Serial passage of foot-and-mouth disease virus in sheep reveals declining levels of viraemia over time

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If an infectious agent is to maintain itself within a closed population by means of an unbroken serial chain of infections, it must maintain the level of infectiousness of individuals through time, or termination of the transmission chain is inevitable. One possible cause of diminution in infectiousness along serial chains of transmission may be that individuals are unable to amplify and transmit comparable levels of the infectious agent. Here, the results are reported of a novel experiment designed specifically to assess the effects of serial passage of foot-and-mouth disease virus (FMDV) in experimental groups of sheep. A virus isolate taken from an epidemic of foot-and-mouth disease (FMD) characterized by rapid fade-out of infection was passed serially through four groups of sheep housed in an isolation unit. Although it was not possible to measure individual infectiousness directly, blood virus load from infected individuals was quantified using a real-time PCR assay and used as an underlying indicator of the level of infection. The results of this assay concurred well with those of the traditional tissue-culture assay and were shown to be highly repeatable. The level of peak viraemia was shown to fall significantly with the time of infection and with passage group, both in terms of the group mean and regression analysis of individual values, suggesting that this isolate of FMDV may, under certain conditions, be unable to maintain itself indefinitely in susceptible sheep populations. The results of these experiments are discussed in terms of the epidemiology of FMD in sheep.

Introduction

Foot-and-mouth disease virus (FMDV; genus Aphthovirus, family Picornaviridae) causes an acute vesicular disease of ruminants and pigs that is considered to be the most economically important disease of livestock. There are seven known immunologically distinct serotypes [O, A, C, SAT (Southern African Territories) 1, SAT 2, SAT 3 and Asia 1], with a spectrum of different strains within each serotype. Type O FMDV is the most commonly isolated serotype at the OIE/FAO World Reference Laboratory for FMD, Pirbright (WRL). Nucleotide sequencing of regions of the viral genome has shown that FMDV exhibits distinct lineages, which are clustered geographically (Samuel & Knowles, 2001). Recently, the PanAsia lineage has displaced existing strains of FMDV in many areas (Knowles et al., 2001). Infection of sheep causes an acute febrile disease in which vesicular lesions can occur on the feet and in the mouth of infected animals. Unlike pigs and cattle, where the disease is usually characterized by overt clinical signs, FMD of sheep is often mild and inapparent (Geering, 1967; Gibson & Donaldson, 1986). Where lesions do occur, they are small and heal quickly. This makes field diagnosis based on clinical signs...
problematic and can result in the spread of infection through infected animals without clinical signs. Although the minimum infectious doses of airborne virus for cattle and sheep are similar, the threshold concentration of virus required to infect sheep is thought to be higher because of their lower inhalation rate (Sørensen et al., 2000). The incubation period for natural infection is normally between 3 and 8 days (Kitching & Mackay, 1994), but can be less than 24 h following experimental inoculation (Sellers et al., 1977). Viraemia is short and coincides with the onset of virus excretion (Donaldson et al., 1970; Sellers & Parker, 1969). Clearance of virus from the blood and a reduction in virus excretion correlate well with seroconversion (Cox et al., 1999; Gibson & Donaldson, 1986).

The mortality rate for adult sheep infected by the virus is usually negligible, but mortality in lambs can reach 90% (Chevskii et al., 1964). Although evidence for persistent infection of sheep with FMDV is well established (Burrows, 1968), the evidence that carrier sheep can be a source of infection for other livestock species is only circumstantial (Stougard, 1983), as attempts to achieve transmission under experimental conditions have failed.

FMDV can naturally infect any even-toed ungulate species, but infection dynamics vary across all these species. Epidemics require that individuals become both infected and infectious, and the relationship between these two states is usually, at best, poorly understood. How infectious an individual becomes may depend on the infectious dose acquired by that individual (Hughes et al., 2002). Under such circumstances, the long-term ability of species to maintain an infection process is critically dependent on animals that arise later in the chain of infection being as infectious as animals infected earlier in the chain. This means that animals must be able to amplify virus and transmit comparable levels of infection, regardless of their temporal position in the epidemic process. Any diminution in the level of infectivity of individuals over time will result in the inability of a species to maintain infection over longer timescales.

There is some empirical evidence to suggest that small ruminant populations in epidemic (Garland et al., 1981) and endemic (Anderson et al., 1976) areas are unable to maintain FMD infection. The epidemic of FMD in Greece during 1994 provided further evidence that sheep may be unable to support FMDV transmission for prolonged periods of time (Mackay et al., 1995). The epidemic began with a high morbidity and seroprevalence within affected sheep flocks but, after less than 1 month, there was a sharp decline in the number of clinical cases and seroprevalence. The virus isolate responsible for the epidemic in Greece during 1994 did not show any predilection for sheep; morbidity and mortality were higher for both cattle and pigs than for sheep and goats (Mackay et al., 1995).

This paper describes the transmission of FMDV between groups of sheep under experimental conditions intended to mimic a non-homogeneously mixed population. The study was designed to test the hypothesis that infectiousness declines along a chain of transmission. To do this, clinical, virological and serological variables were examined for trends over time, asking whether infection later in the chain had characteristics different from those of infection earlier in the chain.

Methods

Experimental design. Thirty-two Polled Dorset sheep were allocated randomly into four groups (denoted G1–G4), each made up of eight individuals. All sheep were from the same flock (Institute for Animal Health, Compton Laboratory, Berks, UK) and between 3 and 6 months old. Each group was housed in a separate room within a high-security isolation building. The experiment was designed such that FMDV, introduced into G1, would be transmitted ‘downstream’ from G1 sequentially to G2, G3 and finally to G4, with a consistent exposure period for sequential groups. For this purpose, the four groups of sheep were moved between boxes in a way that resulted in each group of sheep spending 24 h donating virus to their ‘downstream’ neighbouring group, followed by 24 h receiving virus from their ‘upstream’ neighbouring group (Fig. 1). This ensured that sequential groups were exposed to the same proportion of the previous group’s total virus excretion and removed possible bias from the system. Inoculation of G1 was staggered to increase the probability that a number of G1 sheep would reach peak excretion at the time of donating virus to G2. For this purpose, half of G1 were inoculated on day 0 whilst the other half were moved into a separate animal room in the isolation building. The second half of G1 were inoculated on day 1 and moved back in with the first half on day 2.

All sheep movements took place at the same time each morning, starting with the highest group number. Sheep were moved along a ‘dirty’ corridor in the isolation unit, which was thoroughly disinfected after each movement. Following the movement of animals, all sheep were sampled starting with the highest group number. The personnel involved disinfected themselves thoroughly before moving to a different animal room. The experiment was terminated when 10 days had lapsed without a viraemic animal being detected. The experiment was carried out on two separate occasions.

Inoculum preparation. FMDV type O/Greece/23/94, originally isolated from ovine epithelium using primary calf thyroid (BTY) cells, was obtained from the WRL. This virus was isolated during the peak of the 1994 Greek FMD epidemic. Virus was passed again in BTY cells before inoculation. The experiment was carried out on two separate occasions.

Antigen and antibody detection. All whole-blood samples were examined for the presence of virus using BTY cells (Snowdon, 1966). Cell-culture supernatants were confirmed as containing FMDV by antigen-detection ELISA (Roeder & Le Blanc Smith, 1987). A sample of whole blood was added directly to TRIzol LS (Gibco BRL) for RNA extraction and subsequent quantitative RT–PCR for viral RNA, as described below. Total serum antibody against type O FMDV was measured by liquid-phase blocking ELISA, with titres greater than 1:40 being taken as positive (Kitching et al., 2000).
Serial passage of FMD virus

Quantitative RT–PCR. The assay used here was adapted from that designed to quantify levels of FMDV in porcine tissues (Oleksievicz et al., 2001; Alexandersen et al., 2001). Quantification was performed using the GeneAmp 5700 sequence detection system (Perkin-Elmer Biosystems). Viral RNA was extracted from EDTA-mixed whole blood using TRIzol LS (Gibco BRL). RNA extraction was modified slightly from the manufacturer’s instructions. Initially, 250 µl EDTA-mixed peripheral blood was added to 750 µl TRIzol LS and vortexed vigorously for 20 s. To remove insoluble potential contaminants, samples were then centrifuged at 12000 × g for 15 min at 4 °C and 550 µl of the aqueous layer was removed into a fresh 2 ml Sarstedt tube. To this, 200 µl of sterile PBS was added. Total RNA was then extracted by addition of chloroform for phase separation and precipitation in isopropyl alcohol following the manufacturer’s instructions. RNA was then reverse-transcribed as described by Reid et al. (1998). A 25 µl reaction mixture was used for the PCR. All reactions were carried out in MicroAmp optical 96-well reaction plates (Perkin-Elmer Biosystems). The final reaction contained 12·5 µl 2 × SYBR Green PCR master mix (Perkin-Elmer Biosystems), 8·5 µl nuclease-free water, 1 µl cDNA and 5 pmol of forward and reverse primers. The primers used were IRES1 (5’ CCTGGTCTTTCCAGGTCTAGA 3’) and IRES4 (5’ CTTCTCTAGATCCGAGTGTG T 3’), corresponding to positions 312–332 and 685–665, respectively, of the FMDV genome and generating a 373 bp DNA product. Both primers have been described previously as complementary sequences to the highly conserved FMDV internal ribosome entry site (Forsyth et al., 1998). Plates were sealed using MicroAmp optical caps (Perkin-Elmer Biosystems) and mixed thoroughly before transfer to the thermocycler. DNA was amplified using the following programme: one cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min. Amplification dependence was distinguished using dissociation curves determined for each sample over a temperature range of 60–95 °C. Quantification was determined against a standard curve created using a fourfold dilution series of cDNA from a sample of known titre. The standard curve was generated from an FMDV-positive blood sample that had a very high titre in BTY cells (10⁸ TCID₅₀/ml). The linearity of the standard curve provided a clear indication that no degree of reaction saturation would occur with strong-positive samples (Fig. 2a). All plates were run with the standard dilution series (for generation of the standard curve), eight separate negative controls (four from FMDV-negative blood samples, four nuclease-free water), four strong-positive controls (cDNA derived from RNA extracted from FMDV-positive tissue culture supernatant) and four more negative controls (cDNA derived from RNA extracted from FMDV-negative tissue culture supernatants).

Initially, all blood samples positive by virus isolation were quantified to determine the point of peak viraemia. Peak samples were run a further two times and the mean was calculated. For each PCR, a threshold cycle value (Cₜ) was obtained. This value represents the PCR cycle number at which the fluorescence of the reaction rose above a threshold value. The threshold value for each run was optimized using the four replicates of the strong-positive control. In each case, the threshold value was taken during the linear phase of amplification, above the background levels of negative controls, when the standard deviation of the mean from the four strong-positive control replicates was lowest. Cₜ values are inversely proportional to the log₁₀ of the amount of template in the PCR, with a difference of 1 Cₜ corresponding to a twofold difference in template. For each sample, a Cₜ range for the three replicates was calculated describing the degree of variability of the system. For Experiment 1, this range was 3/15 (95% confidence limit 3·09–2·41; maximum range 4·74; minimum range 2·04) and, for Experiment 2, the range was 2·03 (95% confidence limit 2·32–1·45; maximum range 3·05; minimum range 1·11). The
The severity of clinical FMD was assessed using both the mean number of vesicular lesions per viraemic sheep and the mean number of sites affected by vesicular lesions per viraemic sheep. The data showed no significant departures from normality (using the Kolmogorov–Smirnov test, \( P < 0.05 \)) or homogeneity of variance (using the Levene test, \( P < 0.05 \)). One-way analysis of variance (ANOVA) using the least significant difference (LSD) for multiple comparisons of group means showed no significant difference (\( P > 0.05 \) in all cases) between G2, G3 and G4 in the severity of clinical FMD for viraemic sheep assessed by the number of lesions (Experiment 1: \( F_{2,18} = 0.25; \ P = 0.78 \). Experiment 2: \( F_{2,12} = 0.20; \ P = 0.82 \). Combined: \( F_{2,33} = 0.28; \ P = 0.76 \) and sites affected (Experiment 1: \( F_{2,18} = 0.34; \ P = 0.67 \). Experiment 2: \( F_{2,12} = 0.32; \ P = 0.63 \). Combined: \( F_{2,33} = 0.41; \ P = 0.57 \).

Regression analysis indicated no significant association between either measure of clinical FMD and the time of viraemia (lesions: \( F_{1,34} = 1.87; \ P = 0.18 \); sites: \( F_{1,34} = 1.51; \ P = 0.31 \) for data from the two experiments combined. However, the mild nature of FMD in sheep and the small numbers of clinically infected sheep within each group restrict the statistical power of this analysis.

**Level of viraemia**

The peak titre of FMDV in the blood from viraemic sheep was determined by quantitative RT–PCR (Fig. 4). Quantitative analysis was only performed on blood samples positive by virus isolation in cell culture. For the purpose of analysis, animal UF94 from G2 (Experiment 2) was placed in G4, as infection of this animal was the clear result of back-transmission from G3. Results from G1 individuals are not shown, as a different route of inoculation was used and the results are not comparable with subsequent groups. For analysis, the data were log-transformed to achieve normality and homogeneity of variance. One-way ANOVA using the LSD for multiple comparisons of group means was performed on transformed data and showed a significant difference between the group means of G2 and G4 for Experiment 1 (\( F_{3,24} = 3.48; \ P < 0.05 \)) and Experiment 2 (\( F_{3,19} = 1.31; \ P < 0.05 \)). Only sheep that had a detectable viraemia by virus isolation were included in the statistical analysis. There was no significant difference in the mean length of the viraemic period of G2, G3 and G4 from either experiment and for the combined data (Mann–Whitney U test, all \( P > 0.05 \)).

The effect of the time of onset of viraemia on the level of virus load for contact-infected sheep showed a strongly significant relationship between the two variables (Fig. 5). This analysis makes no assumptions regarding the source of infection, be it from within the same group or from a neighbouring one. The analysis does assume that time of onset of viraemia can be used as a marker for time of exposure to infection and, therefore, that high peak viraemias are not associated with short incubation periods, but no such association is observed in practice (Hughes et al., 2002). A
Table 1. Numbers of sheep in each infection class following serial passage of FMDV

Infections were classified by the severity of FMD. Clinical infections were defined as those where vesicular lesions were found. Inapparent infections were defined as those where viraemia occurred but without the occurrence of vesicular lesions. Subclinical infections were defined as those where seroconversion was the sole indicator of infection. Susceptible (uninfected) animals were seronegative throughout the course of the experiment, but were shown to be susceptible to infection by subsequent intranasal challenge.

<table>
<thead>
<tr>
<th>Infection class</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tr>
<td></td>
<td>G1</td>
<td>G2</td>
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<tr>
<td>Clinical</td>
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<td>4</td>
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<tr>
<td>Inapparent</td>
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<td>2</td>
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<tr>
<td>Subclinical</td>
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<td>1</td>
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<tr>
<td>Total infections</td>
<td>8</td>
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Fig. 4. Individual mean peak viraemic titres determined by RT–PCR for sheep from serial passage Experiments 1 (a) and 2 (b). Only the three contact-infected groups are shown. Error bars show 95% confidence limits of the mean.

Fig. 5. Linear regression analysis for the effect of time of infection on the level of peak viraemia determined by RT–PCR for contact-infected sheep. Data showed no significant departures from normality (using the Kolmogorov–Smirnov test, \( P < 0.05 \)) or homogeneity of variance (using the Levene test, \( P < 0.05 \)). ANOVA using LSD for multiple comparisons of group means showed no significant difference (all \( P > 0.05 \)) between G1, G2 and G3 when all sheep were included, seroconverted animals only and clinically infected sheep only. Analysis was performed for Experiment 1 (all: \( F_{2,21} = 0.68 \), \( P = 0.53 \); seroconverted: \( F_{2,20} = 0.28 \), \( P = 0.76 \); clinical: \( F_{2,18} = 1.05 \), \( P = 0.37 \); Experiment 2 (all: \( F_{2,21} = 0.44 \), \( P = 0.73 \); seroconverted: \( F_{2,17} = 0.44 \), \( P = 0.65 \); clinical: \( F_{2,12} = 0.27 \), \( P = 0.77 \)) and for the two experiments combined (all: \( F_{2,45} = 0.70 \), \( P = 0.50 \); seroconverted: \( F_{2,40} = 0.71 \), \( P = 0.50 \); clinical: \( F_{2,33} = 1.12 \), \( P = 0.52 \)).

In addition, seroconversion did not occur significantly earlier following detectable viraemia for any group (Mann–Whitney U test, \( P < 0.05 \)). For viraemic animals, there was no significant correlation between the level of peak antibody

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response and the time of seroconversion (Spearman’s rank correlation coefficient \( r_s = -0.124; n = 43; P = 0.45 \)).

Subclinically infected sheep had a significantly lower peak antibody response than those clinically or apparently infected (Mann–Whitney U test, \( P < 0.001 \)). It is not clear whether these animals were truly subclinical, in that no viraemia occurred, or whether viraemia was shorter than the intersampling period (24 h) and/or below the detection limits of the tissue-culture detection method employed here. The lower antibody response and lack of viraemia do suggest a lower level of infection for these animals. The influence of such infections on transmission rates of FMDV is unclear.

All animals shown to be uninfected (seronegative against type O FMDV) were challenged by intranasal inoculation at the end of each experiment to confirm their susceptibility to FMDV. All these animals developed clinical FMD within 4 days of challenge.

**Discussion**

The outbreak of FMD in Greece during 1994 suggested that sheep flocks were unable to maintain the transmission of FMDV. This study was designed to investigate experimentally the transmission dynamics of this isolate of FMDV in sheep. The experiment mimicked the spread of infection through a non-homogeneously mixed population. The results show that an important characteristic of FMDV infection, peak viraemia, declines as FMDV is transmitted through a population of sheep. The inferences made from the results of these experiments are based on an a priori hypothesis and as such are potentially falsified by the experimental data.

While we found no significant association between the severity of clinical FMD or antibody response and either group or time of viraemia, the results indicated that a reduction in the level of infection (assessed by peak virus load) occurred over time from the introduction of FMDV. Key to the analysis of these experiments is the hypothesis that the level of peak viraemia is an accurate indication of the overall viral load. Recent studies of FMDV infection have shown that the level of virus in the blood correlates well with: (i) the level of FMDV in tissues of infected pigs, including the pharyngeal and upper respiratory region, where early virus replication and excretion are thought to originate (Alexandersen et al., 2001); (ii) increased transmission to in-contact sheep (Hughes et al., 2002); and (iii) the duration of virus replication in tissues of infected sheep (Alexandersen et al., 2002). Although the level of peak viraemia is not a direct measure of infectiousness, it has been used as a representative measure of the overall level of infection and does correlate with transmissibility under experimental conditions (Hughes et al., 2002).

We considered that only groups exposed to infection from naturally infected individuals would be comparable, as the dynamics of virus excretion have been shown to be dependent on the route and source of infection (Sellers et al., 1977). Thus, the experimental design only permitted legitimate between-group comparisons of G2, G3 and G4. Analysis of the results of these experiments makes no assumption regarding the source of infection. The design of this experiment does not require that all transmission events occur within one group before transmission proceeds to the neighbouring downstream group. The application of a constant exposure period ensures that consistent transmission patterns are not essential for unbiased analysis of a temporal sequence of transmission events.

The transmission of infection between sheep in these experiments could have occurred by several routes, e.g. by inhalation of airborne droplets or droplet nuclei, by ingestion of contaminated food or dust from the floor or by intraocular or percutaneous infection. However, given the high susceptibility of sheep to infection by the respiratory route (Gibson & Donaldson, 1986), the most probable mechanism of transmission would be by the transfer of infectious droplets or droplet nuclei. It is possible that later infection events were preceded by exposure of the individuals to low doses of virus from upstream contacts. Such an effect might potentially have primed immune responses with amounts of virus below that of an infectious dose. Although the serological data from these experiments suggest that no such effect occurred, such a mechanism would be more likely to involve sites of mucosal immunity, measurements of which were not performed during these experiments. It is unclear whether the presence of subclinically infected animals is in any way an indicator of varying exposure doses.

The results of these experiments can be explained by a hypothesis that requires that tentative assumptions be made about the infection dynamics of this isolate of FMDV in sheep. If the infectiousness of an individual is a function of the infectious dose received, and virus dynamics occur such that individuals are unable to transmit doses that quantitatively reflect those with which they themselves became infected, then infectiousness per unit time will decline with the passage number of the infections.

If transmission rates are sufficiently low, chains of infection would terminate without any temporal trends in the severity of infection, although it is possible that exposure doses would fall as the number of infectious animals declined. However, in our experiments, the strongest trend was in the severity of infection, rather than in the number of infected animals.

Here, we have assumed that the gradual reduction in the level of infection with time during these experiments would equate (albeit perhaps not linearly) with a gradual reduction in infectiousness. The duration of an outbreak under these circumstances would depend on the challenge dose to which the sheep flock was exposed. If this was high (e.g. infection from pigs), then more sequential infections might occur before the force of infection declined to subpersistent levels. Early effective contacts and subsequent dispersal (e.g. dissemination of virus at livestock markets followed by numerous diverse...
movements of exposed animals) would create a large number of infection chains. If all FMDV outbreaks in sheep populations were to follow the characteristics of this study, each chain of infection would eventually reach its endpoint unassisted. The time to reach conclusion would be governed by the conditions at the start of the epidemic. A decline in the number of infectious animals could lead to a decrease in the exposure dose and to a potential reduction in the level of infection with time. However, during these experiments, there was no such trend.

It must also be considered that the design of this experiment allows for the possibility that genetic bottlenecks occur. Repeated bottleneck events in vitro have shown that genetic lesions can occur following plaque-to-plaque serial transfer of FMDV (Escarmis et al., 1996). Such lesions can account for reductions in virus fitness. These events may be more probable in vivo when the infectious dose is small, such as with droplet-transmitted diseases (Bergstrom et al., 1999). However, preliminary nucleotide sequence analysis from the experiments described here showed that the consensus sequence of the VP1 gene from the inoculum persisted throughout the two experiments (results not shown). Further genetic analysis is to be undertaken on isolates of FMDV from the two experiments.

The experiments described here have been performed using an isolate of FMDV carefully selected because of the characteristics of its transmission in the field. Further studies must be performed to assess whether other isolates of FMDV exhibit similar properties before any assumptions can be made regarding the generality of this finding. The results of these experiments may also be particular to the experimental design.

If the results of these experiments are shown to be the exception rather than the rule, full sequencing of the genome (and comparison with more-transmissible isolates) may raise interesting questions regarding the genetic determinants of FMDV virulence and transmissibility. Although the capsid region of FMDV is the primary antigenic determinant, and has been shown to be the subject of positive selection (Fares et al., 2001; Haydon et al., 2001), non-structural coding regions of the FMDV genome have been implicated in species adaptation and attenuation (Beard & Mason, 2000; Nunez et al., 2001).

Genetic bottlenecking, isolate-specific infection dynamics and variable transmission rates are only three possible explanations for this phenomenon. The magnitude of individual FMD outbreaks, and the potential for stochastic fade-out, may result from the combination of a number of epidemiological parameters such as host population size, host density, influence of control measures and environmental conditions. The reduction in the level of FMDV infection with time shown here may contribute to the behaviour of FMDV in sheep populations, but the extent to which this effect contributes to the overall epidemiology of the disease requires further investigation.

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