Extent of reduction of foot-and-mouth disease virus RNA load in oesophageal–pharyngeal fluid after peak levels may be a critical determinant of virus persistence in infected cattle

Zhidong Zhang, Ciara Murphy, Melvyn Quan, Jeanette Knight and Soren Alexandersen

Correspondence
Soren Alexandersen
soren.alexandersen@bbsrc.ac.uk

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

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To investigate whether foot-and-mouth disease virus (FMDV) RNA loads in oesophageal–pharyngeal fluid (OP-fluid) in the early course of infection is related to the outcome of virus persistence, viral RNA in OP-fluid samples from cattle experimentally infected with FMDV type O was quantitatively analysed by using a quantitative real-time RT-PCR. Viral RNA was detected within 24 h post-infection (p.i.) in all infected animals. Rapid virus replication led to peak levels of viral RNA load by 30–53 h p.i., and then the load declined at various rates. In some animals (n = 12, so-called non-carriers) viral RNA became undetectable between 7 and 18 days p.i. In contrast, in persistently infected animals (n = 12, so-called carriers) viral RNA persisted in OP-fluid samples at detectable levels beyond 28 days p.i. Analysis of early viral decay/clearance and virus clearance half-life in OP-fluid samples showed that the extent of reduction of viral RNA in OP-fluid samples immediately following peak levels is a critical determinant of the outcome of FMDV persistence.

INTRODUCTION

Foot-and-mouth disease (FMD) virus (FMDV) causes a highly contagious disease of domesticated and wild ruminants and pigs. Of considerable importance in the control of FMD is the persistent infection that can occur following clinical or subclinical infection in both vaccinated and non-vaccinated ruminants exposed to live virus (so-called carriers). There is field evidence to indicate that carrier African buffalo can precipitate new outbreaks of disease, and more anecdotal evidence also implicates carrier cattle and sheep in disease recrudescence or in starting new outbreaks (Alexandersen et al., 2002a, 2003b). A carrier is defined as an animal from which live virus can be recovered for longer than 28 days after exposure (Salt, 1993; Sutmoller & Gaggero, 1965). Persistent FMDV appears eventually to become undetectable in carriers. On the other hand, persistent infection does not occur in all virus-exposed ruminants, i.e. only a proportion of these ruminants become FMDV carriers (Alexandersen et al., 2002a). The mechanism for these phenomena is not fully understood, although the immune status of the animal prior to contact appears not to influence the development of a carrier state.

Bovine pharyngeal tissue has been identified as a primary site for FMDV infection in vivo (Burrows et al., 1971; McVicar & Sutmoller, 1969). Viral RNA has been detected in pharyngeal tissues (Prato Murphy et al., 1994) and has been localized in epithelial cells of the pharyngeal tissues of carrier cattle (Zhang & Kitching, 2001). These studies indicate that FMDV in the pharyngeal area tissues could be a source for the virus present in oesophageal–pharyngeal fluids (OP-fluids). During acute disease and persistence, infectious FMDV can be isolated from OP-fluid samples, but the amounts of virus in OP-fluid samples are generally low during persistence. During the period of persistence, there is considerable variation in the levels of virus recovery from OP-fluid samples (reviewed by Alexandersen et al., 2002a). However, a great deal remains to be learned about quantitative aspects of viral RNA load in OP-fluids and its relationship with the outcome of the infection (persistence or non-persistence). Quantitative analysis of FMDV RNA load is made possible by the recent development of sensitive, quantitative real-time RT-PCR (Alexandersen et al., 2002b; Oleksiewicz et al., 2001; Reid et al., 2002; Zhang & Alexandersen, 2003). Studies have shown that viral RNA copy numbers measured by real-time RT-PCR correlate well with virus infectivity (Alexandersen et al., 2001, 2002b, 2003a, b; Zhang & Alexandersen, 2003). Therefore, the level of viral RNA could be a very useful indicator of the profiles of viral load.

In this study the load of FMDV RNA was quantified in OP-fluid samples from cattle experimentally infected with FMDV type O during the period of acute and persistent
infection. The results suggest that the extent of reduction of viral RNA in OP-fluid samples immediately following peak levels is a critical determinant of the outcome of FMDV persistence.

METHODS

Animals. Twenty-six standard Compton steers (Holsteins) at 6–10 months of age were used and divided into four groups in this study. Cattle in Groups 1, 2 and 4 were infected with FMDV O UK 34/2001 (Alexandersen et al., 2002b; Alexandersen & Donaldson, 2002). Six cattle in Group 1 were infected by subepidermoo-lingual inoculation with 10^9 TCID_{50} and placed in pairs in three boxes. The eight cattle in Group 2 were placed with four animals in each of two boxes, with two cattle in each box inoculated subepidermoo-lingually under the same conditions as the first group and the other two cattle in each box were kept in direct contact. Four cattle in Group 3 were inoculated with FMDV O BFS1860 by subepidermoo-lingual inoculation of 10^7 TCID_{50}. Eight cattle in Group 4 were infected under the same conditions as those of Group 2, with two cattle inoculated subepidermoo-lingually and the other two cattle in each box kept in direct contact [one inoculated animal and another direct contact animal in this group were killed at 5 days post-infection (p.i.) and excluded from this study]. After infection, inoculated and direct contact cattle were monitored for clinical signs of disease and rectal temperatures were recorded daily until 10 days p.i. Samples of OP-fluid, nasal and mouth swabs were collected before the start of the experiment (negative controls) and then twice a day during the first 2–3 days p.i., followed by daily measurements until 7 days p.i., and then taken twice a week or weekly until the end of the experiment. OP-fluid samples were collected using a probang cup (Sutmoller & Gaggero, 1965) and diluted in an equal volume of Eagle’s HEPES medium (pH 7.2) containing 5% FCS and stored at −80°C until required. Nasal and mouth swabs were placed in 1 ml Trizol (Life Technologies) and stored at −80°C.

Assay for virus. The infectivity of OP-fluid samples was determined by inoculation of monolayers of primary bovine thyroid (BTY) cells, as described by Snowdon (1966).

RNA extraction. For automated RNA extraction, 200 μl samples were mixed with 300 μl MagNa Pure LC total nucleic acid lysis buffer (Roche). Total nucleic acids were extracted and eluted in 50 μl elution buffer by using the MagNa Pure LC total nucleic acid isolation kit (Roche) with an automated nucleic acid robotic workstation (Roche), according to the manufacturer’s instructions, and stored at −80°C until used as described previously (Alexandersen et al., 2003a).

Real-time quantitative RT-PCR assay for detection of viral RNA. The level of viral RNA in samples was quantified by real-time RT-PCR as described previously (Alexandersen et al., 2002b, 2003a; Reid et al., 2002; Zhang & Alexandersen, 2003). For the generation of standard curves, standard viral RNA was generated from plasmid pT73S containing full-length FMDV (kindly provided by Dr Andrew King, Institute for Animal Health, UK) by in vitro transcription using a commercially available T7 RNA polymerase kit (Ambion), according to the manufacturer’s instructions, as described previously (Zhang et al., 2002). RNA was resuspended in RNase-free water and quantified by spectrophotometry.

Calculation of the rates of virus growth and decay. Initial virus replication rates were calculated by using the exponential growth rate equation \( \gamma = (\ln Y_1 - \ln Y_2)/(t_1 - t_2) \), where \( Y_1 \) and \( Y_2 \) are the virus load values at times \( t_1 \) and \( t_2 \) (in hours), respectively. The virus load doubling time, \( T_2 \) (in hours), was calculated by using the equation \( T_2 = (\ln 2)/\gamma \). Rates of viral decay or clearance were calculated by using the decay rate equation \( \alpha = (\ln Y_1 - \ln Y_2)/(t_1 - t_2) \), where \( Y_1 \) and \( Y_2 \) are the virus load values at times \( t_1 \) and \( t_2 \) (in hours), respectively. The viral decay/clearance half-life, \( T_{1/2} \) (in hours), was calculated by using the equation \( T_{1/2} = (\ln 2)/\alpha \). Viral decay rates and \( T_{1/2} \) were calculated with the steepest interval of the decay curve within 2 weeks p.i. following the peak.

Statistical analyses. Statistical analyses were performed by using a non-parametric test (Mann–Whitney test using MINITAB release 12.21 software) on log_{10}-transformed FMDV RNA values, or ln values in the case of viral growth and decay rates. \( P < 0.05 \) was considered statistically significant.

RESULTS

Clinical signs and development of carriers

Increased body temperature and early lesions were generally detected in inoculated cattle from around 1 day p.i. and in contact cattle from around 4–5 days p.i. (for more details, see Alexandersen et al., 2003a). The number of carriers in the different groups is shown in Table 1.

Dynamics of viral RNA loads in OP-fluid samples during acute FMDV infection

At 6 h p.i., 4 of 13 cattle inoculated with FMDV O UK 34/2001 (Groups 1, 2 and 4) had FMDV RNA at detectable levels, and by 24 h p.i. all inoculated cattle had a level of FMDV RNA ranging from 5.83 to 9.57 log_{10}(copies ml^{-1}) (mean 7.84 ± 1.13, median 7.59) (Fig. 1a, b, d). Viral RNA levels then peaked from 30 to 53 h p.i. The maximum level of FMDV RNA varied from 7.52 to 9.57 log_{10}(copies ml^{-1}) (mean 8.78 ± 0.66 log_{10}(copies ml^{-1}), median 8.89). Thereafter, FMDV RNA levels decreased at various rates. The contact cattle in Groups 2 and 4 (n = 7) had a somewhat similar kinetic pattern of viral RNA in OP-fluid samples (Fig. 1c, e). However, viral RNA was not detectable before 24–30 h p.i. [ranging from around 2 to 5 log_{10}(copies ml^{-1})] or later and the levels did not peak [mean 8.54 ± 0.68 log_{10}(copies ml^{-1}), median 8.68] until 4 or 5 days after contact. In cattle inoculated with FMDV O BFS 1860 (Group 3) (n = 4), three out of four had FMDV RNA at detectable levels at 24 h p.i. and the fourth animal was

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Inoculated</th>
<th>Contact</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>6</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>Group 2</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Group 3</td>
<td>4</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>Group 4</td>
<td>8*</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

NA, Not applicable as no contact animals were included in the group.

*Two infected cattle in this group were killed at 5 days p.i. and excluded from this study.
positive at 48 h p.i. Viral RNA levels peaked from 48 to 72 h p.i. [mean $7.20 \pm 0.92 \log_{10}\text{(copies ml}^{-1})$, median 6.85] (Fig. 1f). The peak levels of FMDV RNA were lower than those in cattle infected with FMDV O UK 34/2001, but no statistically significant differences were identified (non-parametric test, $P = 0.27$). No correlation was found between viral peak levels and subsequent events (persistent or non-persistent) as there was no significant difference in the viral peak levels between carriers [$8.86 \pm 0.68 \log_{10}\text{(copies ml}^{-1})$, median 9.07, $n = 12$] and non-carriers [$8.60 \pm 0.68 \log_{10}\text{(copies ml}^{-1})$, median 8.53, $n = 12$] (non-parametric test, $P = 0.44$).

The dynamic patterns of viral RNA in the OP-fluid samples exhibited similarities with the dynamics of FMDV RNA observed in nasal and mouth swabs during the first weeks of infection (Fig. 2). The peak levels of viral RNA in nasal and mouth swabs were similar to those in OP-fluid samples; however, in comparison with nasal and mouth swabs, the duration of viral RNA in the OP-fluid samples was longer, even in non-carrier animals (Fig. 2). Beyond 28 days p.i. (latest positive samples detected at 18 days p.i.), nasal and mouth swabs were consistently negative. Fig. 2 shows representative results for Group 1. However, the results for the other groups were essentially similar, i.e. the duration of detectable levels of viral RNA was longer in OP-fluid samples than in nasal and mouth swabs even in non-carriers, and no positive nasal or mouth swab samples were detected later than 18 days p.i. (data not shown). It is important to note that in all of these animals, serum antibodies to FMDV were detected from 4 to 7 days p.i. and afterwards; however, no correlation between antibody titre or kinetics and the development of the carrier state was evident (data not shown). The details of viral RNA levels in nasal and mouth swabs as well as development of serum antibodies are described elsewhere (Alexandersen et al., 2003a).

**Duration of viral RNA in OP-fluid samples**

The levels of viral RNA in OP-fluid samples rose very rapidly to reach peak levels after its initial appearance. OP-fluid samples were consistently positive up to around 7–10 days p.i. However, viral RNA declined at various rates. Some animals could clear virus efficiently even though they had peak viral RNA levels close to those of carriers [close to or greater than $8 \log_{10}\text{(copies ml}^{-1})$]. In these animals ($n = 12$, non-carriers), viral RNA became undetectable....
before 28 days p.i. and remained undetectable until the end of the study (up to 57 days p.i. for Group 1, 51 days p.i. for Group 2, 30 days p.i. for Group 3 and 42 days p.i. for Group 4). In contrast, the persistently infected carrier animals had FMDV RNA at detectable levels beyond 28 days p.i. (n = 12) and up to 57 days p.i. In the single Group 1 carrier animal, levels of viral RNA in OP-fluid samples varied from $4.72 \log_{10}(\text{copies ml}^{-1})$ at 28 days p.i. to $4.4 \log_{10}(\text{copies ml}^{-1})$ at 57 days p.i. In Group 2 carrier animals (inoculated, n = 4), FMDV RNA load varied from $5.54 \pm 1.12 \log_{10}(\text{copies ml}^{-1})$ at 30 days p.i. to $3.29 \pm 0.84 \log_{10}(\text{copies ml}^{-1})$ at 44 days p.i. Of Group 3 carrier animals (n = 2), the viral RNA load varied from $5.13 \pm 1.65 \log_{10}(\text{copies ml}^{-1})$ at 30 days p.i. to $5.15 \log_{10}(\text{copies ml}^{-1})$ at 39 days p.i. (data for one animal only). In Group 4 carrier animals (contact, n = 2), FMDV RNA load varied from $6.28 \pm 0.02 \log_{10}(\text{copies ml}^{-1})$ at 28 days p.i. to $5.21 \pm 0.52 \log_{10}$ at 42 days p.i. All these OP-fluid samples from carriers contained the infectious virus as determined by inoculation onto monolayers of BTY cells (data not shown). For details of the comparative sensitivity of virus isolation and real-time RT-PCR on probang samples, see Zhang & Alexandersen (2003).

**The extent of viral RNA decay after peak levels is associated with the outcome of virus persistence**

To evaluate the possibility that the temporal pattern of viral RNA in OP-fluid samples in the early course of infection is related to the outcome of the infection (persistent or non-persistent), the cattle were divided into carriers and non-carriers, according to persistence status, and then the initial virus growth rate and subsequent decay slopes were calculated. Early virus replication in individual animals was assessed by estimation of the mean rate ($\gamma \pm SD$ of virus growth and the corresponding doubling time, $T_2$, in hours (described in Methods). Growth slopes were calculated by using the two initial quantifiable data points for each animal. As shown in Fig. 3, the mean viral growth rate in carriers ($\gamma = 0.30 \pm 0.28$, $T_2 = 3.84$ h, $n = 11$ (no suitable samples were available from one animal to do the calculations and this animal was therefore excluded)) was slightly higher than those in non-carriers ($\gamma = 0.26 \pm 0.21$, $T_2 = 4.11$ h, $n = 12$), but no statistically significant differences were identified (non-parametric test, $P = 0.93$). Therefore, the outcome of the infection (persistent or non-persistent) appears to be independent of early virus growth rates.

In comparing the initial viral RNA load in the OP-fluid samples before peak levels with the outcome of FMDV infection (persistent or non-persistent), two different patterns of initial viral RNA levels were observed. In the first pattern, the mean initial viral RNA levels did not differ according to persistence status (Table 2, top panel). In the second pattern, the carriers had higher loads of the initial viral RNA than non-carriers (Table 2, middle panel). In Group 2, all four inoculated cattle became carriers (Table 2, bottom panel) and had high levels of viral RNA at day 1. Based on these data, no firm association between initial viral RNA load in OP-fluid samples and the outcome of infection (persistence or non-persistence) could be established, although the data from two of four experiments suggested that the increase in viral RNA in carriers appears to be faster than in non-carriers.

To determine whether the extent of reduction of viral load after peak levels in OP-fluid samples is related to the outcome of the infection, this phase of the FMDV infection was analysed by calculation of the slopes of early viral decay/clearance and virus clearance half-life in OP-fluids during the first 2 weeks p.i. (as described in Methods). A difference in the decay/clearance rate between carriers and non-carriers was observed in all four groups with the early viral decay/clearance in carriers being slower than in non-carriers.

**Fig. 2.** Duration of FMDV RNA in OP-fluid samples (squares), nasal (triangles) and mouth (circle) swabs collected from Group 1 cattle infected with FMDV O UK 2001. Animals were divided into carriers and non-carriers as described in Methods. The viral RNA levels were quantified by real-time RT-PCR. The carrier values are for a single animal while the non-carrier value is the mean of the Group 1 non-carriers ($n = 5$). The results for the other groups were essentially similar (data not shown).
(Table 3). Given these data as a whole, the mean decay rate ($\alpha = 0.039 \pm 0.015$, $T_{1/2} = 21.45$ h, $n = 11$) in carriers is significantly different from that in the non-carriers ($\alpha = 0.084 \pm 0.04$, $T_{1/2} = 10.42$ h, $n = 12$; non-parametric test, $P = 0.0028$) (Fig. 3). These data suggest that the early decay rate of FMDV RNA in OP-fluid samples is a critical determinant of the duration of FMDV persistence. Viral decay rate in nasal and mouth swabs was higher than that in OP-fluid samples and no significant difference was identified between carriers and non-carriers (data not shown).

**DISCUSSION**

The dynamics of FMDV RNA in bovine OP-fluid samples have been quantitatively analysed in this study. The results show that the extent of reduction of FMDV RNA in OP-fluid samples immediately following peak levels has a critical effect on the outcome of FMDV persistence.

To test the hypothesis that the events occurring shortly after infection dictated subsequent infection outcome, frequent monitoring of the levels of viral RNA in bovine OP-fluid samples following FMDV type O infection was performed. We demonstrated that following the peak viral load in OP-fluid samples, the early rate and extent of viral decay was significantly different between carrier and non-carrier animals receiving identical inocula. What determines the magnitude of the reduction of RNA load in OP-fluid samples following the peak load is not fully understood. The differential emergence of viral variants with different replicative capacity within the first week of infection seems unlikely among animals that received identical inocula. Thus, this observation may simply reflect variations in the kinetics of the host response to infection, i.e. clearance of virus or continuing ability to support replication. We are now investigating the quantitative aspects of early events in the host response to infection in cattle, sheep and pigs to determine whether the observed differences in decay rate are caused by differences in the ability to clear virus or by differences in the occurrence or level of continuing virus replication.

**Table 2. Comparison of initial viral RNA levels in OP-fluid samples between carriers and non-carriers**

For the upper panel samples taken at 1 day p.i. had similar levels of viral RNA for carriers and non-carriers, while for the middle panel the carriers had a higher level of viral RNA. For the lower panel, all four animals became carriers and had high levels of viral RNA at 1 day p.i. $n$, Number of animals; NA, not applicable ($n = 0$). All data were taken at 1 day p.i.

<table>
<thead>
<tr>
<th>Animal group (infection route)</th>
<th>Viral RNA levels [log$_{10}$(copies ml$^{-1}$)]</th>
<th>Non-carriers ($n$)</th>
<th>Carriers ($n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (inoculated)</td>
<td>7.78 ± 0.88 (5)</td>
<td>7.78 (1)</td>
<td></td>
</tr>
<tr>
<td>Group 4 (inoculated)</td>
<td>8.40 ± 1.65 (2)</td>
<td>8.49 (1)</td>
<td></td>
</tr>
<tr>
<td>Group 4 (contact)</td>
<td>3.72 ± 0.45 (2)</td>
<td>2.67 ± 0.21 (2)</td>
<td></td>
</tr>
<tr>
<td>Group 2 (contact)</td>
<td>0 ± 0 (2)</td>
<td>4.10 ± 0.15 (2)</td>
<td></td>
</tr>
<tr>
<td>Group 3 (inoculated)</td>
<td>0.66 ± 0.44 (2)</td>
<td>0.66 ± 0.24 (2)</td>
<td></td>
</tr>
<tr>
<td>Group 2 (inoculated)</td>
<td>NA (0)</td>
<td>8.93 ± 0.24 (4)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Comparison of virus decay ($\alpha$)/clearance rate and virus clearance half-life ($T_{1/2}$) in OP-fluid samples between carriers and non-carriers**

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Non-carriers</th>
<th>Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$ ($n$)</td>
<td>$T_{1/2}$ (h)</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.11 ± 0.05 (5)</td>
<td>7.0</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.08 ± 0.05 (2)</td>
<td>12.0</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.06 ± 0.02 (2)</td>
<td>12.8</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.07 ± 0.02 (3)</td>
<td>10.4</td>
</tr>
</tbody>
</table>

*Number of animals.
Virus RNA persisted in OP-fluids for a longer period than in other sample types. The reason for this phenomenon remains to be fully understood. Bovine pharyngeal tissue has been identified as a primary site for FMDV infection and replication in vivo (Burrows et al., 1971; McVicar & Sutmoller, 1969). During persistence, viral RNA has been detected in bovine pharyngeal tissues (Prato Murphy et al., 1994) and has been recently localized in epithelial cells of the bovine pharyngeal area (Zhang & Kitching, 2001). These studies of FMDV infection in experimentally infected cattle have unequivocally shown the importance of pharyngeal area tissues in virus infection and replication. The epithelial cells isolated from pharyngeal tissues of FMDV infected cattle and cultured in vitro have been shown to persistently harbour FMDV and they did not show any cytopathic changes for many weeks; both observations further emphasize the importance of these cells (Zhang et al., 2002). This leads to the hypothesis that the presence of viral RNA and infectious virus in OP-fluids during persistence is because FMDV may be able to continuously replicate in such a specialized cellular site without being efficiently cleared. Therefore, it may be especially important to define those cellular factors and mechanisms involved in the regulation of virus replication and clearance in these cells. In fact, such knowledge would also be extremely helpful in defining selective approaches to the control of FMDV in persistently infected animals.

The experiments described here included experimental infection with FMDV O UK 34/2001 and O BFS1860, and resulted in the development of the carrier state in 50% of the 24 cattle studied for more than 28 days p.i. Other studies using similar conditions (Z. Zhang & S. Alexandersen, unpublished) showed that 0/7 cattle infected with FMDV O SKR 1/2000 in two separate experiments became carriers, while all nine cattle infected with C Oberbayren in two separate experiments became carriers. The O SKR 1/2000 virus only caused mild clinical disease in cattle and the virus was rapidly cleared from all sites, including the pharynx, while C Oberbayren caused severe clinical disease and was cleared slowly from the pharyngeal region with a pattern consistent with the carrier animals described in this study (Z. Zhang & S. Alexandersen, unpublished). Taken together, these findings suggest that the establishment of the carrier state is an early event and that the efficiency or frequency may be dependent on both the virus strain and the host. Furthermore, as the development of the carrier state is associated with a slower clearance rate immediately after the peak viral load, it may be possible to use these findings for practical disease control, i.e. to predict the risk of carriers occurring under certain epidemiological conditions. As discussed above, the exact mechanisms involved in FMDV persistence or clearance in vivo are not clear, but are likely to include both viral and host factors. Levels of circulating antibodies failed to correlate with the development of carriers. As the kinetics of viral load and clearance differed between subsequent carriers and non-carriers very early following infection, the fine details of the innate immune response may prove to be an important host factor.

This study highlights the role of early events in the establishment of persistent FMDV infection. The extent of reduction of viral load after the peak is a critical determinant of the outcome of FMDV infection. Elucidation of the mechanisms that account for these observations will provide insight into the pathogenesis of FMD and may have important practical consequences for the development of an effective vaccine for prevention of carriers.

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