Clinical and virological dynamics of a serotype O 2010 South East Asia lineage foot-and-mouth disease virus in sheep using natural and simulated natural inoculation and exposure systems

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Abstract

Within-host infection dynamics of a recent field isolate of foot-and-mouth disease virus (FMDV), serotype O, topotype South East Asia, lineage Myamar'98 were evaluated in sheep using four different systems for virus exposure. Two novel, simulated natural, inoculation systems consisting of intra-nasopharyngeal (INP) deposition and aerosol inoculation were evaluated in comparison with two conventional systems: coronary band inoculation and direct contact exposure. All four exposure systems were efficient in generating consistently severe, generalized FMD with synchronous clinical characteristics within exposure groups, indicating that this Myamar98 strain is highly virulent in sheep. Clinical and virological dynamics were similarly rapid following INP and coronary band injection, with both systems leading to significantly earlier detection of virus shedding when compared to aerosol inoculation and contact exposure. The data presented herein support application of these simulated natural inoculation systems as valid alternatives to conventionally used exposure systems for studies of FMDV pathogenesis and vaccinology in sheep. Furthermore, these data suggest that targeted exposure of the ovine pharynx is highly efficient for generating consistent FMDV infection in sheep, which supports critical involvement of this anatomic region as a site of primary virus replication in sheep.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease caused by FMD virus (FMDV), an *Aphthovirus* belonging to the family of *Picornaviridae* (Grubman and Baxt, 2004). A wide-range of both domestic and wild cloven-hoofed animal species are susceptible to the infection, although the severity of the clinical disease may vary depending both on virus strain and host species (Alexandersen and Mowat, 2005; Arzt et al., 2011). Although sheep may develop severe FMD, several reports have described clinical signs of FMD in sheep as mild and inconsistent, thereby being unreliable for early detection of disease incursions (Aggarwal et al., 2002; Alexandersen et al., 2003a; Hughes et al., 2002b; Kitching and Hughes, 2002). Despite potentially mild or unapparent clinical disease, sheep may play a critical role in the epidemiology of FMD outbreaks. This was particularly evident during the 2001 FMD epidemic in the UK when transportation and trade with apparently unaffected sheep caused extensive dissemination of the infection before any measures of disease control were initiated (Gibbens et al., 2001; Mansley et al., 2003).

For countries relying on agricultural trade and export, it is important that FMD contingency plans account for the potential of FMD outbreaks initiating in sheep flocks. Experimental studies characterizing clinical disease, infection dynamics, and pathogenesis of relevant (emerging) FMDV strains in sheep are necessary to facilitate appropriate FMD response measures including evaluation of vaccines for potential emergency use. A recent report demonstrated moderate virulence in sheep of a recently emerged FMDV serotype O from the 2010 outbreak in Republic of Korea (Horsington et al., 2015).

The outcome of experimental FMDV studies can vary considerably depending on the system used for virus exposure (Arzt et al., 2014; Fowler et al., 2014; Stenfeldt et al., 2014b). Although

67 injection-based (intra-epithelial) inoculation systems are highly efficient in generating consistent
68 and synchronous clinical FMD, these systems have the disadvantage of utilizing an unnatural
69 manner of virus delivery that bypasses the natural barriers of the mucosal immune system,
70 thereby providing an inaccurate simulation of the initial events of FMDV pathogenesis. Direct
71 contact exposure to infected animals represents a truly natural model for virus challenge,
72 however this system does not allow the ability to control or evaluate the actual exposure dose.
73 Recent investigations of the early events of FMDV pathogenesis in cattle and pigs have localized
74 the sites of primary FMDV infection to the bovine nasopharyngeal mucosa (Arzt et al., 2010)
75 and porcine oropharyngeal tonsils (Stenfeldt et al., 2014a). Simulated-natural inoculation
76 systems designed for optimal delivery of inoculum without disruption of the natural events of
77 early infection were utilized to elucidate these previously obscure events in these two host
78 species (Pacheco et al., 2010; Stenfeldt et al., 2014b).

79 Similarly detailed tissue-based investigations of FMDV in sheep have not been performed,
80 although it has generally been assumed that critical events of FMDV pathogenesis in small
81 ruminants are similar to those described for cattle (Alexandersen et al., 2003b; Arzt et al., 2011).
82 Previous studies have confirmed that sheep are susceptible to FMDV infection via nasal
83 instillation (Hughes et al., 2002a; McVicar, 1971) and exposure to naturally generated aerosols
84 (Aggarwal et al., 2002; Gibson and Donaldson, 1986; Gibson et al., 1984), in addition to
85 coronary band injection and contact exposure (Alexandersen et al., 2002; Burrows, 1968;
86 Horsington et al., 2015). However, the use of different challenge viruses at varying doses
87 generally precludes detailed comparisons of experimental systems based on these works.

88 The objective of the work presented herein was to investigate antemortem (within-host)
89 infection dynamics of a recently emerged field strain of FMDV serotype O in sheep. Ancillary

output consisted of direct comparisons of infection with the same virus delivered via four different exposure systems. The presented data provides important insights into the pathogenesis of FMDV in sheep and facilitates optimization of standardized experimental models for continued studies of FMDV in sheep.

2. Materials and methods

2.1 Virus strain

The serotype O FMDV used for these studies was a South East Asia (SEA) topotype, Myanmar 1998 (Mya98) lineage virus kindly provided by Dr. Kwang-Nyeong Lee, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea. The field virus designated FMDV O/SKR/2010, NVRQS10, isolate 1012_49V was derived from vesicular fluid collected on December 2010 from a bovine with clinical FMD in Paju county, Gyeonggi province, Republic of Korea, (Yoon et al., 2013). The virus was passed once in Holstein cattle before being used to inoculate sheep in the current study. Detailed information of generation of this virus stock is described in a separate publication (Pacheco et al., in preparation).

2.2 Animal experiments

Animal experiments were performed in the BSL-3Ag containment facility at Plum Island Animal Disease Center (PIADC), New York. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), PIADC (USA) and the Animal Ethics Committee, AAHL (Australia) which function to ensure ethical and humane treatment of experimental animals. The sheep used for the study were crossbred castrated males, approximately 5 months old. All animals were delivered in a single batch from a certified vendor.

Four trials were performed in separate isolation rooms to investigate disease generated by the four different inoculation systems. Each trial was comprised of four sheep for characterization of clinical and antemortem infection dynamics. Additional sheep were included in each experiment to allow investigation of tissue distribution of FMDV during early infection (to be reported in a separate publication). Sheep were sedated by intramuscular injection of Xylaxine (1.5 mg/ kg) for direct inoculation and sedation was reversed by intravenous injection of Tolazoline (2 mg/kg)

2.2.1 Inoculation

2.2.1.1 Intra-nasopharyngeal inoculation

For intra-nasopharyngeal (INP) inoculation, sedated sheep were held in an upright (sitting) position while 2ml of inoculum was deposited into the nasopharynx using a 14 gauge flexible plastic catheter (Figure 1A). Inoculum consisted of 10^7 BTID₅₀ (50% bovine tongue infectious dose) diluted in 2ml of minimal essential media with 25mM Hepes. The site of deposition was standardized by adjusting the length of the catheter to match the distance to the nasopharynx.

2.2.1.2 Aerosol inoculation

Aerosol inoculation was performed with an aerosol delivery mask (Equine Aeromask, Medium Size, Trudell Medical) and jet nebulizer (Whisper Jet, Marquest Medical Products) using a system developed for FMDV inoculation of cattle (Pacheco et al., 2010). The jet nebulizer generates aerosols with an average size of 5 μ m by use of compressed air (25 psi). Nebulized inoculum (2 ml total volume; see description above) is delivered into the system holding chamber from where it enters the aerosol delivery mask through a one-way valve upon

inhalation. Sedated sheep were fitted with a mask that covered the muzzle and head up to a level above the eyes (Figure 1B). Nebulization of the complete amount of inoculum took approximately 20 minutes.

2.2.1.3 Coronary band inoculation

Sheep infected by coronary band inoculation received a virus dose of 10^6 BTID₅₀ in a total volume of 0.4ml minimal essential media. Sedated sheep were inoculated by intradermal injection in the coronary bands using a 23 gauge needle. The inoculum was divided between two injection sites on the lateral aspects of the medial and the lateral digits of one front foot.

2.2.1.4 Direct contact exposure

For direct contact exposure, the four sheep subjected to coronary band inoculation were moved into a different room housing eight naïve (recipient) sheep at 48 hours post inoculation (hpi). Four of these contact exposed sheep were euthanized within 72 hours post exposure (hpe) for detailed pathogenesis studies (to be reported in a separate publication). A ratio of 1:2 between donors and recipients was maintained throughout the study by removal of inoculated donor sheep from the contact room as the numbers of contact exposed sheep were reduced. Contact-exposed sheep co-habited with inoculated donors throughout the study period.

2.2.2. Clinical evaluation and sample collection

Samples collected on a daily basis consisted of serum, nasal swabs and oropharyngeal (OP) swabs. The same swab types were also collected directly following INP and aerosol inoculation ('p.i.' = post inoculation in Figures 2 and 3), and at 6 hpe from sheep subjected to INP, aerosol or contact exposure. Swabs were immersed in 2 ml minimal essential media

containing 25mM Hepes directly upon collection. Blood samples were separated through centrifugation, and OP swabs were centrifuged to extract fluid absorbed by the larger swabs used for these samples. All aliquoted samples were immediately frozen at -70°C until further processing.

The progression of the clinical infection (lesion distribution) was quantitated by adaptation of a scoring system originally developed for use in FMDV studies in pigs (Pacheco and Mason, 2010). In brief, vesicular lesions observed at medial or lateral aspects of the two main digits of each foot contributed one point each towards a cumulative score, with additional single points counted for lesions on the dental pad, tongue, lips and nostrils. Using this scoring system, sheep subjected to INP- or aerosol inoculation or contact exposure could reach a maximum score of 20. The highest score possible following coronary band inoculation was 16 as lesions on the foot used for inoculation were not counted. All sheep were monitored and sampled for a period of 9 days after virus exposure.

2.3 FMDV RNA detection

Serum and swab samples were analyzed using quantitative real-time RT-PCR (qRT-PCR), targeting the 3D region of the FMDV genome (Callahan et al., 2002) with forward and reverse primers adapted from Rasmussen et al (2003) and chemistry and cycling conditions as previously described (Stenfeldt et al., 2014c). Cycle threshold values were converted to RNA copies per milliliter using an equation derived from analysis of serial 10-fold dilutions of *in vitro* synthesized FMDV RNA of known concentration. The cycle threshold positivity cutoff of 45 corresponded to a detection threshold value of 2.11 log₁₀ FMDV RNA copies per ml of serum. For nasal and tonsil swabs the inclusion of specific dilution factors corresponding to the

procedures of sample processing resulted in detection thresholds of 3.01, and 2.51 RNA copies/ml respectively.

2.4 Detection of FMDV RNA and antigen in tissue samples

In order to characterize the tissue-specific distribution of FMDV, additional sheep were included in each challenge group and euthanized at 24 or 48 hpe for intensive postmortem tissue sampling (limited description herein). Tissue samples were screened for contents of FMDV RNA using qRT-PCR (Arzt et al., 2010), and subsequent detection of antigen in cryosections was performed by immunohistochemistry (IHC) and multichannel immunofluorescence (MIF) as previously described (Arzt et al., 2009; Arzt et al., 2010). Detection of FMDV antigen was performed using mouse monoclonal antibodies against structural proteins (VP1; F1412SA (Yang et al., 2007b)) and nonstructural proteins(3D; F19-6(302)(Yang et al., 2007a)). MIF experiments included labeling of cell markers using rabbit monoclonal anti-pancytokeratin (Invitrogen, cat. number 180059) and mouse monoclonal anti- sheep MHC II (Serotec MCA2228).

2.5 Statistical analysis

Statistical comparison of FMDV antemortem infection dynamics following different exposure systems was performed by comparing the time (days) from virus exposure to occurrence of specific events (*de-novo* OP shedding of virus, detection of viral RNA in serum and appearance of clinical lesions). Analysis of data was performed through a Cox proportional hazard regression model, with exposure system as explanatory variable, using the package “Survival” included in R software. Statistical significance was determined at $p < 0.05$. All differences in measured values are statistically non-significant unless explicitly described as significant.

3. Results

3.1 Clinical characteristics of FMDV/O/SKR in sheep

Regardless of route of exposure, all sheep developed characteristic FMD lesions within 1 to 4 days of virus exposure (Figures 2–5, Table 1). The most commonly observed lesions consisted of blanching and vesiculation of epithelium within the interdigital cleft or at the coronary bands (Figure 1 C and D). Lesions in the oral cavity consisted of small vesicles on the dorsal surface of the tongue and gingiva or vesicles progressing to erosions on the dental pad (Figure 1 E and F). Foot lesions were often accompanied by mild to marked lameness. A transient increase in body temperature (above 40°C; data not shown) lasting for 1-2 days was observed in all sheep, concurrent with appearance of clinical lesions.

3.2 Within host infection dynamics following INP inoculation

Clinical and virological dynamics in the 4 sheep infected by INP inoculation were highly synchronous (Figure 2). Abundant FMDV RNA was detected in nasal and OP swabs directly following inoculation (Figure 2), interpreted as residual inoculum. However, viral RNA levels in OP swabs substantially increased by 1 dpi in all 4 animals, with peak mean viral RNA levels (7.60 log₁₀ FMDV RNA copies/ml) measured at this early time point (Table 2). The timing of detection of *de-novo* virus replication in OP swabs was significantly earlier than aerosol-inoculated and contact exposed groups, whilst there was no significant difference in timing when compared to sheep infected by coronary band inoculation (Table 1). FMDV RNA quantities in OP swabs remained above post-inoculation values until 8 dpi, whilst detection in nasal swabs was more variable (Figure 2). Viremia (defined as FMDV RNA detection in serum) lasted from 1 to 4 dpi in all four animals (Figure 2), with peak serum concentrations measured at 2 dpi (8.16 log₁₀ FMDV RNA copies/ml; Table 2). Clinical lesions were observed in all sheep at 2 dpi

(Table 1), and all sheep reached close to maximum cumulative lesion scores (≥ 15) by 4 to 6 dpi (Figure 2). The timing of the first appearance of clinical lesions was significantly earlier than contact exposed sheep, whilst no significant difference was detected when compared to the other two groups (Table 1).

3.3 Within-host infection dynamics following aerosol inoculation

Similar to INP inoculation, abundant FMDV RNA was detected in nasal- and OP swabs of aerosol-inoculated sheep directly following inoculation (Figure 3). *De-novo* virus replication, as determined by increasing levels of shedding in OP swabs, was detected between 1 and 3 dpi (mean value= 1.75 dpi), which was significantly later than INP- and coronary band inoculated groups (Table 2). Peak OP shedding occurred at 2 dpi when post-inoculation levels (residual inoculum) were excluded (Table 2). However, in contrast to the INP-inoculated group, virus detection in OP and nasal swabs did not consistently increase above levels measured post-inoculation and at 6 hpi (Figure 3). FMDV RNA was detectable in sera between 1 and 3 or 4 dpi (Figure 3) with peak virus concentration in serum ($6.38 \log_{10}$ FMDV RNA copies/ml) measured at 3 dpi (Table 2). Vesicular lesions appeared at 2-3 dpi, which differed significantly only from the coronary band inoculated group (Table 2). There was, however, a marked variation in cumulative lesion scores within the aerosol inoculated group, with only 1 out of 4 sheep reaching a lesion score above 15 (Figure 3).

3.4 Within-host infection dynamics following coronary band inoculation

OP swabs were not collected earlier than 1 dpi from the coronary band inoculated sheep, preventing direct comparison between groups at earlier time points. However, at 1 dpi, OP levels of viral RNA in coronary band inoculated sheep were higher than in any of the other exposure

groups ($9.11 \log_{10}$ FMDV RNA copies/ml; Table 2). After this early peak in OP shedding of virus, levels decreased steadily throughout the observation period (Figure 4). Nasal shedding followed a pattern similar to OP swabs, but at slightly lower detection levels. Viremia occurred at 1 dpi in all four sheep and lasted for 3-5 days (mean peak serum concentration = $8.95 \log_{10}$ FMDV RNA copies/ml; Table 2). Vesicles at sites distant to the inoculated foot appeared at 1 dpi in one of the animals, and at 2 dpi in the remaining three sheep (Figure 4, Table 1). There was some variation in cumulative lesion scores within this group, with one animal reaching a lower score than the other three (Figure 4). The timing of the first appearance of clinical lesions, as well as of the first detection of OP shedding were significantly earlier than in aerosol inoculated and contact exposed groups, but did not differ from the INP inoculated sheep (Table 1)

3.5 Within-host infection dynamics following contact exposure

All quantitatively examined parameters of FMD were delayed in contact exposed sheep compared to all other routes of infection (Table 1). Mean FMDV RNA detection in OP and nasal swabs increased gradually from 1 to 4 dpe (Figure 5). However, viral RNA was detectable in OP swabs from three out of four sheep at 6 hours after initiation of contact, and at 1 dpe in the remaining animal (Figure 5). After initial detection, OP levels of viral RNA were either maintained at stable (comparatively low) levels, or decreased before markedly increasing concurrent with occurrence of viremia (Figure 5). Mean peak levels of viral RNA in OP swabs reached $8.56 \log_{10}$ FMDV RNA copies/ml at 4 dpe (Table 2), which was higher than peak levels detected in aerosol and INP-inoculated groups, and slightly below peak detection in the coronary band inoculated group. FMDV RNA was detected in serum at 2 dpe in three animals, and at 3 dpe in the fourth individual. Viremia lasted for 4 or 5 days, with mean peak levels measured at 4 dpe ($8.95 \log_{10}$ FMDV RNA copies/ml; Table 2). Clinical lesions appeared at 3 or 4 dpe (Figure

5). Two animals reached cumulative lesion scores of 15, whilst the remaining two had lower scores (Figure 5). The timing of *de-novo* OP shedding and appearance of clinical lesions was significantly delayed when compared to INP and coronary band inoculated sheep, but did not differ significantly from the aerosol inoculated group.

3.7 Microscopic localization of FMDV antigen

Tissue samples from animals euthanized at 24-48 hpi in which FMDV genome could be detected by qRT-PCR (data not shown), were screened for FMDV antigens using IHC and MIF microscopy. In INP-inoculated sheep, at 24 to 48 hpi, FMDV proteins were most consistently detected within crypt epithelial cells (cytokeratin-positive, MHC II-negative) of oropharyngeal tonsils (Figure 6 A-B). By contrast, in sheep subjected to aerosol inoculation FMDV proteins were most consistently detected in the cytokeratin-positive pulmonary epithelial cells of the lungs (Figure 6 C-D) (to be described in greater detail in a following publication).

4. Discussion

The objectives of the work described herein were to characterize clinical and virological dynamics of an emergent FMDV, serotype O of SEA topotype, lineage Mya98 in sheep, by comparative investigation of clinical and virological dynamics subsequent to virus exposure via one of four different routes. Previous works have investigated infection dynamics of FMDV in sheep using a variety of exposure systems, including combinations of coronary band inoculation and direct contact exposure (Alexandersen et al., 2002; Burrows, 1968), intra-nasal instillation (Hughes et al., 2002a; McVicar, 1971), and exposure to natural aerosol delivered either through a mask (Gibson and Donaldson, 1986) or by indirect contact to infected pigs (Aggarwal et al., 2002; Gibson et al., 1984). Although these publications provide a useful overview of certain

characteristics of FMD in sheep, comparative investigations contrasting the relationship between exposure system and infection dynamics have been lacking.

The FMDV serotype O strain derived from the 2010 Republic of Korea outbreak induced consistently severe clinical disease and synchronous within-host infection dynamics via four different routes of exposure in sheep: intra-nasopharyngeal (INP)-, aerosol-, and coronary band inoculation, as well as direct contact exposure. There were some route-dependent variations in infection dynamics, as defined by significant differences in timing of specific events, between groups that were exposed to FMDV through different exposure systems. *De-novo* virus replication in OP swabs occurred significantly earlier in INP- and coronary band- inoculated sheep when compared to aerosol-inoculated and contact exposed groups. There was a similar difference in the first appearance of clinical lesions, with earlier detection in INP- and coronary band inoculated sheep compared to the other groups, although this difference was not statistically significant between INP- and aerosol inoculated groups.

All animals included in this study developed clearly visible, characteristic FMD lesions with the infectious dose used. Furthermore, a subset of animals developed severe and acute lameness culminating in partial loosening of the hoof wall due to expansive coronary band vesiculation. The severity of the clinical syndrome, with the occurrence of striking lesions, contrasts the common descriptions of FMD in sheep, which generally state that clinical lesions may be scarce or absent even when infection can be confirmed by detection of viremia and viral shedding (Hughes et al., 2002b; Kitching and Hughes, 2002).

The current study clearly demonstrated that the recently emerged Korean strain of serotype O FMDV used for these investigations is highly virulent in sheep under the conditions

applied. The only other study of experimental inoculation of a similar virus in sheep reported a less virulent phenotype following both coronary band inoculation and contact exposure (Horsington et al., 2015). Multiple factors may have contributed to these distinct findings; the two studies were performed in different physical settings with study animals of different genetic background. Furthermore, there were differences in the challenge doses received by the inoculated sheep, and the precise origin and passage history of the virus stocks used were different. Notably, the virus stock used for inoculation by Horsington et al (2015) had been through four passages in tissue culture, whereas the virus used in this current study was derived directly from vesicular epithelium of an infected cow.

In the current study, a 10-fold higher inoculation dose was used for INP- and aerosol inoculation compared to coronary band injection. This is justified by demonstrated intrinsic differences between simulated natural exposure systems (INP- and aerosol) and needle inoculation. Whereas INP- and aerosol inoculation comprise deposition of inoculum (either as liquid or droplets) on intact mucosal surfaces, coronary band inoculation implies injection of virus directly into a tissue known to be highly susceptible to infection. Thus, virus injected in the coronary band will undergo substantial amplification at the site of inoculation, resulting in a higher effective challenge dose. In contrast, virus deposited through INP- or aerosol inoculation needs to overcome the host mucosal immune defenses before substantial amplification can occur. Additionally, abundant quantities of FMDV RNA in nasal and OP swabs collected shortly after inoculation suggest that substantial amounts of the inoculum deposited are lost following both INP- and aerosol inoculation

Official outbreak reports from the 2010 Republic of Korea FMDV serotype O outbreak do not indicate any sheep premises being affected by the outbreak or related countermeasures.

However, according to animal population data derived from the World Animal Health Information Database (OIE, 2014) the total population of sheep in The Republic of Korea in 2010 consisted of 3216 individuals, compared to more than 3 million cattle and close to 10 million pigs. These data suggest that the apparent lack of infected sheep during the Korean outbreak may be explained by low (or absent) exposure due to the scarce ovine population in the country.

In contrast to cattle and pigs, the pathogenesis of FMDV in sheep has not been investigated in great detail. However, it is generally believed that critical events of FMDV pathogenesis in small ruminants are similar to those that have been described in cattle (reviewed in Arzt et al., 2011). Studies in cattle have demonstrated that primary FMDV replication occurs in the nasopharynx prior to subsequent dissemination and generalization of infection (Arzt et al., 2010; Burrows et al., 1981). Similar to the sheep included in this current study, it has also been established that cattle are highly susceptible to both aerosol and INP-inoculation (Donaldson et al., 1987; Gloster et al., 1981; Pacheco et al., 2010); Pacheco et al, unpublished).

The finding that sheep were efficiently infected by both INP and aerosol systems suggests that highly permissive tissues to FMDV infection are present within the ovine upper respiratory tract. However, microscopic evidence of FMDV replication was found most consistently in the oropharynx of INP-inoculated sheep. This is similar to recent reports indicating that in pigs primary FMDV infection occurs in oropharyngeal tonsils (Stenfeldt et al., 2014a) yet differs from cattle in which the epithelial cells of the nasopharyngeal mucosa have been identified as the primary site of infection (Arzt et al., 2010). However, the current study also demonstrated that large quantities of FMDV proteins could be detected in the lungs of sheep exposed to aerosol inoculation, confirming that, similar to cattle, the ovine lower respiratory tract

is capable of supporting substantial FMDV replication. Thus, there are aspects of FMD pathogenesis in sheep that are similar to processes reported in both cattle and in pigs. Ongoing studies in our laboratory are pursuing further elucidation of the tissue-specific events of FMDV pathogenesis in sheep.

It is also noteworthy that in the current study, FMDV RNA detection in OP swabs was consistently high at 1 dpi even in the sheep that were infected by a route that bypasses the upper respiratory and gastrointestinal tracts (coronary band inoculation). This finding, in combination with consistently higher and less variable quantities of FMDV RNA recovered from OP swabs in comparison to nasal swabs, further supports the importance of the ovine oropharynx (specifically the oropharyngeal tonsils) in the pathogenesis of FMDV in this host species.

The results herein confirm that all sheep included in this study were successfully infected. Significant differences between groups subjected to different exposure systems consisted of variations in time elapsed from virus exposure to onset of clinical disease and shedding of virus. Determination of the relative appropriateness of these different inoculation systems would depend on the precise objectives of the experimental study to be conducted. Whilst the conventionally used system of coronary band inoculation offers control and consistency in dosage and timing of virus challenge, this system does not provide an accurate simulation of natural exposure conditions as the host mucosal immune system is largely bypassed. In contrast, the novel INP inoculation system described herein utilizes what is believed to be the natural route of FMDV exposure (respiratory), whilst still maintaining control of the inoculation dose and time. Furthermore, our findings confirm that FMDV infection dynamics in sheep subjected to INP inoculation are similarly rapid and severe as compared to coronary band inoculation.

Direct contact exposure provides a truly natural route of virus exposure, but at the cost of excluding the ability to control or evaluate the actual challenge dose. Furthermore, experimental models based on contact exposure require the use of additional animals to function as virus donors, which may be inconvenient for large scale investigations with regards to space requirements in containment facilities, direct costs and ethics considerations. Infection dynamics in the aerosol-inoculated sheep of the current study closely resembled the contact-exposed animals. Similar to the INP-inoculation system, aerosol inoculation utilizes a natural route of exposure, but adds a level of control that is not possible in contact based exposure systems. The variability in dynamics within the aerosol-inoculated group was somewhat higher than within the INP inoculated group, although this could also be interpreted as demonstrating a greater resemblance to a truly natural (contact) exposure system.

5. Conclusions

The current study demonstrated consistent and severe clinical FMD in sheep exposed via contact or inoculation of the 2010 Korean field isolate of serotype O FMDV. Two newly developed simulated-natural exposure systems; INP- and aerosol inoculation were efficient in generating consistent and synchronous disease comparable to conventional systems of coronary band inoculation and direct contact exposure. FMDV infection dynamics following INP inoculation were very similar to coronary band inoculation, whereas disease dynamics following aerosol inoculation more closely resembled direct contact exposure. The consistency and simplicity of the optimized INP-inoculation technique, in combination with clear advantages of utilizing a natural route of virus exposure, support the utility of this system. However, further evaluation using different strains of FMDV and larger numbers of sheep are required before extrapolating these conclusions as generally applicable to ovine FMD pathogenesis.

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Conflicts of interest

None

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Figure legends

Figure 1. Images characterizing experimental systems and clinical lesions. A) Intra-nasopharyngeal inoculation; Inoculum is deposited in the nasopharynx using a flexible plastic catheter fitted onto a syringe. B) Aerosol inoculation using a system consisting of nebulizer and an (equine-) aerosol delivery mask. C) Epithelial blanching and vesiculation (arrows) on the medial aspects of two main digits of a hind foot, four days post contact exposure. D) Vesiculation at the coronary band of a hind foot (arrow) four days post aerosol inoculation. E) Multiple small vesicular lesions on the dorsal surface of the tongue (arrowheads) with blanching of epithelium (arrow) three days post INP inoculation. F) Dental pad erosion occurring subsequent to rupture of a vesicular lesion five days post contact exposure.

Figure 2. Antemortem infection dynamics of FMDV in sheep following INP inoculation. FMDV RNA detection in serum, nasal and oropharyngeal (OP) swabs was performed using qRT-PCR, and is presented as Log_{10} genome copy numbers (GCN) / ml. Clinical score provides a cumulative index of FMD lesion distribution. p.i. (post inoculation) indicates nasal- and OP swabs collected immediately after inoculation. Limits of detection: serum = $2.11 \text{ Log}_{10} \text{ GCN/ml}$, nasal swabs = $3.01 \text{ Log}_{10} \text{ GCN/ml}$, and OP swabs = $2.51 \text{ Log}_{10} \text{ GCN/ml}$.

Figure 3. Antemortem infection dynamics of FMDV in sheep following aerosol inoculation. FMDV RNA detection in serum, nasal and oropharyngeal (OP) swabs was performed using qRT-PCR, and is presented as Log_{10} genome copy numbers (GCN) / ml. Clinical score provides a cumulative index of FMD lesion distribution. p.i. (post inoculation) indicates nasal- and OP swabs collected immediately after inoculation. Limits of detection: serum = $2.11 \text{ Log}_{10} \text{ GCN/ml}$, nasal swabs = $3.01 \text{ Log}_{10} \text{ GCN/ml}$, and OP swabs = $2.51 \text{ Log}_{10} \text{ GCN/ml}$.

Figure 4. Antemortem infection dynamics of FMDV in sheep following coronary band inoculation. FMDV RNA detection in serum, nasal and oropharyngeal (OP) swabs was performed using qRT-PCR, and is presented as Log₁₀ genome copy numbers (GCN) / ml. Clinical score provides a cumulative index of FMD lesion distribution. Limits of detection: serum =2.11 Log₁₀ GCN/ml, nasal swabs= 3.01 Log₁₀ GCN/ml, and OP swabs= 2.51 Log₁₀ GCN/ml.

Figure 5. Antemortem infection dynamics of FMDV in sheep following direct contact exposure. FMDV RNA detection in serum, nasal and oropharyngeal (OP) swabs was performed using qRT-PCR, and is presented as Log₁₀ genome copy numbers (GCN) / ml. Clinical score provides a cumulative index of FMD lesion distribution. Limits of detection: serum =2.11 Log₁₀ GCN/ml, nasal swabs= 3.01 Log₁₀ GCN/ml, and OP swabs= 2.51 Log₁₀ GCN/ml.

Figure 6. Immuno-microscopical detection of FMDV in ovine tissues following INP- and aerosol inoculation. **A)** Immunohistochemical localization of FMDV structural (VP1) protein (blue chromogen, arrows) within epithelial crypts of the palatine tonsil 48 hours post INP-inoculation. 20x magnification. Anti-FMDV capsid monoclonal antibody. Micropolymer alkaline phosphatase, BCIP/NBT chromogen and Nuclear Fast-Red counterstain. **B.** Multichannel immunofluorescence (MIF) image of serial section of region identified in Figure 6A. 20x Magnification. FMDV capsid antigens (red) within cytokeratin-positive (green) epithelial cells. Anti-FMDV capsid and anti-cytokeratin monoclonal antibodies. **C)** Immunohistochemical localization of FMDV structural (VP1) protein (blue chromogen, arrows) in ovine pulmonary tissue 48 hours post aerosol inoculation. 20x magnification. Anti-FMDV capsid monoclonal antibody. Micropolymer alkaline phosphatase, BCIP/NBT chromogen and Nuclear Fast-Red counterstain. **D.** Multichannel immunofluorescence (MIF) image of serial

563 section of region identified in Figure 6C. 20x Magnification. FMDV capsid antigens (red) within
564 cytokeratin-positive (green) epithelial cells. MHC-II –positive cells (blue) in close proximity of
565 infected cells but with no co-localization with viral proteins. Anti-FMDV capsid, anti-
566 cytokeratin and anti- sheep MHC II monoclonal antibodies. Scale bars (6A-D); 50µm

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Table1. Comparison of FMDV infection dynamics across different routes of virus exposure; tabulation of mean time (days) from exposure to first detection of specific events

Exposure system	<i>de-novo</i> FMDV RNA shedding in OP swabs*	FMDV RNA in serum	Clinical lesions
INP	1 ^{AER, DC, #}	1	2 ^{DC}
Aerosol	1.75 ^{INP, CB}	1	2.75 ^{CB}
Coronary band	1 ^{AER, DC}	1	1.75 ^{AER, DC}
Direct contact	2.5 ^{INP, CB}	2.25	3.75 ^{INP, CB}

* defined as first day of increasing FMDV RNA levels subsequent clearance of residual inoculum.

#Superscripts indicate significant difference from these groups; INP= intra-nasopharyngeal inoculation, AER= aerosol inoculation, CB = Coronaryband injection, DC= Direct Contact.

Table 2. Mean peak clinical scores and levels of FMDV RNA in OP swabs and serum in sheep infected with FMDV via different exposure systems.

Exposure System	OP shedding (GCN/ml)	RNA in serum (GCN/ml)	Clinical lesions (score)
INP	7.60 (1*)	8.16 (2)	18 (7)
Aerosol	6.27 [#] (2)	6.38 (3)	11 (8)
Coronary band	9.11 (1)	8.43 (2)	11.75 (8)
Direct contact	8.96 (4)	8.58 (5)	12.5 (9)

*Numbers in bracket indicate the day (dpe) at which peak values were observed.

[#] post inoculation and 6 hpe data points excluded; interpreted as residual inoculum

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6