMDV RNA was hybridised with a cocktail of biotin-labelled oligo probes. Detection was performed using biotin TSA. FMDV RNA was localised in the stratum germinativum of the epithelium of pharynx tissue (A). Pre-treatment with RNase A abolished the positive signal (B). Hybridisation of uninfected tissue with antisense probe resulted in no signal (C). Stained using Vector Red with Vector Methyl Green counterstain (original magnification x 200).

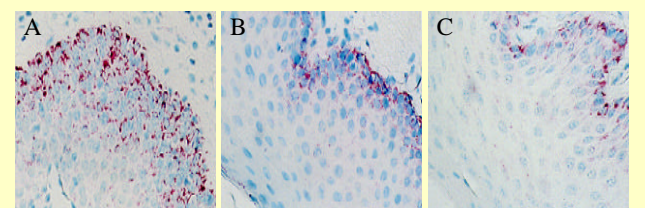


Figure3

ISH-TSA detection of FMDV RNA in persistently infected bovine soft palate tissues. Bovine soft palate tissues were collected at 42 (A), 72 (B), and 82 (C) days after infection with type C FMDV and fixed with 10% neutral buffered formalin. FMDV RNA was hybridised with a cocktail of biotin-labelled oligo probes. Detection was performed using biotin TSA. FMDV RNA was localised in the epithelium of soft palate tissues. Stained using Vector Red with Vector Methyl Green counterstain (original magnification x 600).

Table 1 Sequences of oligo probes used for ISH

Oligo designation	Oligo sequences (5')	(3')	mer
FMDV-2AR	antisense	TCGGAGAAAAAGAGGGCCACGGGTT	26
FMDV-3BR	antisense	GCTTTGTGTGCCATGACCATCTTTTGC	29
FMDV-3CR	antisense	GCGTCTGAGAGCATGTCTGTCTTTTACT	30
FMDV-3DR	antisense	GCGACGGTGGGTGCAAGCTTGGTTTT	26