Infection dynamics of foot-and-mouth disease virus in cattle following intra-nasopharyngeal inoculation or contact exposure

Juan M Pacheco^{1,✉}, Carolina Stenfeldt^{1,2,✉}, Luis L Rodriguez¹, Jonathan Arzt^{*1}

Summary

For the purpose of developing an improved experimental model for studies of foot-and-mouth disease virus (FMDV) infection in cattle, three different experimental systems based on natural or simulated-natural virus exposure were compared under standardized experimental conditions. Antemortem infection dynamics were characterized in cattle exposed to FMDV through a novel, simulated-natural intra-nasopharyngeal (INP) inoculation system or through standardized and controlled systems of within- or between- species direct contact exposure (cattle-to-cattle or pig-to-cattle). All three systems were efficient in causing synchronous, generalized foot-and-mouth disease in cattle exposed to one of three different strains of FMDV representing serotypes O, A and Asia1. There was more within-group variation in the timing of clinical infection following natural and simulated-natural virus exposure systems when compared to the conventionally used system of needle inoculation (intraepithelial lingual inoculation). However, the three optimized exposure systems described herein have the advantage of closely simulating field conditions by utilizing natural routes of primary infection, thereby facilitating engagement of mucosal host-defense mechanisms. Overall it is concluded that INP inoculation and standardized systems of direct contact exposure provide effective alternatives to conventional (needle) inoculation systems for studies in which it is desirable to simulate the natural biology of FMDV infection.

Keywords: FMDV, foot-and-mouth disease virus, pathogenesis, animal models

Introduction

Foot-and-mouth disease virus (FMDV), a single stranded positive sense RNA virus belonging to the *Aphthovirus* genus of the *Picornaviridae* family, is the causative agent of foot-and-mouth disease (FMD), a highly infectious vesicular disease of cloven hoofed animals. FMD is a disease of substantial socio-economic impact because the FMD status of any geographic region defines access to international markets for trade in agricultural products. The economic implications of this disease combined with its' capacity for rapid transmission within and between a wide range of susceptible host species demonstrate the importance of FMD as a constant threat to multinational livestock industries as well as small-scale farmers (Arzt *et al.*, 2010b; Knight-Jones and Rushton, 2013)

Multiple experimental models for FMD studies in cattle have been developed with the goals of closely simulating naturally occurring disease mechanisms. Additional requirements for optimal experimental systems include providing consistent and reproducible results with minimal variation in temporal or quantitative measures of infection dynamics across experimental subjects. Conventional experimental models most often involve virus challenge through intraepithelial injection in the tongue (intraepithelial lingual, IEL, often described incorrectly as intradermal lingual (IDL)) (Arzt *et al.*, 2014; Charleston *et al.*, 2011; Henderson, 1949). Although this approach provides a highly consistent and reproducible model for the disease, there are disadvantages with using this system for studies of FMD pathogenesis and vaccinology as it by-passes the host's natural mucosal barrier. Even though it can be argued that viral entry through pre-existing abrasions in the oral cavity may occur during natural conditions, it has been demonstrated that the more common route of infection in cattle consists of viral entry through the upper respiratory tract (Donaldson and Alexandersen, 2002; Donaldson *et al.*, 2001) with

primary infection occurring within the nasopharyngeal mucosa (Arzt *et al.*, 2010a; Burrows *et al.*, 1981; Pacheco *et al.*, 2010; Stenfeldt *et al.*, 2015a).

Experimental systems based on contact transmission provide the closest simulation of truly natural routes of virus exposure. However, contact systems have two major drawbacks: 1) dose and timing of virus exposure cannot be precisely controlled, and 2) additional experimental animals are required as virus donors which is undesirable in consideration of cost and ethical concerns. Thus, experimental studies based on contact exposure need to be carefully executed in order to minimize the effects of any extrinsic factors that might influence the interaction between animals and thereby alter the resulting “dose” of virus exposure.

Contact transmission has been achieved by co-housing experimental animals with animals of the same or different species that have previously been infected by direct inoculation. Published studies have included cattle-to-cattle (Graves *et al.*, 1971; Howey *et al.*, 2012; Orsel *et al.*, 2005) and/or pig to pig transmission (Alexandersen *et al.*, 2001; Eble *et al.*, 2004; Pacheco and Mason, 2010; Pacheco *et al.*, 2012), with fewer studies describing interspecies transmission, i.e., pig-to-cattle or cattle-to-pig (Blackwell *et al.*, 1982; Gomes and Auge de Mello, 1994; Pacheco *et al.*, 2016). Within any contact system, efficiency of FMD transmission will depend on the biological characteristics of the specific FMDV strain involved (Pacheco and Mason, 2010) and the conditions of contact and interaction between animals (Pacheco *et al.*, 2012; Quan *et al.*, 2009). Additionally, relevant intrinsic viral properties that may affect transmission include possible variations in host range (Brooksby, 1950; Dunn and Donaldson, 1997), virulence, and environmental stability of the specific virus strain. Additional variations in exposure conditions may be adjusted to simulate distinct transmission scenarios; for instance, continuous direct contact (Stenfeldt *et al.*, 2011; Zhang *et al.*, 2006) would be expected to deliver a higher

effective challenge dose compared to time-limited exposure of hours (Burrows *et al.*, 1981; Quan *et al.*, 2009) or days duration (Cox *et al.*, 2005; Juleff *et al.*, 2013). Animals may be separated by gates or unoccupied space to simulate indirect exposure (Bouma *et al.*, 2004; Donaldson and Ferris, 1980). Some investigators have utilized 3-fold (tertiary) contact systems to ensure that the experimental subjects are exposed to donors that were infected by natural contact exposure (Charleston *et al.*, 2011).

Similar to contact-based challenge systems, simulated natural exposure systems strive to closely simulate the natural biology of primary FMDV infection. Such systems allow precise control of timing and dosage of the virus challenge while preserving the natural engagement of the mucosal immune system and reducing the numbers of study-animals required. For cattle, FMDV inoculation by a natural (aerogenous) route has been demonstrated by delivery of naturally generated aerosol using an improvised apparatus (Donaldson *et al.*, 1987), artificially generated aerosol using improvised apparatus (Brown *et al.*, 1992; Brown *et al.*, 1996; Donaldson *et al.*, 1987; McVicar and Eisner, 1983), artificially generated aerosol using standardized apparatus (Arzt *et al.*, 2010a; Pacheco *et al.*, 2010), or by virus-moistened gauze deposition on the nasal planum (Korn, 1957). Other investigators have inoculated FMDV using an intranasal delivery system developed for use in humans (Pacheco *et al.*, 2010) or deposition of virus suspension using tubing with a length of 5 cm (Sutmoller *et al.*, 1968) or 10 cm (Graves *et al.*, 1971; McVicar *et al.*, 1970). Other authors have described using intranasal inoculation without giving specific details of depth of instillation (Bouma *et al.*, 2004; McVicar and Sutmoller, 1974; Orsel *et al.*, 2007; Orsel *et al.*, 2005; Salt *et al.*, 1996; Sanz-Parra *et al.*, 1999).

In the current study, recently acquired knowledge gained from studies of FMDV dynamics in cattle (Arzt *et al.*, 2010a; Arzt *et al.*, 2014; Pacheco *et al.*, 2010) and contact

transmission in pigs and cattle (Pacheco *et al.*, 2016; Pacheco and Mason, 2010; Pacheco *et al.*, 2012) were elaborated upon in order to develop standardized FMDV challenge systems for cattle based on time-limited direct contact with infected pigs or continuous contact with infected cattle. Furthermore, a novel system based on direct deposition of virus within the bovine nasopharynx is described as an alternative, simulated-natural system that provides precise control of the challenge dose whilst also engaging natural mucosal defenses at the primary site of infection. This system was designed to target the specific region of the upper respiratory tract that has been demonstrated to be the primary site of infection after inoculation of cattle by aerosol inoculation (Arzt *et al.*, 2010a; Pacheco *et al.*, 2010). All three systems were evaluated in studies performed with three different strains of FMDV representing serotypes O, A and Asia1. Infection dynamics in exposed cattle were monitored through the first nine days of clinical infection and the comparative utility of each model is discussed in relation to experimental objectives.

2. Materials and Methods

Viruses

FMDV strains used were Asia1 Shamir, O1 Manisa and A24 Cruzeiro. Generation of pig-adapted virus stocks of the three FMDV strains for inoculation of pigs has been described previously (Pacheco *et al.*, 2012). An infectious clone was utilized for direct inoculation of cattle with A24 Cruzeiro (Rieder *et al.*, 2005). A natural bovine-derived isolate was used for inoculation of cattle with FMDV O1 Manisa (Golde *et al.*, 2005), while the pig-derived strain of FMDV Asia1 Shamir (described above) was also used for cattle studies. Inoculated doses for each particular inoculation route and virus strain are described below.

Animal experiments

Animal experiments were performed at Plum Island Animal Disease Center, New York. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), which functions to ensure ethical and humane treatment of experimental animals. The pigs used as virus donors were castrated male Yorkshire pigs, weighing 25-30 kg. Cattle were Holstein steers of approximately 200 kg. All animals were allowed one to two weeks of acclimation before initiation of experiments. For inoculation, pigs were sedated by intramuscular (IM) injection of a mixture of telazole, ketamine and xylazine, (3, 8 and 4 mg/kg, respectively). Steers that received IEL or intra-nasopharyngeal (INP) inoculation were sedated by IM injection of xylazine (0.22 mg/kg) and placed in sternal recumbency. Sedation in steers was reversed by intravenous (IV) injection of Tolazoline (2 mg/kg). Euthanasia for both species was performed by IV injection of pentobarbital sodium (Fatal-plus®, 10 ml/45.3 kg) after sedation as described above.

Exposure and inoculation systems

Intra-nasopharyngeal inoculation

For INP inoculation, 2 ml of inoculum containing 10^6 or 10^7 infectious units (Table 1) was prepared by diluting the original high titer virus stocks in minimal essential media with 25 mM hepes. Steers were sedated and the inoculum was deposited into the nasopharynx through the nares and nasal cavities using a 3 cc syringe and a 30 cm length, 2 mm diameter flexible plastic catheter (Fig. 1). The site of deposition was standardized by adjusting the length of the catheter to match the distance from the nares to the medial canthus of the eye for each animal.

Earlier dissections of other animals had indicated that this external measurement consistently corresponded with the internal distance to the nasopharynx.

Direct inoculation of pigs and pig-to-cattle contact challenge

For each FMDV strain, four steers were exposed by direct contact to four pigs that served as virus donors. Donor pigs were infected by intra-dermal heel-bulb (IDHB) injection (Pacheco and Mason, 2010) with 100 PHID₅₀ (50% pig-heel infectious doses) as previously described (Pacheco *et al.*, 2012). At 2 days post inoculation (dpi), corresponding to the time when vesicular lesions were first observed, the pigs were moved into a room housing the naïve steers where they remained through 24 h of cohabitation. The room was not washed during the contact period. After completion of 24 h of direct-contact exposure (corresponding to 3 dpi for the pigs), pigs were removed from the room and were either euthanized or housed in a separate isolation unit for continued observations. Cattle were monitored up to 9 days post contact (dpc).

Direct inoculation of cattle and cattle-to-cattle contact challenge

For each virus strain, two steers were directly inoculated and used as virus donors for two contact exposed steers. Direct inoculation with 10⁴ infectious units (Table 1) was performed by the IEL route (Henderson 1949) with the donor steers in isolation. At 24 hpi, the directly inoculated steers were moved to the room housing the two naïve steers and the 4 cattle cohabitated in direct-contact throughout the evaluation period (9 dpi / 8 dpc).

Clinical evaluation and sampling

Clinical evaluation and sampling were performed daily starting at 0 dpi, before direct-inoculation or contact-exposure, continuing for 3 dpi for donor pigs and 8-9 dpi or dpc for steers. Samples collected from cattle were blood and secretions (oral and nasal swabs), with the exception that nasal swabs were not collected in A24 Cruzeiro or O1 Manisa steer-to-steer experiments. Additional oral and nasal swabs were collected from animals exposed by INP inoculation immediately after inoculation, as well as at 6 hpi, to monitor clearance of residual inoculum. Samples from pigs consisted of blood as well as nasal and oropharyngeal (OP) swabs. Nasal swabs were harvested using small cotton-tipped swabs, while OP swabs were collected by targeting the tonsil of the soft palate using larger cotton swabs. These swabs were subsequently immersed in 2 ml of MEM with 25 mM Hepes. For cattle, tampons were used for harvest of oral and nasal secretions, with no further dilution in media. Serum was separated from blood collected from the jugular vein from both host species. All samples were frozen at -70°C immediately after collection. Clinical signs of FMD in pigs were scored as previously described (Pacheco and Mason, 2010) with a maximum potential score of 16 as the inoculated foot was not used for scoring. Clinical signs in steers were determined as previously described (Arzt *et al.*, 2010a) with a maximum possible score of 20 points for INP or contact exposed steers and 16 for IEL-inoculated steers, since vesicles of the oral cavity were not used for scoring.

FMDV RNA detection

Serum and secretion samples from animals infected with FMDV serotypes O and A were analyzed using a qRT-PCR system, targeting the 3D region of the FMDV genome (Callahan *et al.*, 2002) following a protocol previously described (Arzt *et al.*, 2010a; Pacheco *et al.*, 2010). As

the limit of detection of this system was suboptimal for detection of FMDV Asia1 Shamir, all samples obtained from animals infected with this strain were processed using alternative forward and reverse primers adapted from Rasmussen et al (Rasmussen *et al.*, 2003), following a previously described protocol (Stenfeldt *et al.*, 2014b). Cycle threshold values were converted into FMDV genome copy numbers (GCN) per milliliter by use of standard curves based on analysis of 10-fold dilutions of in-vitro synthesized FMDV RNA for each of the virus strains investigated. Based on these standard curves, a distinct lower limit of detection was determined for each strain (Fig 2-4).

3. Results

FMDV Asia1 Shamir

Intra-nasopharyngeal (INP) inoculation

Four steers were subjected to INP inoculation with FMDV Asia1 Shamir with either of two different doses: low dose (10^6 TCID₅₀) or high dose (10^7 TCID₅₀) per animal (Fig. 2A). Regardless of the dose delivered, all four steers developed fulminant FMD with highly synchronous virological and clinical parameters (Fig. 2A). De-novo shedding of FMDV RNA in oral and nasal swabs was detectable at 1 dpi; viral RNA was detected in serum at 2 or 3 dpi, with peak serum concentrations of FMDV RNA detected at 4 or 5 dpi. Clinical lesions first appeared at 3-4 dpi and all four animals reached full clinical score within 1-3 days after the first observed lesion.

Between-species (pig-to-cattle) contact exposure

Donor-pigs inoculated with FMDV Asia1 Shamir had detectable levels of FMDV RNA in serum and swabs at 1 dpi with peak viral loads detected in all sample types at 2 to 3 dpi, (Fig. 2B1). Clinical lesions in donor pigs were first observed at 2 dpi, corresponding to the time point for initiation of the 24 h cohabitation with recipient cattle. By the end of the 24 h contact period all pigs' clinical scores had increased to near-maximum values.

Overall, the clinical progression in steers exposed to FMDV Asia1 Shamir through direct contact with donor pigs was highly synchronous and marginally more rapid than observed in INP-inoculated cattle. At the end of the contact exposure period (1 dpc, Fig. 2B2) FMDV RNA was detected in oral and nasal secretions of all contact exposed steers and 3 out of 4 steers were viremic; the remaining steer (B368) became viremic at 2 dpc. Peak viremia in contact-exposed cattle occurred at 3 or 5 dpc, whereas the peak of FMDV RNA detection in secretions occurred between 2 and 7 dpc. The first detection of clinical FMD lesions occurred between 2 and 4 dpc with 3 out of 4 steers reaching maximum clinical score at 1 -3 dpc.

Within-species (cattle-to-cattle) contact exposure

The two steers directly inoculated (by the IEL route) with FMDV Asia1 Shamir were viremic at 1 dpi, with concurrent detection of FMDV RNA in both saliva and nasal swabs. Thus, detection of viral RNA in serum and swabs coincided with the initiation of contact exposure. Virus shedding in donor-steers peaked at 2 or 3 dpi with peak viremia detected at 3 dpi (B100 and B101, Fig. 2C). Vesicular lesions in directly inoculated steers appeared at 2 dpi, with one of the two steers immediately reaching full clinical score and the second steer progressing to full

score one day later. The clinical progression in steers exposed to FMDV Asia1 Shamir through cattle-to-cattle contact exposure was highly synchronous but with a somewhat slower disease progression compared to INP-inoculated cattle and pig-to-cattle contact exposed steers. The two contact exposed steers shed detectable amounts of FMDV RNA in oral/nasal fluids starting at 1 dpc, with viremia detected in both animals at 3 dpc, and peak viral loads detected at 5 or 7 dpc (B102 and B103, Fig. 2C). Clinical lesions were first detected at 4 dpc, with one steer (B103) progressing to full clinical score within three days whilst clinical FMD was slightly less severe in the second contact exposed steer (B102).

FMDV O1 Manisa

INP inoculation

Four steers were INP inoculated with 10^6 BTID₅₀ of FMDV O1 Manisa. One of these animals (B352) was euthanized at 3 dpi due to reasons unrelated to the study. In all four steers, the virological parameters were nearly identical, and for the 3 surviving steers clinical progression was highly synchronous (Fig. 3A). FMDV RNA was first detected in secretions and serum at 1 to 2 dpi and peaked at 3 to 4 dpi. Clinical lesions appeared at 2 or 3 dpi in the three animals that survived beyond 3 dpi and had reached full clinical score at time of first lesion detection or 24 h later. No clinical signs were detected in the fourth steer up until the time of euthanasia, 3 dpi (B352).

Between-species (pig-to-cattle) contact exposure

At 1 dpi, all donor-pigs inoculated with O1 Manisa had detectable levels of FMDV RNA in serum and nasal swabs, with concurrent detection of virus in OP swabs from two out of four pigs. FMDV RNA loads peaked in both serum and swabs at 2-3 dpi (Fig. 3B1). All pigs had vesicular lesions at 2 dpi, corresponding to the initiation of the 24 h cohabitation with recipient cattle. By the end of the contact period all pigs had maximum clinical scores.

In three of the contact-exposed steers, the clinical progression and virological parameters were highly synchronous, while, the onset of FMD was somewhat delayed in the fourth steer (B356) (Fig. 3B2). At the end of the contact exposure period (1 dpc), all contact exposed steers had FMDV RNA in secretions and 3 out of 4 were viremic, with viremia occurring at 4 dpc in the remaining steer (B356). Peak viremia occurred at 3 or 5 dpi, respectively and detection in secretion peaked at 2 to 5 dpi (Fig. 2B2). Clinical lesions was first detected at 2 dpc in 3 steers and at 5 dpc in B356.

Within-species (cattle-to-cattle) contact exposure

Clinical progression and virological parameters were highly synchronous in IEL-inoculated donors and contact exposed steers infected with FMDV O1 Manisa. FMDV RNA was detected in serum and oral secretions of the two donor steers at 1 dpi, corresponding to the initiation of contact exposure (B624 and B625, Fig. 3C). Vesicular lesions in donor steers were detected at 2 dpi, with both animals reaching full clinical score at 3 dpi. The two contact exposed steers had detectable FMDV RNA in saliva at 1 dpc, with viremia occurring at 2 or 3 dpc (B626 and B627, Fig. 3C). For both contact exposed steers, clinical lesions were detected at 3 dpc with maximum clinical score achieved at 3 to 4 dpc.

283

284 FMDV A24 Cruzeiro

285 *INP inoculation*

286   Four steers were subjected to either low (10^6 BTID₅₀) or high (10^7 BTID₅₀) dose INP
287 inoculation using FMDV A24 Cruzeiro. The two steers in the low-dose group had detectable
288 levels of FMDV RNA in serum at 2 dpi, with *de-novo* shedding of virus in oral and nasal swabs
289 (after clearance of residual inoculum) at 1 dpi (Fig. 4A). The dynamics of virus shedding were
290 similar in the two steers that received the high-dose inoculum, however in one of these two
291 animals there was neither viremia nor clinical signs of FMD detected (B319, Fig. 4A). Vesicular
292 lesions appeared at 4 dpi in both steers of the low dose group. One of these two steers reached
293 full cumulative lesion score, whereas the second animal only developed a single large vesicle on
294 the tongue. The non-viremic steer in the high-dose group did not develop any visible FMD
295 lesions. The second steer in this group had a tongue-vesicle on day 5 pi, with an additional foot-
296 vesicle appearing at 6 dpi. Overall, clinical FMD and virological parameters were highly
297 synchronous in three of the steers INP-inoculated with the A24 Cruzeiro virus. However, onset
298 of clinical FMD was slightly delayed in this cohort compared to cattle subjected to INP-
299 inoculated with FMDV O1 Manisa or Asia1 Shamir (Fig. 4A vs. Fig. 2A and 3A).

300 *Between-species (pig-to-cattle) contact transmission*

301   Donor-pigs inoculated with FMDV A24 Cruzeiro had detectable levels of FMDV RNA
302 in serum and swabs at 1 dpi and peak viral loads were measured at 2 dpi (Fig. 4B1). Clinical
303 lesions were first observed in these pigs at 2 dpi, corresponding to the time of initiation of the 24
304 h co-habitation with recipient cattle.

Despite synchronous and progressively severe FMD in inoculated donor-pigs, the onset and progression of disease in cattle exposed to FMDV A24 Cruzeiro through pig-to-cattle contact exposure was slower and less synchronous compared to the other virus strains and exposure systems investigated. When the contact exposure period ended (1 dpc, Fig. 4B2), FMDV RNA was detected in swabs from all contact exposed steers (with the exception of nasal swabs for B371). All four steers had detectable levels of FMDV RNA in serum at 2-3 dpc with clinical lesions first appearing between 3 and 6 dpc. Three out of the four steers reached full clinical score, whereas one steer only developed a single large vesicle on a hind-foot. In two steers (B370 and B372), FMDV RNA levels in saliva continued to increase directly following removal of the donor pigs from the room, whereas in the two remaining steers (B371 and B373) there was a transient decrease in oral detection of FMDV RNA before levels started to increase again (Fig. 4B). The dynamics of FMDV detection in nasal swabs was comparable to oral swabs, but at slightly lower levels throughout the observation period. Viral dynamics were not substantially different in the two steers in which onset of clinical signs was delayed.

Within-species (cattle-to-cattle) contact exposure

The onset and progression of clinical FMD was rapid and synchronous in both IEL-inoculated and contact-exposed steers infected with FMDV A24 Cruzeiro. FMDV RNA loads in serum and saliva peaked at 1 dpi in both IEL-inoculated donor steers (B147 and B174, Fig. 4C). Vesicles were detected at 3 dpi, with both reaching a 10/16 score by 7-8 dpi. Despite generalization of clinical FMD with vesicles present in all four feet, the clinical scores of these animals were moderate as the majority of detected vesicles were less than 2 cm in diameter.

FMDV RNA was detected in saliva of the two contact exposed starting at 1 dpc with viral loads increasing progressively until 4 or 6 dpc. Viremia was first detected at 3 dpc, with peak levels detected at 3 or 4 dpc (B161 and B175, Fig. 4C). One of the two steers (B175) reached full clinical score whereas the clinical score was lower in the other animal (B161)

4. Discussion

Various experimental systems have been used to successfully infect cattle with FMDV for pathogenesis and vaccine studies. The World Organization for Animal Health (OIE) indicates that for the purpose of vaccine efficacy validation, cattle should be challenged by intraepithelial (IEL) (*sic* intradermal) lingual inoculation (OIE, 2012). This system is the most consistent, rapid, and most stringent manner of evaluating vaccine-induced protection. However, IEL inoculation is a largely unnatural manner of exposing cattle to FMDV as this system bypasses the mucosal defenses which are engaged during natural FMDV infection. Thus, for investigation of FMD pathogenesis and immunity it is necessary to utilize standardized and validated exposure systems that closely simulate natural exposure conditions. Furthermore, close emulation of natural disease mechanisms also facilitates accurate elucidation of host-virus interactions for the ultimate goal of enhancing or manipulating naturally occurring processes of the host defense to promote protection against infection.

In the current study, a series of experiments was performed to compare and contrast three simulated natural challenge systems for studies of FMDV infection in cattle. A novel system of intra-nasopharyngeal (INP) inoculation was developed and evaluated in comparison with IEL inoculation and two different contact exposure systems based on either pig-to-cattle or cattle-to-

cattle direct contact. All three challenge systems were efficient in generating infection in cattle exposed to three different strains of FMDV representing serotypes O, A and Asia1. However, there was some variability in synchrony of the onset of clinical FMD across infection systems and virus strains. The numbers of animals included in the current study were not sufficient for statistically significant conclusions. Thus, the findings described herein are presented as proof of concept and further investigations should be pursued for full validation of any observed trends.

As expected, infection dynamics were less synchronous within groups of cattle infected through natural (direct contact) or simulated natural (INP) exposure systems when compared to the donor animals that were infected with the same viruses through injection based inoculation systems (IEL inoculated cattle and IDHB inoculated pigs). Intraepithelial inoculation consists of direct injection of virus into the epithelium which is the tissue type with demonstrated capability of supporting the highest levels of FMDV replication (tongue for cattle (Henderson, 1952) and heel bulb for pigs (Burrows, 1966)). As a consequence of the high susceptibility of these epithelial cells to FMDV infection, the injected challenge dose rapidly amplifies at the inoculation site resulting in a substantially increased effective challenge dose.

When using the natural or simulated-natural exposure systems described herein, FMDV must engage and overcome the host mucosal immune system before it can amplify to levels needed for generalization of infection. The resulting effective inoculation dose when utilizing simulated natural inoculation systems is therefore less than the actual deposited dose as a substantial amount of the inoculum will be diluted in secretions and shed directly back to the environment. Additionally, FMDV amplification within the pharyngeal tissues does not reach the amplitudes of virus replication at lesion sites (Arzt *et al.*, 2010a; Pacheco *et al.*, 2010; Stenfeldt *et al.*, 2015a); thus, there is no immediate amplification of inoculum as occurs with needle-based

inoculation systems. While these factors undoubtedly contribute to a larger degree of variation, these simulated natural inoculation systems provide more accurate simulations of field conditions by retaining and promoting critical events of natural FMDV pathogenesis.

The system of needleless INP inoculation used in the current study was developed as a simulated-natural inoculation system based upon localization of the primary site of FMDV infection to the nasopharyngeal epithelium in previous studies (Arzt *et al.*, 2010a; Burrows *et al.*, 1981; Pacheco *et al.*, 2010; Stenfeldt *et al.*, 2015a). The system consists of direct deposition of virus within the bovine nasopharynx, but without injection through the mucosal surface. Thus, this system allows for accurate control of both dose and timing of inoculation, whilst still engaging the intact mucosal barrier, thus triggering the natural host-response to infection. INP inoculation has additional advantages of rapidity and ease of inoculation over the previously described aerosol system (Pacheco *et al.*, 2010). Recent works from our laboratory have demonstrated that this method of inoculation is similarly effective in sheep (Stenfeldt *et al.*, 2015b), but ineffective in pigs which are more effectively inoculated by virus deposition in the oropharynx (Stenfeldt *et al.*, 2014a).

In the current study, clinical and virological parameters of FMD were similar in INP-inoculated cattle when compared to cattle exposed by contact exposure. The greatest disparity between INP and contact experiments occurred with the A24 Cruzeiro infectious clone virus wherein the progression of FMD was delayed more than for the other viruses. Overall, antemortem infection dynamics and lesion scores were more synchronous within groups of cattle exposed to viruses of known higher virulence in cattle (in this case O1 Manisa and Asia1 Shamir), while less synchronous and with milder clinical disease in steers infected by the less virulent A24 Cruzeiro infectious clone. Subsequent work in our laboratory has indicated that a

bovine-derived strain of A24 Cruzeiro was highly synchronous and virulent when inoculated by INP in cattle, even at a lower dose than utilized herein (10^5 BTID₅₀) (Eschbaumer *et al.*, 2016; Stenfeldt *et al.*, 2015a; Stenfeldt *et al.*, 2016). These results indicate that a virus of comparatively low virulence may be more affected by the host immune defenses, and may thus generate more variable infection dynamics when used in natural or simulated-natural exposure systems. This observation is also consistent with recent work performed in our laboratory in which the pathogenesis and host response of an attenuated FMDV strain was contrasted to the parental wild type virus (Arzt *et al.*, 2014). This study demonstrated that while the attenuated virus was capable of causing generalized clinical disease when administered by needle inoculation, this virus was not able to overcome the innate host defense and cause clinical disease when delivered by aerosol inoculation.

The studies described herein demonstrate that either pigs or cattle may be used effectively as a source of infectious virus in direct contact systems for FMDV studies in cattle, as has been demonstrated in previous studies. (Alexandersen *et al.*, 2001; Charleston *et al.*, 2011; Eble *et al.*, 2004; Pacheco *et al.*, 2016; Stenfeldt *et al.*, 2012). For two viruses (Asia1 Shamir and O1 Manisa), use of pigs as donors led to more rapid onset of clinical signs, whereas use of cattle donors resulted in more synchronous progression. Contrastingly, for A24, the onset of FMD was more rapid and more synchronous in the cattle-to-cattle system. Direct contact systems have the advantage of providing the most natural conditions of infection, but disadvantages include added cost of additional animals and ethical concerns related to the requirements of additional animals to function as virus donors. In this regard, the use of pigs as donors may be advantageous due to reduced space requirements for smaller animals within containment facilities as well as lower costs for purchase of animals.

Different routes of virus exposure can result in substantially different outcomes of experimental FMDV studies. It is therefore important that the precise objectives of any experimental study, including the impact of potential confounding factors, are carefully considered before deciding on the study design. Needle-injection-based, inoculation systems provide highly repeatable models of infection and are currently required by OIE for vaccine testing; however, the natural and simulated natural exposure systems described in the current study provide more accurate representations of field conditions. We have demonstrated herein how systems based on direct contact exposure can be standardized to minimize variation across studies. Additionally, a novel system of intra-nasopharyngeal deposition of virus (INP-inoculation) was developed as an alternative, simulated-natural exposure system that enables control of inoculation dose while retaining the natural route of exposure and minimizing the numbers of animals required. This inoculation system has subsequently been used in numerous studies in our laboratory with larger numbers of animals, providing further demonstration of the system's utility as a challenge method for studies of FMDV in cattle (Eschbaumer *et al.*, 2016; Stenfeldt *et al.*, 2015a; Stenfeldt *et al.*, 2016).

4. Acknowledgments

This work was funded in part by CRIS project 1940-32000-057-00D (USDA, Agricultural Research Service), as well as through an interagency agreement with the Science and Technology Directorate of the U.S. Department of Homeland Security under Award Number HSHQDC-11-X-00189. CS was recipient of PIADC Research Participation Program fellowships, administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement with the U.S. Department of Energy. The sponsors had no involvement in the study design, in the collection, analysis and interpretation of data, in the

writing of the manuscript, or in the decision to submit the manuscript for publication. The authors acknowledge and appreciate expert laboratory support provided by George R. Smoliga, Ethan J. Hartwig, Steven J. Pauszek, and Elizabeth Bishop.

5. Conflict of interest

None.

6. References

- Alexandersen, S., Oleksiewicz, M. B. and Donaldson, A. I. (2001). The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. *J Gen Virol*, **82**, 747-755.
- Arzt, J., Pacheco, J. M. and Rodriguez, L. L. (2010a). The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation. Identification of the nasopharynx as the primary site of infection. *Vet Pathol*, **47**, 1048-1063.
- Arzt, J., Pacheco, J. M., Smoliga, G. R., Tucker, M. T., Bishop, E., Pauszek, S. J., Hartwig, E. J., de los Santos, T. and Rodriguez, L. L. (2014). Foot-and-mouth disease virus virulence in cattle is co-determined by viral replication dynamics and route of infection. *Virology*, **452-453**, 12-22.
- Arzt, J., White, W. R., Thomsen, B. V. and Brown, C. C. (2010b). Agricultural diseases on the move early in the third millennium. *Vet Pathol*, **47**, 15-27.
- Blackwell, J. H., McKercher, P. D., Kosikowski, F. V., Carmichael, L. E. and Gorewit, R. C. (1982). Concentration of foot-and-mouth disease virus in milk of cows infected under simulated field conditions. *J Dairy Sci*, **65**, 1624-1631.
- Bouma, A., Dekker, A. and de Jong, M. C. (2004). No foot-and-mouth disease virus transmission between individually housed calves. *Vet Microbiol*, **98**, 29-36.
- Brooksby, J. B. (1950). Strains of the virus of foot-and-mouth disease showing natural adaptation to swine. *J Hyg (Lond)*, **48**, 184-195.

468 Brown, C. C., Meyer, R. F., Olander, H. J., House, C. and Mebus, C. A. (1992). A pathogenesis
469 study of foot-and-mouth disease in cattle, using in situ hybridization. *Can J Vet Res*, **56**,
470 189-193.

471 Brown, C. C., Piccone, M. E., Mason, P. W., McKenna, T. S. and Grubman, M. J. (1996).
472 Pathogenesis of wild-type and leaderless foot-and-mouth disease virus in cattle. *J Virol*,
473 **70**, 5638-5641.

474 Burrows, R. (1966). The infectivity assay of foot-and-mouth disease virus in pigs. *J Hyg (Lond)*,
475 **64**, 419-429.

476 Burrows, R., Mann, J. A., Garland, A. J., Greig, A. and Goodridge, D. (1981). The pathogenesis
477 of natural and simulated natural foot-and-mouth disease infection in cattle. *J Comp*
478 *Pathol*, **91**, 599-609.

479 Callahan, J. D., Brown, F., Osorio, F. A., Sur, J. H., Kramer, E., Long, G. W., Lubroth, J., Ellis,
480 S. J., Shoulars, K. S., Gaffney, K. L., Rock, D. L. and Nelson, W. M. (2002). Use of a
481 portable real-time reverse transcriptase-polymerase chain reaction assay for rapid
482 detection of foot-and-mouth disease virus. *J Am Vet Med Assoc*, **220**, 1636-1642.

483 Charleston, B., Bankowski, B. M., Gubbins, S., Chase-Topping, M. E., Schley, D., Howey, R.,
484 Barnett, P. V., Gibson, D., Juleff, N. D. and Woolhouse, M. E. (2011). Relationship
485 between clinical signs and transmission of an infectious disease and the implications for
486 control. *Science*, **332**, 726-729.

487 Cox, S. J., Voyce, C., Parida, S., Reid, S. M., Hamblin, P. A., Paton, D. J. and Barnett, P. V.
488 (2005). Protection against direct-contact challenge following emergency FMD
489 vaccination of cattle and the effect on virus excretion from the oropharynx. *Vaccine*, **23**,
490 1106-1113.

491 Donaldson, A. I. and Alexandersen, S. (2002). Predicting the spread of foot and mouth disease
492 by airborne virus. *Rev Sci Tech*, **21**, 569-575.

493 Donaldson, A. I., Alexandersen, S., Sorensen, J. H. and Mikkelsen, T. (2001). Relative risks of
494 the uncontrollable (airborne) spread of FMD by different species. *Vet Rec*, **148**, 602-604.

495 Donaldson, A. I. and Ferris, N. P. (1980). Sites of release of airborne foot-and-mouth disease
496 virus from infected pigs. *Res Vet Sci*, **29**, 315-319.

497 Donaldson, A. I., Gibson, C. F., Oliver, R., Hamblin, C. and Kitching, R. P. (1987). Infection of
498 cattle by airborne foot-and-mouth disease virus: minimal doses with O1 and SAT 2
499 strains. *Res Vet Sci*, **43**, 339-346.

500 Dunn, C. S. and Donaldson, A. I. (1997). Natural adaption to pigs of a Taiwanese isolate of foot-
501 and-mouth disease virus. *Vet Rec*, **141**, 174-175.

502 Eble, P. L., Bouma, A., de Bruin, M. G., van Hemert-Kluitenberg, F., van Oirschot, J. T. and
503 Dekker, A. (2004). Vaccination of pigs two weeks before infection significantly reduces
504 transmission of foot-and-mouth disease virus. *Vaccine*, **22**, 1372-1378.

505 Eschbaumer, M., Stenfeldt, C., Pacheco, J. M., Rekant, S. I. and Arzt, J. (2016). Effect of storage
506 conditions on subpopulations of peripheral blood T lymphocytes isolated from naive
507 cattle and cattle infected with foot-and-mouth disease virus. *Vet Clin Pathol*, **45**, 110-115.

508 Golde, W. T., Pacheco, J. M., Duque, H., Doel, T., Penfold, B., Ferman, G. S., Gregg, D. R. and
509 Rodriguez, L. L. (2005). Vaccination against foot-and-mouth disease virus confers
510 complete clinical protection in 7 days and partial protection in 4 days: Use in emergency
511 outbreak response. *Vaccine*, **23**, 5775-5782.

512 Gomes, I. and Auge de Mello, P. (1994). Ensaios de infecciosidade do vírus da febre aftosa. I.
513 Transmissão entre bovinos infectados e contatos e suínos. . *Boletín del Centro*
514 *Panamericano de Fiebre Aftosa*, **60**, 36-48.

515 Graves, J. H., McVicar, J. W., Suttmoller, P. and Trautman, R. (1971). Contact transmission of
516 foot-and-mouth disease from infected to susceptible cattle. *J Infect Dis*, **123**, 386-391.

517 Henderson, W. M. (1949). The quantitative study of foot-and-mouth disease virus. *Report Series*
518 *Agricultural Research Council, London, no. 8, Her Majesty's Stationery Office*.

519 Henderson, W. M. (1952). A comparison of different routes of inoculation of cattle for detection
520 of the virus of foot-and-mouth disease. *J Hyg (Lond)*, **50**, 182-194.

521 Howey, R., Bankowski, B., Juleff, N., Savill, N. J., Gibson, D., Fazakerley, J., Charleston, B. and
522 Woolhouse, M. E. (2012). Modelling the within-host dynamics of the foot-and-mouth
523 disease virus in cattle. *Epidemics*, **4**, 93-103.

524 Juleff, N., Valdazo-Gonzalez, B., Wadsworth, J., Wright, C. F., Charleston, B., Paton, D. J.,
525 King, D. P. and Knowles, N. J. (2013). Accumulation of nucleotide substitutions
526 occurring during experimental transmission of foot-and-mouth disease virus. *J Gen Virol*,
527 **94**, 108-119.

528 Knight-Jones, T. J. and Rushton, J. (2013). The economic impacts of foot and mouth disease -
529 what are they, how big are they and where do they occur? *Prev Vet Med*, **112**, 161-173.

530 Korn, G. (1957). Experimentelle untersuchungen zum virusnachweis im inkubationsstadium der
531 maul-und klauenseuche und zu ihrer pathogenese. *Archiv für experimentelle*
532 *veterinarmedizin*, **11**, 637-649.

533 McVicar, J. W. and Eisner, R. J. (1983). Aerosol exposure of cattle to foot-and-mouth disease
534 virus. *J Hyg (Lond)*, **91**, 319-328.

535 McVicar, J. W., J.H., G. and Suttmoller, P. (1970). Growth of foot-and-mouth disease virus in the
536 bovine pharynx. *Proceedings of the 74th Annual Meeting of the United States Animal*
537 *Health Association*, 230-234.

538 McVicar, J. W. and Suttmoller, P. (1974). Neutralizing activity in the serum and oesophageal-
539 pharyngeal fluid of cattle after exposure to foot-and-mouth disease virus and subsequent
540 re-exposure. *Arch Gesamte Virusforsch*, **44**, 173-176.

541 OIE. (2012). In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, World
542 Organization for Animal Health, Paris, France, pp. 145-173.

543 Orsel, K., de Jong, M. C., Bouma, A., Stegeman, J. A. and Dekker, A. (2007). The effect of
544 vaccination on foot and mouth disease virus transmission among dairy cows. *Vaccine*, **25**,
545 327-335.

546 Orsel, K., Dekker, A., Bouma, A., Stegeman, J. A. and de Jong, M. C. (2005). Vaccination
547 against foot and mouth disease reduces virus transmission in groups of calves. *Vaccine*,
548 **23**, 4887-4894.

549 Pacheco, J. M., Arzt, J. and Rodriguez, L. L. (2010). Early events in the pathogenesis of foot-
550 and-mouth disease in cattle after controlled aerosol exposure. *Vet J*, **183**, 46-53.

551 Pacheco, J. M., Lee, K. N., Eschbaumer, M., Bishop, E. A., Hartwig, E. J., Pauszek, S. J.,
552 Smoliga, G. R., Kim, S. M., Park, J. H., Ko, Y. J., Lee, H. S., Tark, D., Cho, I. S., Kim,
553 B., Rodriguez, L. L. and Arzt, J. (2016). Evaluation of Infectivity, Virulence and
554 Transmission of FDMV Field Strains of Serotypes O and A Isolated In 2010 from
555 Outbreaks in the Republic of Korea. *PLoS One*, **11**, e0146445.

556 Pacheco, J. M. and Mason, P. W. (2010). Evaluation of infectivity and transmission of different
557 Asian foot-and-mouth disease viruses in swine. *J Vet Sci*, **11**, 133-142.

558 Pacheco, J. M., Tucker, M., Hartwig, E., Bishop, E., Arzt, J. and Rodriguez, L. L. (2012). Direct
559 contact transmission of three different foot-and-mouth disease virus strains in swine
560 demonstrates important strain-specific differences. *Vet J*, **193**, 456-463.

561 Quan, M., Murphy, C. M., Zhang, Z., Durand, S., Esteves, I., Doel, C. and Alexandersen, S.
562 (2009). Influence of exposure intensity on the efficiency and speed of transmission of
563 Foot-and-mouth disease. *J Comp Pathol*, **140**, 225-237.

564 Rasmussen, T. B., Uttenthal, A., de Stricker, K., Belak, S. and Storgaard, T. (2003).
565 Development of a novel quantitative real-time RT-PCR assay for the simultaneous
566 detection of all serotypes of foot-and-mouth disease virus. *Arch Virol*, **148**, 2005-2021.

567 Rieder, E., Henry, T., Duque, H. and Baxt, B. (2005). Analysis of a foot-and-mouth disease virus
568 type A24 isolate containing an SGD receptor recognition site in vitro and its pathogenesis
569 in cattle. *J Virol*, **79**, 12989-12998.

570 Salt, J. S., Mulcahy, G. and Kitching, R. P. (1996). Isotype-specific antibody responses to foot-
571 and-mouth disease virus in sera and secretions of 'carrier' and 'non-carrier' cattle.
572 *Epidemiol Infect*, **117**, 349-360.

573 Sanz-Parra, A., Vazquez, B., Sobrino, F., Cox, S. J., Ley, V. and Salt, J. S. (1999). Evidence of
574 partial protection against foot-and-mouth disease in cattle immunized with a recombinant
575 adenovirus vector expressing the precursor polypeptide (P1) of foot-and-mouth disease
576 virus capsid proteins. *J Gen Virol*, **80** (Pt 3), 671-679.

- Stenfeldt, C., Eschbaumer, M., Pacheco, J. M., Rekant, S. I., Rodriguez, L. L. and Arzt, J. (2015a). Pathogenesis of Primary Foot-and-Mouth Disease Virus Infection in the Nasopharynx of Vaccinated and Non-Vaccinated Cattle. *PLoS One*, **10**, e0143666.
- Stenfeldt, C., Eschbaumer, M., Rekant, S. I., Pacheco, J. M., Smoliga, G. R., Hartwig, E. J., Rodriguez, L. L. and Arzt, J. (2016). The foot-and-mouth disease carrier state divergence in cattle. *J Virol*.
- Stenfeldt, C., Heegaard, P. M., Stockmarr, A. and Belsham, G. J. (2012). Modulation of cytokine mRNA expression in pharyngeal epithelial samples obtained from cattle infected with foot-and-mouth disease virus. *J Comp Pathol*, **146**, 243-252.
- Stenfeldt, C., Heegaard, P. M., Stockmarr, A., Tjornehoj, K. and Belsham, G. J. (2011). Analysis of the acute phase responses of Serum Amyloid A, Haptoglobin and Type 1 Interferon in cattle experimentally infected with foot-and-mouth disease virus serotype O. *Vet Res*, **42**, 66.
- Stenfeldt, C., Pacheco, J. M., Rodriguez, L. L. and Arzt, J. (2014a). Infection dynamics of foot-and-mouth disease virus in pigs using two novel simulated-natural inoculation methods. *Res Vet Sci*, **96**, 396-405.
- Stenfeldt, C., Pacheco, J. M., Singanallur, N. B., Ferreira, H. C., Vosloo, W., Rodriguez, L. L. and Arzt, J. (2015b). 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. *Vet Microbiol*, **178**, 50-60.
- Stenfeldt, C., Pacheco, J. M., Smoliga, G. R., Bishop, E., Pauszek, S. J., Hartwig, E. J., Rodriguez, L. L. and Arzt, J. (2014b). Detection of Foot-and-mouth Disease Virus RNA and Capsid Protein in Lymphoid Tissues of Convalescent Pigs Does Not Indicate Existence of a Carrier State. *Transbound Emerg Dis*.
- Sutmoller, P., McVicar, J. W. and Cottral, G. E. (1968). The epizootiological importance of foot-and-mouth disease carriers. I. Experimentally produced foot-and-mouth disease carriers in susceptible and immune cattle. *Arch Gesamte Virusforsch*, **23**, 227-235.
- Zhang, Z., Bashiruddin, J. B., Doel, C., Horsington, J., Durand, S. and Alexandersen, S. (2006). Cytokine and Toll-like receptor mRNAs in the nasal-associated lymphoid tissues of cattle during foot-and-mouth disease virus infection. *J Comp Pathol*, **134**, 56-62.

Figure 1.

Demonstration of procedure and relevant anatomy of intra-nasopharyngeal inoculation with catheter inserted through one nostril into the nasopharynx. A) Length of catheter insertion estimated by measuring distance from tip of the nose to the eye. B) Position of steer's head and

syringe/catheter placement for inoculation. C) Sagittal section of steer's head to demonstrate internal anatomic position of the catheter during inoculation. The nasopharynx is the space bound by the dorsal surface of the soft palate (green arrowheads), nasopharyngeal tonsil (NT), and dorsal surface of nasopharynx (orange arrowheads). PT (palatine tonsil), LT (lingual tonsil), E (epiglottis), Eso (esophagus). D) Magnification of nasopharyngeal region facilitates viewing of the catheter position.

Figure 2.

Graphs illustrating the dynamics of FMDV infection with the Asia1 Shamir strain by five routes of exposure: intra-nasopharyngeal inoculation of cattle (2A), intradermal inoculation of pigs (2B1), pig-to-cattle contact exposure (2B2), and cattle-to-cattle contact exposure (2C) including direct-inoculated donors (DI) and contact exposed (CE) recipients. Animal ID is indicated on the top left corner of each figure, preceded with a B for bovine or a P for porcine. Number in parenthesis in animals belonging to group 2A indicates the dose of virus inoculated. Time on the X-axes is in days post inoculation or days post contact, except for 'p.i.' which indicates samples collected immediately post inoculation and '6 h' that indicates samples were collected 6 hours post inoculation. Charted lines represent $\log_{10}$ RNA copy numbers per ml of FMDV RNA expressed in the left Y-axes. Bars represent a cumulative index of FMD lesion score expressed in the right Y-axes. Shaded yellow areas in Fig. 2B and 2C indicate the period of direct contact between donor and recipient animals.

Figure 3.

Graphs illustrating the dynamics of FMDV infection with the O1 Manisa strain by five routes of exposure: intra-nasopharyngeal inoculation of cattle (3A), intradermal inoculation of pigs (3B1), pig-to-cattle contact exposure (3B2), and cattle-to-cattle contact exposure (3C) including direct-inoculated donors (DI) and contact exposed (CE) recipients. Animal ID is indicated on the top left corner of each figure, preceded with a B for bovine or a P for porcine. Number in parenthesis in animals belonging to group 3A indicates the dose of virus inoculated. Time on the X-axes is in days post inoculation or days post contact, except for 'p.i.' which indicates samples collected immediately post inoculation and '6 h' that indicates samples were collected 6 hours post inoculation. Charted lines represent $\log_{10}$ RNA copy numbers per ml of FMDV RNA expressed in the left Y-axes. Bars represent a cumulative index of FMD lesion score expressed in the right Y-axes. Shaded yellow areas in Fig. 3B and 3C indicate the period of direct contact between donor and recipient animals.

Figure 4.

Graphs illustrating the dynamics of FMDV infection with the A24 Cruzeiro strain by five routes of exposure: intra-nasopharyngeal inoculation of cattle (4A), intradermal inoculation of pigs (4B1), pig-to-cattle contact exposure (4B2), and cattle-to-cattle contact exposure (4C) including direct-inoculated donors (DI) and contact exposed (CE) recipients. Animal ID is indicated on the top left corner of each figure, preceded with a B for bovine or a P for porcine. Number in parenthesis in animals belonging to group 4A indicates the dose of virus inoculated. Time on the X-axes is in days post inoculation or days post contact, except for 'p.i.' which indicates samples collected immediately post inoculation and '6 h' that indicates samples were collected 6 hours post inoculation. Charted lines represent $\log_{10}$ RNA copy numbers per ml of FMDV RNA expressed in the left Y-axes. Bars represent a cumulative index of FMD lesion score expressed in

656 the right Y-axes. Shaded yellow areas in Fig. 4B and 4C indicate the period of direct contact
657 between donor and recipient animals.

658

Table 1**Doses of FMDV used for direct inoculation of cattle**

		<i>Inoculation route</i>	
		<i>INP</i> [*]	<i>IEL</i> [†]
FMDV strain	Asia1 Shamir	10 ⁶ or 10 ⁷ TCID ₅₀ [‡]	10 ⁴ TCID ₅₀
	O1 Manisa	10 ⁶ BTID ₅₀ [§]	10 ⁴ BTID ₅₀
	A24 Cruzeiro	10 ⁶ or 10 ⁷ TCID ₅₀	10 ⁴ TCID ₅₀

^{*}INP, intranasopharyngeal

[†]IEL, intraepitheliolinguar

[‡]TCID₅₀, tissue culture infectious doses 50%, calculated by the Spearman-Kärber method, performed in BHK cells, as previously described (Pacheco *et al.*, 2012).

[§]BTID₅₀, bovine tongue infectious doses 50%, calculated by the Reed and Muench method, as previously described (Golde *et al.*, 2005).

Figure1

Figure

FMDV Asia1 Shamir

Figure

FMDV O1 Manisa

Figure

FMDV A24 Cruzeiro