The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT–PCR

Soren Alexandersen, Martin B. Oleksiewicz† and Alex I. Donaldson

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

Foot-and-mouth disease (FMD) is a highly contagious, economically important virus disease of cloven-hoofed animals. The objective of the present study was to examine the early pathogenesis of FMD in pigs by a quantitative time-course study. Under experimental conditions, recipient pigs were infected by contact with donor pigs affected by FMD. Every 24 h from day 1 to day 4 after exposure, two recipient pigs were selected randomly, killed and necropsied. A range of tissues were analysed by a quantitative TaqMan RT–PCR method and by titration of FMD virus on primary bovine thyroid cells. The titres of virus determined by assay in cell culture and calculated from the quantitative TaqMan data correlated strongly \((r > 0.9)\), thereby establishing the validity of the TaqMan calculations. The data indicated that the replication of virus in the lungs contributes only in small part to airborne virus excretion. Sites in the pharynx, trachea and nasal mucosa are probably more important in that regard. The sites of earliest virus infection and possibly replication in recipient pigs appeared to be in the pharynx (soft palate, tonsil and floor of pharynx). The data indicated that FMD virus replication in pigs is rapid and that the majority of virus amplification occurs in the skin. A model for the progression of infection is proposed, indicating initial spread from the pharyngeal region, through regional lymph nodes and via the blood to epithelial cells, resulting in several cycles of virus amplification and spread.

Introduction

Foot-and-mouth disease (FMD) is a virus disease of cloven-hoofed animals characterized by the appearance of vesicles on the feet and in and around the mouth. FMD virus (FMDV) belongs to the genus *Aphthovirus* in the family *Picornaviridae* (Belsham, 1993). FMD is the most contagious disease of animals and the major constraint to international trade in livestock and animal products. Features of FMD that contribute to its highly contagious nature include the very small quantities of virus that can initiate infection, the short ‘generation time’ of virus in infected animals, the large quantities of virus excreted by infected animals and the multiplicity of routes by which the virus can infect its hosts. As a consequence, FMD can be transmitted by a variety of mechanisms including, under certain climatic and epidemiological conditions, by the wind (Anonymous, 1969; Donaldson et al., 1982; Gloster et al., 1982). The pattern of windborne spread of FMD over more than 10 km is invariably from pigs at source to cattle downwind (Donaldson, 1979, 1986, 1987; Gloster et al., 1982). This is because infected pigs are powerful emitters of airborne FMDV and cattle are highly susceptible to infection by inhalation (Donaldson et al., 1970, 1987; Donaldson & Ferris, 1980; Sellers, 1971; Sellers & Parker, 1969).

The objectives of the present study were to determine the early pathogenesis of FMD in pigs by a time-course study using a quantitative TaqMan assay (Oleksiewicz et al., 2001) and to correlate these findings with results obtained using virus isolation in cell culture and histopathological examination of selected tissues.

Methods

**Animals.** Four donor, eight recipient and two non-infected control Landrace crossbred Large White pigs, each weighing 20–30 kg, were used in the experiment. They were housed in biosecure isolation...
buildings. After exposure to virus, the recipient and donor pigs were observed daily for clinical signs of FMD by visual inspection only. The donor pigs were killed (by exsanguination following captive bolt stunning) immediately after the period of contact with the recipient pigs. Blood samples were taken each day from two recipient pigs (pigs UD94 and UD95) to monitor the progression of infection. These two pigs were clinically examined each day at the time of blood sampling. Every 24 h from day 1 to day 4 post-exposure (p.e.), two recipient pigs were selected randomly, clinically examined and then killed. At 4 days p.e., two freshly introduced, unexposed pigs were included as non-infected controls. Blood samples for sera were taken when the pigs were being exsanguinated and a range of other tissues were collected during necropsy. One series of tissues was immersed immediately in RNA later (Ambion) for RT–PCR analysis. Conversions to tissue-content equivalents (TCID<sub>50</sub> equivalents/g) were made with the assumption that 1 g tissue contained approximately 10<sup>8</sup> cells (Alexandersen et al., 1988) and that a cell contained 6 pg total RNA on average (Ausubel et al., 1988; Maniatis et al., 1982). Another series was snap-frozen over solid CO<sub>2</sub>, transferred to the laboratory and stored at −70 °C for virus titration. Sera were stored at −20 °C. Selected tissues were fixed in 10% neutral buffered formalin for 24 h, paraffin embedded, sectioned, stained with haematoxylin and eosin and examined by light microscopy. Specimens collected during previous experiments were also examined (Oleksiewicz et al., 2001).

**Virus.** A stock preparation of FMDV strain O I, Lausanne Sve/65 was obtained from the International Vaccine Bank, IAH, Pirbright, UK. It had been passaged once in cattle and then grown in porcine IB-RS-2 cells (De Castro, 1964). For the experiments, a pig-adapted inoculum (S. Alexandersen, I. Brotherhood & A. I. Donaldson, unpublished) was made by passaging the original stock virus sequentially through a series of three pigs. The first passage was by intradermal inoculation in the heel pads, the second by experimental aerosol transmission and the third by intradermal/subdermal heel-pad inoculation of an epithelial suspension prepared from foot lesions. The experimental inoculum was a 10% (w/v) suspension of epithelial tissue from a single pig with multiple vesicular lesions (S. Alexandersen, I. Brotherhood & A. I. Donaldson, unpublished). The inoculum was stored at −70 °C. It had a titre of 10<sup>8.7</sup> TCID<sub>50</sub> /ml when assayed by end-point dilution in primary bovine thyroid (BTV) cells (Snowdon, 1966) and 10<sup>8.4</sup> TCID<sub>50</sub>/ml when assayed in IB-RS-2 cells.

**Infection of donor pigs and contact exposure of recipients.** The virus suspension used to infect the donor pigs was the pig-adapted inoculum diluted 1:10 immediately before use in Eagle’s minimal essential medium (MEM) with 20 mM HEPES buffer and 2× antibiotics. Each of four donor pigs received approximately 0.5 ml of the suspension (i.e. approximately 10<sup>5</sup> BTV TCID<sub>50</sub>) by the intradermal/subdermal route in the heel bulbs (Burrows, 1966) of the left fore foot. Two pigs were inoculated on day 3 and two more on day 2 before being used as donors. The eight recipient pigs were exposed to virus by moving them into the isolation room containing the four donor pigs for a 2 h period, i.e. direct contact exposure. The donor pigs had severe, generalized lesions of FMD at this time and remained recumbent. The isolation room was within a biosecure isolation compound. After exposure, the recipient pigs were returned to their respective isolation rooms, where they were housed in individual cubicles, two animals per room, to prevent direct contact between room-mates. A detailed description of the cubicles and the isolation rooms has been submitted for publication and is available from the corresponding author.

The donor pigs were killed immediately after the 2 h exposure period. The personnel then thoroughly cleansed and disinfected their protective clothing and all other materials that had been in contact with the pigs. In order to avoid cross-infection, the recipient pigs (except pigs UD 94 and UD95, from which blood was sampled daily) were not handled until they were killed for necropsy.

**Air sampling.** During exposure of the recipient pigs, air samples were collected by passive sedimentation into 18 ml tubes with a diameter of 9 mm containing 1 ml Trizol and 0·1 ml Eagle’s MEM with 20 mM HEPES and antibiotics, which were held in a rack placed at a height of about 1 m from the floor directly above the recumbent pigs. The caps were removed from the tubes during the 2 h exposure period.

**Assay for virus.** Tissue suspensions were made as 10% (w/v) suspensions in maintenance medium by careful grinding with sterile sand in a mortar followed by low-speed centrifugation. Tissue suspensions and serum samples were assayed for virus in monolayer cell cultures of primary BTV cells in roller tubes after making a 10-fold dilution series of each sample and inoculating each dilution into five tubes (Donaldson et al., 1987; Gibbon & Donaldson, 1986; Snowdon, 1966). Infectivity titres were calculated according to Karber (as described by Lennette, 1964). The specificity of CPE was confirmed by antigen-detection ELISA (Ferris et al., 1988; Ferris & Dawson, 1988; Hamblin et al., 1984; Roeder & Le Blanc Smith, 1987).

**Assay for antibodies.** Serum samples were assayed for antibodies to FMDV by liquid-phase blocking ELISA as described previously (Hamblin et al., 1986a, b).

**Quantitative RT–PCR.** A quantitative RT–PCR method was used to determine the amount of FMDV RNA in extracts of RNA from blood and tissue samples (Oleksiewicz et al., 2001). We decided to use an assay employing primers located in the internal ribosome entry site (IRES) (Belsham, 1993) because (i) the best primer set available in the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) for diagnosis of many isolates of FMDV is in the IRES region (see Reid et al., 2000) and (ii) we have recently developed a hybridization-based RT–PCR ELISA capable of detecting nearly all isolates of FMDV by using primers and oligonucleotide probes based in the IRES region (see Alexandersen et al., 2000). All tissue samples were subjected to RNA extraction and cDNA preparation after 1–3 days in RNA later. Four 4-fold RNA dilutions (3–200 ng RNA) and the minimal C<sub>50</sub> method (the C<sub>50</sub> method scoring the lowest C<sub>50</sub> value, i.e. highest virus RNA content, obtained in the four dilutions) were used in combination with specific primers (SAIR 89F, 5′ CTGTCTCAGCGGAGCAGTG 3′, and SAIR 151R, 5′ GCCCCGTG-GGCTCTCTG 3′) designed specifically for the O<sub>1</sub> strain (Kaufbeuren/01, Lausanne strains of FMDV, as detailed previously (Oleksiewicz et al., 2001). The C<sub>50</sub> method was developed specifically to overcome the problems of large variation in RNA yields and tissue- specific RT–PCR inhibitors experienced when extracting RNA from solid tissue. On serum and impinger samples, we used a simplified version of the protocol, essentially omitting the absorbance measurement and RNA dilution steps and using RNase-free oyster glycogen as co-precipitant. RNA was extracted from 100 μl aliquots of the samples using the Trizol procedure. All estimations, including standard reactions using tissue samples with a known content of FMDV (as determined by virus titration in cell culture), and furthermore all quantifications were based on a comparison of the minimal C<sub>50</sub> of samples with standard curves based on: (i) a dilution series of cell culture supernatant harvested by vigorous shaking of the flasks at 40 h after infection with FMDV (infectious titre determined by virus titration); (ii) comparison with pig sera (infectious titre determined by virus titration); and (iii) comparison with the infectivity titre of selected tissue suspensions assayed on BTV cells. The method is influenced minimally by tissue type and has a sensitivity (still
in the quantitative range) of approximately 0.1 TCID₅₀/ml of either serum or cell culture or 1–10 TCID₅₀/g tissue suspension for quantitative estimation. Although they were outside the range of quantitative estimation, tissue suspensions containing as little as 1 TCID₅₀ equivalent/g could consistently be detected as weak positives in comparison with non-infected tissues and cell cultures. One additional advantage of real-time PCR is that potential contamination with PCR product is greatly diminished, because the PCRs are never opened after cycling. Also, the dUTP/UNG contamination prevention system was used in TaqMan PCRs (PE Applied Biosystems). However, even in the absence of contamination with PCR product, unavoidable formation of unspecific PCR product and primer dimers will invariably cause an increase in TaqMan signal at very high cycle numbers (not shown). Thus, we have observed that, at high cycle numbers, negative TaqMan PCRs will, in a very small number of reactions, produce false-positive fluorescence (around 1.5% false-positive TaqMan PCRs in the C₅₀ 40–50 region when tested on a large number of negative controls). Nevertheless, detection and quantification of very low levels of virus is still possible, because no false-negative reactions were seen in the C₅₀ 30–40 range. Moreover, we observed that true weak-positive cDNAs that yielded C₅₀ values in the range 40 to 50 did so reproducibly in repeated PCRs. By contrast, negative cDNAs that yielded false-positive C₅₀ values in the range 40 to 50 were negative on repeated PCR (not shown). We believe that such unrepeatable false-positive TaqMan PCRs may not be due to contamination, but might relate to evaporation of PCRs or non-specific hydrolysis of TaqMan probe at very high cycle numbers. In effect, the very low level (1.5%) and non-reproducibility of false-positives makes it possible to use the C₅₀ 40–50 range for detection as well as quantification of very low levels of FMDV by TaqMan PCR, provided that several replicate PCRs are made from each cDNA. Consequently, it is considered that, since two pigs were examined at each time-point and data were obtained for days 1–4 p.e., the results shown in Figs 1–5 are valid, with samples being positive at a cut-off point or at above 0.1 TCID₅₀ equivalents/ml serum or 1 TCID₅₀ equivalent/g tissue.

**Results**

**Initial experiments to determine whether there was a correlation between quantitative RT–PCR results and titres estimated by virus titration in cell cultures**

Initially, 12 of the 16 serum samples collected in the experiment and six serum samples from other experiments (S. Alexandersen, I. Brotherhood & A. I. Donaldson, unpublished) were analysed both by quantitative RT–PCR and by virus titration and the values were plotted (Fig. 1). The linear correlation coefficient (r) between FMDV RNA content and infectious titre exceeded 0.95 over 7 log units, thus establishing the validity of converting TaqMan results obtained from serum to TCID₅₀ equivalents by comparison with standard curves (at least up to day 3, and in our example even including two day 3 samples among the 18 samples). The kinetics of the viraemia results from the present study plotted as infectivity titres in BTY cell cultures and as titres converted from TaqMan data are shown in Fig. 2. Viraemia peaked at around 10⁴ TCID₅₀/ml at 3 days p.e. and decreased at 4 days p.e. in one animal.

In addition, selected tissues were tested both by quantitative RT–PCR and by virus titration and values were plotted (Fig. 3 a, b). The correlation coefficient (r) was 0.91 for tissue samples tested on day 3 (pig UD93) and, moreover, the starting point of the curve was very close to zero (actually an overestimation by TaqMan calculation of around 0.1 log) and the slope of the curve was very close to 1 (1.04). The titrations were performed twice and gave almost identical results. The results shown are the means of two experiments. Tissues from a pig killed on day 4 (pig UD94) were also titrated and compared to TaqMan-calculated equivalents. Again, the linear correlation between infectious titre and the TaqMan estimate was strong (r = 0.96). However, the TaqMan results on these day 4 tissues were apparently about 2 log units higher than those obtained by virus titration (Fig. 3 b). This is probably because the pigs examined at 4 days p.e. had already produced local antibodies (especially pig UD94; see later) that may have altered the correlation by partly neutralizing the virus. Thus, evaluation by t-test supported the null hypothesis, i.e. that the mean titres of 18 serum samples were the same in TaqMan as in infectivity assays for the day 3 tissue samples. The day 4 tissue samples, containing low levels of neutralizing antibodies, did not support the null hypothesis; instead, a t-test indicated that the TaqMan estimate was around 2 log units higher than the infectivity result (P < 0.05). Thus, the data support the conclusion that the TaqMan assay is highly reliable for determining FMDV content, despite the reduction of virus infectivity, which was presumably due to neutralization by circulating antibodies. In order to get the best possible quantifications based on the data available, we used the curve from the UD93 samples (Fig. 3 a) to calculate the TCID₅₀ equivalents from the TaqMan data from tissue samples.

**Quantitative RT–PCR results and calculation of equivalents to infectivity titres (TCID₅₀ equivalents)**

Based on the data above, we assayed all of the tissue samples in this pathogenesis study by quantitative RT–PCR.
and converted the C\textsubscript{T} values obtained to estimated TCID\textsubscript{50} equivalents. The results are presented as scatter diagrams showing the content of each sample (Fig. 4). In general, the results differed very little between individual pigs (usually less than 10-fold variation). The data are shown on a log\textsubscript{10} scale to be comparable with the way infectivity data are usually depicted. The discriminating power of the assay is 5- to 10-fold (i.e., a log\textsubscript{10} variation). The data are shown on a log\textsubscript{10} scale to be comparable with the way infectivity data are usually depicted.

Fig. 4 shows that significant amounts of virus could be detected in several tissues as early as 1 day p.e. However, the concentrations were usually low on days 1 and 2 p.e. and then rose on days 3 and 4 p.e. Some tissues, including liver, spleen, lung and bronchial lymph node, contained very low virus concentrations on days 1 and 2 p.e., which then increased on days 3 and 4 p.e. However, the levels did not exceed those found in the corresponding serum samples. Two sites in the lung were examined: the frontal lobe (lung 1) and the accessory lobe (lung 2). The virus contents of the two were found to be very similar. In a previous study, we had quantified the FMDV in five different areas of the lungs of two pigs. The virus contents of the two were found to be very similar. In a previous study, we had quantified the FMDV in five different areas of the lungs of two pigs. The virus contents of the two were found to be very similar. In a previous study, we had quantified the FMDV in five different areas of the lungs of two pigs. The virus contents of the two were found to be very similar. In a previous study, we had quantified the FMDV in five different areas of the lungs of two pigs. The virus contents of the two were found to be very similar. In a previous study, we had quantified the FMDV in five different areas of the lungs of two pigs.
Correlation of virus titres in selected tissue samples subjected to titration on BTY cells and calculated from TaqMan data. Titres are given on log_{10} scales. (a) Tissue samples from pig UD93 at 3 days p.e. The correlation coefficient (r) is greater than 0.91. The mean values from two virus titration experiments are shown. (b) Tissue samples from pig UD94 at 4 days p.e. The correlation coefficient (r) is greater than 0.96, although infectivity is reduced by approximately 2 log units (P < 0.05) by the presence of specific antibodies. Abbreviations: Lung 1, frontal lobe of lung; Lung 2, accessory lobe; Man LN, mandibular lymph node; VFPH, ventral floor of pharynx; w, with; w/o, without.

lungs, soft palate or tonsil. Therefore, histopathological examination did not indicate any significant cytopathogenic or inflammatory changes in any internal organs associated with the FMDV infection.

Tests on serum samples taken during the experiment revealed that one day-4 pig (UD94) had an ELISA antibody titre of 1:362 on day 4 while the other day-4 pig (UD95) had a titre of 1:128. This last pig (UD95) had a borderline positive titre of 1:45 (1:40 being the cut-off point; Donaldson et al., 1996) on day 2. All other sera were negative for specific antibodies.

Discussion

In the present study, the kinetics of the amplification and accumulation of FMDV RNA during the early stages of incubation and clinical FMD in pigs infected by contact exposure were investigated. Virus assays on a selected range of tissues were performed by using a recently described TaqMan RT–PCR assay for quantifying FMDV RNA in specimens containing the O_1 Kaufbeuren/Lausanne strain of FMDV (Oleksiewicz et al., 2001). When the data were compared with those from other recent investigations, in which pigs were infected by other routes, it was found that the kinetics of FMDV replication were very rapid under all circumstances, but the data may suggest that the rate of development of peak titres can be influenced significantly by the dose of challenge virus and the route of exposure.

The TaqMan RT–PCR assay used had a quantitative range of more than eight orders of magnitude and the results correlated strongly with infectivity titres obtained from virus grown in cell culture preparations. We applied the method in the present work and obtained a strong correlation between the quantities of FMDV RNA and virus infectivity in serum and tissue samples. However, for certain tissue samples, estimates by RT–PCR were approximately 2 log units higher than those determined by virus titration, depending on the actual sample. This is most likely due to the presence of low levels of FMDV-neutralizing antibodies in the day 4 p.e. tissue samples. Nevertheless, estimation of FMDV contents in tissue samples on the basis of TaqMan RT–PCR and standard curves gave consistent results, irrespective of the presence of antibodies.

The time-course showed that the appearance of vesicular lesions was coincident with the peak of viraemia and high concentrations of virus in sites where there were clinical lesions. Histopathological abnormalities were found only in areas where there were macroscopic lesions. However, samples of tissue from histopathologically normal areas in the tongue and skin contained significant amounts of virus, albeit 1–2 log units less than similar sites with macroscopic lesions. The amount of virus was up to 10^8 TCID_{50} equivalents/g and 10^7–10^8 TCID_{50} equivalents/g in normal skin and pharynx, respectively, during the period of the investigation (days 1–4 p.e.). Relatively high titres of FMDV have been described previously in macroscopically normal skin from cattle, although microscopic lesions, i.e. microvesicles, were observable in the sections (Gailiusas, 1968; Gailiusas & Cottral, 1966, 1967). It is possible that there is a difference between the replication of FMDV in the skin of cattle and pigs, since in situ hybridization studies have previously identified FMDV RNA in the normal skin of pigs without associated lesions (Brown et al., 1995).

In order to compare the pathogenesis of FMDV in pigs that had been infected with the same inoculum but by the intradermal/subdermal route of inoculation, data obtained in a previous study expressed as relative FMDV RNA content (Oleksiewicz et al., 2001) were converted to TCID_{50} equivalents by reference to a standard curve (Fig. 5). Interestingly, the contact-infected pigs in the present study took 1–2 days longer than those that were inoculated artificially (Oleksiewicz et al., 2001) to reach peak levels of viraemia and tissue-localized virus and, moreover, the peak levels were approximately 1–2 log units lower, except for epithelial lesions, which had very
high and comparable levels in both groups. This may indicate that, when a longer period is required for virus amplification, the host response may reduce virus replication more effectively. However, if host factors are responsible for this reduction, seen as early as 3 days p.e. in intradermally challenged pigs, these factors most likely include antibodies (circulating antibodies detectable from day 4) and possibly interferons or certain cytokines, perhaps TNF-α or IL-1. This is supported by the severe pyrexia seen in affected pigs. Nevertheless, a single pig, infected by exposure to airborne virus and included for comparison, had FMDV RNA levels in tissue samples at 5 days p.e. that were comparable to those in contact-infected animals at days 3–4 p.e., even though this pig had developed antibodies against the virus (ELISA titre of 1:64). This may indicate differences in virus replication and accumulation kinetics and perhaps in host reaction in pigs infected by a virus aerosol; however, this is speculative and more data are needed for confirmation.

The dose that infected the recipient pigs exposed by contact was not determined but it can be presumed that it was high, since the donor pigs had severe generalized disease and were in close contact for 2 h. The airborne virus they excreted would have been associated with heterogeneously sized particles (Donaldson et al., 1981; Sellers & Parker, 1969).
Therefore, all parts of the respiratory tract of the recipient pigs would have been exposed to infectivity (Hatch & Gross, 1964). The presence of virus in the tubes placed above the floor during the exposure period confirms that airborne virus was present, but does not give an accurate indication of the exposure dose, since this sampling method would have selectively collected the more rapidly sedimenting particles. While the recipient pigs are most likely to have been infected by the aerogenous route, the possibility that additional routes were involved, e.g. the alimentary or conjunctival route, cannot be excluded. It is interesting, however, that, when the distribution of virus in pigs infected by different routes, i.e. contact and intradermal/subdermal heel pad inoculation (and selected samples from a single pig infected by airborne virus), were compared, similar patterns of tissue distribution were found. However, virus accumulation in the tonsil and perhaps the lung in late infection was unexpectedly high in the pig infected by the aerogenous route. This may suggest that the initial distribution of FMDV in pigs is determined in part by the predilection of virus for certain sites in the pig, as has been established for cattle (Burrows et al., 1981).

Only tissues from the pharynx (ventral floor of pharynx, soft palate and tonsil) had intermediate concentrations of virus, fluctuating from \(10^4\) to \(10^6\) TCID\(_{50}\) equivalents/g, throughout the study period (days 1–4), i.e. 100-fold or more higher than the other tissues at days 1 and 2. By contrast, the virus concentrations in other tissues were low initially and, for certain tissues, increased sharply at days 3 and 4 p.e. Thus, our data are consistent with tissues of the pharynx being the most likely initial sites of virus replication or deposition. Whether FMDV replicates initially in the pharynx or reaches that site by the aerogenous or haematogenous route is currently unknown. However, our hypothesis, which would explain the differences observed in the kinetics of FMDV replication and accumulation between pigs infected by natural routes and those infected by artificial routes, is that natural FMDV infection in pigs is initiated by the deposition of inhaled virus in the pharynx, in particular the soft palate and the tonsil. After passage through local lymph nodes, the virus enters the bloodstream at a low, not yet measurable, level of infectivity. This initial viraemia results in the infection of a few cells in the stratified squamous epithelia, perhaps including Langerhan’s cells (David et al., 1995), resulting in a significant amplification of virus, and higher viraemia then results in the infection of a larger number of epithelial cells. In susceptible hosts, this cycle continues until sufficient epithelial cells are infected in predestination sites to cause the development of vesicular lesions and signs typical of the disease or until controlled by the host response. Based on our data, we propose that a ‘cycle of FMDV replication’ in pigs is of 12–24 h duration, so heel pad-inoculated pigs take about 48 h to develop severe disease (and actually around 72 h if given a small dose), while pigs infected by contact require 72–96 h to become severely ill. In contrast, pigs exposed to natural aerosol (receiving a minimal infectious dose) required about 120 h to develop severe disease. If the infective dose were to be reduced further, we would expect that even more time (more replication cycles) would be required to reach high virus levels and the development of clinical disease. However, at low doses, the host response (including antibodies) is likely to prevent the development of high levels of virus. Such a model of multiple cycles may help in the design of more effective FMD vaccines for pigs, since current vaccines are less efficient for pigs than for other livestock species (Salt et al., 1998). This could be through the provocation of a more effective local immunity in the pharynx or, alternatively, by the production of a better circulating immune response to cause the sequestration of circulating virus into non-replicating compartments.

The lungs yielded only small amounts of virus, indicating that they probably play a minor role in FMDV replication. This is in agreement with earlier studies on the source of airborne FMDV excreted in the breath of infected pigs, which showed that, in the early stages of infection, most airborne virus originates from the upper part of the respiratory tract but, as infection proceeds, the lower respiratory tract becomes more involved (Donaldson & Ferris, 1980).

Although the data presented here suggest that there was little or no replication of FMDV in the lungs during the 1–4 day period of investigation, the possibility that replication occurs in lung epithelia or macrophages later in infection cannot be excluded. Replication in the lungs has been suggested by others, based on In situ hybridization and virus isolation (Brown et al., 1995; Terpstra, 1972). However, the actual levels of replication and/or accumulation at those sites were not determined. In our experiment, the virus concentrations were near maximal and vesicular lesions were evident from day 3 p.e., making that time the most likely point of peak airborne virus excretion (Davidson, 1997). This is consistent with our hypothesis that stratified squamous epithelial cells, especially those in the skin, the oral mucosa and the pharynx, are those primarily responsible for the amplification of virus. Thus, the airborne virus excreted most likely originated from stratified squamous epithelial cells, with subsequent release of particles into the lumen of the respiratory tract and then transport in breath through the trachea, larynx, pharynx, nasal cavities and mouth to the exterior. However, the respiratory epithelium as well as lungs may play an indirect role in this excretion, because the large surface area may release virus that has been produced elsewhere and transported to these sites by mucus-ciliary movement or the bloodstream.

Lymph nodes and the spleen appeared to accumulate virus at days 3 and 4 p.e. This virus was most likely produced elsewhere and filtered from the lymph and blood. The liver contained virus at a level that could be explained by the concentration of virus in the bloodstream.

In conclusion, the present study increases knowledge of the early pathogenesis of FMDV infection and disease in pigs. We focused on the pharyngeal area, the nasal mucosa, trachea and
lungs; however, in future studies we plan to examine excretion from the epithelia in more detail using sensitive molecular techniques combined with continued quantitative analysis of data.

We thank Martin Broomfield and Natasha Smith for their careful management of the experimental animals. Geoffrey Hutchings, Linda Turner, Nigel Ferris and Teli Rendle are thanked for excellent technical assistance. Sue Hacker at IAH, Compton, is thanked for preparing and providing histopathological sections. Paul Kitching made helpful comments on the manuscript. We gratefully acknowledge the financial support provided by the UK Ministry of Agriculture, Fisheries and Food and by the Danish Ministry of Food, Agriculture and Fisheries. M.B.O. was on leave from the Danish Veterinary Institute for Virus Research.

References


Received 23 October 2000; Accepted 4 January 2001