RESEARCH REPORT
SWINE HEALTH

Title: Investigating potential existence of chronic, persistent foot-and-mouth disease virus infection in domestic pigs; implications for disease control strategies - **NPB # 11-174**

Investigator: Jonathan Arzt

Institution: Agricultural Research Service, USDA, Plum Island Animal Disease Center

Support Scientists: Carolina Stenfeldt, Juan Pacheco

Date Submitted: February 18, 2014

Industry Summary

The severe measures routinely enacted for control of foot-and-mouth disease (FMD) outbreaks are based upon extreme contagiousness of the disease, rapidity of spread, and fear of residual contagion associated with long-term asymptomatic virus carriers. It is well established that many ruminant species are capable of this prolonged carrier state; however, it is largely believed that pigs are incapable of becoming FMD virus (FMDV) carriers. The purpose of the work described herein was to investigate the potential for pigs to become long-term carriers of FMDV with the ultimate goal of using this information to design rational approaches to species-specific outbreak response planning.

A large proportion of FMD research has focused on pathogenesis and prevention in cattle. The work performed within this project contributes detailed knowledge of the early and late stages of FMDV infection in pigs, which is critical to development of improved FMDV countermeasures for use in pigs.

The FMDV carrier state in ruminants (cattle, sheep, etc) is a period subsequent to clinical disease in which animals carry and shed infectious FMDV for long periods without showing any symptoms of the disease. Because of this, an animal that has recovered from the disease, or that has been protected from developing disease by vaccination, is treated as if it may still be capable of spreading the virus. This means that large numbers of healthy animals are routinely destroyed during FMD outbreaks in countries that are normally free of the disease (USA, UK, France, etc). Based on limited data, it has become generally accepted as 'conventional wisdom' that pigs are not capable of becoming long term carriers of FMDV. However, during recent years, there have been a limited number of scientific publications that have suggested otherwise. The main purpose of the work performed within this project has been to thoroughly investigate if pigs are capable of harboring infectious FMDV for extended time after recovery from the disease.

To achieve these goals, pigs were experimentally inoculated with one of five distinct strains of FMDV. Infection dynamics were characterized for periods ranging from 6 hours to 100 days after which pigs were euthanized for tissue collection. These investigations demonstrated that infectious virus could not be detected in blood or secretions from pigs once the clinical signs of disease had disappeared. Furthermore, live virus was not detected in tissue samples obtained at 35-100 days after infection from pigs that had recovered from the disease.

Although live virus was not recovered, viral degradation products (RNA and structural proteins) could be detected in select lymph nodes harvested at 35 days after infection. This detection of viral remnants declined markedly by 60 days after infection, and was completely absent at 100 days post infection.

The data described herein provides the most extensive investigation of potential FMDV persistence in pigs. The overall conclusion is that domestic pigs are unlikely to carry infectious FMDV for prolonged periods after recovery from clinical disease. The significance of detection of viral degradation products in lymph nodes is debatable and has not been thoroughly investigated in the current studies. Overall, this work provides a basis for considering species-specific control plans. However, further research and validation would be required to enact such policy.

Keywords: Foot-and-mouth disease; FMD, FMDV, Virus; Persistence; Carriers; Pigs

Scientific Abstract

A systematic study was performed to investigate the potential of pigs to maintain persistent FMDV infection. The first step in the path to understand late phase of infection was to determine the infection dynamics of the early stages of FMD. Tissue distribution of virus at early time points indicated that oropharyngeal tonsils (specifically the lingual tonsils and paraepiglottic tonsils) function as initial sites of viral replication. Viremia and substantial shedding of virus in oral and nasal secretions was detected 24 hours prior to the appearance of clinical lesions. Following development of viremia, there was extensive dissemination, and infectious virus could be recovered from essentially all tissues analyzed.

After resolution of clinical infection, infectious virus could not be recovered from sera, oral, nasal- or oropharyngeal fluids. Furthermore, there was no isolation of live virus from tissue samples harvested at 28-100 days post infection from convalescent pigs recovered from clinical or subclinical FMD. Despite lack of detection of infectious FMDV, there was a high prevalence of FMDV RNA detection in lymph nodes draining lesion sites harvested at 35 days post infection (dpi), with the most frequent detection recorded in popliteal lymph nodes (positive detection in 88% of samples obtained from non-vaccinated pigs). Similarly, at 35 dpi, FMDV capsid antigen was localized within draining lymph nodes, but without concurrent detection of FMDV non-structural protein. There was a marked decline in detection of FMDV RNA and antigen in tissue samples by 60 dpi, and no antigen or viral RNA could not be detected in samples obtained at 100 dpi.

The work performed within this project provides information of the mechanisms of early FMDV infection in pigs that is critical for future development of improved products for FMDV countermeasure. Moreover, the data provides the most extensive investigation of FMDV persistence in pigs. The overall conclusion is that domestic pigs are unlikely to be competent long term carriers of infectious FMDV; however, transient persistence of FMDV degradation products in lymphoid tissues is common following clinical or subclinical infection.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious infection of cloven-hoofed animals with a renowned ability of rapid transmission amongst susceptible hosts. An outbreak of FMD in FMD-free countries leads to an immediate standstill of agricultural production, with prolonged restrictions on export of animal products.

The characteristic clinical manifestations of FMD, which includes blanching and vesiculation of cornified epithelium within the oral cavity and in areas of non-haired skin, are similar across a wide range of susceptible host-species, including domestic and wild ruminants and pigs [1-3]. Despite several commonalties of the clinical

infection, there are certain features of FMDV pathogenesis that differ across distinct host-species. In contrast to recently gained knowledge characterizing acute FMDV-infection in cattle [4, 5], precise determination of the initial sites of virus entry and subsequent mechanisms involved in generalization of infection, have not yet been fully elucidated in pigs [3]. Improved knowledge of the mechanisms involved in the initial events of FMD infection in pigs is critical to facilitate future development of vaccines and biotherapeutic countermeasures to improve protection against infection in this species.

Another important factor in the control of FMD is the occurrence of persistently infected, asymptomatic, so called "carriers" amongst ruminant species. Experimental studies have concluded that up to 50- 100% of vaccinated and naïve cattle, can develop into FMDV-carriers following either clinical or subclinical infection [6-9] . The epidemiological importance of the FMDV carrier state has been a subject of some controversy [3, 10-14]. However, the occurrence of persistently infected animals, which may constitute an enduring source of infectious virus following a disease outbreak, is of considerable importance for international trade regulations. Specifically, in order to regain "FMD-free" status following an outbreak, affected countries must prove freedom of infection through substantial diagnostic screening of susceptible livestock populations. Due to the existence of a subclinical FMD carrier state in ruminants, any animal with antibodies against non-structural FMDV proteins, indicating previous exposure to replicating virus, is considered a potential carrier of infectious virus. Consequently, the FMD carrier state is a factor that highly influences national policies directing FMD countermeasures in areas in which the disease is not endemic.

No study has ever demonstrated a true FMDV carrier state in pigs, and it is generally accepted that suids are not competent hosts for FMDV persistence [3, 15]. However, this conventional wisdom has been challenged by a limited number of experimental studies which have demonstrated detection of FMDV RNA in serum or lymphoid tissues for periods extending the accepted threshold for FMDV persistence of 28 dpi [16-19].

Substantiated evidence that swine cannot support prolonged persistence of infectious FMDV could justify implementation of species-specific FMDV response measures. Specifically, such adaptations in national policies and international trade regulations could save valuable breeding stock and mitigate the financial impacts of FMD outbreaks. Additionally, elucidation of the possibility of persistence of FMDV in swine is important for countries in which growing populations of wild suids could potentially become a wildlife reservoir of FMDV upon incursion of the virus.

The work performed within this project included initial development of an experimental model optimized for studies of FMDV pathogenesis in pigs. This model is based on a simulated-natural inoculation system that mimics natural exposure conditions and enables precise control of timing and dose of exposure whilst also reducing the number of animals needed for the investigations.

The system of intra-oropharyngeal (IOP) inoculation was utilized to investigate the initial events of FMDV infection in pigs. Infection dynamics through the early phase of disease were characterized through intensive monitoring of viral detection in sera and oral and nasal secretions. Tissue distribution of virus during the early phase of infection was determined through necropsies performed at pre-determined time points corresponding to 6, 12, 24, 48 and 78 hours post infection (hpi).

The potential of pigs to support long term persistence of FMDV was investigated through experimental studies in which pigs were infected with either of five different FMDV strains, representing serotypes O, A and Asia1. The data presented includes analyses of infection dynamics through experimental periods of 28 to 100 dpi as well as the outcome of a systematic investigation of FMDV persistence in porcine tissue samples harvested during what has been defined for cattle as the persistent phase of infection (beyond 28 dpi).

Stated Objectives from original proposal

(a) Determine optimal route of direct inoculation of donor pigs for contact experiments; compare efficacy of intra-oral (IO) and heel bulb intradermal (HBI) FMDV inoculation as administration route using FMDV, serotype O.

(b) Characterize FMDV acute pathogenesis parameters (shedding, viremia, tissue-specific distribution) of infection in contact transmission studies using FMDV, serotype O.

(c) Characterize FMDV post-acute (i.e. suspect persistent) pathogenesis parameters (shedding, tissue-specific distribution) of infection in contact transmission studies using FMDV, serotype O.

(d) Characterize FMDV post-acute (i.e. suspect persistent) pathogenesis parameters (shedding, tissue-specific distribution) of infection in contact transmission studies using FMDV, serotypes A, and Asia1.

Materials and Methods

Animal experiments

Initial experimental studies included development and optimization of novel systems for intra-oropharyngeal (IOP) and intra-nasopharyngeal (INP) inoculation of swine with FMDV serotypes O and A. Results of these trials indicated that the IOP system was highly efficient in generating reproducible and synchronous clinical infection suitable for experimental studies. This simulated-natural inoculation system preserves the natural route of FMDV infection whilst allowing the advantages of precise control of dose and timing of challenge. Following modifications of the original project plans (included in the Annual report submitted December 2012) it was decided that the new IOP inoculation system should replace contact challenge for the majority of the remaining challenge studies.

Experimental studies with the objective of investigating the acute pathogenesis of FMDV with detailed investigations of antemortem infection dynamics have been performed using both FMDV serotypes O and A. Following initial trials, it was decided to focus on continued experimental studies using FMDV serotype A for the objective of achieving a map of the temporal distribution of FMDV in porcine tissues during acute infection, as this would reduce variability and thereby generate a data set of superior power.

Investigations of the early pathogenesis of FMDV A infection in pigs, including characterization of early phase infection dynamics and tissue distribution of virus was performed using a total number of 19 pigs. To facilitate intensive monitoring of antemortem infection dynamics, as well as harvest of tissue samples at desired time points, animal experiments were performed following a staggered design, with 2-6 pigs in separate groups that were subjected to the exact same experimental protocol. Two-three pigs were euthanized on each of the following time points; 6, 12, 24, 48 and 78 hpi. An additional 4 pigs were kept alive through 96 hpi to enable monitoring of infection dynamics throughout the peak of clinical infection. Pigs were subjected to standardized necropsies with collection of up to 47 distinct tissue samples per pig and time point.

FMDV late phase infection dynamics were investigated through 35 dpi in animals infected with FMDV O1 Manisa (12 pigs), FMDV A24 Cruzeiro (8 pigs), FMDV O/SKR/2010 (6 pigs), FMDV A/SKR/2010 (4 pigs), and FMDV Asia-1 Shamir (4 pigs). Additional studies spanning experimental periods of 60 dpi (2 pigs) and 100 dpi (4 pigs) respectively, were performed with pigs infected with FMDV A24 Cruzeiro. A separate study investigated antemortem infection dynamics through 28-30 dpi in pigs that had been vaccinated and subsequently challenged with FMDV A24 Cruzeiro (7 pigs). Tissue distribution of FMDV at the time of euthanasia (28 to 100dpi) was investigated in all pigs included in the studies mentioned above.

In order to evaluate potential infectivity of pigs recovered from clinical FMD infection, two pigs that had recovered from clinical infection with FMDV A24 Cruzeiro were co-mingled for 21 days, from 40 to 61 dpi, with a group of four age-matched naïve sentinels.

Antemortem sample collection

A standardized protocol for sampling and clinical evaluation was followed throughout the experiments. Blood samples were collected from the jugular vein, nasal swabs were obtained using small cotton swabs, and tonsil swabs were obtained through direct targeting of the tonsil of the soft palate using a larger cotton swab. The progression of the clinical infection (lesion distribution) was quantitated using a previously described scoring system [20, 21]. In brief, each of 16 digits showing a characteristic FMDV lesion contributed one point towards a cumulative score, with additional single points counted for lesions within the oral cavity, on the snout, on the lower lip, and on carpal / tarsal skin giving a maximum score of 20.

In order to monitor acute phase infection dynamics and development of clinical disease, pigs included in these experiments were intensively sampled and clinically evaluated through the first 96 hpi. Blood samples were collected at 6 hour intervals from 0 to 24 hpi, and at 12 h intervals subsequently. Collection of nasal and tonsil swabs, together with clinical evaluations, were performed directly before and after inoculation and subsequently at 2 hour intervals from 4 to 12 hpi, at 3 hour intervals from 12 to 24 hpi, and at 12 hour intervals thereafter.

In studies of longer duration (investigating late phase infection dynamics) blood samples were collected prior to inoculation, once daily for the first 10 days after inoculation, and once weekly thereafter. Swabs were collected prior to inoculation, once daily for 10 days, twice weekly from 14 to 35 dpi and once weekly subsequently for the studies of longer duration. Samples of oropharyngeal fluid (probang samples) were collected using a small probang cup [22], from animals infected with FMDV O1 Manisa, Asia-1 Shamir and A24 Cruzeiro at similar same intervals as outlined for swabs, but from14 dpi until termination of the experiments.

Post mortem sample collection

All pigs were subjected to a standardized necropsy protocol immediately following euthanasia, with collection of up to 47 distinct tissue samples. Each tissue sample was divided into three 30mg aliquots, which were placed in individual tubes before being frozen on liquid nitrogen. An adjacent specimen was collected from each tissue sample, that was embedded in optimal cutting temperature media and frozen above a bath of liquid nitrogen. Frozen tissue samples were transferred to the lab within two hours after collection, and were stored at -70^{o} C until further processing.

FMDV RNA detection

Two aliquots of each tissue sample collected at necropsy were individually thawed and macerated. Tissue macerates, serum swabs and probang samples were analyzed using qRT-PCR, targeting the 3D region of the FMDV genome as previously described [4, 5].

Virus isolation

Macerated tissue sample were cleared from debris and potential bacterial contamination by centrifugation through filter columns. Samples were subsequently analyzed for infectious FMDV through virus isolation on LFBK αvβ6 cells, a cell line with demonstrated high sensitivity for FMDV isolation [23, 24], following a protocol previously described [5]. If cytopathic effect was detected, FMDV positivity was further investigated by qRT-PCR analysis of cell culture supernatants. When no cytopathic effect was observed, two blind passages were performed with the supernatants from the final passage analyzed by qRT-PCR before the samples were deemed negative. In addition to this, a subset of tissue samples, in which virus isolation was negative despite relatively high contents of FMDV RNA, were subjected to treatment with Trichlorotrifluorotethane (TTE) to evaluate if dissociation of immune-complexes could improve the ability to recover infectious virus [25].

Immunomicroscopy

Following screening for content of FMDV RNA using qRT-PCR, subsequent detection of antigen in cryosections was performed on select specimens by immunohistochemistry (IHC) and multichannel immunoflourescense (MIF) as previously described [4, 26].

Results

Objective A

Optimization of inoculation system

Initial animal experiments were performed with the objective of determining an optimal route of FMDV inoculation of pigs for use in experimental studies. Results of these investigations indicated that the simulated-natural inoculation system of intra-oropharyngeal (IOP) inoculation was highly efficient in generating reproducible and synchronous clinical infection suitable for experimental studies. This simulated-natural inoculation system preserves the natural route of FMDV infection whilst allowing the advantages of precise control of dose and timing of challenge.

Objective B

Acute phase infection dynamics and tissue distribution of virus

The optimized system of IOP inoculation was utilized to investigate acute phase FMDV infection dynamics in pigs infected with FMDV strains O1 Manisa and A24 Cruzeiro. The purpose of characterizing FMDV distribution in the acute phase was two-fold:

1. To provide anatomic landmarks to be further pursued as sites of persistence in the post-acute phase
2. To generate the most extensive characterization of tissue-specific FMDV-loads in pigs ever documented

Clinical infection

The progression of the clinical infection was highly synchronous in all animals that were kept alive long enough to develop clinical disease. Vesicular lesions were first detected in the coronary bands of the hind feet, where they were observed as early as 36 hpi in one animal. Vesicular lesions appeared approximately 8 hours before increased rectal temperature was observed (above 104^{o}F). All animals had coronary band vesicles on one or more feet, and rectal temperatures above 104^{o}F at 48 hpi. There was a substantial progression in lesion distribution from 48 to 96 hpi, with additional vesicles appearing on accessory digits, in the oral mucosa, on the snout and on the skin covering the tarsal and carpal joints. Coronary band vesicles spread to include the solar epithelium of the heel bulb which was sloughed and replaced with hyperkeratinized scar tissue in the animals that survived the acute phase of infection.

Acute phase infection dynamics

High levels of FMDV RNA could be detected in tonsil swabs collected directly following IOP inoculation (”p.i.” in Figure 1), interpreted as residual inoculum. Tonsil detection decreased during the initial 8 hpi, after which tonsil shedding gradually increased through the onset of clinical infection (at 36 to 48 hpi) to then maintain stable levels through the clinical phase of infection. Shedding in nasal swabs followed a pattern similar to that of tonsil swabs, but with a less pronounced peak following inoculation and lower detection levels during the pre-

clinical phase of infection. The difference in viral RNA levels between tonsil and nasal swabs decreased at the onset of clinical disease, with only marginally lower RNA quantities measured in nasal swabs through the clinical phase of infection (Figure 1A). FMDV RNA and infectious virus could be detected in serum from 18 hpi. There was an increase in viral RNA levels in serum until peak viremia was reached at 60 hpi, after which levels gradually declined.

Figure 1A

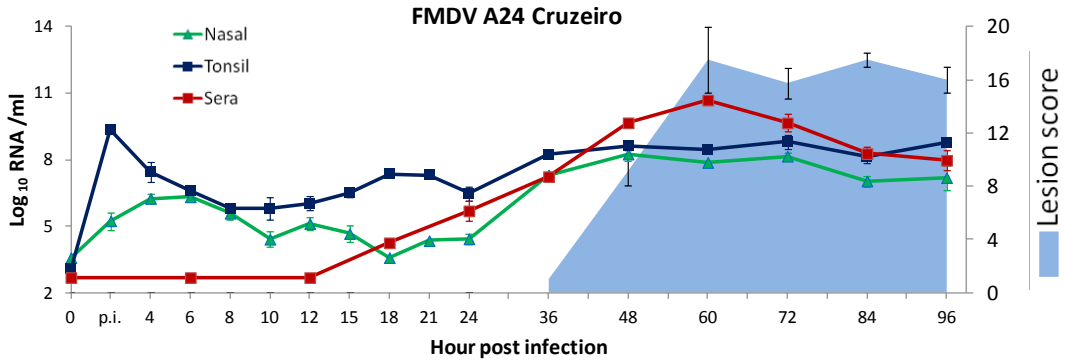


Figure 1B

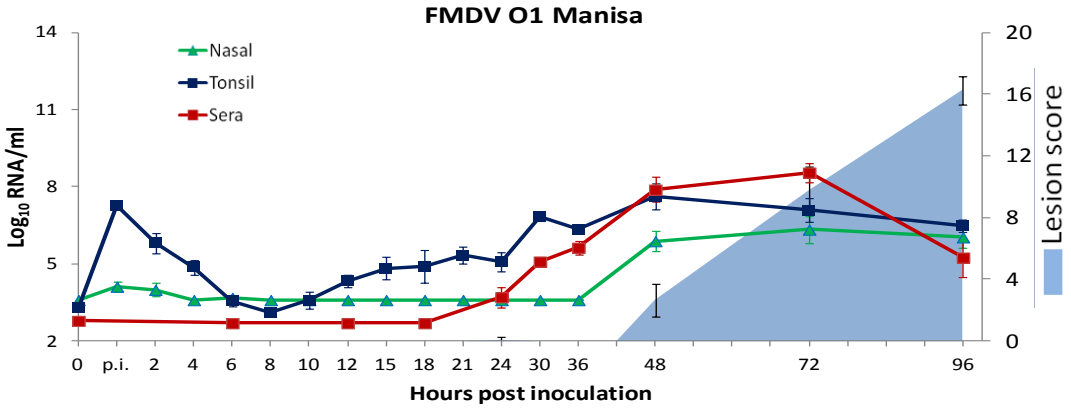


Figure 1 A-B. Antemortem dynamics of FMDV serotype A (a) and serotype O (b). Data presented are averaged values obtained from several pigs sampled at each time point. The pigs were infected through intra-oropharyngeal inoculation. Virus shedding in oral swabs, nasal swabs and virus presence in serum has been measured through quantitative realtime (qRT-PCR), and is presented as average log10 RNA-copies/ ml. Clinical score provides a quantitative index of FMD lesion distribution. p.i. = post inoculation indicates samples collected directly following inoculation

Acute phase tissue distribution of FMDV

Pigs were euthanized at pre-determined time points regardless of the progression of the clinical infection. Post mortem examinations included collection of up to 47 distinct tissue samples (table 1). In the 2 pigs that were

euthanized at 6 hpi, FMDV RNA and infectious virus was consistently detected in the lingual- and paraepiglottic tonsils. The dorsal soft palate contained low amounts of FMDV RNA in one of the two pigs, and infectious virus (without concurrent RNA detection) was detected at this site in the other pig. It is noteworthy that no virus or viral RNA was detected in the tonsil of the soft palate at this time point, despite this tonsil constituting the anatomical site targeted during inoculation. At 12 hpi, FMDV RNA could also be detected in the proximal mid lung from 1 out of 3 animals, whilst FMDV infectivity was still restricted to the pharyngeal region.

In order to determine which tissues were the primary sites of viral replication, the prevalence of virus detection, for both qRT-PCR and virus isolation, was determined in the pre-viremic pigs (including all pigs euthanized at 6 and 12 hpi). The highest detection prevalence was found in the paraepiglottic tonsil, with FMDV RNA detected in 4 out of 5 pigs (80%), and infectious virus in all 5 pigs investigated (100%). The detection prevalence for both viral RNA and infectious virus reached 60% for the lingual tonsil, whilst the corresponding number for the tonsil of the soft palate was only 20%.

There was a marked dissemination of virus detection in tissue samples following detection of viremia and onset of clinical infection (table 2). Due to the high levels of virus that are present in the blood during viremia, it is difficult to discern if detection of virus at distinct anatomic sites is evidence of viral replication or merely a result of contamination from the blood in viremic animals. The highest quantities of FMDV RNA were recovered from the coronary bands during the clinical phase of infection (up to 9.91 $\log_{10}$ GCN/mg). Neither viral RNA nor infectious virus could be recovered from this site in pigs that had not yet developed clinical lesions (despite some of these animals being early viremic).

Table 1. Tissue distribution of FMDV in pre-viremic pigs. Numbers represent genome copy numbers (GCN)/ mg of tissue. Bold numbers indicate that samples were positive for both FMDV RNA (qRT-PCR) and virus isolation, + indicates that virus isolation was positive but FMDV RNA content was below the limit of detection, - indicates double negative samples. * Visceral organs included liver, spleen, kidney, pancreas, ileum and peyers patches. Other tissues analyzed with consistently negative results were epiglottis, larynx, pharyngeal diverticulum salivary glands, adrenal glands, bone marrow

	6 HPI		12 HPI			% Positive		
Animal ID	**37400**	**37401**	**37896**	**37897**	**37403**	**qRT-PCR**	**VI**	**Mean GCN**
Sample identification								
Oral cavity/ oropharynx								
Tongue- anterior	-	-	3.04	-	-	20	0	3.04
Lingual tonsil	**3.48**	**3.08**	-	-	**4.96**	60	60	3.84
Paraepiglottic tonsil	**3.00**	**4.61**	**3.37**	+	**4.93**	80	100	3.98
Tonsil of the soft palate	-	-	**4.00**	-	-	20	20	4.00
Nasal cavity/ Nasopharynx								
Dorsal soft palate -Rostral	2.56	+	-	-	**3.08**	40	40	2.82
Dorsal soft palate -Caudal	-	+	-	-	**4.01**	20	40	4.01
Nasal turbinates	-	-	-	-	-	0	0	-
Nasopharyngeal tonsil	-	-	-			0	20	-
Dorsal nasopharynx	-	-	-	+	**3.42**	20	40	3.42
Lungs/ Trachea								
Trachea	-	-	-	-	-	0	0	-
Cranial lung	-	-	-	-	-	0	0	-
Mid lung	-	-	-	-	2.87	20	0	2.87
Caudal lung	-	-	-	-	-	0	0	-
Additional tissues								
Visceral organs*	-	-	-	-	-	0	0	-
Medial Retropharyngeal LN	-	-	-	-	-	0	0	-
Submandibular LN	-	-	-	-	-	0	0	-
Hilar LN	-	-	-	-	-	0	0	-
Popliteal LN	-	-	-	-	-	0	0	-
Adrenal gland	-	-	-	-	-	0	0	-
Bone marrow	-	-	-	-	-	0	0	-
Myocardium	-	-	-	-	-	0	0	-
Psoas Muscle	-	-	-	-	-	0	0	-
Semimembraneous muscle	-	-	-	-	-	0	0	-
Shoulder Muscle	-	-	-	-	-	0	0	-
Neck skin	-	-	-	-	-	0	0	-
Snout skin	-	-	-	-	-	0	0	-
Coronary band	-	-	-	-	-	0	0	-

Table 2. Tissue distribution of FMDV in viremic pigs (acute infection). Numbers represent genome copy numbers (GCN)/ mg of tissue. Bold numbers indicate that samples were positive for both FMDV RNA (qRT-PCR) and virus isolation, + indicates that virus isolation was positive but FMDV RNA content was below the limit of detection, - indicates double negative samples.

	24 HPI		**48 HPI**		**78 HPI**		**Mean**
Animal ID	*34257*	*34256*	*34258*	*34255*	*37404*	*37405*	**GCN**
Sample identification							
Oral cavity/ oropharynx							
Tongue	2.81	**4.63**	**5.71**	**6.46**	**6.85**	**7.08**	5.59
Paraepiglottic tonsil	**5.71**	**6.29**	**5.59**	**5.30**	**5.91**	**5.03**	5.64
Tonsil of the soft palate	+	+	**4.78**	**4.22**	**6.05**	**5.95**	5.25
Nasal cavity/ Nasopharynx							
Dorsal soft palate -Rostral	**3.46**	**4.87**	**5.70**	**6.09**	**5.44**	**5.03**	5.10
Dorsal soft palate -Caudal	**5.10**	**4.02**	**6.32**	**6.13**	**5.53**	**4.72**	5.30
Nasal turbinates	-	**2.80**	**4.15**	**6.28**	**5.63**	**5.09**	4.79
Nasopharyngeal tonsil	**2.71**	+	**6.21**	**6.08**	**3.89**	**4.94**	4.77
Dorsal nasopharynx -Caudal	**3.96**	**4.98**	**5.62**	**5.78**	**5.58**	**5.28**	5.20
Dorsal nasopharynx -Rostral	**4.41**	**3.35**	**6.35**	**5.67**	**6.02**	**4.92**	5.12
Lungs/ Trachea							
Trachea	+	-	**5.69**	**6.98**	**4.66**	**5.71**	5.76
Proximal cranial lobe	-	**3.22**	**5.49**	**6.33**	**4.41**	**4.83**	4.86
Mid cranial lobe	-	**3.04**	**2.61**	**3.98**	**5.68**	**4.37**	3.94
Distal cranial lobe	-	**2.94**	**3.30**	**4.42**	**4.38**	**4.13**	3.83
Proximal mid lobe	-	**2.85**	**4.67**	**5.78**	**4.85**	**4.01**	4.43
Mid mid lobe	-	**3.33**	**3.74**	**5.34**	**4.80**	**4.37**	4.32
Distal mid lobe	-	**3.35**	**3.92**	**4.96**	**5.19**	**4.42**	4.37
Proximal caudal lobe	-	**2.67**	**3.87**	**6.96**	**3.83**	**4.72**	4.41
Mid caudal lobe	-	**3.02**	**3.87**	**5.81**	**4.22**	**4.50**	4.28
Distal caudal lobe	-	**3.34**	**3.93**	**5.33**	**4.81**	**4.80**	4.44
Visceral organs							
Liver	-	+	+	**4.10**	**3.45**	**4.66**	4.07
Spleen	-	+	**2.99**	**4.79**	**5.93**	**4.97**	4.67
Kidney	-	+	**3.55**	**4.06**	*NA*	*NA*	3.81
Pancreas	-	+	**3.81**	**4.52**	-		4.16
Peyers Patches	+	+	**4.28**	**5.21**	**4.27**	**3.39**	4.29
Additional tissues							
Medial Retropharyngeal LN	+	+	**3.28**	**4.49**	**5.61**	**3.40**	4.19
Submandibular LN	+	-	**3.87**	**4.19**	**5.03**	**2.81**	3.98
Hilar LN	-	+	**3.58**	**4.84**	**5.59**	**4.71**	4.68
Renal LN	-	-	**2.91**	**6.36**	**6.08**	**3.67**	4.76
Popliteal LN	+	**3.81**	**5.69**	**7.78**	**6.20**	**5.18**	5.73
Heart -right ventricle	-	-	**5.10**	**5.99**	**4.68**	**4.23**	5.00
Heart- left ventricle	-	-	**4.85**	**5.46**	**6.01**	**4.16**	5.12
Psoas Muscle	-	-	**4.44**	**5.79**	**3.70**	**6.04**	4.99
Semimembraneous muscle	+	-	**6.04**	**6.92**	**6.87**	**5.51**	6.33
Shoulder Muscle	-	+	**5.75**	**6.32**	**5.48**	**5.23**	5.70
Neck skin	-	-	**4.99**	**5.61**	**6.21**	**5.38**	5.55
Snout skin	-	-	**5.94**	**7.00**	**8.40**	**8.07**	7.35
Coronary band	-	-	**9.66**	**9.91**	**7.41**	**8.46**	8.86

Objective C & D

Post-acute infection dynamics and tissue distribution of virus

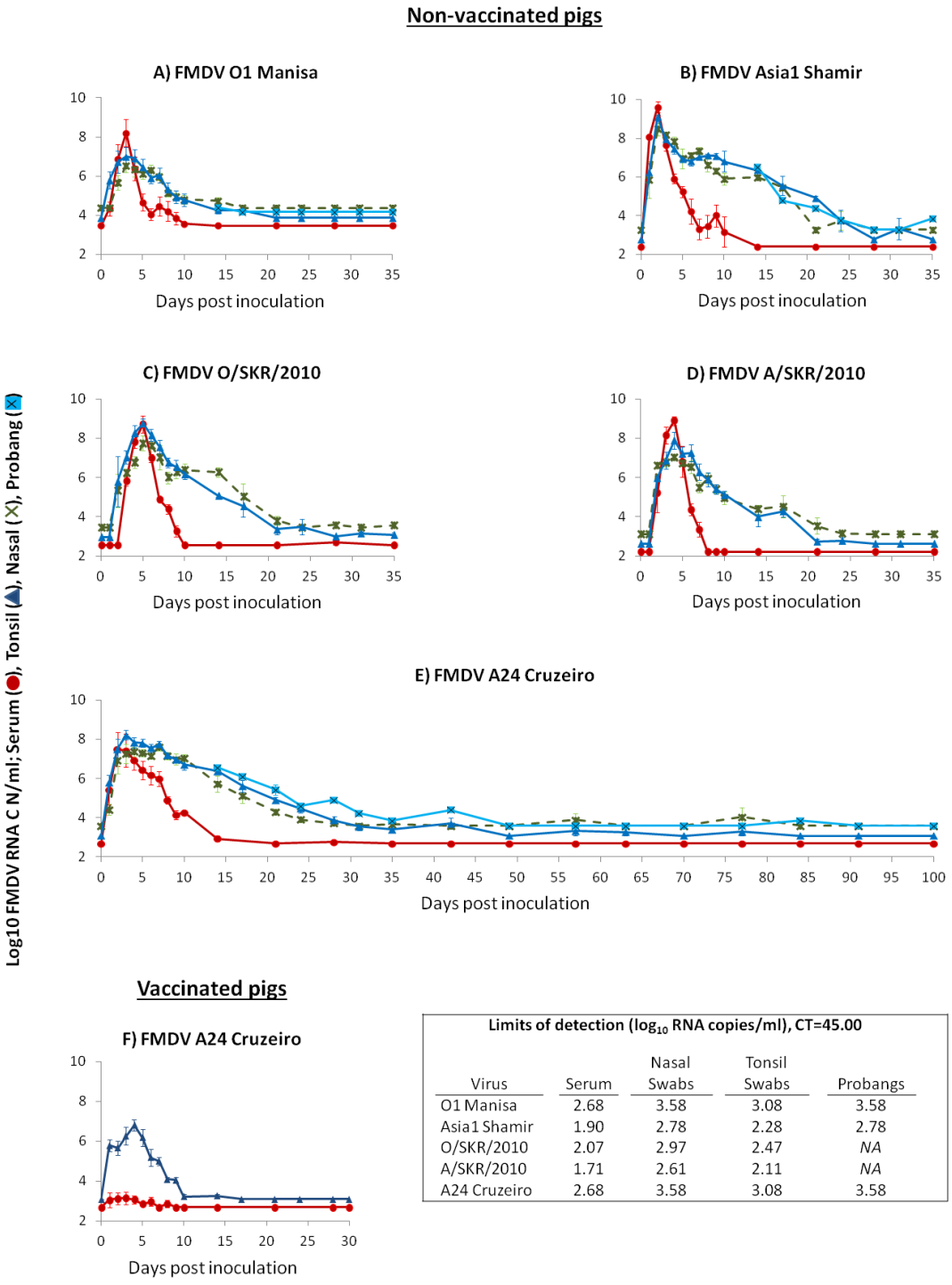
Viral dynamics and shedding during late infection

In naïve pigs infected with FMDV O1 Manisa, detection of viral RNA in swabs and probang samples continued until approximately 14 dpi (Figure 2 A). The extinction of detection of FMDV RNA shedding was somewhat slower in pigs infected with FMDV strains Asia-1 Shamir, O SKR 2010, A SKR/2010 and A24 Cruzeiro (Figure 2 B-E). The latest time point at which viral RNA was detected in nasal and tonsil swabs from pigs infected with FMDV A SKR/2010 was 21 dpi, whereas a subset of pigs infected with FMDV Asia-1 Shamir, O SKR/2010 and A24 Cruzeiro shed low levels of RNA (4.20- 4.71 $\log_{10}$ GCN/ml) in tonsil swabs and/or probang samples beyond 28 dpi (Figure 2B-E). There was a marked decline in this late phase RNA detection by approximately 50 dpi in the pigs infected with FMDV A24 Cruzeiro that were kept for longer periods of observation (Figure 2 E). It was however, still possible to detect low levels of FMDV RNA (3.94-4.70 $\log_{10}$ GCN/ml) in a small subset of tonsil swabs and probang samples harvested as late as 84 dpi (Figure 2 E). It was not possible to isolate infectious FMDV from any antemortem samples obtained beyond the accepted threshold of FMDV persistence of 28 dpi (data not shown).

Pigs that were vaccinated prior to virus challenge were protected against generalized clinical FMD. However, low and fluctuating levels of FMDV RNA were detected in serum up to 6 days post challenge (dpc) and viral shedding in tonsil swabs was detectable for up to 10 dpc, indicating subclinical FMDV infection in all pigs included in the study [21].

Late phase transmission experiment

In order to assess potential transmission of FMDV during the persistent phase of infection, four naïve sentinels were co-mingled with two pigs recovered from infection with FMDV A24 Cruzeiro. Over the course of 21 days of cohabitation, starting at 40 dpi for the two seeder pigs, none of the four sentinels developed any clinical signs of FMD. Additionally, the naïve sentinels did not develop neutralizing anti-FMDV antibodies, and FMDV RNA was never detected in serum-, probang- or swab samples collected from donors or sentinels during the co-mingling period. FMDV RNA was detected in the popliteal lymph nodes of the two seeder pigs at 60 dpi (Table 2)



Limits of detection (log_{10} RNA copies/ml), CT=45.00

Virus	Serum	Nasal Swabs	Tonsil Swabs	Probangs
O1 Manisa	2.68	3.58	3.08	3.58
Asia1 Shamir	1.90	2.78	2.28	2.78
O/SKR/2010	2.07	2.97	2.47	*NA*
A/SKR/2010	1.71	2.61	2.11	*NA*
A24 Cruzeiro	2.68	3.58	3.08	3.58

Figure 2. Antemortem infection dynamics in non-vaccinated pigs infected by FMDV O1 Manisa (A; 12 pigs), FMDV Asia1-Shamir (B; 4 pigs), FMDV O SKR/2010 (C; 6 pigs) and FMDV A SKR/2010 (D; 4 pigs) through experimental periods of 35 dpi, FMDV A24 Cruzeiro through 100 dpi (E; 14 pigs ≤35 dpi, 6 pigs >35≤60 dpi, 4 pigs >60≤100dpi), and vaccinated pigs challenged with FMDV A24 Cruzeiro and observed through 30 days post challenge. FMDV RNA detection in serum, probang samples and nasal and tonsil swabs was performed using quantitative realtime RT-PCR, and is presented as Log_{10} genome copy numbers (CN) / ml. Data presented are averaged values (mean +/- SEM).

Post-acute tissue distribution of virus

In order to precisely determine the extent of possible FMDV persistence in porcine tissues, all pigs were subjected to extensive postmortem tissue collection and all tissues were screened by qRT-PCR and virus isolation (Tables 3 and 4, Supplementary tables 1-6).

Infectious FMDV was not isolated from any of the tissue samples collected (range 28-100dpi). In order to determine if FMDV-specific immune complexes were precluding recovery of infectious FMDV from tissue samples, several tissue samples with high FMDV RNA loads were subjected to TTE-treatment followed by repeat of virus isolation [25]. In all cases, TTE treatment did not alter the lack of detection of infectious FMDV in persistent phase tissues in LFBK-αvβ6 cells.

Despite the absence of infectious FMDV, viral RNA could be detected in a substantial proportion of persistent phase tissue samples (Table 3). Mean detection prevalences were similar across all virus strains (Table 3; Supplementary tables 1-6). In general, the highest detection prevalences of FMDV RNA occurred within lymph nodes draining lesion sites, followed by oropharyngeal tonsils and nasopharyngeal tissues. FMDV RNA was found at lower prevalences in other tissues including coronary band, visceral lymph nodes, and muscle. The highest overall prevalence of FMDV RNA detection in post-FMD convalescent pigs was found in popliteal (88%) and submandibular (41.7%) lymph nodes obtained from naïve infected pigs euthanized at35 dpi (Table 3; Supplementary tables 1-6). There was a general trend towards clearance of FMDV RNA from tissues over increasing elapsed time (dpi). In tissue samples obtained from pigs euthanized at 60 dpi, viral RNA was only detected in the popliteal lymph nodes and the mean GCN was lower than at 35dpi (Table 3). No FMDV RNA was detected in any tissue samples harvested from pigs at 100 dpi (Table 3).

FMDV RNA was also detected in lymph nodes harvested at 28 dpi from pigs that had been vaccinated prior to virus challenge and which had not developed generalized clinical infection (Table 4). FMDV RNA detection in tissue samples from these animals reflected the route by which the pigs had been inoculated, with consistent detection in popliteal lymph nodes of pigs inoculated by IDHB route, but not in those that had received virus challenge through the oropharyngeal route. RNA detection in pharyngeal tonsils and associated lymph nodes was observed in two out of four vaccinated and IOP challenged pigs, and in one out of the three vaccinated and IDHB challenged pigs (Table 4, Supplementary table 6).

Table 3 Detection prevalence (**bold**) and log_{10} mean genome copy number (GCN)/ mg of FMDV RNA (expressed as mean log_{10}GCN/mg; underscored) in tissue samples harvested at 35 dpi from pigs infected with either of 5 FMDV isolates. Additional tissue samples from pigs infected with FMDV Cruzeiro were harvested at 60 dpi (n=2) and 100 dpi (n=4).

	Detection prevalence / log_{10} GCN/mg, 35 dpi					***Over-all detection prevalence/ mean GCN/mg 35 dpi (n=34)***	**Detection prevalence / log_{10} GCN/mg, ≥60dpi**		***Over-all detection prevalence / mean GCN/mg ≥60 dpi (n=6)***
	O1 Manisa *(n=12)*	***Asia1 Shamir*** *(n=4)*	***SKR O/2010*** *(n=6)*	***SKR A/ 2010*** *(n=4)*	***A24 Cruzeiro*** *(n=8)*		***A24 Cruzeiro*** *(60dpi; n=2)*	***A24 Cruzeiro*** *(100dpi; n=4)*	
Tonsil of the soft palate	**8.3/** 3.60	**25/** 2.97	**50/** 2.46	**25/** 2.38	**75.0/** 3.73	**35.3/ 3.22**	-	-	-
Para-epiglottic tonsil	**8.3/** 4.08	-	*NA*	*NA*	**75.0/** 3.31	**26.9/ 3.42**	-	-	-
Nasal tonsil	**8.3/** 3.45	-	**50/** 2.52	-	-	**11.8/ 2.75**	-	-	-
Nasal turbinates	-	-	*NA*	*NA*	**12.5/** 3.27	**3.9/ 3.27**	-	-	-
Pharyngeal diverticulum	-	-	*NA*	*NA*	**12.5/** 2.60	**6.3/ 2.60**	-	-	-
Ventral epiglottis	-	-	-	-	**25.0/** 3.05	**7.7/ 3.05**	*NA*	*NA*	***NA***
Tongue epithelium	-	-	-	**25/** 1.96	-	**2.9/ 1.96**	-	-	-
Neck skin	-	-	-	**25/** 1.84	-	**2.9/ 1.84**	*NA*	*NA*	***NA***
Larynx	-	*NA*	-	**25/** 2.09	-	**3.8/ 2.09**	*NA*	*NA*	***NA***
Dorsal soft palate	**8.3/** 3.31	**25/** 2.07	**33.3/** 2.70	**25/** 1.65	**25.0/** 2.98	**20.6/ 2.62**	-	-	-
Dorsal nasopharynx	**8.3/** 3.50	-	**16.7/** 1.95	-	**12.5/** 2.84	**8.8/ 2.76**	-	-	-
Proximal Lung	**8.3/** 3.47	-	-	-	-	**2.9/ 3.47**	-	-	-
Spleen	-	-	-	-	**37.5/** 3.00	**8.8/ 3.0**	-	-	-
Myocardium	-	-	**16.7/** 2.49	-	**12.5/** 2.68	**7.7/ 2.59**	-	-	-
Skeletal muscle	-	-	-	**50/** 1.91	**12.5/** 2.52	**8.8/ 2.11**	-	-	-
Coronary band	-	-	**33.3/** 1.96	**25/** 1.92	**12.5/** 2.59	**11.8/ 2.11**	-	-	-
Popliteal LN	**75.0/** 4.07	**75/** 4.13	**100/** 3.87	**100/** 3.28	**100/** 4.37	**88.0/ 4.01**	**100/** 3.56	-	**33.3/3.56**
Submandibular LN	**25.0/** 3.63	-	-	-	**87.5/** 3.02	**41.7/ 3.20**	-	-	-
Hilar LN	**8.3/** 3.59	-	-	-	**37.5/** 3.01	**11.8/ 3.16**	-	-	-
Renal LN	**16.7/** 3.34	-	-	**50/** 2.00	**75.0/** 3.02	**29.4/ 2.92**	-	-	-
Retropharyngeal LN	**25.0/** 3.53	-	**16.7/** 2.32	-	**62.5/** 4.00	**26.5/ 3.66**	-	-	-

Table 4. Detection prevalence (**bold**) and mean genome copy number (GCN)/ mg of FMDV RNA (expressed as mean $\log_{10}$ GCN/mg; underscored) in tissue samples obtained at 28 dpi from pigs subclincally infected with FMDV A24 Cruzeiro following vaccination and challenge through either intra-oropharyngeal (IOP) or intradermal heelbulb (IDHB) inoculation.

	Detection prevalence / log10 GCN/mg, 28 dpi	
	Ad5-FMDV A + FMDV A24 Cruzeiro	
	Intra-orophayngeal	***Intradermal heelbulb***
	(n=4)	**(**n=3)
Tonsil of the soft palate	**25/** 2.52	-
Para-epiglottic tonsil	**25/** 3.92	-
Nasal tonsil	-	**33.3/** 2.71
Nasal turbinates	-	-
Pharyngeal diverticulum	-	-
Ventral epiglottis	-	-
Tongue epithelium	-	-
Neck skin	-	-
Larynx	-	-
Dorsal soft palate	-	-
Dorsal nasopharynx	-	-
Lung	-	-
Spleen	-	-
Myocardium	-	-
Skeletal muscle	-	-
Coronary band	-	-
Popliteal LN*	-	**100/** 5.14
Submandibular LN	**50/** 4.10	-
Hilar LN	-	-
Renal LN	-	-
Retropharyngeal LN	-	-

Detection of FMDV structural antigen by immuno-microscopy

FMDV capsid antigen (VP1) was detected within follicles of lymph nodes draining lesion sites, most consistently within popliteal lymph nodes harvested at 35 dpi from animals infected with FMDV O1 Manisa, FMDV A24 Cruzeiro (Figure 3) and FMDV Asia-1 Shamir. FMDV capsid protein was generally detected in a subset of lymphoid follicles and co-localized with CD21-immunopositive cells. FMDV antigen detection was not associated with surface markers identifying T-cells (CD3) or cells of monocyte/macrophage lineage (CD172a, CD163, CD16). Overall, the phenotypic and anatomic characterization of cells containing FMDV antigens was most suggestive of follicular dendritic cells. It was not possible to detect FMDV nonstructural protein (3D) in any of the tissue sections analyzed. FMDV antigen was not detected in tissue samples collected at 60 or 100 dpi.

Discussion

The objectives of the work performed within this project included optimization of an experimental model for studies of FMDV pathogenesis in pigs (objective a). The developed system was subsequently utilized for thorough and extensive investigations of infection dynamics and tissue distribution of virus during both acute (objective b) and post-acute (objectives c-d) FMDV infection.

Antemortem infection dynamics was investigated by monitoring of FMDV RNA quantities in serum, probang samples and tonsil and nasal swabs through the experimental studies. To investigate the progressive tissue distribution of virus during the early phase of infection, two or three pigs infected with FMDV A24 Cruzeiro were euthanized at each of five pre-determined time points; 6, 12, 24, 48 and 78 hpi.

Investigation of late phase (suspect persistent) tissue detection of FMDV included experimental studies performed with 5 different strains of FMDV, comprising serotypes O, A and Asia-1. These animals were euthanized at time points from 28 to 100 dpi, corresponding to what has been defined as the persistent phase of FMDV infection in ruminants.

All necropsies performed included extensive collection of tissue samples which were subsequently analyzed for contents of FMDV RNA, infectious virus as well as structural and non-structural proteins.

Acute infection

The IOP inoculation system that was utilized for the major part of the studies performed involves direct deposition of inoculum on the surface of the tonsil of the soft palate in deeply sedated pigs. Despite this, it is noteworthy that in pre-viremic pigs, the proportions of positive detection of both FMDV RNA and infectious virus were significantly higher in the paraepiglottic tonsils when compared to the tonsil of the soft palate. The paraepiglottic tonsils are small aggregates of tonsillar follicles located bilaterally within the epiglottic fold at the base of the epiglottis [27].

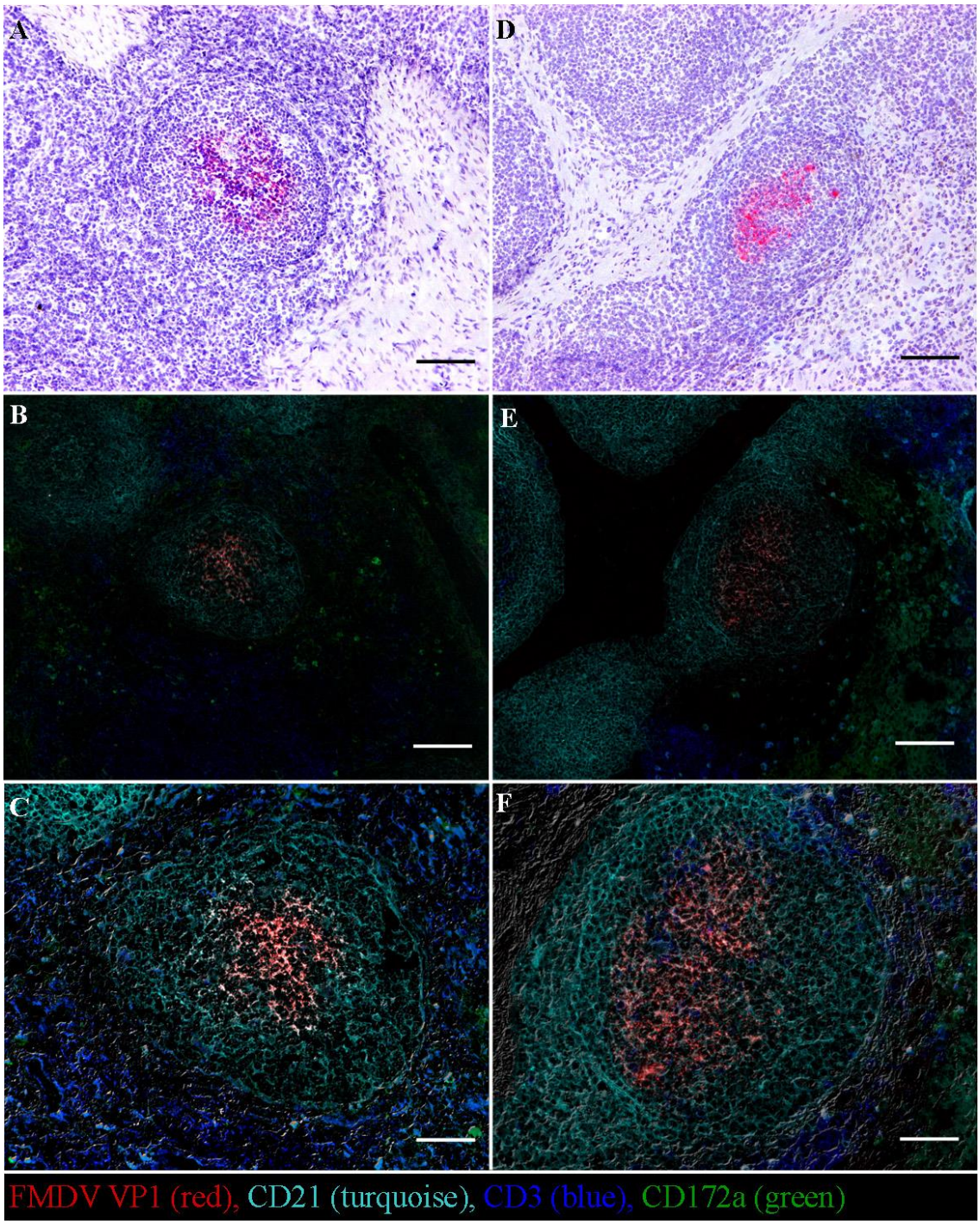


Figure 3 Localization of FMDV capsid antigen in lymphoid follicles in popliteal lymph nodes harvested at 35 dpi from pigs infected with FMDV O1 Manisa (A-C) or FMDV A24 Cruzeiro (D-E). A+D: Immunohistochemical detection of FMDV structural protein. Anti-FMDV capsid monoclonal antibody. Micropolymer alkaline phosphatase. Gill's hematoxylin counterstain. 10x magnification, scale bars 100µm. B+E: Multichannel immunoflourescence images of serial sections of regions identified in Figure 2A+D. 10x Magnification, scale bars 100µm. FMDV capsid antigen (red), co-localizes with CD21 (turquoise) within lymphoid follicles. CD3 (blue), CD172a (green) . C+F: 20x magnification of regions identified in Figure 2 C-D, scale bars 50µm

Similarly to the tonsil of the soft palate and the lingual tonsils, the paraepiglottic tonsils are exposed to material and pathogens entering through the oral route, whilst the anatomical location makes them more protected from exposure to pathogens entering through the nasal/ respiratory route. It was demonstrated during initial animal studies, whilst optimizing the IOP inoculation system, that presence of viral RNA within the oropharynx following inoculation was a prerequisite for successful infection[21]. E.g., in pigs that were inoculated through deposition of virus within the nasopharynx, only the individuals that had a detectable 'spill-over' of inoculum into the oropharynx would develop clinical disease. These findings together indicate that the oropharynx, and therein specifically the paraepiglottic tonsils, constitute anatomical sites critical for the initial phase of FMDV infection in pigs.

This finding contrasts recently gained knowledge regarding FMDV pathogenesis in cattle, for which it has been demonstrated that the initial focus of FMDV replication is located within lymphoid associated epithelial cells of the dorsal nasopharynx [4]. However, these contrasting discoveries are in general agreement with the 'conventional wisdom' of cattle being more susceptible to FMDV infection through inhalation, whereas pigs are more likely to become infected through the oral route [3, 15, 28].

In pre-viremic pigs, detection of infectious virus was restricted to the pharyx, initially with predominant location within the oropharynx, and subsequently spreading to also involve the nasopharynx. Viral distribution in early viremic (pre-clinical) pigs was more variable, with a general pattern of pharyngeal distribution (oropharynx and nasopharynx) subsequently disseminating to pulmonary sites. Due to the high quantities of virus within the blood of viremic pigs, it is difficult to draw conclusions of the relative involvement of different tissues as sites of active viral replication during the more advanced stages of infection. It is, however, clear that from 48 hpi and later, the highest mean quantities of FMDV RNA were found at predilect lesion sites including coronary bands and tongue, which have also previously been identified as sites harboring substantial viral amplification [29, 30].

Post-acute infection

A large part of the work performed in this project was focused on performing a thorough, prospective investigation of the extent to which infectious FMDV, or remnants thereof, could be detected in porcine tissues harvested beyond 28dpi (corresponding to the accepted threshold for FMDV persistence).

It is thoroughly established that both naïve and vaccinated cattle may become persistently infected carriers following FMDV exposure [31-33], and current strategies for deployment of FMD countermeasures within non-endemic regions are largely dictated by this perceived threat. In the current study, after duplicate screening of well over one thousand (n=1140) distinct tissues using a highly FMDV-sensitive cell line [23], there was no detection of infectious FMDV in tissue samples obtained during the persistent phase of infection from naïve or vaccinated pigs. Furthermore, attempted dissociation of immune-complexed virus through TTE treatment of tissue macerates prior to virus isolation did not alter the lack of detection of infectious FMDV.

Despite absence of infectious virus, FMDV RNA was detected in a substantial proportion of tissue samples harvested from 28 to 35 dpi. The anatomical distribution of viral RNA reflected the pathogenesis of the clinical infection, in that highest quantities of viral RNA were found at viral

replication sites and associated lymphoid tissues. Specifically, during acute infection massive viral amplification occurs in the coronary band and foot epithelium that drain to the popliteal lymph nodes, which are thus exposed to large amounts of virus and viral degradation products. Lower amounts of viral RNA were detected in other tissues, including tonsils, pharyngeal mucosal sites, and lymph nodes draining the oral cavity and pharynx, suggesting involvement of these tissues during the acute phase of FMD. The detection of FMDV RNA in tissue samples was markedly reduced from 35 dpi to 60 dpi and was absent at 100 dpi, further indicating that the observed persistence of viral RNA in porcine tissues is a transient process preceding complete clearance of viral degradation products.

A subset of animals infected with FMDV Asia-1 Shamir, O/SKR/2010 and A24 Cruzeiro, shed low, but detectable, levels of viral RNA in tonsil swabs and/or probang samples beyond the accepted threshold of FMDV persistence of 28 dpi. However, it was never possible to isolate live virus from any of these samples, and there was no transmission of infection from pigs recovered from clinical infection to naïve sentinels. There was a marked decline in FMDV RNA shedding in swabs and probang samples by approximately 50 dpi in animals that were kept through longer periods of observation. However, low levels of FMDV RNA could still be detected in occasional probang or tonsil swab samples harvested as late as 84 dpi.

Since FMDV is a positive sense RNA virus, the full length, genomic RNA may be considered an infectious RNA molecule [34]. However, the qRT-PCR assay used for RNA detection in the current study, targets a limited sequence within the 3D coding region of the viral genome [35, 36]. Thus, detection of FMDV RNA by qRT-PCR does not imply the presence of intact, full-length and infectious FMDV genome, or of viable virus. The overall interpretation of the detection of FMDV RNA by qRT-PCR in the convalescent pigs in the current study is that the risk of contagion from these animals is extremely low and continuously decreases over time.

In addition to viral RNA, FMDV capsid antigen was commonly detected within lymphoid follicles of lymph nodes draining lesion predilection sites in FMDV-convalescent pigs. Similar findings have been described in relation to persistent FMDV infection in cattle [37], as well as in porcine circovirus infection, where antigen detection in lymphoid follicles has been described without concurrent signs of active infection [38]. In the current study, FMDV antigen was detected within a subset of lymphoid follicles in lymph nodes that drain lesion sites, co-localizing with CD21, but not with phenotypic markers for cells of monocyte/ macrophage lineage. CD21 is expressed on the surface of both B-cells and follicular dendritic cells (FDCs) within lymphoid follicles [39]. A major function of the FDC network consists of continuous presentation of unprocessed antigen to B-cells to stimulate and maintain a long lasting and specific immunity to encountered pathogens [40-42]. Based on this information, it is feasible that FMDV protein could be retained in this anatomic compartment, without persistence of infectious virus.

Summary

Work performed within this project has generated novel information regarding both acute and late-phase FMDV pathogenesis in pigs. Studies performed in pre-viremic pigs have indicated that the porcine oropharyngeal tonsils are critical as initial points of entry for FMDV. This finding is in contrast with previous findings from studies in cattle, in which initial FMDV replication has been localized to the nasopharynx. These contrasting findings are in agreement with the

conventional wisdom that cattle are more susceptible to FMDV infection through the respiratory route, whilst pigs are more likely to become infected via the oral route. This knowledge is of critical importance for future development of improved vaccines and biotherapeutic countermeasures aimed at protecting pigs against FMDV exposure.

The studies of late-phase infection dynamics and tissue distribution of FMDV in convalescent pigs provide the most comprehensive evidence compiled to date that domestic pigs are not competent long term carriers of FMDV. Further studies would be needed to validate these findings in order to support potential development of differential, species-specific FMD-control policies for swine and ruminants. Requisite data for such validation would include testing a wider range of FMDV strains and diverse host genetic backgrounds.

Supplementary material

Supplementary tables 1 -6

FMDV RNA genome copy number (GCN)/ mg (expressed as $\log_{10}$ GCN/mg) in tissue samples obtained at 28 - 100 dpi from pigs infected with FMDV. Results are presented as $\log_{10}$ GCN/mg for each distinct tissue sample for individual pigs as well as mean $\log_{10}$ GCN/mg calculated within positive samples for each distinct tissue within the different groups of pigs. Presented values reflect the higher RNA content/mg, out of two samples processed for each tissue. The set cut-off value for positivity represented by a cycle threshold of 40 corresponded to following detection limits; FMDV O1 Manisa =3.28 $\log_{10}$ GCN/mg, FMDV Asia1-Shamir = 1.88 $\log_{10}$ GCN/mg, FMDV O SKR/2010= 1.95 $\log_{10}$ GCN/mg, FMDV A SKR/2010= 1.60 GCN/mg and FMDV A24 Cruzeiro= 2.26 $\log_{10}$ GCN/mg

Supplement 1: Naïve pigs infected with FMDV O1 Manisa (12 pigs, 35 dpi)
Supplement 2: Naïve pigs infected with FMDV A24 Cruzeiro (8 pigs 35 dpi; 2 pigs 60 dpi; 4 pigs 100dpi)
Supplement 3: Naïve pigs infected with FMDV Asia1-Shamir (4 pigs, 35 dpi)
Supplement 4: Naïve pigs infected with FMDV O SKR/2010 (6 pigs, 35 dpi)
Supplement 5: Naïve pigs infected with FMDV A SKR/2010 (4 pigs, 35 dpi)
Supplement 6: Vaccinated pigs subclinically infected with FMDV A24 Cruzeiro (7 pigs, 28-30 dpi)

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