



Transmission of Foot-and-Mouth Disease from Persistently Infected Carrier Cattle to Naive Cattle via Transfer of Oropharyngeal Fluid

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ABSTRACT Control and eradication of foot-and-mouth disease (FMD) are impeded by the existence of a persistent, subclinical phase of infection in ruminants; animals with this status are referred to as carriers. However, the epidemiological significance of these FMD virus (FMDV) carriers is uncertain. In the current investigation, the contagion associated with FMDV carrier cattle was investigated by exposure of susceptible cattle and pigs to oropharyngeal fluid (OPF) samples or tissues harvested from persistently infected cattle. Naive cattle were inoculated through intranasopharyngeal deposition of unprocessed OPF samples that had been collected from FMDV carriers at 30 days postinfection. These inoculated cattle developed clinical FMD, and the severity of disease they developed was similar to that of animals that had been infected with a high-titer inoculum. In contrast, pigs exposed via intraoropharyngeal inoculation of the same OPF samples or via ingestion of nasopharyngeal tissues harvested from the same cohort of persistently infected cattle did not develop FMD. These findings indicate that there is demonstrable contagion associated with FMDV carrier cattle despite the lack of evidence for transmission by direct contact. The findings presented herein provide novel information that should be considered for FMD risk mitigation strategies.

IMPORTANCE Foot-and-mouth disease (FMD) is a viral disease of livestock with substantial impact on agricultural production and subsistence farming on a global scale. Control of FMD is impeded by the existence of a prolonged asymptomatic carrier phase during which infected cattle shed low quantities of infectious virus in oropharyngeal fluid (OPF) for months to years after infection. The epidemiological significance of FMD virus (FMDV) carriers is unresolved. However, the existence of the FMDV carrier state has substantial impact on international trade in animal products. The current investigation demonstrated that transfer of OPF from persistently infected FMDV carrier cattle to naive cattle led to fulminant clinical FMD. It was thus demonstrated that, although the risk for disease transmission under natural conditions is considered to be low, there is detectable contagion associated with FMDV carrier cattle. This finding is important for optimization of FMD risk mitigation strategies.

KEYWORDS FMDV, carrier, cow, foot-and-mouth disease, foot-and-mouth disease virus, risk, transmission, virus

Foot-and-mouth disease (FMD), caused by infection with FMD virus (FMDV; family *Picornaviridae*, genus *Aphthovirus*), is an economically important disease of livestock (1, 2). Large regions of the developed world, including Europe, North America, and Australia, are kept free of FMD through strict regulations on the importation of animals


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 Contagion associated with persistently infected carriers of foot-and-mouth disease; new study suggests that the risk of disease transmission from these animals cannot be discounted. @carostenfeldt

and related products. However, the disease is still endemic in most of Africa and Asia (3–5). FMDV can infect a wide range of cloven-hoofed animals and can cause severe disease in domestic ruminants and pigs (6, 7). The endemic presence of FMD is problematic, as it prevents access to international markets for trade in animal products. Additionally, measures required for disease control, including repeated vaccination campaigns and control of animal movements, are economically and logistically challenging in many affected regions. In countries that are normally free of FMD, incursions of the disease cause major disruptions in agricultural production and severe losses in revenue due to interrupted trade (8–11). Control and eradication of FMD are further impeded by the existence of a subclinical persistent phase of infection in ruminant species (7, 12). This FMDV carrier state has traditionally been defined by detection of infectious FMDV in oropharyngeal fluid (OPF) more than 28 days postinfection (dpi), and it is reported to occur in $\geq 50\%$ of infected cattle (12). However, recent work has demonstrated that carriers may be identified as early as 15 dpi in vaccinated cattle and 21 dpi in cattle that were not vaccinated (13). Persistent FMDV infection occurs in both vaccinated and naive cattle, regardless of the occurrence of clinical disease (14, 15). This is of particular concern in relation to the use of emergency vaccination to control FMD outbreaks in regions that are normally free of FMD, as infection in vaccinated animals may go unnoticed.

Cattle are highly sensitive to FMDV infection via the respiratory route, and the site of initial virus replication has been localized to specific regions of epithelium of the nasopharyngeal mucosa that contain mucosa-associated lymphoid tissue (MALT) (16–18). The clinical phase of disease includes fever, transient viremia, and the occurrence of characteristic vesicular lesions in and around the mouth, on the feet, and in other areas of nonhaired skin (1). Affected animals may develop severe lameness; in addition, both milk yield and growth rates may be reduced. Mortality rates are low in adult animals, which generally recover from the clinical phase of FMD within approximately 2 weeks. Persistent FMDV infection in cattle has, similar to early infection, also been localized to lymphoid-associated epithelium of the nasopharynx (13, 19). During the persistent phase of infection, FMDV can be detected in nasopharyngeal tissues and in OPF sampled using a probang (sputum) cup (20), but it is usually not detected in oral or nasal swab samples (13, 21, 22).

The existence of the FMDV carrier state has had a profound impact upon legislation concerning international trade in animal products (6, 23). However, there is still uncertainty regarding the actual level of risk associated with FMDV carriers as well as their potential role in the ecology of FMDV in regions where FMD is endemic (24, 25). Historical records have reported FMD outbreaks that were believed to have been seeded by transmission of FMDV from a persistently infected carrier to a susceptible animal (14, 24). However, multiple, small-scale, experimental investigations have failed to demonstrate transmission of FMDV from persistently infected carrier cattle to contact-exposed sentinels (22, 26, 27). A meta-analysis of experimental attempts to transmit FMDV from carrier cattle to naive cattle or pigs demonstrated that transmission had occurred only once among 26 published attempts (28). The original record from that transmission study reported confirmed seroconversion, without clinical signs of FMD, in two pigs after 75 days of contact exposure to carrier cattle (29). It has been speculated that the apparent lack of contagion associated with FMDV carrier cattle may be due to neutralization of virus shed in OPF by secreted anti-FMDV IgA. This theory is supported by a demonstrated increase in infectivity in tissue culture of OPF samples that have been treated with a fluorocarbon compound to dissociate immunoglobulin-bound virus (13, 30). There is experimental evidence that supports transmission from persistently infected African buffalo (*Syncerus caffer*) to in-contact cattle (31, 32). Additionally, in an experimental investigation from 1968, it was shown that it is possible to infect naive cattle by intrapharyngeal inoculation of OPF samples harvested from FMDV carriers (33). However, the published details of the experiment lack explicit details.

The objective of this current study was to investigate the infectivity of unprocessed

OPF samples and pharyngeal tissues harvested from persistently infected FMDV carriers. For this purpose, naive calves and pigs were exposed to OPF samples harvested from FMDV carriers using simulated-natural inoculation systems. Additionally, samples of nasopharyngeal mucosal tissues harvested from the same cohort of FMDV carriers were macerated and fed to naive pigs. The findings presented herein demonstrate that OPF from FMDV carrier cattle is indeed infectious and may, under specific conditions, cause disease in susceptible cattle. Lack of disease in exposed pigs is consistent with previous reports (34) suggesting that higher quantities of virus may be required to infect pigs with FMDV via natural exposure routes.

RESULTS

Animal experiments. The experiments reported herein consisted of three distinct phases. Phase I included nine steers that were infected with a high-titer FMDV inoculum and monitored to the persistent phase of infection. Experimental phase II consisted of eight steers that were challenged by intranasopharyngeal inoculation of oropharyngeal fluid (OPF) samples that had been harvested from the persistently infected steers of phase I at 30 days postinfection (dpi). Phase III consisted of two groups of five pigs; one group was challenged by intraoropharyngeal inoculation of the pooled OPF obtained from the phase I cattle, and the other group was fed macerated nasopharyngeal tissues from the same persistently infected cattle.

Clinical progression and infection dynamics in phase I cattle. The main purpose of the first phase of the experiment was to produce carrier cattle and harvest appropriate material for inoculation of cattle and pigs in phases II and III. Following intranasopharyngeal (INP) deposition of FMDV A24 Cruzeiro, all nine animals in phase I of the study developed clinical FMD, consistent with previous investigations using the same virus strain, dose, and exposure route (13, 18, 35). Vesicular lesions were observed in the mouth as well as on one or more feet of all animals, with initial detection of lesions occurring between 3 to 6 dpi (Fig. 1). Four animals in phase I reached the maximum lesion score, with lesions in the mouth and on all four feet. The remaining five animals had one or more feet without lesions (Fig. 1). Viremia, defined as detection of FMDV RNA in serum, was apparent in all nine animals and lasted for 2 to 5 days. Abundant quantities of FMDV RNA were detected in nasal fluids throughout the clinical phase of infection. Nasal FMDV detection ceased between 7 and 10 dpi. Low quantities of FMDV RNA were detected in at least two OPF samples in all animals. However, two animals (animals 02 and 09) had two or more FMDV RNA-negative OPF samples, including the sample harvested at 28 dpi (Fig. 1). Therefore, OPF from these two animals was not included in the pooled inoculum that was harvested at 30 dpi and used for challenge of animals in phase II. The persistently infected animals of phase I were euthanized for tissue harvest at 31 dpi.

Infectivity of OPF inoculum and pooled tissue macerate. The titer of FMDV in the unprocessed, pooled OPF (i.e., the material that was used to challenge cattle and pigs in phases II and III, respectively) was 10^1 50% tissue culture infectious doses (TCID₅₀) per ml on LFBK- α v β 6 cells and below the detection level on BHK-21 cells (Table 1). Trichlorotrifluoroethane (TTE) treatment of the OPF increased the titer to $10^{2.5}$ TCID₅₀/ml on LFBK- α v β 6 cells, but the infectivity was still below the detection limit when using BHK-21 cells (Table 1). All titrations of the nasopharyngeal tissue macerate were below detection limits for both cell lines. However, infectious virus was isolated when a higher volume (1 ml) of the same tissue macerate was inoculated onto LFBK- α v β 6 cells in unfiltered form (Table 1). Calculating the challenge doses using the titer of the non-TTE-treated OPF derived from the highly sensitive LFBK- α v β 6 cells indicated that cattle in phase II received a dose of 10^2 TCID₅₀, and the pigs in phase III that were challenged by intraoropharyngeal (IOP) inoculation received 5×10^1 TCID₅₀.

Clinical progression and infection dynamics in phase II cattle. The second phase of the experiment included eight cattle that were challenged by INP deposition of 10 ml of pooled OPF that had been harvested at 30 dpi from seven phase I animals that had been identified as being persistently infected carriers. The first sign of FMD in the phase

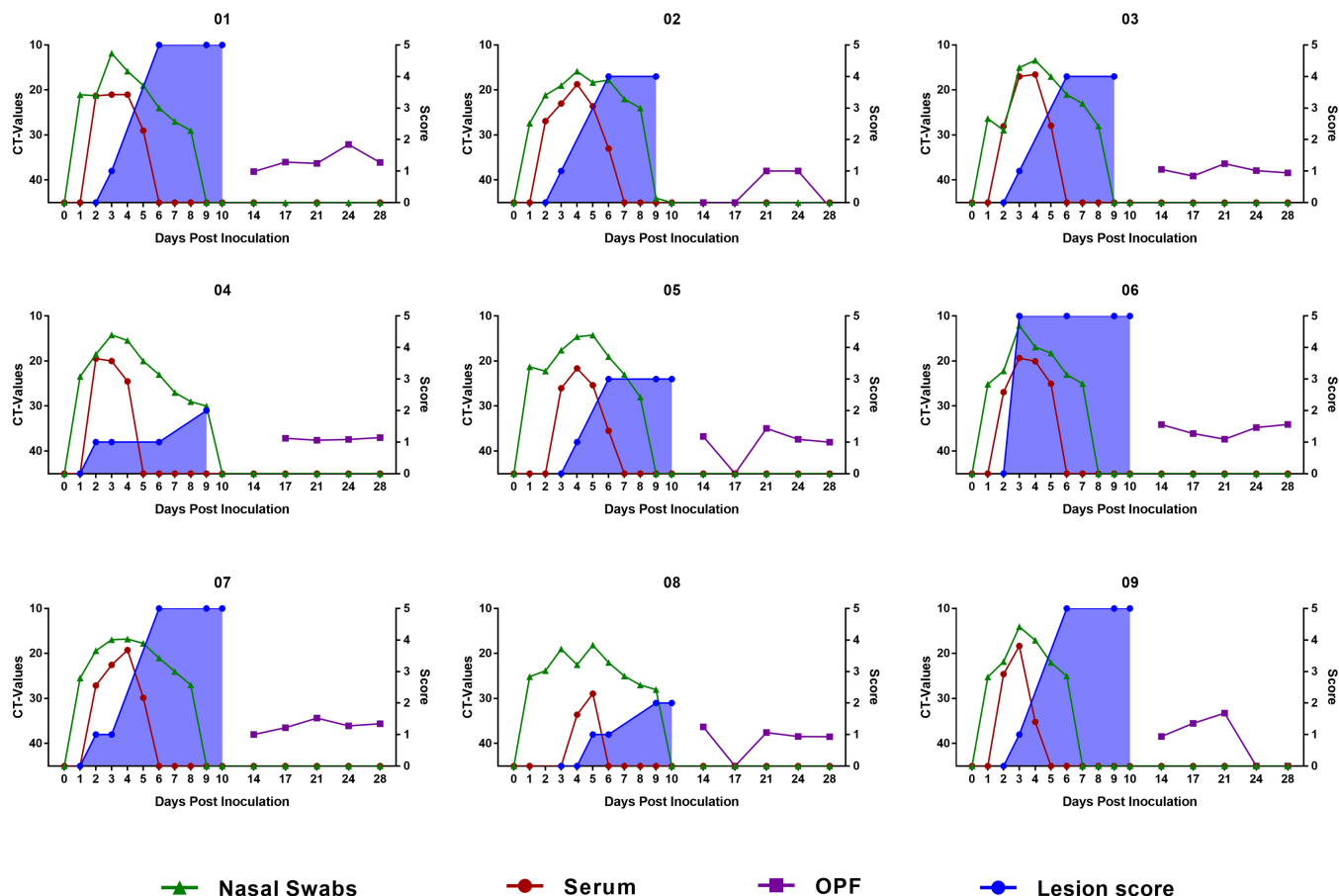


FIG 1 FMDV infection dynamics in cattle in phase I of the study. Detection of FMDV RNA by RT-qPCR in nasal swabs and serum and oropharyngeal fluid (OPF) samples collected from cattle infected with 10^5 TCID₅₀ of FMDV A24 Cruzeiro following intranasopharyngeal inoculation. Time (in days postinoculation) is shown on the x axes, C_T values are shown on the left-hand y axes, and lesion scores are shown on the right-hand y axes. The blue shaded area represents cumulative lesion score, which was recorded up to 10 days postinfection (dpi). OPF was collected twice weekly from 14 dpi. Pooled OPF used for challenge of cattle in experimental phase II and pigs in phase III was harvested at 30 dpi, from all animals except animals 02 and 09 (the FMDV carrier status of these two animals was undetermined).

II cohort consisted of a large vesicle on the tongue of animal 13 at 4 dpi (Fig. 2). On the subsequent day, an interdigital cleft lesion was detected in this same animal, and oral vesicles were observed in five additional animals (Fig. 2). The remaining two cattle had developed oral lesions by day 6 and 7, respectively. Seven out of the eight animals developed lesions on one or more feet subsequent to the initial oral lesions. All animals became viremic, although FMDV RNA was detected in only one serum sample from animal 17. High quantities of FMDV RNA were detected in nasal secretions from all eight cattle starting from 2 to 4 dpi until 7 to 10 dpi (Fig. 2). The severity and progression of clinical FMD were similar to those observed in phase I cattle.

Clinical monitoring and infection dynamics in phase III pigs. The pigs in the third phase of the study were challenged by either intraoropharyngeal deposition ($n = 5$) of

TABLE 1 FMDV detection in pooled OPF and nasopharyngeal tissues from persistently infected carriers

Sample	C _T value by FMDV RT-qPCR	FMDV isolation in LFBK- α v β 6 cells ^{a,b}	FMDV titer (TCID ₅₀ /ml) in ^b :			
			BHK-21 cells		LFBK- α v β 6 cells	
			Unprocessed	TTE-treated	Unprocessed	TTE-treated
OPF	31.8	Pos	Neg	Neg	10 ¹	10 ^{2.5}
Tissue	32.0	Pos	Neg	Neg	Neg	Neg

^aVirus isolation on LFBK- α v β 6 cells in T25 flask using unprocessed and unfiltered material.

^bPos, positive (observed cytopathic effect [CPE] with FMDV replication confirmed by RT-qPCR); Neg, negative.

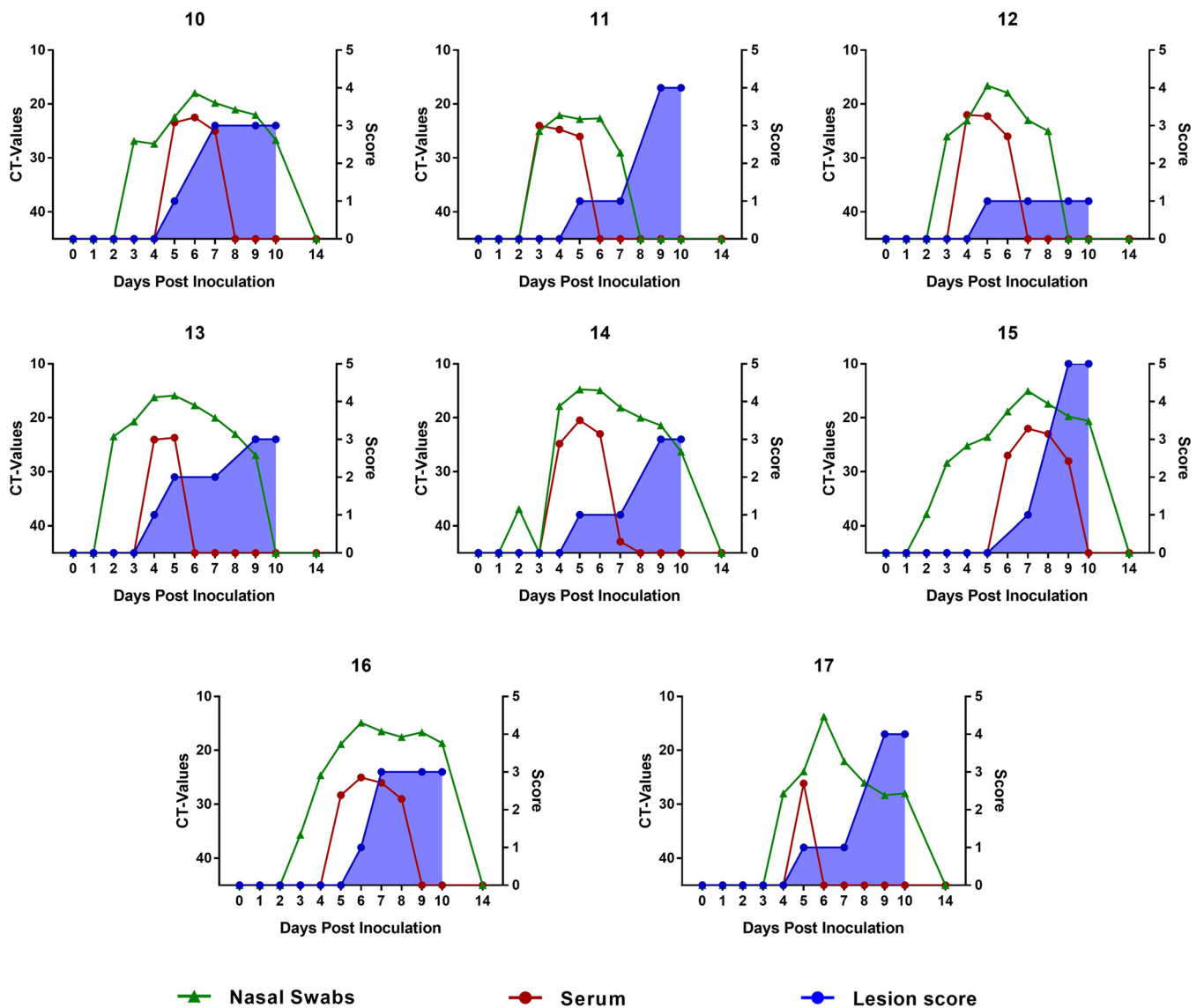


FIG 2 FMDV infection dynamics in cattle in phase II of the study. Detection of FMDV RNA by RT-qPCR in nasal swabs and serum samples in cattle infected through intranasopharyngeal inoculation of pooled OPF obtained from cattle in experimental phase I at 30 dpi. The blue shaded area represents cumulative lesion score, which was recorded up to 10 days postinfection (dpi). The challenge dose was determined to have been 10^2 TCID₅₀ per animal.

the same pooled OPF as was used to challenge cattle in phase II or by feeding macerated nasopharyngeal tissues ($n = 5$) harvested from the persistently infected carriers of phase I at 31 dpi. There were no signs of FMD in any of the pigs included in the third phase of the experiment. Similarly, FMDV RNA was not detected in any oropharyngeal (OP) swabs or serum samples (not shown), and no antibodies against FMDV were detected in sera from each of these pigs collected at 14 dpi (not shown).

FMDV sequence analysis. In order to assess the region specificity of genomic changes during the course of these studies, near-complete FMDV genome sequences were acquired from the original virus inoculum, as well as from nasal swab samples obtained during the clinical phase of infection from one calf from experimental phase I and from one calf from phase II (not shown). The majority of the observed changes within the genome occurred within the VP1 coding region. On this basis, further genomic analysis was focused upon the VP1-2A coding sequence in FMDV RNA derived from nasal swab samples from all animals in phase I and almost all animals from phase II (not including one animal). Samples for sequence analysis ranged from 3 to 4 dpi for

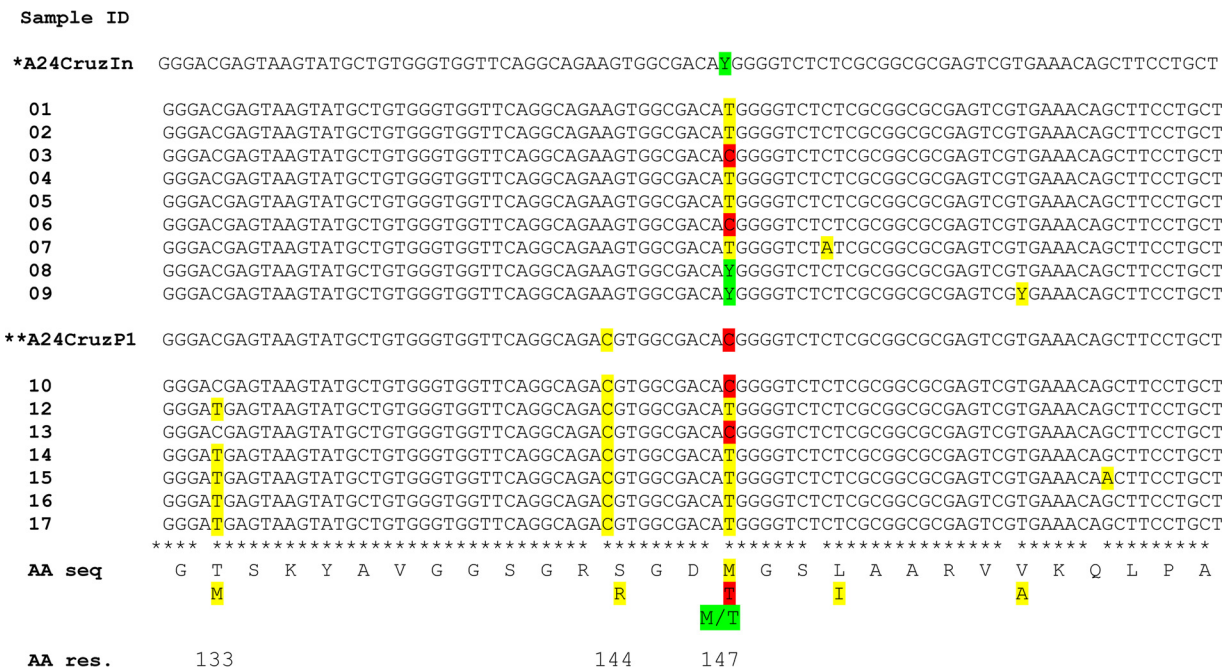


FIG 3 Nucleotide sequences encoding part of the VP1 capsid protein of FMDVs derived from infected cattle in phase I and phase II of the study. Partial VP1 coding sequence obtained from nasal swab samples collected during the clinical phase of FMD from cattle of experimental phases I (animal identifiers [IDs] 01 to 09) at 3 or 4 dpi and phase II (animal IDs 10 to 17) at 6 or 7 dpi. The top row (*A24CruzIn for A24 Cruzeiro given INP) represents the consensus sequence of the inoculum used to infect cattle in phase I. The middle row (**A24CruzP1) is the consensus sequence of the pooled OPF inoculum (as determined after a single passage in cell culture; see text) that was used to infect cattle in phase II. Nucleotide changes and the corresponding amino acid substitutions are marked using the same color code. The variations in coding sequence suggest that the inoculums used for both phase I and II consisted of heterogeneous viral populations. Additionally, the sequences obtained from phase II animals suggest that infection of this group of animals was seeded by at least two different virus populations, as the samples from animals 10 and 13 are distinct from the remaining samples at amino acid residues 133 and 147 of VP1. Y = mixture of C and T (pyrimidines). AA seq, amino acid sequence; AA res., amino acid residue.

phase I and 6 to 7 dpi for phase II. Specific samples were selected based on the presence of high levels of FMDV RNA (as judged by a low threshold cycle [C_T] value in real-time quantitative PCR [RT-qPCR] assays) and corresponded to the peak of clinical FMD for both phase I animals and phase II animals. Nonsynonymous changes in the coding sequence were found predominantly at amino acid residues 133, 144, and 147 (Fig. 3) of VP1 which includes the integrin-binding motif (RGD) within the G-H loop. While the original inoculum and all phase I samples included amino acid sequences RSGDM or RSGDT at this site, all samples from the phase II animals were either RRGDM or RRGDT (Fig. 3). Due to low RNA content, it was not possible to obtain the VP1 coding sequence from the pooled OPF inoculum used for challenge of phase II cattle without one passage of the virus in tissue culture. However, the passaged phase II inoculum had a sequence encoding RRGDT at the specified site, indicating that the S-to-R substitution at residue 144 had occurred during the later stages of infection of the phase I cattle. Additionally, the sequences obtained from the phase II samples suggested that infection of this group was seeded by at least two major viral variants; one with methionine (M) at residues 133 and 147 and another with threonine (T) at both of these sites. The two variants at residue 147 had been present in different cattle in phase I. This suggested that virus from at least two different cattle from phase I initiated infection in phase II.

DISCUSSION

Although the FMDV carrier state was first described in cattle almost 60 years ago (36), the epidemiological significance of FMDV carriers remains undetermined and controversial. Although numerous reports have characterized the carrier state under natural and laboratory conditions, the crucial issue of whether carrier cattle pose a

significant risk of contagion remains incompletely resolved. Despite a lack of substantiated experimental evidence of transmission from FMDV carrier cattle (28), the FMDV carrier state has had an impact upon the regulation of global trade in animal products (24, 37). The objective of this current investigation was to use recent knowledge about the initial and persistent sites of virus replication (13, 18) to investigate the potential risk of disease transmission associated with biological materials (OPF samples and tissues) from persistently infected FMDV carrier cattle.

Although the manner of transfer of OPF samples to naive cattle used herein could not occur under natural conditions, the finding of transmission of FMD from unprocessed OPF samples from carrier cattle indicates that the potential risk of disease outbreaks being initiated from these animals is real and cannot be discounted. Naive cattle and pigs were exposed to OPF samples from persistently infected carriers via direct intranasopharyngeal (INP) (cattle) or intraoropharyngeal (IOP) (pigs) deposition. These challenge systems have been developed to simulate natural, species-specific, exposure conditions while maintaining control of the dose and timing of virus challenge (38, 39). These challenge systems are needle-free inoculation systems that do not breach the mucosal barrier but rely on natural mechanisms of virus entry into susceptible cells at the documented primary infection sites. Additionally, one group of pigs was fed nasopharyngeal tissues from FMDV carriers to test the hypothesis that feeding pigs offal from persistently infected animals may constitute a risk for initiation of FMD outbreaks.

Titration of the pooled OPF on highly sensitive LFBK- α v β 6 cells suggested that the INP-challenged cattle had each received a total dose of 10^2 TCID₅₀, whereas the IOP-challenged pigs had each received a dose of 5×10^1 TCID₅₀. However, the infectivity of the pooled OPF was below the detection limit when titration was attempted using the cell line most commonly used for isolation of FMDV (BHK-21 cells). The small (twofold) difference in inoculation dose between cattle and pigs was purely a practical consequence of not being able to deposit a larger volume of fluid onto the porcine tonsil of the soft palate. It was not possible to measure an infectivity titer in the macerated tissue that was fed to the pigs in experimental phase III. However, RT-qPCR analysis and successful virus isolation on LFBK- α v β 6 cells indicated the presence of low quantities of infectious FMDV in this material.

Viral sequence analysis was utilized to determine whether multiple animals had been infected by the phase II inoculum, as opposed to a single animal being infected by inoculation and subsequently transmitting the disease to the other phase II animals. The predicted VP1 amino acid sequence encoded by the FMDV RNA isolated from nasal swab samples obtained from the clinical phase of cattle in phase II indicated that infection of that group of cattle was seeded by at least two distinct viral variants. Specifically, the viral sequence obtained from animal 13, which had developed clinical FMD 1 day earlier than the majority of animals of the group, was similar to only one other sample obtained from that group (animal 10). This suggests that animals 12, 14, 15, 16, and 17 were not infected by animal 13, but rather through at least one additional distinct primary infection directly from the inoculum. Interestingly, all sequences obtained from phase II samples differed from the phase I samples within the integrin-binding region of the G-H loop (RSGD in phase I samples versus RRGD in phase II samples). However, since only samples from the clinical phase of infection were included in this analysis, it is likely that this substitution had occurred during the later stages of infection of the phase I animals, as suggested by the sequence obtained from the pooled OPF inoculum (Fig. 3).

The use of OPF and the simulated natural INP system in this study had advantages but also intrinsic limitations. Under natural conditions, the probability that a sufficient quantity of FMDV shed from a carrier animal would reach the susceptible cells within the nasopharynx of a naive animal is likely very small. However, the successful transmission under these experimental conditions substantiates the perceived risk of contagion associated with persistently infected FMDV carrier cattle. Furthermore, these

findings confirm that the infectious dose of FMDV required to infect cattle through nasopharyngeal exposure is very low.

There has been speculation on the mechanisms responsible for the low infectiousness of FMDV carrier cattle, but the mechanisms have not been definitively elucidated. It has been postulated that FMDV in OPF from persistently infected animals may be shed either as immunoglobulin-bound (cell-free) virus or within detached cells or cellular debris (2). Oropharyngeal fluid samples from FMDV carriers contain secreted anti-FMDV IgA (13, 40, 41), which may decrease infectivity. Furthermore, it has been demonstrated that the apparent infectivity of OPF samples increases substantially for tissue culture cells if these samples are pretreated with a fluorocarbon compound (1,1,2-trichlorotrifluoroethane or trichlorotrifluoroethane [TTE]), which is believed to dissociate immunoglobulins bound to FMDV (30, 42). The advantages of using TTE treatment of OPF samples prior to virus isolation have been utilized in multiple large-scale investigations (13, 19, 43, 44) and were confirmed in this investigation by an increased titer measured in the TTE-treated OPF. However, it is also possible that the increased virus titers detected after TTE treatment of OPF may be caused by disruption of cellular membranes and thereby release of virus from cells or cellular debris present within the OPF. Shedding of persistent FMDV in association with sloughing of superficial nasopharyngeal epithelial cells is consistent with findings from investigations using immunomicroscopy in our laboratory (13). Additionally, previous *in vitro* investigations have suggested that FMDV may be released from infected cells within “cytoplasmic blebs” (45). This may be similar to the nonlytic cellular escape mechanisms that have been described for other picornaviruses (46). Specifically, hepatitis A virus and some members of the *Enterovirus* genus have been reported to be released from infected cells in a “quasi-enveloped” state within membranous vesicles (47–49). To our knowledge, such nonlytic cellular escape of FMDV has not yet been described *in vivo*. Although the mechanism of shedding of FMDV into OPF in persistently infected animals is not known, the possible release of virus within membranous compartments could potentially prevent immediate binding of secreted IgA to FMDV and thus protect the infectivity of shed virus.

The pigs exposed to the OPF from the carrier cattle in this investigation did not become infected. This is in agreement with previous reports that have concluded that higher infectious doses are required to initiate FMD in this species (2, 34). Feeding unprocessed food waste to pigs has been associated with initiation of FMD epidemics (50). However, the current findings suggest that the amounts of FMDV present in OPF or nasopharyngeal tissues of FMDV carriers may not be sufficient for disease dissemination to pigs to occur readily via this route. Further investigations are necessary to determine a minimum infectious dose to infect pigs via oropharyngeal exposure.

Conclusions. This current investigation demonstrated that it is possible to transmit FMDV from persistently infected cattle to naive recipient cattle via mechanical transfer of unprocessed oropharyngeal fluid. Pigs exposed to the same material, under similar conditions, did not develop FMD, which suggests that a higher infectious dose and/or distinct mechanisms are required to infect this species by this route. These results contribute to improved understanding of the FMDV carrier state and provide novel information that is relevant for FMD risk analyses.

MATERIALS AND METHODS

Virus. The virus used for this investigation was an isolate of FMDV strain A24 Cruzeiro that had been passaged twice in cattle as used previously (13).

Authentication of cell lines. The cell lines used for virus isolation were LFBK- $\alpha\text{v}\beta\text{6}$ (51), which are porcine kidney cells transfected with a bovine integrin receptor, and BHK-21 cells originally obtained from ATCC (Manassas, VA, USA). Species specificity and absence of cross-contamination of cell lines were confirmed by validated conventional PCR assays detecting DNA from pig, cow, hamster, and monkey, corresponding to the species origin of cell lines routinely used in the laboratory.

Animals. All animal experiments were performed in the CL3Ag FMDV research facility on Lindholm Island, which is part of the National Veterinary Institute of the Technical University of Denmark (DTU). This study was conducted with approval from the Danish Animal Experimentation Inspectorate (license 201415-0201-00173) in accordance with Danish and European Union (EU) legislation (Consolidation Act

474 15/05/2014 and EU Directive 2010/63/EU). Cattle were Holstein bull calves weighing approximately 150 to 200 kg upon arrival. Pigs were conventionally bred Landrace/Yorkshire mixed males and females weighing approximately 20 kg at delivery. All animals were allowed 1 week of acclimatization in the facility before the start of the experiments.

Study design. Phases I and II of the study were carried out sequentially, using separate isolation units, while phase III was performed several weeks later after cleaning, disinfection, and fumigation of the unit.

Phase I. The first phase of the experiments included nine cattle that were inoculated with 10^5 infectious doses (titrated in bovine tongue epithelium) of FMDV A24 via intranasopharyngeal (INP) inoculation as previously described (38). Briefly, cattle were sedated and placed in sternal recumbency, and the virus inoculum (2 ml), diluted in minimal essential medium (MEM) with 25 mM HEPES, was deposited within the nasopharynx using a flexible 30-cm catheter. The cattle in the first phase of the study were monitored for 30 days postinfection (dpi). Oropharyngeal fluid (OPF) samples for challenge of cattle and pigs in phases II and III were collected at 30 dpi, and tissues for challenge of pigs in phase III were harvested at 31 dpi immediately after euthanasia. Nasopharyngeal tissue samples for feeding to pigs consisted of the dorsal surface of the soft palate and the dorsal surface of the nasopharynx as previously described (13, 16). Tissue samples were macerated using forceps and scissors to facilitate feeding to pigs in phase III. Clinical monitoring and sample collection were carried out as described below.

Phase II. The second phase of the experiment included eight cattle that were subjected to INP inoculation as described above, but with each animal receiving 10 ml of pooled OPF that had been harvested at 30 dpi from seven phase I animals that had been identified as being persistently infected carriers. Prior to inoculation of phase II animals, the pooled OPF was homogenized using a 16-gauge metal cannula attached to a 50-ml syringe in order to disrupt mucous and potential epithelium within the sample. There was no further processing or freezing of this material prior to inoculation of the phase II cattle. Challenge of phase II cattle (0 dpi) was performed on the same day as the OPF samples were harvested from phase I animals (30 dpi). Animals in this second phase of the experiment were monitored for a further 14 days.

Phase III. The third phase of the investigation included two groups of five pigs each. The pigs in the first group were inoculated with OPF from the phase I carrier cattle (stored at -80°C following collection) by intraoropharyngeal (IOP) inoculation as previously described (39). Briefly, sedated pigs were placed in dorsal recumbency, and the inoculum (5 ml) was deposited onto the surface of the tonsil of the soft palate using a blunt-ended metal cannula. Pigs were kept on their backs for approximately 1 min before they were placed in sternal position and left to recover from the sedation. The pigs in the second group were each fed approximately 45 g of nasopharyngeal tissue harvested from the phase I cattle. The macerated tissues were mixed with a small amount of the pig's standard feed, and each pig received the measured ration in a separate feeding bucket. Both groups of pigs were monitored for the following 14 days.

Clinical monitoring and sample collection. Samples collected to evaluate infection dynamics in cattle were nasal swabs and blood and OPF samples. Nasal swabs were collected using tampons inserted into each nostril, and blood samples were collected through jugular venipuncture. Blood samples and tampons were centrifuged at $2,075 \times g$ for 10 min to harvest serum and nasal fluid, respectively. Blood samples were collected daily from 0 to 10 dpi, and once per week thereafter. Nasal swabs were collected daily from 0 to 10 dpi and twice per week thereafter. OPF was collected using a probang cup. Probang sampling of phase I animals was conducted twice weekly from 14 to 28 dpi, with an additional final sampling at 30 dpi in order to harvest OPF for challenge of phase II animals. The OPF was diluted with an equal volume of MEM with 25 mM HEPES, except for the 30 dpi sample for which the amount of added medium was reduced to half (giving a 2:1 ratio). OPF samples were homogenized using a syringe and cannula as described above. FMDV carrier status of phase I animals was determined on the basis of consistent detection (by real-time quantitative PCR [RT-qPCR]) of FMDV RNA in OPF from 14 dpi to 28 dpi. At 30 dpi, OPF samples were collected only from the seven cattle that had been identified as FMDV carriers.

Samples collected from pigs were whole blood and oropharyngeal (OP) swabs as previously described (39). The OP swab samples were harvested using a large cotton swab applied to the tonsil of the soft palate and subsequently immersed in MEM (2 ml) with 25 mM HEPES. Swabs and blood samples were centrifuged as described above to retrieve the OPF and serum. Swabs were collected daily from 0 to 10 dpi and again at 14 dpi. Blood samples were collected on 0, 2, 3, 4, 6, 8, 10, and 14 dpi.

Daily clinical examinations of both cattle and pigs included monitoring for any signs of FMD, such as fever, lameness, and vesicular lesions. The occurrence and progression of clinical FMD were recorded using a cumulative lesion score for which any lesion on the head contributed 1 point and foot lesions contributed 1 point per affected foot, giving a maximum score of 5. For cattle, the head and oral cavity were inspected daily from 0 to 10 dpi. Feet were inspected twice weekly in sedated cattle (0, 3, 6 and 10 dpi for phase I cattle and 0, 3, 5, 7, and 10 dpi for phase II cattle). For pigs, the head and feet were examined daily from 0 to 10 dpi and again after euthanasia at 14 dpi.

FMDV RNA detection. Total RNA was extracted from serum, nasal fluid, and OPF samples using a MagNA Pure LC total nucleic acid isolation kit (product no. 03038505001; Roche) with an automated robotic workstation (Roche), according to the manufacturer's instructions. The level of FMDV RNA in serum and nasal fluid samples was determined using a one-step quantitative RT-PCR assay targeting the 3D-coding region of the FMDV genome (52) as modified by Vandenbussche et al. (53). OPF samples were analyzed using similar primers and probes, but in a two-step assay. Briefly, cDNA was produced using

TABLE 2 Primers used for RT-PCRs and sequencing

Primer	Primer sequence (5'–3') ^a	Orientation	Location (nt) ^b
8-APN35	GAGAAAXGGGACGTCXGCGC	Forward	522
8-APN2	GTCXCCTATTACAGGCXTAGAAG	Reverse	990
8-APN3	GGCTAAGGATGCCCTTCAG	Forward	894
9-XPN18	TTXGAXAACCAXTCXTTXXTGXGTGT	Reverse	1832
14-CPN63	CCGTTGGAGGTGACACACG	Reverse	1503
14-CPN7	ATGCCATCAGTGGAGGCTCC	Forward	1773
14-CPN6	GTCCAACAGGTTGGTAAGC	Reverse	2679
14-CPN5	ATGGCAAGGTGTACAACCCG	Forward	2637
11-FPN35	GAARGGCCRRGGTTGGAC	Reverse	3898
14-CPN61	GGAGGCGCAACTCAAAGTC	Reverse	3185
14-CPN3	TACAACAAGGCACCATTCACG	Forward	3542
11-SPN3	ACACTGTCGCCAGCACACG	Reverse	3593
14-CPN4	CCAGACCCTGTTGGCAATAG	Forward	3783
8-APN45	GGAAGAACTCGAGGCGAC	Reverse	4316
8-APN22	AAGGACCCXGTCCTTGTGGC	Forward	4151
1-XPN28	GTTGTAGCCGTCXAAGTGGTC	Reverse	4808
8-APN46	TGGTCGTTTCCCTCCGTGG	Forward	4683
8-APN87	CTCAAAGAATCAATTGCTGC	Reverse	5387
8-APN13	GXCCTTCTXAACGGXATGGC	Forward	5171
8-APN68	GGGTCCTTCAGCTGGTGG	Reverse	5774
8-APN113	CGCGAXACTCGCAAGAGAC	Forward	5555
9-XPN11	AGCATGTCCTGCTTTTACT	Reverse	6223
14-CPN2	CCATTTGCTGTGCTACTGGA	Forward	6084
8-APN114	CAGGGTTGAACACCCGTG	Reverse	6716
9-XPN2	AATGAAGGCACACXTXGAXCCXGA	Forward	6601
14-CPN1	AGGGTTACAACCGACCGCG	Reverse	7276
8-APN17	CTGAAGGACGAXTXCGXCC	Forward	7124
8-APN52	GGAXTGACCAAGAACAACCC	Reverse	7754
9-XPN23	TGGACACXTACCATGATCTC	Forward	7620
NVT27	TTTTTTTTTTTTTTTTTTTTTTVN	Reverse	8140

^aX = inosine.

^bThe primer locations are based on the sequence of FMDV A24 Cruzeiro (accession no. [AY593768.1](https://www.ncbi.nlm.nih.gov/nuclot/AY593768.1)). For forward primers, the 5'-terminal nucleotide (nt) is used, while for the reverse primers, the position of the nt that is complementary to the 3' nt of the primer is given.

reverse transcriptase (TaqMan reverse transcriptase; Applied Biosystems) with random hexamer primers (Roche). The quantitative PCR was performed using AmpliTaq (Applied Biosystems) using the primers and probes described by Callahan et al. (52).

Quantitation of infectivity in OPF and tissue macerate. The *in vitro* infectivity of the OPF and nasopharyngeal tissue samples obtained from the seven persistently infected carriers at 30 dpi was evaluated through titration on BHK-21 and LFBK- $\alpha\text{v}\beta\text{6}$ cells (51) as previously described (17). Two aliquots of 30 mg of tissue macerate were thawed, MEM (900 μl) and two steel beads (catalog no. 69989; Qiagen) were added to each aliquot, and the samples were homogenized using a TissueLyser bead beater (Qiagen) (2 min; frequency, 22/s). The processed macerates were pooled and clarified through centrifugation at $1,000 \times g$ for 2 min at 4°C. For both the processed tissue macerate and the pooled OPF sample, one aliquot (1 ml) was treated with 1,1,2-trichlorotrifluoroethane (TTE) for dissociation of immunocomplex-bound virus as previously described (54). All four sample aliquots (TTE-treated and nontreated OPF and processed tissue macerate) were cleared of debris and potential bacterial contamination by centrifugation through Spin-X filter columns (pore size, 0.45 μm ; catalog no. 8163; Costar) and were subsequently inoculated onto the two different cell types. Calculations of infectivity (as 50% tissue culture infectious doses [TCID₅₀] per milliliter) were performed by standard methods (55). An additional attempt to isolate FMDV from the tissue macerate was performed by inoculating T25 flasks of both LFBK- $\alpha\text{v}\beta\text{6}$ and BHK-21 cells with 1 ml of non-TTE-treated and nonfiltered macerate. All supernatants were analyzed by RT-qPCR to determine the occurrence of FMDV replication.

Detection of anti-FMDV antibodies in serum. Serum samples obtained at 0 and 14 dpi from the pigs in experimental phase III were analyzed for the presence of anti-FMDV antibodies using a solid-phase blocking enzyme-linked immunosorbent assay (ELISA) as previously described (56).

FMDV sequence assembly and analysis. RNA was extracted from nasal swab samples from individual calves (collected on preselected days) and also from the FMDV A24 inoculum using the MagNA Pure system (Roche) as described by the manufacturer. The cDNA synthesis was carried out using Ready-To-Go You-Prime First-Strand beads (GE Healthcare Life Sciences, Uppsala, Sweden) with a mixture of random hexamer primers [pd(N)6] and oligo(dT). The cDNA was diluted to 150 μl from which an aliquot (5 μl) was used to amplify the VP1 coding region using primers 10-PPN6 (5'-TAGCGCCGGCAAA GACTTTGA) and 1-OPN20 (5'-GACATGTCCTCTGCATCTG). The products (ca. 810 bp) were purified using a QIAquick gel extraction and purification kit (Qiagen, Hilden, Germany) and quantified using a Nanodrop 1000 spectrophotometer (Fisher Scientific, Waltham, MA, USA). Sequencing of the PCR products was

performed using a BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), using the same primers as used in the PCRs, according to the manufacturer's instructions, and ran on an automated DNA sequencer (ABI PRISM 3730 DNA analyzer; Applied Biosystems, Foster City, CA, USA). The resulting consensus sequences were compared to others using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and translated into amino acid sequences using ExpAsy (https://web.expasy.org/cgi-bin/translate/dna_aa). Near-full-length genome sequences, from selected samples, were assembled using Seqman Pro 14 based on the production and sequencing of 15 separate PCR products, using primers (Table 2) to produce overlapping amplicons.

Accession number(s). The sequence of the inoculum used to infect the animals, and to which all other sequences were compared, is available at GenBank under accession no. [MH746921](https://www.ncbi.nlm.nih.gov/nuccore/MH746921).

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