# MODELLING THE MAINTENANCE AND TRANSMISSION OF FOOT-AND-MOUTH DISEASE VIRUS IN SHEEP

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# ABSTRACT

The aim of this study was to investigate the characteristics of an outbreak of foot-and-mouth disease (FMD) in Greece during 1994. This epidemic suggested that under certain circumstances transmission of foot-and-mouth disease virus (FMDV) may not be as efficient as is classically thought. Here, analysis of field data from the epidemic has shown that conditions at the source of the epidemic may have provided an initial amplification of the force of infection, enabling the virus to cause a limited number of outbreaks elsewhere. These favourable conditions included a high density and large number of sheep flocks. Later in the epidemic, the distribution of sheep flocks was more dispersed and led to an epidemic that was termed "self-limiting". Seemingly, without the influence of control measures, the infection rate of FMDV within sheep flocks fell dramatically until no cases of clinical FMD occurred. The virus was not sheep-adapted.

Serial passage experiments described here have been designed to ensure a consistent exposure period for sequential groups of sheep, such that each group was exposed to the same proportion of the previous group's total excretion. Two identical experiments were performed with four groups of sheep. Serial passage of this isolate showed a significant reduction in the viral load of infected animals after passage of virus through two groups of contact infected sheep. This reduction corresponds with a decrease in the estimated force of infection. Transmission of this isolate through contact infected sheep failed to amplify or maintain the level and force of infection.

Virological factors contributing to "self-limitation" have also been investigated. Experiments described here show that dose-dependent transmission of this isolate is a complicated process. Immunologically, inoculated sheep responded well to this isolate, such that with higher doses immune mechanisms reduced the infectiousness of inoculated animals. Sheep inoculated with the highest dose of virus did not transmit infection to in-contact animals. Above the threshold dose for infection there was a decline in the infectiousness of inoculated animals and a corresponding reduction in the level of infection for in-contact animals. Principal component analysis of infection parameters for inoculated animals shows that characteristics of infection group well with dose.

The results of the experiments described here suggest that isolates of FMD that are not adapted to sheep may be unable to maintain themselves in sheep populations. FMDV in cattle and pigs transmits rapidly and has the potential to create concentrated foci of infection. The potentially different behavior of FMDV in sheep should be carefully considered when attempting to control FMD epidemics.

## AUTHOR'S DECLARATION

I declare that the work in this thesis was carried out in accordance with the Regulations of the University of Edinburgh. The work is all my own except where indicated by special reference in the text and no part of the thesis has been submitted for any other degree. Any views expressed in the thesis are those of the author and in no way represent those of the University of Edinburgh. The dissertation has not been presented to any other University for examination in the United Kingdom or overseas.

**Signed:** **Date:** 22/08/07

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## ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| BTY | Primary bovine thyroid cells |
| ELISA | Enzyme linked immunosorbent assay |
| FMD | Foot-and-mouth disease |
| FMDV | Foot-and-mouth disease virus |
| IFN-$\gamma$ | Interferon gamma |
| IRES | Internal ribosome entry site |
| $\lambda$ | Force of infection |
| LSD | Least significant difference |
| OPF | Oesophageal-pharyngeal fluid |
| PCA | Principal component analysis |
| R | Reproduction ratio |
| RT-PCR | Reverse transcription polymerase chain reaction |
| $TCID_{50}$ | 50 per cent tissue culture infective dose |
| $T_{I-I}$ | Time interval between inoculation and peak IFN-$\gamma$ production |
| $T_{I-S}$ | Time interval between inoculation and seroconversion |
| $T_{V-S}$ | Time interval between viraemia and seroconversion |

# CHAPTER 1: INTRODUCTION

## 1.1 Foot-and-mouth disease

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cattle, sheep, goats, pigs and wild ruminants. Infection of a wide range of species including impala, red kangaroos, wombats, hedgehogs, coypu, water voles, moles, agouti, some species of deer and rarely man has been demonstrated.

### 1.1.1 Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV), of the family *Picornaviridae*, is a member of the *aphthoviridae* genus. There are seven serotypes O, A, C, SAT (South African Territories) 1, SAT 2, SAT 3 and Asia 1. Within each serotype there is a large range of antigenically distinct strains. Types O, A and C are the most widely distributed of the seven serotypes. SAT 1, SAT 2, and SAT 3 are restricted to Africa although SAT 1 and SAT 2 have occasionally spread to the Middle East. Asia 1 has been restricted to Asia with a recent incursion into Greece (2000).

FMDV is an icosahedral, non-enveloped virus with a diameter of 28nm. The virus is extremely pH sensitive with a very narrow stability range, the virus being quickly

inactivated outside the pH range of 6.0-9.0. The capsid is made up of 60 protomers each made up of one copy of the four viral proteins (VP1, VP2, VP3 and VP4) and surrounds a single strand of RNA approximately 8.45 kilo-bases in length with a molecular mass of $2.8\times10^6$ Da. The surface of the virion is relatively smooth compared to other picornaviruses, having no canyons or pits although the surface of VP1 has a number of loops (Acharya *et al.*, 1989) which are thought to be essential for cell attachment (Strohmaier *et al.*, 1982). VP4 is completely internalised.

Attached to the 5' end of the viral genome is a small virus basic glycoprotein (VPg) in place of the methylated cap present in eukaryotic mRNA. The FMDV genome is divided into four distinct regions. The first region (about 400 bases) from the 5' terminus is known as the S fragment. Formation of a hairpin loop within this segment has been predicted and may be involved with RNA replication (Belsham, 1993). The second region consists of a homopolymeric tract of cytidyl residues termed the poly C tract whose function is unknown. Following the poly C tract there is a non-coding region (about 720 bases) which contains the internal ribosome entry sequence (IRES) and has been predicted to form a number of pseudoknots whose function is also unknown. The FMDV IRES directs cap-independent initiation of protein synthesis and is highly conserved among FMDV isolates. The majority of the genome consists of a single, large open reading frame that in type O viruses is 6996 nucleotides long and encodes for a single polyprotein. In common with other picornaviruses, FMDV has a poly-adenosine

tail at the 3' end. The single large polyprotein is cleaved by viral proteases to form structural and non-structural proteins after translation of viral RNA. The virus has associated RNA polymerase, for replication, and protein kinase for phosphorylation of structural proteins.

For type $O_1$ strains of FMDV five neutralising antibody sites have been characterised (Kitson *et al.*, 1990; Crowther *et al.*, 1993). Two of these sites, including the major site A within the G-H loop, form part of VP1. The function of site A as the immunodominant antigenic site has been tested in pigs where varying degrees of immunodominance were found (Mateu *et al.*, 1995). Antigenic epitopes on the surface of the virion can be screened using panels of monoclonal antibodies. Such methods enable characterisation of strains of FMDV into subtypes (Crowther, 1986).

VP1 is the only structural protein capable of inducing a serotype specific neutralising antibody response (Bachrach *et al.*, 1975) and when administered alone can confer protection to challenge by homologous virus (Crowther, 1986). Despite this, FMDV sub-units containing VP1, as well as VP2 and VP3, are not as immunogenic as 146S whole particles which are essential for a successful vaccine (Crowther, 1986).

### 1.1.2 History

FMD was first described by Fracastorious (1545) in Northern Italy. FMD was recorded in Germany in 1751 and quickly spread throughout Europe. A major breakthrough in the understanding of FMD came in 1898 when the causative agent was discovered to be a virus (Loeffler & Frosch, 1898). FMD is one of the most economically important diseases of livestock. The outbreak in Great Britain during 1967-8 resulted in compensation in excess of £35 million (Anon, 1978). The disease reached Britain in 1839 and there followed a succession of outbreaks, which resulted in the disease being made notifiable in 1871. The difficulty in controlling the disease was mainly due to a poor understanding of the epidemiology of FMD. For example, it is thought that the spread of FMD between farms by visitors facilitated a major epidemic in America between 1915 and 1916. In 1922, Vallee and Carre demonstrated there were antigenic variants of FMDV and named them Oise (O) and Allemange (A). Type C was discovered in 1926 and SAT 1-3 in 1948. Asia 1 was not recognised until 1954.

FMD is endemic in countries situated in Africa, the middle and far east, Asia and South America. Australia, USA, Canada and Scandinavia are all free of FMD. The majority of continental Europe is also free of FMD although there are occasional outbreaks in the countries of southern and eastern Europe (Kitching, 1998). The adoption of a non-vaccination policy by the European Union (EU) in 1991 has created a livestock

population that is completely susceptible to FMD. Since implementation of the non-vaccination policy outbreaks of FMD have occurred within the EU (Italy, 1993; Greece, 1994, 1996 and 2000; Great Britain, France and Holland, 2001). The possibility of vaccinated cattle developing persistent infection following contact with field virus during an outbreak ensures there will be a continued reluctance to use vaccination in favour of stamping out procedures. The EU remains at risk of FMD from the Middle East, Eastern Europe and North Africa. Major outbreaks occurred in Britain in 1922, 1942, 1952, 1967 and recently in 2001. In 1967, the introduction of FMDV in infected meat from Argentina resulted in 2364 outbreaks and the slaughter of almost half a million animals (Hugh-Jones & Wright, 1970). Variation in price differentials could lead to the incursion of FMD into the EU through illegal movements of infected animals, particularly sheep and goats in which the disease is often mild or inapparent. With the continued development of fast modern transport systems infected animals can travel the entire length and breadth of the EU in two or three days.

### 1.1.3 Pathogenesis

Sheep are highly susceptible to FMDV infection through the respiratory route and breaks in the epithelium. Although sheep and cattle have the same minimum infectious dose, the threshold concentration of virus required to infect sheep is much higher due to the lower inhalation rate (Sorenson *et al.*, 2000). After virus has entered the host, primary

replication occurs at the site of entry, particularly the upper respiratory tract and pharynx (Burrows *et al.*, 1981). Following initial replication there is dissemination of virus to other areas of the body. Although virus has been detected in sites of predilection prior to the onset of viraemia (Brown *et al.*, 1992) transport of virus via the bloodstream no doubt plays an active role in spread within the body. It has been postulated that the occurrence of vesicular lesions in the absence of viraemia could represent local infection through abrasions in the epithelium (McVicar & Sutmoller, 1972). Sites of predilection include the epithelia of the oral cavity, coronary band and interdigital space and the mucosa of the pharynx (Burrows *et al.*, 1981).

Three serotypes of type C FMDV utilise a common receptor on bovine kidney cells (Baxt & Bachrach, 1980) and monoclonal antibodies against viral surface receptors can block binding of other picornaviruses (Shepley *et al.*, 1988). The conserved arginine-glycine-aspartic acid (RGD) sequence on the loop of VP1 is a motif used by many viruses to attach to the integrin molecules on mucosal epithelial cells (Bai *et al.*, 1994, Roivainen *et al.*, 1994). Enzymatic digestion of the RGD region of VP1 (residues 133-158) inhibits attachment of FMDV to certain cell lines (Strohmaier *et al.*, 1982). FMDV has been shown to be capable of binding to heparan sulphate on the surface of cells (Jackson *et al.*, 1996) although this is thought to be a consequence of tissue culture adaptation (Sa-Carvalho *et al.*, 1997). FMDV utilises the $\alpha_V\beta_3$ integrin as a primary receptor (Neff *et al.*, 1998).

### 1.1.4 Diagnosis

Diagnosis of FMD is based on the recognition of clinical signs, virus isolation and antigen detection. Seroconversion, where there is no history of vaccination, can also serve as an indicator of infection (Hamblin *et al.*, 1986). ELISA testing has replaced complement fixation and virus neutralisation tests in the laboratory diagnosis of FMD. ELISA tests are commercially available for the detection of FMDV antigens and antibodies against FMDV (Ferris and Dawson, 1988). Field samples (epithelium or blood) from suspect animals, can be tested for infection against all seven serotypes and several strains. If the initial sample does not contain sufficient viral material then samples can be amplified in tissue culture e.g. primary bovine thyroid cells (BTY) or a continuous swine kidney (IB-RS2) cell line.

#### 1.1.4.1 RT-PCR as a diagnostic tool

The use of reverse transcription polymerase chain reaction (RT-PCR) as a diagnostic tool to detect and serotype subclinical FMD has been investigated (Reid *et al.*, 1998; Vangrysperre & De Clerq, 1996). To increase the sensitivity of RT-PCR assays above that of virus isolation a second PCR step is needed. Nested PCR (RT-nPCR) has been shown to have a sensitivity greater than virus isolation (Forsyth *et al.*, 1998) and plaque testing (Moss & Haas, 1999) but also has a higher risk of contamination leading to false

positive results (Erlich *et al.*, 1991). Reid *et al.* (1998) advised that RT-PCR for the routine diagnosis of FMD should be in addition to the conventional methods already in use as PCR methods sometimes fail to diagnose samples that show positive results by virus isolation.

RT-PCR to detect FMDV RNA in various secretions and tissue samples, including oesophageal-pharyngeal fluid (OPF) (Moss & Haas, 1999), throat swabs (Callens *et al.*, 1998) and nasal swabs (Forsyth *et al.*, 1998; Moss & Haas, 1999) has yielded varying results. From nasal swabs, RT-nPCR is able to detect viral RNA prior to the onset of clinical signs (Moss & Haas, 1999). Detection of digoxigenin labelled PCR amplicons by ELISA (RT-PCR-ELISA) increases the sensitivity of tests (Forsyth*et al.*, 1998). RT-PCR-ELISA on throat swabs taken from experimental sheep has been shown to detect subclinical infection of in-contact sheep (Callens *et al.*, 1998). Inhibitors of reverse transcription and PCR within sample fluid (Moss & Haas, 1999) and the intermittent detection of virus in OPF (Doel *et al.*, 1994) may limit the use of RT-nPCR on probang samples. Conversely, for the detection of persistent infection, probang samples have been shown to be more reliable than nasal swabs (Forsyth *et al.*, 1998). The variable detection rates of viral RNA in both throat swabs (Callens *et al.*, 1998) and OPF (Moss & Haas, 1999) suggests that reliable diagnosis of infected and persistently infected animals may require continuous sampling regimes. Recently, a quantitative RT-PCR

method has been successfully applied to measure the levels of FMDV in various porcine tissues following infection (Oleksiewicz *et al.*, 2001).

### 1.1.5 Control

For successful international trade of livestock, movements of live animals and animal products must follow the guidelines of the Office International des Epizooties (OIE) code. Effective control of outbreaks is through measures such as slaughter of infected, in-contact and seropositive animals, coupled with stringent movement and other zoosanitary policies. These measures remain the most effective mechanisms in restricting spread of FMD. Since the realisation that wind borne virus could prove an important factor in FMD outbreaks, a computer programme has been developed to model the effects meteorological conditions can have on an epidemic (Donaldson, 1988). This allows ring vaccination to be planned around the area predicted to be affected. Ring vaccination strategies aim to reduce susceptible animals around a primary outbreak. Strategies of this type have a number of problems inherent with their implementation. These include restrictions on the movement of vaccinated animals from within the ring zone, loss of export from within the region and the risk of fomite transmission originating from infected vaccinated animals (Donaldson & Doel, 1992).

### 1.1.6 Vaccination

Vaccination against FMD is determined by regional or national control policies. Since 1991, the European Union has adopted a non-vaccination policy in an attempt to allow greater movement of livestock within the Single Market (Directive 90/423/EEC). The decision to cease vaccination within the EU also followed evidence that improper inactivation of vaccine virus had led to a number of outbreaks in Europe during the 1980s (Beck & Strohmaier, 1987). However, the option to vaccinate is retained should other control measures prove ineffective, and emergency antigen banks are available should an EU country require vaccine. Immunisation against one serotype of FMDV does not confer resistance against others. Emergency vaccines should induce a very early protective immune response as well as having a broad antigenic spectrum.

#### 1.1.6.1 Response of sheep to immunisation

Emergency vaccination has been shown to protect cattle from airborne challenge between 2 and 4 days post immunisation (Doel *et al.*, 1994, Salt *et al.*, 1994), pigs within 4 days of immunisation (Salt *et al.*, 1998) and sheep within 4 days of immunisation (Cox *et al.*, 1999). In cattle, early humoral responses are similar to those seen in natural infection but the peak titres of IgM, $IgG_1$ and $IgG_2$ are delayed with a reduced titre and avidity (Abu Elzein & Crowther, 1981). Immunisation of sheep with

inactivated whole virus particles (146S) protects against clinical disease and has been shown to elicit a local neutralising antibody response in the respiratory tract of vaccinated sheep, and may reduce the duration of excretion (Cox *et al.*, 1999; Gibson *et al.*, 1984; Sellers *et al.*, 1977). Sheep are vaccinated with a third or a half of the bovine dose with conventional saponin aluminium hydroxide-inactivated vaccine. Responses to inactivated vaccines have been shown to be antigen dose related (Pay & Hingley, 1987). Vaccination can induce protection from challenge for 3-6 months (Panina, 1990). Primary courses of vaccine are given in the form of two inoculations within 3-4 weeks with the use of booster doses depending on the potency of the vaccine used. Sheep show good serological responses to such booster doses (Pay, 1988). In endemic areas, particularly those with a high density of susceptible animals, such as farms in Saudi Arabia and Israel, vaccination of cattle can take place up to five times a year.

### 1.1.7 Foot-and-mouth disease in sheep

Although sheep represent the major proportion of the world's FMD susceptible livestock (Anon, 1993) studies on the nature of the disease in small ruminants remain limited. Sheep and goats are often overlooked as a potential source of FMD dissemination (Panina, 1990) despite experimental evidence to the contrary (Gibson & Donaldson, 1986). The effects of the disease are often mild or inapparent and easily missed by the farmer or veterinarian. Geering (1967) concluded that unless there was suspicion of

FMD and careful examination, the disease would go undetected. It is generally accepted that naturally acquired infection of FMD is milder in small ruminants than in cattle and pigs. One set of findings showed, using serology, that 27% of experimentally infected sheep failed to develop lesions (Gibson *et al.*, 1984). To ensure the regular occurrence of viraemia in infected sheep, serial passage was shown to be required for one isolate of FMDV (Fontaine *et al.*, 1966).

The incubation period for natural infection is normally between 3 and 8 days (Kitching & Mackay, 1994) but can be less than 24 hours following experimental inoculation (Sellers *et al.*, 1977). The first sign of infection within a susceptible flock is lameness, which develops quickly and spreads. The feet of affected animals are hot and painful when handled. In severe cases classic signs of FMD are seen: lassitude, anorexia, fever and vesicle formation. Foot lesions in sheep are more common than oral lesions and occur in the interdigital cleft, on the coronary band and on the bulb of the heels (Pay, 1988). If mouth lesions do occur they are transient, and quickly heal. Lesions may also occur on the gums, hard palate, lips and tongue. The serotype and strain of virus has been shown to influence the virulence of disease in sheep (see Pay, 1988). The mortality rate in ovine epidemics is normally negligible for adult animals and a figure of 0.38% has been recorded for an outbreak amongst sheep in Turkey during 1963 (Pay, 1988). Infection of lambs is normally without vesicles but mortality can reach up to 100% due to myocarditis (Chevskii *et al.*, 1964).

## 1.2 Host immune responses to foot-and-mouth disease virus

If a virus is successful in evading non-specific host immune defences such as physical barriers, non-specific phagocytes and natural killer cells, then it encounters specific immune responses. Picornavirus immunity is led by an isotype-specific antibody response, the levels of virus specific antibody correlating well with clearance and recovery from infection.

### 1.2.1 Serum antibody responses

Infection of susceptible cattle induces an antibody response with early IgM production (around 3.5 days post-infection) which peaks at 10 days post infection. The onset of seroconversion coincides with the cessation of viraemia and a reduction in excretion (Cox *et al.*, 1999; Gibson *et al.*, 1984). Early IgM production is followed by an isotype switch to $IgG_1$ between four and seven days post-infection with smaller amounts of $IgG_2$ and IgA also detectable (Abu Elzein & Crowther, 1981; Brown *et al.*, 1964). Cuncliffe (1964) detected neutralising antibody in infected cattle for up to 4.5 years. Several cattle from the study resisted challenge with homologous and heterologous virus 11 months after infection.

Subcutaneous injection of sheep with FMDV produces virus specific antibodies within 3 days, which rise to a peak after 10 days. Between 10 and 35 days the levels decrease and plateau for around 150 days (Panina, 1990). Exposure to virus produces detectable antibody within 5 days (Dellers & Hyde, 1964). Production of large amounts of antibody corresponds to the onset of clinical signs, a reduction in excretion and a cessation of viraemia. Serum antibody produced by infected sheep provides protection against challenge from homologous virus for up to 1.5 years.

Antibody mediated clearance of virus is accepted as the major immunological response to FMDV (McCullough *et al.*, 1992). The $IgG_1$ titre appears to be crucial for a successful immune response (Mulcahy *et al.*, 1990). Both total serum antibody and levels of $IgG_1$ correlate well with recovery from FMDV infection (Salt, 1993b). Serum IgG may prevent the spread of virus to potential sites of persistence in the infected animal. For animals with high serum antibody levels virus may persist in the pharynx or utilise a cell-associated transport system to pass throughout the body unhindered (Brown *et al.*, 1992).

### 1.2.2 Secretory antibody responses

In response to viral infection of tissues of the respiratory system, specific secretory and serum antibody responses are produced (Welliver & Ogra, 1988). The ruminant mucosal

immune system, responsible for large secretory antibody production, has been shown to be similar to other mammalian species (Husband, 1987). Mucosal antibodies are predominantly IgA, levels of which are greater than all other isotypes of immunoglobulin combined (Mazanec *et al.*, 1993). The bronchus associated lymphoid tissue (BALT) includes specialised cells that take up and transport antigen (McGhee *et al.*, 1992). Induction of a good immune response requires large amounts of antigen present at the site of infection to aid the expansion of B-cells into isotype specific plasma cells. The stimulated B and T lymphoblasts then enter circulation and target infected tissues. Terminal differentiation occurs within the local microenvironment.

BALT activation by antigen induces stimulation and dissemination of B- and T-cells to infected mucosal sites (McGhee *et al.*, 1992). Secretory antibody responses are isotype-specific (Heckert *et al.*, 1991) and in a number of viral infections immunity to infection correlates to the titre of the secretory antibody response (Welliver & Ogra, 1988). During FMDV infection a secretory response can last up to five months post-infection and is predominantly local secretory IgA (Matsumoto *et al.*, 1978). The lymphoid tissue of the mucosa contains memory and virgin cells for the production of IgA (McGhee *et al.*, 1992). Scicchitano *et al.* (1986) found that 95% of IgA in the upper respiratory tract of sheep was mucosa derived, levels being approximately 25 times those in plasma. Serum derived antibody can be selectively transported to the infected epithelium whereas IgA is produced locally within the mucosa as dimeric secretory IgA (Butler,

1986). Levels of IgG become at least equal to those of IgA in the lower regions such as the bronchi and alveoli (Welliver & Ogra, 1988).

Experimental observations have shown animals persistently infected with FMDV to produce a longer secretory antibody response (Matsumoto *et al.*, 1978). Convalescent carrier animals show marked resistance to viral replication in the pharynx where IgA responses are high (Salt, 1993b). Antigen specific IgA, when extracted from the respiratory secretions of cattle, has been shown to neutralise FMDV *in vitro*. It is unknown whether IgA actively protects against subsequent homologous infection or whether a related arm of the local immune system plays a contributory role in clearance. Garland (1974) found that secretory antibody produced by previous vaccination, could reduce the titre of FMDV in OPF. Cattle with established carrier status have shown both high serum antibody titres to FMDV antigens (McVicar & Sutmoller, 1974) and non-detectable levels (Hedger, 1968) indicating there is considerable individual variation. Salt (1993a) found no discernible differences in the titres of secretory IgA between cattle that had successfully cleared infection and those that developed persistence. Pigs produce a serum response very similar in profile to that of the secretory one (Francis & Black, 1983) and do not develop into carriers of FMDV.

### 1.2.3 Role of cytotoxic T-cells and cell mediated immunity

Despite the correlation of recovery from infection with serum antibody production, there are recorded anomalies that suggest there is a role for cellular arms of the immune system in immunity to FMDV. During potency testing of virus vaccine, some animals, with seemingly protective antibody titres, remain susceptible whilst others with almost non-detectable levels following vaccination resist challenge with live virus (Sigal *et al.*, 1992). Animals viraemic for FMDV have also been shown to clear infection without seroconversion (Donaldson & Kitching, 1989). The poor protection produced with peptide vaccines could be due to a failure to correctly induce antibody production or a failure to activate necessary arms of the cellular immune system, or both (Collen, 1994). The role of specific cellular immune mechanisms in immunity to FMDV is largely unknown. Cytotoxic T-lymphocytes have been implicated as the more active component of the immune system in combating persistent viral infections (Oldstone, 1991) although expression of surface proteins during persistent infection is minimal (Oldstone, 1991) and such mechanisms have been previously discounted in immunity to FMDV (McCullough *et al.*, 1987). Recently, $CD8^+$ T-cell responses following experimental infection with FMDV have only been detectable after the acute stage of infection (Childerstone *et al.*, 1999).

Cell-mediated immune mechanisms can act upon cells expressing very small amounts of antigen on their surface (Demotz *et al.*, 1990) and can destroy infected cells during the latent phase of infection (Oldstone, 1991). Proteolytic viral epitopes (5-9 amino acids) are presented on the surface of infected cells and recognised by $CD8^+$ T-cells, via MHC class I expression, and by $CD4^+$ T-cells, via MHC class II expression. Elaboration of cytokines selecting for a T-helper cell type 1 (Th1) response, such as IFN-γ and IL-2, leads to activation of cellular arms of the immune system and intracellular killing through macrophage activation. Amadori *et al.* (1992) found that IL-2 and IFN-γ correlated well with successful immunity to FMD in cattle. The saliva and nasal fluid of cattle possessing anti-viral activity, in the acute stage of FMD infection, has been shown to contain IFN-γ. There is limited evidence of a Th1 type response in FMDV infected guinea pigs (Knudsen *et al.*, 1979) and buffalo (Sharma *et al.*, 1985). NK cells, large producers of IFN-γ, have been shown to influence the regulation of T-helper cell differentiation and such an activity has been described in FMDV infected cells (Amadori *et al.*, 1992). Although there are clear divisions in the cytokines responsible for the Th1/Th2 dichotomy *in vitro* the situation remains unclear *in vivo*. Th1 and Th2 responses are cross regulatory (Scott, 1993) and a number of human and mouse clones have been shown to co-express both Th1 and Th2 cytokines (Wood & Seow, 1996). The factors leading to differentiation of naive $CD4^+$ T-cells remains unclear. In some animal models the epitopes present on the antigen presenting cell have no effect on the proliferation to either a Th1 or Th2 type response but *in vitro* studies have demonstrated

otherwise (Scott, 1993). The antigen dose has been demonstrated to influence this dichotomy. At present it is unknown which factors determine levels of regulatory cytokines in FMD infection.

### 1.2.4 Neutralisation and clearance of virus

Neutralisation of FMDV *in vivo* is antibody mediated and potentially utilises a number of mechanisms. McCullough *et al.* (1987) demonstrated that monoclonal antibodies to a type O FMDV could induce conformational change in the capsid that neutralised greater than 99% of virus due to loss of the RNA genome and formation of unstable capsids. Aggregation of virus, prevention of viral uncoating and destabilisation of the capsid (Mason *et al.*, 1993) can also neutralise virus *in vivo*. Clearance of FMDV in the mouse model is reduced when either the Fc portion of the specific antibody or the macrophage system is absent (McCullough *et al.*, 1990). For successful clearance of virus, phagocytosis is assisted by antibody-dependent opsonisation of virus. During FMDV infection it has been shown that in the absence of phagocytosis, antibody-virus complexes are still infectious (McCullough *et al.*, 1992).

## 1.3 Persistence and the carrier state

Persistent infection may develop following immune failure to clear virus at the acute stage of infection. This situation is common for many picornavirus infections including poliovirus, Theiler's virus and echovirus 6 (Borzakian *et al.*, 1992). For a lytic virus such as FMDV to persist within an immunologically uncompromised host, replication must occur in non-lytic cycles with suppression of immune responses. This pressure may select for variants which utilise one of two recognised mechanisms for the development of persistence: expression of a non-lytic phenotype (or development of a non-lytic phase of viral replication) or diminished viral gene expression through interaction of viral and cellular transcription factors (Oldstone, 1991). Persistent virus in tissue culture can take one of four forms (Mahy, 1985): steady state viruses (cells infected with a non-lytic virus), carrier culture virus (lytic virus replicating at a very low rate), virus showing intra-cytoplasmic persistence (immature uninfectious virus passes from cell to cell) and latent virus (transcription of viral genome is severely restricted). The implications and classification of these classes *in vivo* is unclear.

Persistent FMDV infection is suspected as the cause of a number of outbreaks of FMD including the 1922 UK epidemic (Hedger & Stubbins, 1971) and the introduction of FMD into Mexico from Brazil in the 1940s (Van Bekkum, 1973). Van Bekkum *et al.* (1959) demonstrated carrier status under experimental conditions when virus was

detected in samples from experimental cattle up to 5 months post-infection. For FMD a carrier is classified as any animal where virus can be isolated after 28 days post-infection (Sutmoller *et al.*, 1968). Carrier animals of FMDV develop following infection and virus persists in the pharynx (cattle) and tonsillar region (sheep) (Burrows, 1968b). Detection of persistent infection uses the probang 'sampling cup' to extract OPF for virus isolation. Virus detection is intermittent and sampling techniques produce inconsistent results. Virus is not found in the saliva of carrier cattle (Wittman & Eissner, 1966) suggesting that mucus and cellular material are the critical components of OPF for virus recovery. Experimental studies have shown that sheep can become carriers of FMDV following exposure to infected cattle, sheep or pigs (Burrows, 1968b; Fondevila *et al.*, 1996; McVicar & Sutmoller, 1968) and virus may be recovered from OPF for up to 9 months post infection (McVicar & Sutmoller, 1968).

### 1.3.1 Methods of persistence

#### 1.3.1.1 Ineffectual immune response

For cattle the immune status (vaccinated, passively immunised or naive) of the animal has no influence on the probability of development of a persistent infection following contact with live virus (Burrows, 1966) although Salt (1993b) found a marginally greater incidence amongst non-vaccinated cattle. Field evidence shows that the probability of

detecting carriers of FMDV increases if the FMDV-specific serum antibody titre of the herd or flock is high (McVicar & Sutmoller, 1974), despite evidence from experimental animals which shows no such relationship (Hedger, 1970; Van Bekkum, 1973). Vaccination reduces the frequency of clinical cases, raising levels of immunity and therefore reducing levels of FMDV in the environment and the potential of new carriers developing (Anderson *et al.*, 1976). Cattle herds with low mean serum neutralising antibody titres are unlikely to harbour carriers (Hedger, 1970).

The early $IgG_1$ peak in cattle may be responsible for clearance of virus from the pharynx due to efficient uptake of $IgG_1$ complexed virus by macrophages (Salt, 1993b). Large amounts of sIgA produced by mucosal tissues may restrict the spread of FMDV to the bloodstream. Resistance to reinfection has been shown to correlate with a persistent infection of homologous virus (McVicar & Sutmoller, 1974) and the presence of IgA in secretory fluids (Salt, 1993b). There is no evidence of a failure to elicit local or systemic immune responses in persistent infection with FMDV.

### 1.3.1.2 Persistence in privileged sites

Persistent viral infection can occur at immunologically privileged sites. Epithelium lined secretory cells are known sites of persistence for immune evading viruses of vertebrates (Mims, 1988). These cells face into a lumen which is outside the body and from which virus can be released without cytolysis (Salt, 1998). Other frequently used sites for viral persistence are cellular elements of the immune system (monocyte/macrophage lineage) (Oldstone, 1991) but there is a lack of evidence linking this method to FMDV persistence. FMDV immune complexes have been shown to infect pig macrophages (Baxt & Mason, 1993). Yilma (1980) implicated mononuclear cells as a possible privileged site for FMDV in the pre-viraemic stages of infection and as a potential transport system for virus although this has not been proven.

### 1.3.1.3 Trans-encapsidation with bovine enterovirus

FMDV has been seen encapsulated with the coat protein of bovine enterovirus (BEV) *in vitro* (Trautman & Sutmoller, 1971) and *in vivo* (Graves *et al.*, 1971). It has been suggested that encapsulation could stimulate a low level localised, non-specific immune response sufficient to prevent the spread of FMDV but too small to bring about a destructive immune response (Sutmoller *et al.*, 1970). There is no evidence from carrier studies to support this hypothesis.

### 1.3.1.4 Defective interfering particles

Persistent infection by a lytic virus can develop through the formation of replication defective genomes which become incorporated into defective interfering particles (DIPs) or viruses (DIVs). DIPs exist as a separate population within an infected host, dependent on the homologous parental virus for replication and causing disruption to the biology of the host-parasite relationship. This method is employed successfully by paramyxoviruses and rhabdoviruses. DIP's have been observed for almost every virus, including members of the pircornaviridae. Non-infectious FMDV RNA fragment production has been demonstrated at the latter stage of persistent infections *in vivo* and *in vitro* (de la Torre *et al.*, 1985; Rossi *et al.*, 1988). The role of DIP's in FMDV persistence is unclear.

### 1.3.1.5. Antigenic variation

Through variation in antigenic make-up, viruses may persist by avoiding specific immune responses. Antigenic variance of FMDV is thought to occur at rates similar to other RNA viruses (McCahon, 1986) as a result of point-mutations caused by errors in copying of the genome and recombination events between FMDV genomes present in the same cell (Domingo *et al.*, 1985; Fellowes & Sutmoller, 1970). The mutation rate has been estimated at between $10^{-3}$ to $10^{-4}$ base pairs per replication (Smith & Inglis, 1987). Domingo *et al.* (1993) defined FMDV populations as quasispecies with the

potential to undergo rapid antigenic change. This has been shown to take place irrespective of immune status (Domingo *et al.*, 1993) but the selective pressure produced by cellular and humoral arms of the immune system may provide an ideal environment for the selection of variants. Antigenic drift is common *in vitro* and was first shown by Hyslop (1965). Antigenic variation has been demonstrated in tissue culture (Diez *et al.*, 1990) and after only a single passage in pigs (Carrillo *et al.*, 1990). In a state of persistence large variations in antigenic structure (Fox *et al.*, 1989) and, conversely, antigenic stability in cattle (Vosloo *et al.*, 1996) and sheep (Sharma *et al.*, 1982) has been shown to occur for FMDV.

Rowlands *et al.* (1983) found that serial passage of FMDV resulted in an alteration of antigenicity through amino acid substitutions. Within an isolate of virus there is more than one variant, leading to what has been termed a 'master' sequence with an a equilibrium distribution of variant sequences (Domingo *et al.*, 1985). Under selective pressure from the immune system *in vivo* a virus population may under go antigenic drift until a new master sequence becomes established (Domingo *et al.*, 1990). Studies on genome evolution in beef herds in Zimbabwe has shown that FMDV antigenic change is very slow (Knowles & Bosch, 1990). This makes this method of persistence unlikely for FMDV although antigenic variation may have implications for virulence, pathogenicity and transmissibility (Haydon & Woolhouse, 1998).

### 1.3.1.6 Attenuation of virulence

The high mutation rate for FMDV during replication creates a potential for genetic alteration that may lead to a virus population capable of invading host cells and producing little or no cytopathic effect. The scope of genetic variation has been demonstrated *in vitro* for FMDV and co-evolved BHK cells where only nine fixed amino acid substitutions resulted in significant phenotypic alteration (Diez *et al.*, 1990). Numerous strains of carrier virus have shown reduced virulence in homologous and heterologous species (Burrows, 1966; Kaaden *et al.*, 1970; Straver *et al.*, 1970). Diez *et al.* (1990) found a strain of FMDV which, after 100 passages through BHK cells, showed high attenuation of virulence for mice and cattle.

The polyribocytidilic acid tract (poly C) of FMDV is a distinctive feature of the viral genome. Located approximately 400 nucleotides from the 5' end of the RNA genome, the tract is 100-300 nucleotides in length and has been investigated in attenuation studies. During replication of FMDV the length of the poly C tract can vary (Zibert *et al.*, 1990) and heterogeneity of the tract has been linked to persistence of FMDV. In tissue culture, alteration of the poly C tract corresponds to attenuation (Costa Giomi *et al.*, 1984; Escarmis *et al.*, 1992) and cattle isolates have been shown to exhibit a longer poly C tract than the parental strain (Costa Giomi *et al.*, 1988). Escarmis *et al.* (1992)

suggested that deviation in the length of the non-coding region could lead to the formation of a genetic lesion capable of interfering with host cell replication procedures.

## 1.4 Epidemiology of foot-and-mouth disease

FMDV can spread by a number of mechanisms of which the most common is direct contact following animal movement. Disease can also be introduced through contaminated animal products, fomites, vehicles, people, domestic or wild animals and by airborne spread. The occurrence of secondary outbreaks is determined by a number of factors including the species infected, species in-contact, local density of livestock, movement patterns, the number of effective contacts between infected and in-contact susceptible animals, speed of diagnosis and the efficiency of control measures.

Spread of FMD over large distances is possible through the generation of large plumes of virus excreted by infected animals. Pigs are by far the most significant species with regard to airborne spread of FMDV. The total excretion from one infected pig is equivalent to that of 3000 infected cattle (Sellers, 1971). The risk of spread of FMD by airborne virus from the continent has risen with the recent increase in the size and density of pig herds in the Benelux countries and northern France (Donaldson & Doel, 1992). Historically, it has been suggested that outbreaks of FMD in Great Britain have

been caused by spread of airborne virus across the channel when meteorological conditions have proved favourable (Donaldson *et al.*, 1982; Gloster *et al.*, 1982).

### 1.4.1 The role of sheep in epidemics

Infectious sheep are a potential source of FMDV with infection of susceptible sheep occurring following exposure to as little as ten infectious units (Gibson & Donaldson, 1986). This dose-dependent response is also strain-dependent. In 1972, sheep and goats were recognised as a frequently inapparent source of spread and recommendations were made to include prophylactic vaccination of sheep in vaccine campaigns (Pay, 1988).

The large population of small ruminants in North Africa and the Middle East has created a reservoir for FMDV. The threat of incursions of disease to surrounding FMD free areas is constant. Outbreaks of FMD with a predilection for sheep occurred in North Africa between 1989 and 1992 (Mackay, 1994) and in the Middle East during 1996 (Taylor & Tufan, 1996). Outbreaks involving large numbers of small ruminants occurred in Turkey during 1995 and 1996 and Greece in 1994 and 1996 (Kitching, 1998). Recently, the outbreak of type O FMDV in Great Britain predominantly affected sheep flocks. Serological surveys were undertaken following the outbreaks in Morocco (Mackay, 1994; Mackay & Rendle, 1996) and Greece (Mackay *et al.*, 1995). The results of these surveys revealed a low level of seroconversion within sheep flocks towards the

end of the epizootic. This low-level seroconversion followed an initial period of highly transmissible clinical disease. In Morocco, vaccination, through an increase in the level of herd immunity, seemed to reduce the spread of infection whereas slaughter of seropositive animals had no impact on subsequent transmission. This may be due to the fact that following the onset of seroconversion sheep are no longer infectious. Thus the slaughter of seropositive animals will have no effect on the levels of circulating virus although it will decrease the probability of carrier animals developing.

#### 1.4.1.1 Excretion by infected sheep

Cattle, pigs and sheep infected with FMDV have been shown to excrete large amounts of infectious virus (Donaldson *et al.*, 1970; Sellers & Parker, 1969; Sellers, 1971). The most common route of transmission of FMDV from sheep is through the production of infectious droplets and aerosols excreted in the breath of sheep (Donaldson, 2000). In some studies, detection methods have lacked the sensitivity necessary to accurately quantify excretion (Sellers & Parker, 1969). In the two studies of FMDV excretion using infected sheep, the point of peak excretion was found between 24 and 0 hours prior to the onset of vesicular lesions. FMDV has been isolated from infected sheep for up to five days prior to the onset of clinical signs (Burrows, 1968a) but this has not been correlated with infectiousness. Existing data for FMDV excretion by sheep was collated for the EpiMAN-FMD epidemiological information system (Sanson *et al.*, 1999) and

has been adapted for atmospheric models to predict the airborne spread of FMDV (Sorenson *et al.*, 2000). The serotype and strain of virus has been shown to influence the excretion profile of infected animals (Donaldson *et al.*, 1970).

#### 1.4.1.2 Sheep as maintenance hosts

Although there is substantial experimental evidence to suggest that carrier sheep are of epidemiological importance, field evidence is scarce and to the contrary. With the possible exception of a type O infection in a sheep flock in Brazil where within the same flock new cases of clinical disease was recorded 2 months following the first case and a number of sheep developed into carriers (Hancock & Prado, 1993). Interestingly, the virus in Brazil showed a very high predilection for sheep. Studies in epidemic (Garland *et al.*, 1981) and endemic areas (Anderson *et al.*, 1976) suggest small ruminant populations are unable to maintain infectious virus for longer than the duration of the period of clinical disease within the flock. Similar observations in Greece followed the 1994 epidemic where a period of subclinical transmission followed a period of clinical disease. There were no further cases of clinical disease once the initial burst of transmission ceased (Mackay *et al.*, 1995). Field evidence from Saudi Arabia suggests type O FMDV may continue to circulate within small ruminants with up to 40% of animals subclinically infected (Farag *et al.*, 1998). Similar observations have been recorded for sheep and goat flocks in India (Shankar *et al.*, 1998). Transmission from

carrier animals (cattle or sheep) to susceptibles, despite numerous attempts, has not been demonstrated experimentally. Titres of infectious virus from carrier cattle are low and often below what is thought to be the necessary level required for transmission (Donaldson & Kitching, 1989).

### 1.4.2 Species adaptation

Replication of FMDV within small ruminant hosts may result in an alteration of virulence for both the small ruminant host and in-contact cattle and pigs. There are numerous field reports of a variation in virulence of FMDV for different hosts. Observations on pig adapted viruses in the field (Brooksby, 1950) have recently been confirmed by experimental evidence of natural adaptation of an isolate of FMDV to pigs (Dunn & Donaldson, 1997). The most recent, and well-characterised, species adapted virus is the type O virus that caused the hugely devastating epidemic in Taiwan during 1997 (Dunn & Donaldson, 1997). This virus was unable to cause generalised disease in cattle and a deletion in the 3A non-structural protein and a number of surrounding point mutations have been shown to be the major genetic determinants of the altered host range of this virus both *in vitro* and *in vivo* (Beard & Mason, 2000). Field isolates from the same area that have replicated within different host species has been shown to be genetically different (Donaldson, 1998). Strains of type O FMDV with a predilection for sheep were isolated from the outbreak of FMD in North Africa from 1989-1992. FMDV

had maintained itself for 4 years, largely in small ruminants, exhibiting a very low morbidity for cattle. In Morocco, the attack rate for sheep was 97% but only 0.3% for cattle (Samuel *et al.*, 1999). Studies on the receptors for FMDV have indicated that receptor switching may be involved in adaptation of strains to different hosts (Neff *et al.*, 1998).

## 1.5 Molecular epidemiology

The genetic relationship between strains of FMDV can be compared using nucleotide sequencing of the genome (Beck & Strohmaier, 1987). Although other viral proteins contribute to the overall antigenicity of isolates (Kitson *et al.*, 1990), VP1 phylogenetic grouping does correlate with serotyping (Dopazo *et al.*, 1988). This technique has been used to study the molecular epidemiology and evolution of a wide range of isolates of FMDV (Samuel *et al.*, 1999). Nucleotide sequencing at the World Reference Laboratory for FMD, Pirbright (WRL) has resulted in a database comprising over 1500 partial or complete VP1 sequences of FMDV isolates (Kitching *et al.*, 1989). Serotypes of FMDV have been clustered together phylogenetically and divided into lineages of closely related genotypes. When a genotype of FMDV exhibits geographical restriction they have been termed topotypes. For type O FMDV there are 6 recognised topotypes (Europe/South America, South Asia, South-East Asia, China/Hong Kong, East Africa and Indonesia).

## 1.6. Molecular evolution of foot-and-mouth disease virus

RNA viruses have a high mutation rate during copying of the genome due to the low fidelity and lack of proof-reading activity of RNA polymerases (Domingo & Holland, 1994). The mutation rate of RNA viruses has been estimated as between $10^{-3}$ and $10^{-4}$ per single base site per replication cycle. The mutant progeny form a dynamic distribution in sequence space termed the quasispecies (Domingo *et al.*, 1999). This spectrum of heterogeneous genotypes has a high potential adaptation to different environments. Clones of FMDV exhibit varying fitness and heterogeneity of viral populations has been found during acute infection (Carrillo *et al.*, 1990). Selection for a more transmissible variant does not assume selection of a more virulent variant. The trade off between transmissibility, virulence and resistance may favour a reduction in virulence through selection (Anderson & May, 1991). Reductions in viral fitness through stochastic loss of the least mutated clones, known as Muller's ratchet, can occur (Chao, 1990; Bergstrom *et al.*, 1999). Alteration in the virulence of picornaviruses has been seen to associate with a number of regions of the genome including capsid proteins through altered interaction with cellular receptors (Samuel *et al.*, 1990), the internal ribosome entry site (Gromeier *et al.*, 1999) and the poly C tract (Duke *et al.*, 1990; Escarmis *et al.*, 1992). Positive selection of FMDV capsid proteins has recently been shown to occur with field isolates (Haydon *et al.*, 2001).

## 1.7 Quantitative epidemiology

Simple mathematical models can be developed to estimate transmission rates after introduction of a number of infectious individuals into a closed population of susceptible animals. Such models make the assumption that the animal population is homogenous and equally mixed i.e. each susceptible animal has equal contact with infectious individuals. Estimations of transmission rates rely on the fundamental concept of mathematical epidemiology that the course of an epidemic depends on the rate of contact between susceptible and infectious individuals. This is the principle of mass-action. The principle assumes there is a threshold density or number of susceptibles below which an epidemic cannot maintain itself.

### 1.7.1 Basic reproduction numbers

Rates of transmission for infectious diseases can be measured using estimates of the basic reproduction number ($R_0$). $R_0$ is the number of new infections, or the average number of secondary cases, caused by one infectious individual introduced into a population of susceptible animals (Diekmann *et al.*, 1990). For an infection to spread, $R_0$ must be greater than unity in value. $R_0$ can also be estimated using a livestock unit (herd or flock) as the epidemiological unit, in which case $R_0$ is expressed as the ratio of secondary outbreaks to primary outbreaks. Such a method has been successfully applied

in estimation of $R_0$ for FMDV epidemics among herds (Haydon *et al.*, 1997). Movement of individuals between disease classes can be modelled using differential equations describing the rate of change for each class over time (Anderson and May, 1992). Each equation is controlled by infection parameters. The rate that individuals become infected is described as the force of infection ($\lambda$) and can be estimated from experimental transmission data.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Preparation of cell cultures

All virus isolation and titrations were performed using primary calf thyroid cells (BTY) prepared fortnightly at the World Reference Laboratory for foot-and-mouth disease, Pirbright (WRL). Bovine thyroid cultures were prepared from glands obtained from young calves less than two months old which were trimmed of fat and rinsed twice in 100 ml of calcium and magnesium free phosphate buffered saline (PBS; see appendix). Glands were then finely chopped and covered with Eagle's medium (see appendix) with 10% adult bovine serum and left overnight at 4°C. Chopped glands were washed three times with PBS before addition of 100 ml of Dispase (neutral protease from *Bacillus polymixa*, Grade II, Boehringer Mannheim) followed by stirring for 1 hour at 37°C to dissociate the cells. Cell suspensions were then clarified at 1000 g for 10 min. This process was repeated 2-3 times until all cells were thoroughly dissociated. Cells were resuspended in Eagle's medium with 10% normal bovine serum, counted and diluted to $1\times10^5$ cells/ml. Cells were seeded in either flat sided tissue culture tubes (Nunc, Denmark) or Falcon tissue culture flasks (Becton Dickinson Labware). After 4-5 days growth at 37°C in Eagle's medium with 2% foetal calf serum, cells were transferred to 32°C. BTY tubes were ready for use within two weeks of preparation.

## 2.2 Virus isolation and growth in bovine thyroid cells

Cell sheets were washed with 2 ml of PBS before addition of 200 µl of sample. Five tubes were used for each sample tested. Tubes were then left at 37°C for 1 hour to enable virus adsorption. Following incubation, cell sheets were washed three times with 2 ml of PBS and 2 ml Eagle's-HEPES (see appendix) medium added. Tubes were returned to 37°C, placed on rollers and examined after 24 and 48 hours for evidence for cytopathic effect (CPE). After clarification at 3000 g for 10 min all suspected positive samples were confirmed as type O FMDV by antigen detection ELISA (as described in section 2.4). If amplification of the titre of isolated virus was required, virus was passed again in BTY cells by inoculation of cells with harvested tissue culture supernatant. An adsorption step was not required for this procedure.

## 2.3 Virus titration in bovine thyroid cells

Five separate dilutions from a 10-fold dilution series were used for virus titrations. Samples were diluted in M25 buffer (see appendix) and 200 µl added to each of four BTY tubes as described in section 2.2. Titres were calculated as the Karber method (Karber, 1931). Tissue culture infective dose 50 ($TCID_{50}$) was calculated using the following formula:

$$\log TCID_{50}/ml = (\log \text{highest virus concentration}) - \text{total \% CPE}/100 - 0.5) + 0.7$$

## 2.4 Antigen detection

Tissue culture supernatants were confirmed as containing FMDV type O by ELISA (Ferris & Dawson, 1988). Nunc Maxisorp 96 well ELISA plates (Nunc, Denmark) were coated overnight at 4°C with anti-FMDV type O hyperimmune rabbit serum (1:5000) in coating buffer (0.05 M $Na_2CO_3$, 0.05 M $NaHCO_3$; pH 9.6). Wells were washed three times with PBS using a Denley platewasher before addition of 50 µl of sample and positive controls. Plates were incubated at 37°C for 1 hour on an orbital shaker and following incubation washed as before prior to addition of preblocked guinea-pig anti-FMDV type O immune serum (1:1000 in blocking buffer; see appendix) and incubation at 37°C on an orbital shaker. Following incubation, plates were washed as previously before addition of horseradish-peroxidase (HRPO) conjugated rabbit immunoglobulin anti guinea pig (1:200 in blocking buffer). Plates were returned to the orbital shaker at 37°C for 45 min, washed as before and an additional wash included which involved flooding the plate with PBS to ensure all excess conjugate had been removed. Activated substrate (*O*-phenylenediamine dihydrochloride (OPD) in phosphate-citrate buffer (pH 5.0) with 5% $H_2O_2$) was added to each well and all plates left for 15 min at room temperature for colour to develop. Reactions were stopped by the addition of 50 µl of 1.25M $H_2SO_4$ to each well. Optical densities were read at 492 nm using a Dynex MRX plate reader.

## 2.5 Serology

Total serum antibody titres against FMDV type O were measured using a liquid phase blocking ELISA (Hamblin *et al.*, 1986). Duplicate two-fold dilution series of each serum sample were incubated at 4°C overnight on an orbital shaker with an equal volume of FMDV type $O_1$ Manisa antigen (previously determined dilution in PBST; see appendix) in 96-well U bottomed plates (Sterilin). ELISA plates (Nunc, Denmark) were coated with FMDV type O specific hyperimmune rabbit serum (1:5000) in coating buffer (0.05 M $Na_2CO_3$, 0.05 M $NaHCO_3$; pH 9.6) and left overnight at room temperature in a humid box. The following morning, coated plates were washed three times with PBS using a Denley plate washer and 50 µl of test sample/antigen transferred to corresponding wells on the coated plates. ELISA plates were then incubated at 37°C for 1 hour on an orbital shaker. Following incubation, plates were washed three times with PBS and 50 µl FMDV type O specific guinea pig serum added to each well (1:1000 in blocking buffer; 85% PBST, 10% normal bovine serum, 5% normal rabbit serum). Plates were returned to the orbital shaker at 37°C for 1 hour. Following incubation, plates were washed as before prior to addition of 50 µl of horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin to each well (1:1000 in blocking buffer). Plates were then returned to the orbital shaker at 37°C for 1 hour. Following incubation, plates were washed as before with an additional flooding of the plate with PBS to ensure excess conjugate was removed. After incubation, 50 µl of OPD with 5% $H_2O_2$ was added to each well and left

for 15 min at room temperature for colour to develop. Reactions were stopped by the addition of 50 µl of 1.25M $H_2SO_4$ to each well and optical densities read at 492 nm using a Dynex MRX plate reader. All plates included strong and weak positive controls (bovine serum), negative controls (normal bovine serum) and antigen controls (PBST). Results were assessed as percentage inhibition of the mean of four antigen control wells. From this, strict boundaries were set for the control samples (strong positive >85% inhibition, weak positive between 50 and 85% inhibition, negative <40% inhibition). If these control conditions were not met the plate was rejected and repeated. Antibody titres were calculated as the reciprocal of the final dilution of serum that gave an OD value of 50% of the mean antigen control. Mean antibody titres were calculated as described in Thrusfield (1995). Geometric mean titres were calculated by back-transformation of $\log_2$ transformed individual titres.

## 2.6 Oesophageal-pharyngeal samples

Oesophageal-pharyngeal fluid (OPF) was collected using the probang sampling cup (Sutmoller & Gaggero, 1965). Samples collected from animals were immediately diluted in the isolation unit with 5 ml of Eagle's-HEPES medium with 5% foetal calf serum. In the laboratory, samples were immediately placed on ice before processing. 2.5 ml of the diluted sample was added to 2.5 ml of Freon (1,1,2-trichloro-tirflourethane; Sigma). The sample-Freon mix was vortexed for 2 min. The homogenate was then spun at 3000 g for

10 min. Following separation the supernatant was carefully removed and 250 µl added directly to 750 µl of TRIzol LS (Gibco, BRL) and 200 µl added to each of five BTY tubes for virus isolation. The remaining volume of Freon treated OPF sample was stored at -70°C.

## 2.7 RNA extraction

### 2.7.1 Throat swabs and probang samples

RNA was extracted using TRIzol and TRIzol LS (both Gibco, BRL) according to the manufacturer's instructions. Throat swabs and TRIzol were transferred to a 2 ml skirted Sarstedt tube using a sterile needle and 200 µl sterile PBS added to each tube followed by 200 µl of chloroform. For probang samples, 250 µl of Freon treated sample was added to 750 µl of TRIzol LS and vortexed. Following complete mixing, 200 µl of chloroform was added. After addition of chloroform for both sample types, samples were vortexed for 15 seconds, left at room temperature for 5 min and spun at 12000 g for 15 min at 4°C. Following separation, 500 µl of the aqueous phase was transferred to a 1.5 ml Eppendorf centrifuge tube containing 1 µl of glycogen and 500 µl of isopropanol added. Samples were vortexed briefly and left at room temperature for 10 min to facilitate RNA precipitation. After centrifugation at 12000 g for 10 min at 4°C, supernatants were aspirated and 1 ml of 70% ethanol added. Samples were vortexed

briefly and centrifuged at 12000 g for 10 min at 4°C. Following centrifugation, supernatants were aspirated and RNA pellets dried for 5 min at room temperature. Pellets were redissolved in 20 µl of nuclease free water (Promega) by repeat pipetting and stored immediately at -70°C.

### 2.7.2 Blood

Prior to the addition of chloroform, samples were vortexed for 20 seconds to ensure complete homogenisation and spun at 12000 g for 15 min at 4°C. Following separation, 550 µl of the aqueous layer was removed into a fresh 2 ml Sarstedt and 200 µl of sterile PBS added. 200 µl of chloroform was then added to each tube and RNA extracted as described in section 2.7.1.

## 2.8 Reverse transcription

Five µl of extracted RNA was heated with 100 ng of random hexanucleotides (Boehringer Mannheim) at 70°C for 5 min. After cooling at room temperature for 10 min, the primer template mixture was reverse transcribed at 37°C for 45 min in a 20 µl reaction mix. The mix contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ (Gibco, BRL; supplied with reverse transcriptase), 0.5 mM (each) dNTP (Promega), 10

mM dithiothreitol and 200 units of Moloney-murine leukemia virus reverse transcriptase (Gibco, BRL).

## 2.9 Nested polymerase chain reaction

Primers for both rounds of the PCR procedure had been previously designed (Forsyth *et al.*, 1998) as complementary sequences to the highly conserved FMDV internal ribosome entry site (IRES) and are shown in table 2.1.

| Primer | Sequence | Nucleotide positions |
|---|---|---|
| IRES1 | 5'-CCTGGTCTTTCCAGGTCTAGA-3' | 312-332 |
| IRES2 | 5'-CCTCCTTGGTAACAAGGACCC-3' | 435-455 |
| IRES3 | 5'-CCTTCTCAGATCCCGAGTGT-3' | 631-612 |
| IRES4 | 5'-CTTCTCAGATCCCGAGTTGT-3' | 685-665 |

**Table 2.1. Oligonucleotide primers used for polymerase chain reaction (PCR).** All primers have been previously designed and used for detection of type O FMDV (Forsyth *et al.*, 1998).

First round, external primers used were IRES1 and IRES4 to give a 373bp DNA product. For the internal nested PCR, primers used were and IRES3 and IRES4 giving a 196bp product.

A 5 µl RT reaction mix was amplified in a 50 µl total PCR reaction mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTP, 10 pmol of

IRES1 and IRES4 and 1 *Taq*Bead Hot Start Polymerase wax bead containing 1.25 units *Taq* DNA polymerase (all Gibco, BRL). Viral DNA was amplified using the following thermocycler program: 94°C for 5 min, 1 cycle; 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, 30 cycles; 72°C for 7 min, 1 cycle. Amplification was performed using a PTC-100 thermocycler machine (MJ Research Inc., USA). Second round nested PCR was carried out under the same conditions using 5 µl of first round product as a template and 10 pmol of each internal primer (IRES2 and IRES3). Nested PCR utilised the same thermocycler program but for only 20 cycles. PCR products were visualised by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml). Product size was measured using a 100bp DNA ladder (Gibco, BRL).

## 2.10 Quantitative reverse transcription polymerase chain reaction

Quantification of viral loads was carried out using the GeneAmp 5700 sequence detection system (Perkin-Elmer Biosystems). Viral RNA was extracted from whole blood using TRIzol LS (as described in section 2.7) and reverse transcribed as described in section 2.8. A 25 µl total reaction volume was used for PCR and reactions carried out in MicroAmp optical 96-well reaction plates (Perkin-Elmer Biosystems). 24 µl of master mix was added to each well (5 pmol IRES1, 5 pmol IRES4, 12.5 µl 2×SYBR Green PCR master mix (Perkin-Elmer Biosystems) and 8.5 µl nuclease free water (Promega)) and 1 µl of cDNA added. Plates were sealed using MicroAmp optical caps (Perkin-

Elmer Biosystems) and mixed thoroughly before transfer to the thermocycler. Viral DNA was amplified using the following program: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 seconds, 58°C for 30 seconds, 72°C for 1 min, 50 cycles. Amplicon independent amplification was distinguished using dissociation curves determined for each sample from 60-95°C. Quantification was determined against a standard curve created using a 4-fold dilution series of cDNA from a known titre sample. All plates were run with the standard dilution series (for generation of the standard curve), eight separate negative controls (4 from FMDV negative blood samples, 4 nuclease free water), 4 strong positive controls (RNA extracted from FMDV positive tissue culture supernatant) and 4 negative controls from FMDV negative tissue culture supernatants. All samples were run three times and a mean calculated from these values. The threshold value for each run was optimised using the 4 replicates of the positive controls. In each case, the threshold value was taken during the linear phase of amplification when the standard deviation of the mean from the 4 positive control replicates was lowest.

## 2.11 Interferon-gamma enzyme immunoassay

Interferon-gamma (IFN-γ) levels in plasma from experimental animals were assayed using a commercially available monoclonal antibody-based sandwich enzyme immunoassay (BOVIGAM™; CSL Veterinary, Australia). The 96% homology between

ovine and bovine IFN-γ allows the immunoassay to be used to detect ovine IFN-γ (Rothel *et al.*, 1990). Samples were assayed as the manufacturers protocol. Briefly, 50 µl of test sample was added to 50 µl of diluent in wells of microplates coated with antibody to bovine IFN-γ. Plates were mixed for 1 min on a plate shaker and incubated at room temperature for 60 min. Following incubation, all wells were washed 6 times with wash buffer before addition of 100 µl of horseradish peroxidase labeled anti-bovine IFN-γ conjugate. Plates were incubated at room temperature for 60 min, washed as before and 100 µl of enzyme substrate buffer (containing $H_2O_2$) containing chromogen (tetramethylbenzidine (TMB) in dimethyl-sulphoxide) added. The reaction was terminated after 30 min by the addition of 50 µl 0.5M $H_2SO_4$ and read at 450nm using a Dynex MRX platereader. A cut-off for positive samples was determined as the mean of the three negative control wells plus two standard deviations of the mean. To assess the repeatability of the assay, samples were tested in duplicate (figure 2.1). The results show the system to be very repeatable.

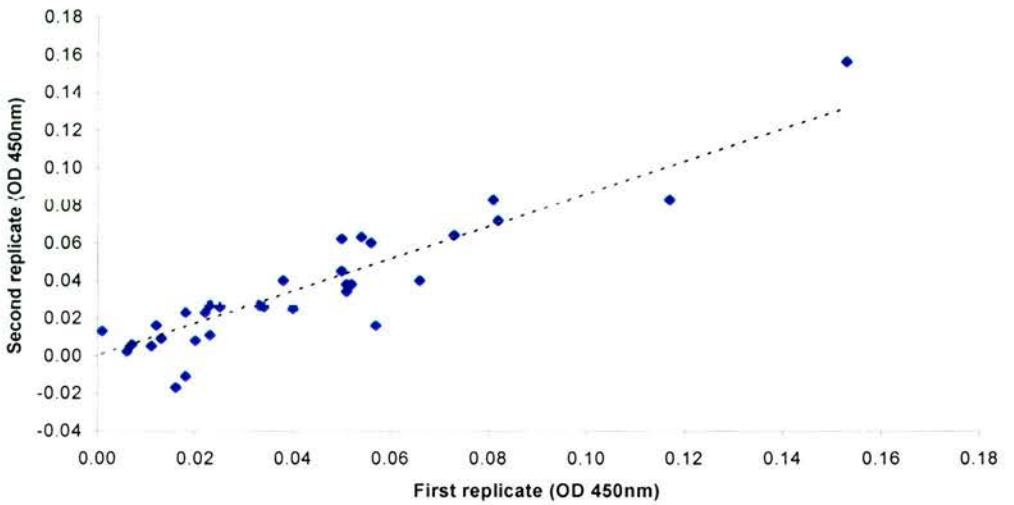


**Figure 2.1. Repeatability of the IFN-γ enzyme immunoassay.** Duplicate samples were tested as section 2.11. Pearson's linear correlation: $R^2$= 0.828, N=32, p<0.001.

## 2.12 Definitions of infection status

For all experimental animals the final infection class has been defined as follows. Clinical infections are defined as those where vesicular lesions occurred. Inapparent infections are defined as those where viraemia occurred but without the occurrence of clinical signs. Subclinical infections are defined as those where seroconversion was the sole indicator of FMDV infection.

# CHAPTER 3: THE EPIZOOTIC OF FOOT-AND-MOUTH DISEASE IN GREECE DURING 1994

## 3.1 The epizootic

Between August and October of 1994 an epizootic of foot-and-mouth disease (FMD) occurred in Greece. The last outbreak of FMD had occurred 10 years previously in the "buffer" zone along the Evros river where Greece borders Turkey (Tsaglas, 1995). The implementation of a non-vaccination policy by the European Union in 1991 created an entirely susceptible population of small ruminants in Greece. A total of 96 outbreaks of clinical FMD occurred in 6 separate prefectures although a retrospective serological survey revealed that foot-and-mouth disease virus (FMDV) infection had spread to 4 other areas (figure 3.1).

Despite the predominant numbers of small ruminants on affected holdings compared to other FMD susceptible species (13 sheep/goats to every 1 cattle/pig), the virus showed no predilection for small ruminants (Mackay *et al.*, 1995). The morbidity and mortality for cattle and pigs combined were both higher than for sheep and goats combined (49.5% of cattle and pigs infected compared to 18.0% of small ruminants within clinically infected prefectures; 4.5% mortality for infected cattle and pigs compared to 0.6% for infected sheep and goats; Mackay *et al.*, 1995). This evidence suggests that the virus was not sheep-adapted.

Control measures adopted during the epizootic were the stamping out of seropositive animals and the slaughter of in-contact livestock within affected holdings. The implementation of control measures was limited by the efficiency of the clinical surveillance programme which was in many cases inadequate (P Kitching, Pers. comm.) and hindered by the mild clinical signs of FMD in small ruminants (Geering, 1967). Post-epizootic serology indicated that infection had spread more intensively than was originally thought.

### 3.2 Source and spread

The primary route of infection was infected sheep moved from the island of Lesbos to the mainland. Illegal trade of animals had been occurring on Lesbos between Turkish farmers and Greek farmers. The island is situated close (4 km) to the Turkish mainland and due to the lower prices of Turkish sheep (Tsaglas, 1995) it is suspected that FMD infected sheep were moved onto Lesbos sometime before the beginning of May (Davies, 1995). Greek farmers receive a headage count payment from the EU at the end of May based on the size of their flock, and the cheap prices of Turkish sheep encouraged farmers on Lesbos to augment the size of their flocks by illegally importing sheep from Turkey (Davies, 1995). Nucleotide sequencing of the major capsid gene (VP1) showed that the virus circulating in Greece was closely related to that circulating in Turkey between 1991 and 1994 (Knowles & Samuel, 1995). FMD was only confirmed on Lesbos following confirmation of FMD in Xanthi. The conditions on Lesbos seem to have enabled FMDV to successfully maintain itself for

a period of possibly 2 months before recognition by the veterinary authorities. This is the time period between when it is assumed sheep were being smuggled onto Lesbos (to increase the headage count) and confirmation of the first case of FMD. The number of outbreaks of clinical FMD on Lesbos will have been under-reported but serosurveillance data do indicate an increased prevalence of FMDV infection on Lesbos when compared to areas of the mainland.

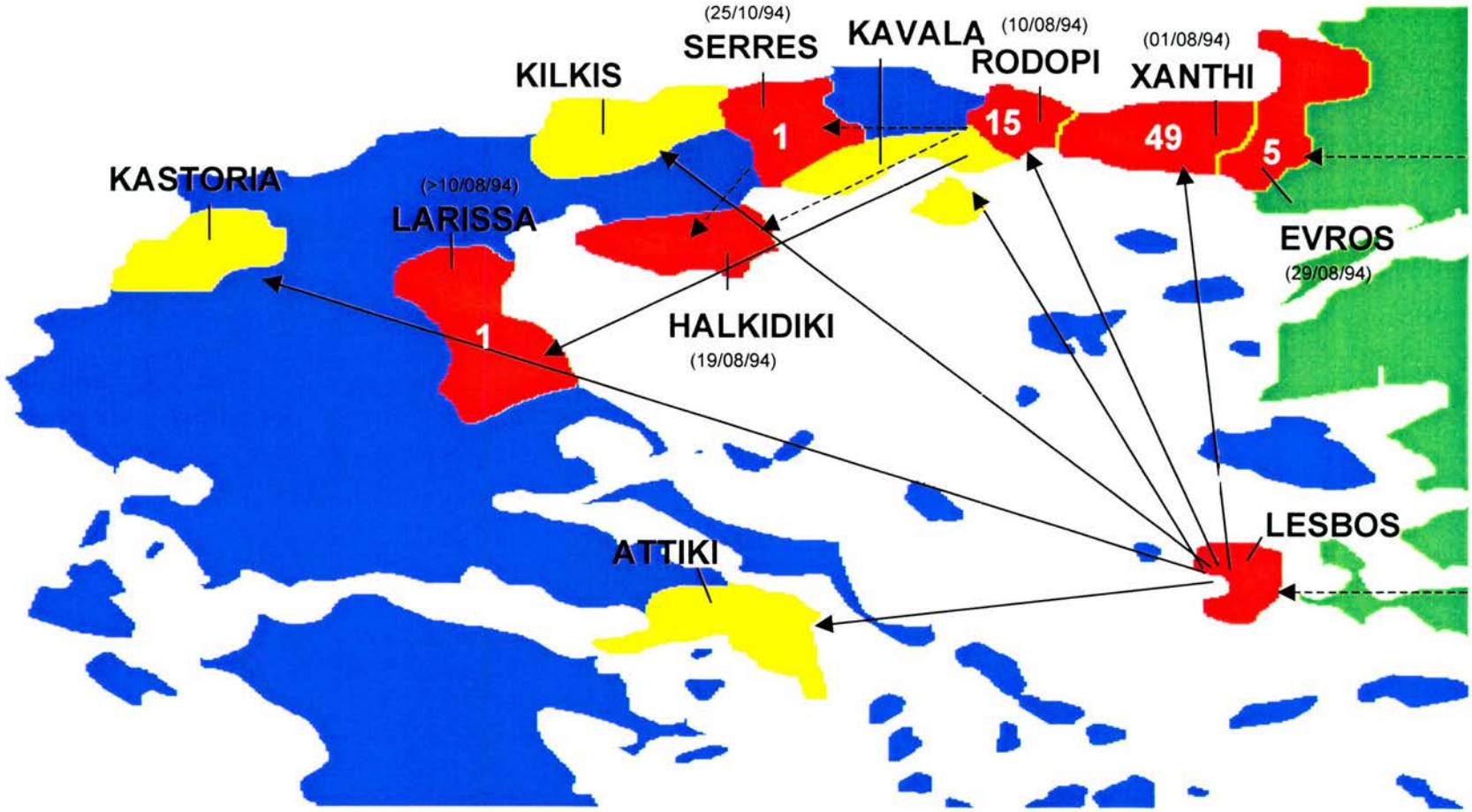


**Figure 3.1. The epizootic of foot-and-mouth disease (FMD) in Greece during 1994.** Red and yellow areas show prefectures with clinical and subclinical FMD respectively. Dates show the time of the first outbreak. Unbroken lines show known movements of infected sheep. Broken lines show possible routes of transmission. Values within regions show the numbers of clinical FMD outbreaks. Adapted from Tsaglas (1995).

## 3.3 Analysis of clinical outbreaks

The course of clinical FMD during the epizootic is shown graphically in figure 3.2. For the purpose of this analysis the course of the epizootic has been divided into three distinct phases.

1. **The introduction of infected sheep onto the island of Lesbos.** Four outbreaks of FMD on Lesbos were due to illegal importation of sheep from Turkey (Mackay *et al.*, 1995). Five outbreaks occurred in Evros due to similar illegal animal movements but no secondary outbreaks occurred. In all probability the number of clinical outbreaks occurring on Lesbos will have been much higher. Outbreaks of FMD will have occurred during the 2 months following movement of infected sheep from Turkey and confirmation of FMD.
2. **Movement of infected animals from Lesbos to Rodopi and Xanthi.** Epidemiological investigations discovered that 14 consignments of infected sheep had moved from Lesbos to various areas of northern Greece (Davies, 1995). One consignment is known to have reached each of Attiki, Kastoria, Kilkis and Kavala. The remaining 10 consignments arrived at either Rodopi or Xanthi. These 10 foci of infection resulted in 35 secondary outbreaks (Mackay *et al.*, 1995).
3. **Spread of FMD on mainland Greece.** The 35 outbreaks due to direct contact with infected animals from Lesbos caused a further 29 outbreaks of FMD within Rodopi and Xanthi. The remaining 4 outbreaks of clinical FMD occurred in

regions not neighbouring infected prefectures. The outbreak in Larissa is known to have occurred following movement of infected sheep from Rodopi (Tsaglas, 1995) but the source of virus for the 1 outbreak in Serres and 2 outbreaks in Halkidiki is unknown. The Greek Veterinary Services reported that 4 outbreaks of FMD were due to transport of infected animals on the mainland (Mackay *et al.*, 1995). All satellite outbreaks occurred after the outbreaks in Rodopi and Xanthi (disease outbreaks reported to the Office International des Epizooties in July, August, September and October 1994). For the purpose of this analysis, all three foci of outlying outbreaks (Serres, Halkidiki and Larissa) are assumed to be part of the same phase and caused by contact with the 35 primary outbreaks in Rodopi and Xanthi. Although the outbreaks of FMD in Kilkis and Kavala were termed subclinical by the reporting authorities, it is possible that mild clinical disease occurred undetected within these areas. If this was the case, spread of FMD from these areas to Serres and Halkidiki can not be ruled out.

The numbers of primary and secondary outbreaks for the three phases are shown in figure 3.3. Reproduction ratios (R) for the three phases above have been calculated as the ratio of secondary outbreaks to primary outbreaks (Haydon *et al.*, 1997) and are shown graphically in figure 3.4. The threshold value of 1 for R (Diekmann *et al.*, 1990) has been added to figure 3.4. If R falls below this threshold each primary case fails to replace itself and the epidemic will fade. For the purpose of this study the epidemiological unit is considered a livestock holding rather then an individual

animal as is used in standard applications of theory. Reproduction ratios are upper estimates.

Figure 3.4 shows that R fell below the threshold level required for successful invasion and maintenance of infection within a susceptible population, shortly after reaching the mainland. The morbidity rate for sheep on affected prefectures was only 18% (Mackay *et al.*, 1995). This suggests that the number of remaining susceptible hosts, due to the infection process, was unlikely to be solely responsible for any diminution in R seen during the epizootic, as the ratio of susceptible hosts to infectious hosts would still have remained high.

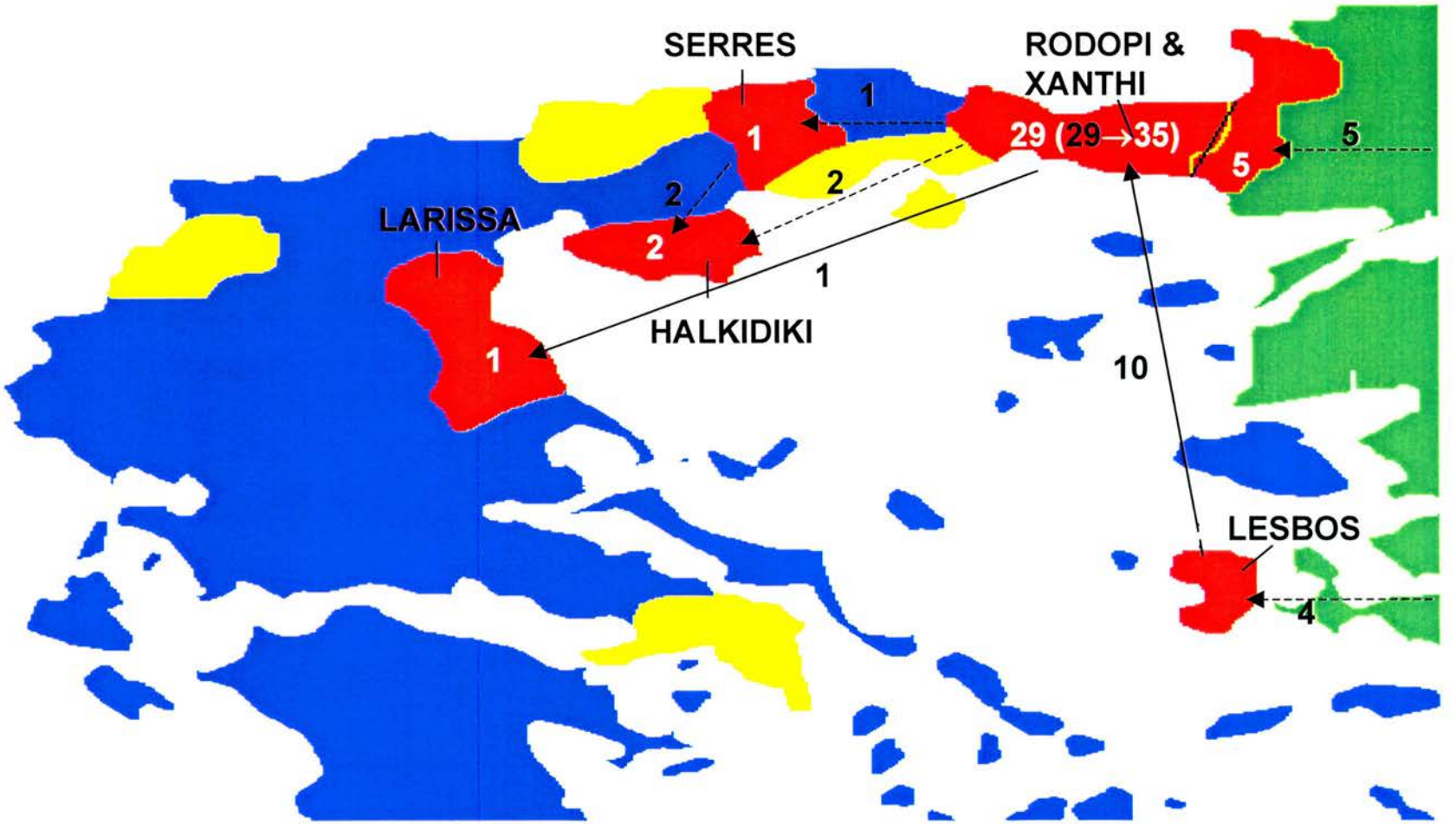


**Figure 3.2. Sources of outbreaks for the epizootic of foot-and-mouth disease (FMD) in Greece during 1994.** Red and yellow areas show prefectures with clinical and subclinical FMD respectively. Unbroken lines show known movements of infected sheep. Broken lines show possible routes of transmission. By far the most probable route of transmission is the movement of infected animals. Black numbers show the number of outbreaks caused by introduction of infected animals (primary outbreaks). White values show the number of outbreaks as a result of contact with primary sources (secondary outbreaks). Data assembled from Tsaglas (1995) and Mackay *et al.* (1995). Details of outbreaks numbers are given in section 3.3.

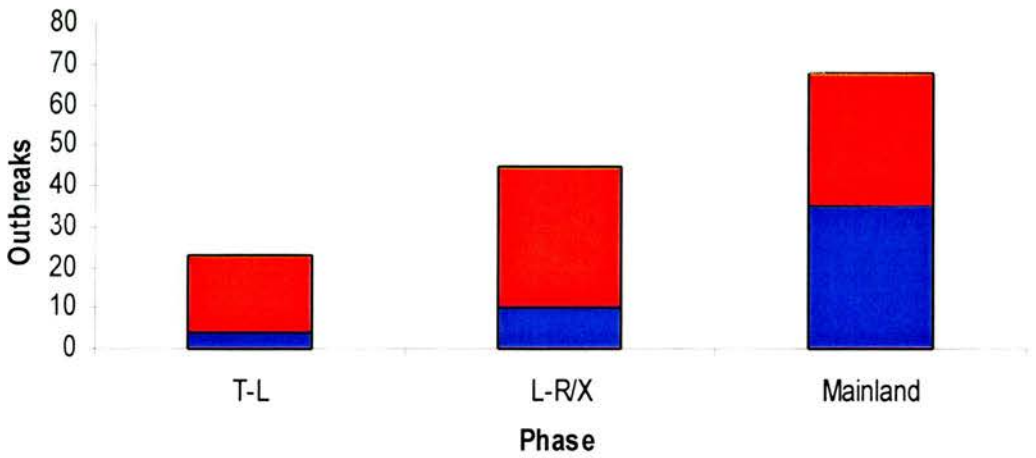


**Figure 3.3. Primary and secondary outbreaks of foot-and-mouth disease in Greece during 1994.** Blue bars show primary outbreaks, red bars secondary outbreaks. T-L = following introduction of FMDV to Lesbos from Turkey. L-R/X = following movement of infected sheep to mainland Greece (Rodopi and Xanthi). Mainland = spread of FMDV on mainland Greece from primary outbreaks in Rodopi and Xanthi.

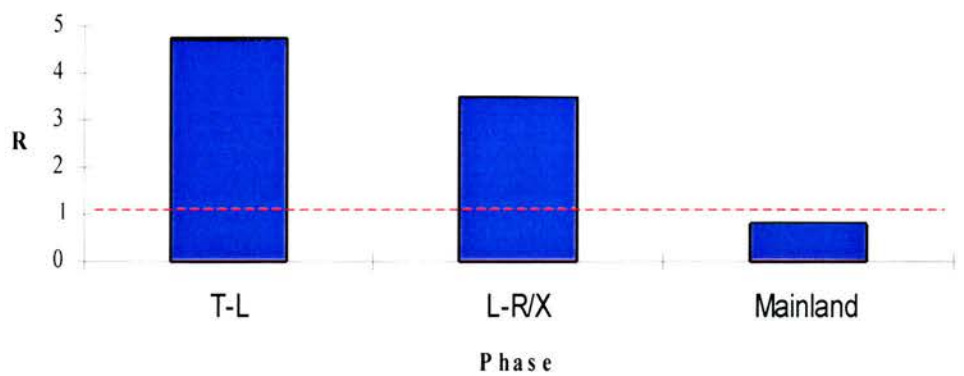


**Figure 3.4. Reproduction ratios (R) for the epizootic of foot-and-mouth disease in Greece during 1994.** R values were calculated as the ratio of secondary outbreaks to primary outbreaks using the data shown in figure 3.3. The broken line shows the threshold value of 1. T-L = following introduction of FMDV to Lesbos from Turkey. L-R/X = following movement infected sheep to mainland Greece (Rodopi and Xanthi). Mainland = spread of FMDV on mainland Greece from primary outbreaks in Rodopi and Xanthi.

## 3.4 Serological survey

The epidemiological pattern within affected prefectures was in some cases extremely complicated due to the density of animal holdings. This made successful tracking of infected animal movements difficult for the Greek authorities (Mackay *et al.*, 1995). It was also suspected that undetected FMD may have spread from infected prefectures, particularly where a large number of outbreaks had occured. The nature of FMD in sheep is mild and in many cases asymptomatic (Geering, 1967) and the possibility remained that, throughout the course of the epizootic, outbreaks of FMD had been drastically under reported. A serological survey was performed in 10 km areas around sites of either (a) clinical disease, (b) where consignments of infected sheep had arrived and (c) in a number of "random" holdings within each prefecture. Holdings classified as "random" were selected after the establishment of epidemiological links with known outbreaks of disease and it has been suggested that the holdings should have been classified as in-contact but insufficient data were available for all holdings to allow this classification (Mackay *et al.*, 1995).

Random sampling of 14 small ruminants was performed on each holding to provide a 95% confidence of detecting infection given 20% prevalence of seropositive animals on each holding (Mackay *et al.*, 1995). Animals sampled were individually identified and sera tested for antibodies against FMDV at one of five laboratories: the World Reference Laboratory for FMD, Pirbright, UK; the Institute for Animal Science and Health, Lelystad, Netherlands; the Danish Veterinary Institute for Virus Research,

Lindholm, Denmark; the Bundesforshungsanstalt fur Viruskrankheiten der Tiere, Tübingen, Germany and the FMD Institute, Athens, Greece.

Samples were examined by liquid-phase blocking ELISA (Hamblin *et al.*, 1986) with an elevated threshold level for positive samples due to the large numbers of samples that appeared borderline positives (Mackay *et al.*, 1995). If 1 or more samples from a holding tested positive the holding was revisited and a further 28 samples collected. This provided a 95% confidence of detecting infection given only 10% prevalence of seropositive animals on each holding (Canon & Roe, 1982).

### 3.4.1 Results

Table 3.1 shows the results of serological testing by prefecture. Data from table 3.1 are shown graphically in figure 3.5 as proportions of positive samples with 95% confidence intervals.

| Prefecture | Clinical disease | Sera tested | Number positive | % positive (prefectural attack rate) |
|---|---|---|---|---|
| Lesbos | + | 964 | 150 | 15.6 |
| Xanthi | + | 10928 | 730 | 6.7 |
| Evros | + | 3033 | 151 | 5.0 |
| Serres | + | 748 | 36 | 4.8 |
| Rodopi | + | 8567 | 350 | 4.1 |
| Halkidiki | + | 831 | 17 | 2.0 |
| Kastoria | - | 640 | 50 | 7.8 |
| Kilkis | - | 2283 | 114 | 5.0 |
| Kavala | - | 3797 | 183 | 4.8 |
| Attiki | - | 494 | 2 | 0.4 |

**Table 3.1. Results of the serological survey performed following the epizootic of foot-and-mouth disease in Greece during 1994.**

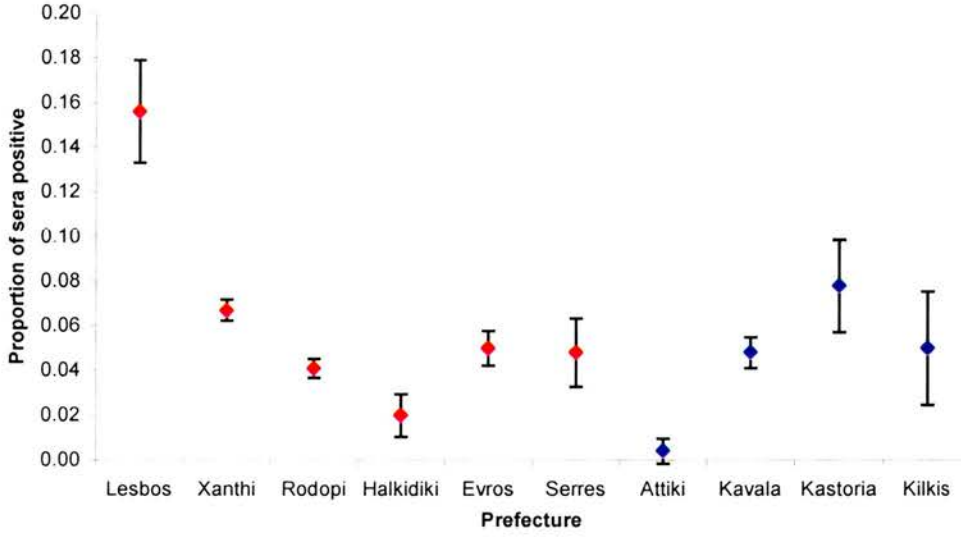


**Figure 3.5. Results of the serological survey performed following the epizootic of foot-and-mouth disease in Greece during 1994.** Binomial error bars show 95% confidence limits of the proportion positive. Red markers indicate prefectures were clinical FMD was reported and are shown in chronological order of occurrence, starting with Lesbos. Blue markers show prefectures where clinical FMD was not detected.

## 3.5 Population effects on transmission

To assess the correlation between host population demographics and prefectural attack rate, livestock numbers were obtained from a census of Greece taken in 1996

(supplied by Greek Veterinary Services). Although farming practices are known to be similar throughout Greece (P Kitching, Pers. comm.) there is considerable variation in the total number of livestock, average flock size and the density of livestock within prefectures (table 3.2). Sheep make up the vast majority of the FMDV susceptible livestock in all prefectures. For calculation of livestock densities, data from the 1996 survey of livestock numbers has been combined with land area data for prefectures of Greece (supplied by IGD Group, Athens, Greece).

| Prefecture | Number of livestock | | Number of flocks | Average flock size | Flock density (per sq km) |
|---|---|---|---|---|---|
| | thousands | % sheep | | | |
| Evros | 243 | 94 | 3278 | 176 | 0.31 |
| Rodopi | 298 | 94 | 5815 | 142 | 0.78 |
| Xanthi | 160 | 88 | 3533 | 105 | 0.74 |
| Lesbos | 320 | 98 | 12052 | 28 | 5.27 |
| Attiki | 183 | 95 | N/A | N/A | N/A |
| Kavala | 179 | 94 | 1424 | 284 | 0.28 |
| Halkidiki | 180 | 98 | 1110 | 212 | 0.22 |
| Kilkis | 218 | 86 | 3587 | 206 | 0.35 |
| Serres | 328 | 88 | 4890 | 165 | 0.44 |
| Kastoria | 77 | 94 | 77 | 126 | 0.33 |

**Table 3.2. Livestock data for prefectures of Greece in 1996.**

### 3.5.1 Population size

Figure 3.6 shows the relationship between population size (total number of livestock) and prefectural attack rate (percentage seroconversion). Table 3.2 shows that sheep make up the vast majority of FMDV susceptible livestock within each prefecture. Non-parametric correlation analysis shows no significant correlation between population size and prefectural attack rate (Spearman's rank correlation coefficient $r_s$=-0.012; N=10; p=0.973).

### 3.5.2 Number of sheep flocks

Figure 3.7 shows the relationship between the number of sheep flocks and prefectural attack rate (percentage seroconversion). Non-parametric correlation analysis shows no significant correlation between the number of sheep flocks and prefectural attack rate (Spearman's rank correlation coefficient $r_s$=0.101; N=9; p=0.769). The data point for Lesbos is an extreme outlier and so the results of analysis are difficult to interpret.

### 3.5.3 Flock size

Figure 3.8 shows the relationship between average size of flock and prefectural attack rate (percentage seroconversion). Non-parametric analysis of the data shows a significant negative correlation between flock size and prefectural attack rate (Spearman's rank correlation coefficient $r_s$=0.698; N=9; p=0.037) but when the data point for Lesbos is excluded gives no significant negative correlation (Spearman's rank correlation coefficient $r_s$=-0.563, N=8, p=0.143).

### 3.5.4 Host density

Figure 3.9 shows the relationship between the density of sheep flocks and prefectural attack rate (percentage seroconversion). Non-parametric analysis of the data shows no significant correlation (Spearman's rank correlation coefficient $r_s$=0.437; N=9;

p=0.240). The density of sheep flocks and cattle herds combined, cattle herds only, total number of sheep, total number of cattle and the density of all livestock correlates less with prefectural attack rate than the density of sheep flocks. The data point for Lesbos is an extreme outlier and so the results of analysis are difficult to interpret.

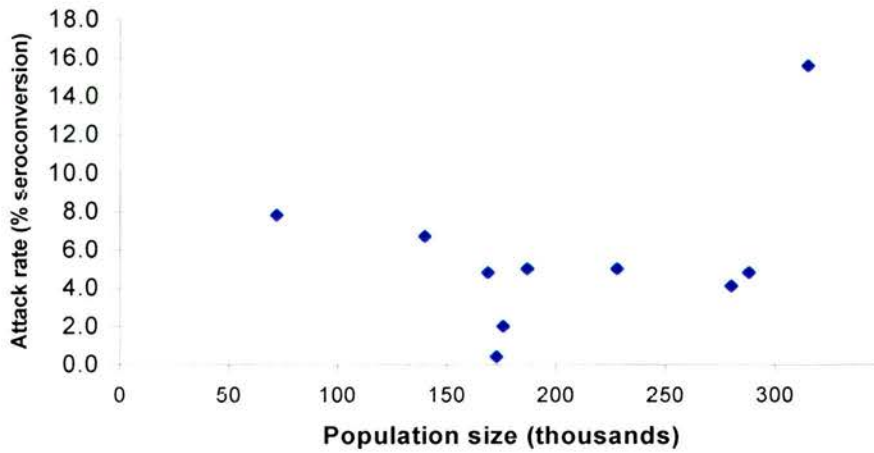


**Figure 3.6. Relationship between population size (total FMDV susceptible livestock) and the prefectural attack rate of FMDV for the epizootic in Greece during 1994.** Spearman's rank correlation coefficient $r_s$=-0.012; N=10; p=0.973.

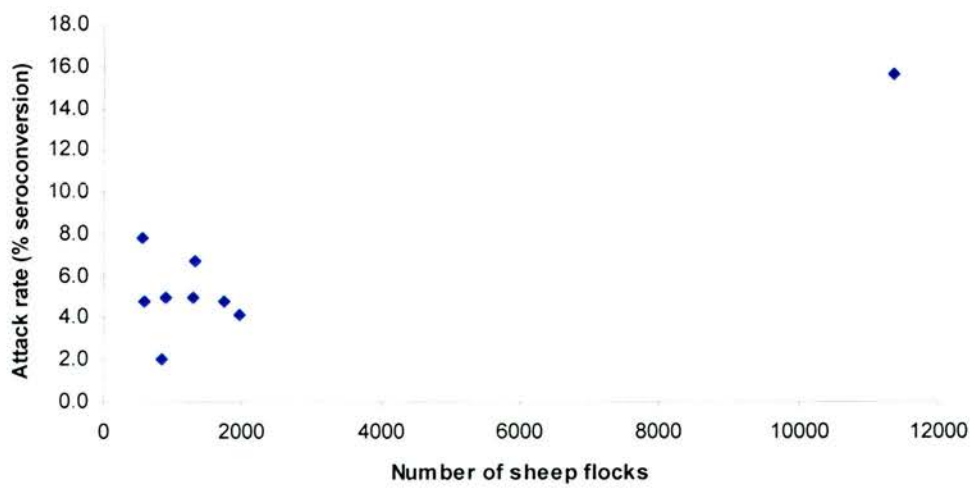


**Figure 3.7. Relationship between the number of sheep flocks and the prefectural attack rate of FMDV for the epizootic of Greece during 1994.** Spearman's rank correlation coefficient $r_s$=0.101; N=9; p=0.796.

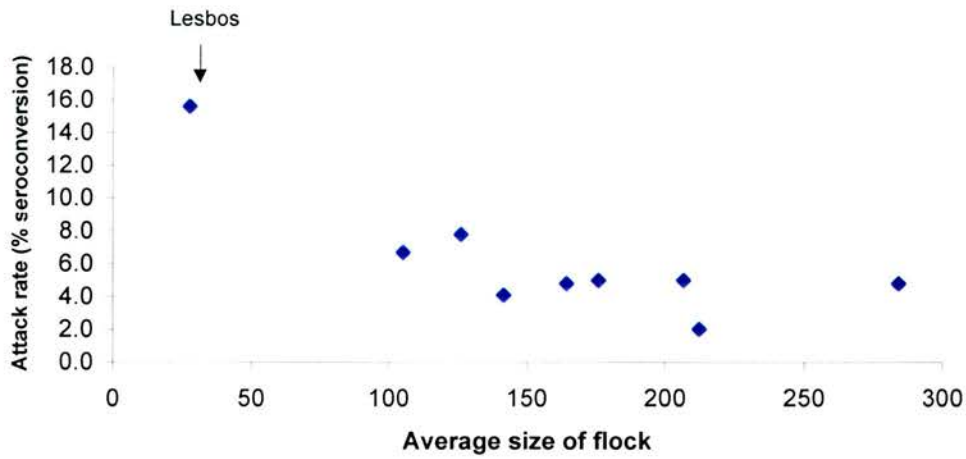


**Figure 3.8. Relationship between the average size of flock and the prefectural attack rate of FMDV for the epizootic in Greece during 1994.** Spearman's rank correlation coefficient $r_s$=-0.698; N=9; p=0.037 (all points), $r_s$=-0.563; N=8; p=0.143 (excluding Lesbos)

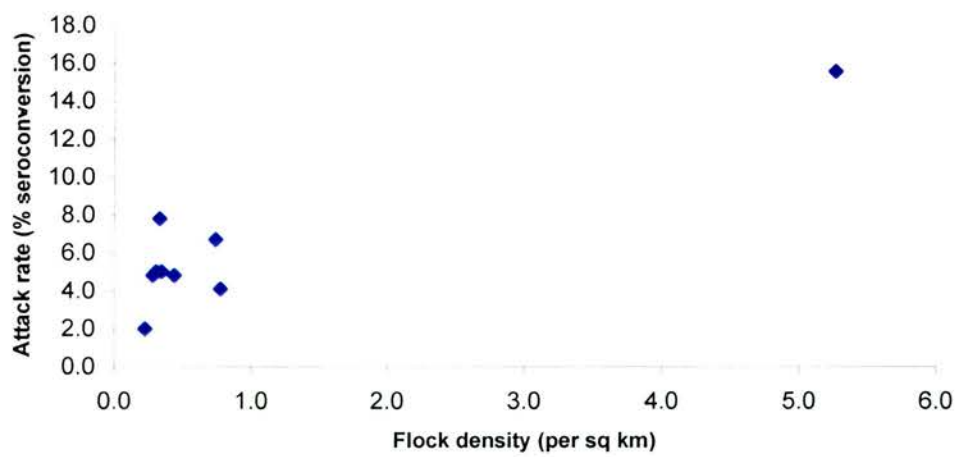


**Figure 3.9. Relationship between the density of sheep and the prefectural attack rate of FMDV for the epizootic in Greece during 1994.** Spearman's rank correlation coefficient $r_s$=0.437, N=9, p=0.240.

## 3.6 Discussion

Of the characteristics of this epidemic, the significantly greater attack rate of FMDV on Lesbos compared to the rest of the mainland is perhaps the most intriguing. The conditions on Lesbos greatly favoured the transmission of FMDV and provided an ideal environment for maintenance. The density of sheep flocks on Lesbos was a minimum of 6.8 times higher than in any of the other regions of Greece affected by the epizootic and the total number of sheep flocks a minimum of 2.3 times higher. Both the density of susceptible sheep and the numbers of flocks have assisted transmission of this isolate within and between sheep flocks. The density of sheep in different areas provides an indication of the likelihood of an infected flock making a successful contact (contact in which transmission of FMDV occurs) with a naïve flock. Not only was this likelihood considerably higher on Lesbos, but the large number of flocks ensured there was a greater pool of susceptible flocks of sheep. The size of the recipient flock may not influence the degree to which FMD disseminates through the susceptible population. Above a minimum threshold, the low morbidity of this virus for sheep means that saturation of a flock with infected sheep is unlikely to occur. Although average flock size and prefectural attack rate are significantly negatively correlated, this only applies when the data point for Lesbos is included. Removal of this outlier removes any significant correlation. The critical factor for transmission may not be the size of the flock but the number of individual distinct flocks. Minimum flock size would only influence transmission if the flock were small enough for a certain proportion of the population to become infected quickly

and thus reduce the size of the in-contact susceptible population so that the flock was partially saturated with infected individuals, and the density of susceptible hosts had fallen below the optimum.

Once FMDV reached mainland Greece there was a reduction in flock-to-flock transmission (below the threshold level necessary for successful maintenance) and attack rate. R has been estimated for distinct phases of the epizootic and all outbreaks not known to have been caused as a result of direct contact with infected shipments of sheep, have been classified as secondary outbreaks. In reality a number of these will have been tertiary or greater outbreaks. Also, the 35 outbreaks that occurred due to contact with the 29 outbreaks caused by contact with infected sheep introduced to Xanthi/Rodopi, could have been classified as tertiary outbreaks within that phase. This distinction does not effect the decline in R below the threshold of 1. Some of the variation in R can be attributed to differences in the parameters that contribute towards R (Haydon *et al.*, 1997). These include environmental factors that are know to contribute to transmission of FMDV such as temperature and atmospheric relative humidity (Sorenson *et al.*, 2000) and demograhical parameters such as livestock density and flock size described above. For those prefectures where clinical FMD occurred and where the dates that outbreaks began are known (Xanthi, Rodopi and Halkidiki), there was a gradual reduction in attack rate upon reaching the mainland. The five outbreaks in Evros are known to have occurred through direct contact with infected sheep from Turkish Thrace and as such make up a separate arm of the epidemic. The four outbreaks where clinical disease was not reported (Attiki, Kavala,

Kastoria and Kilkis) are all known to have received infected sheep directly from Lesbos. The dates of these shipments are unknown so it is difficult to interpret the serological data in terms of the rest of the epizootic. It is also difficult to assess changes in the clinical severity of FMD throughout the epizootic, as clinical surveillance was inadequate.

With successive generations of infection, reduction in both the number of new outbreaks and the attack rate of FMDV within infected prefectures led to an epizootic that displayed "self-limiting" properties. Such an outbreak may be defined as an epidemic during which, without external intervention, the pathogen is unable to maintain the force of infection necessary for passage through successive fully susceptible populations. The outbreak control strategies applied during this epizootic attempted to reduce the spread of virus by restricting transmission opportunities and reducing the infectious population. Stamping-out of FMD during this epidemic consisted of removing seropositive and in-contact animals. Removal of seropositive animals would not have influenced attack rates as these animals are no longer infectious and may even have increased the contact rate between infectious and susceptible sheep (by increasing the probability that an infectious individual makes contact with a susceptible sheep rather than a recovered one). The slaughter of in-contact animals may have restricted intra-flock transmission through the removal of incubating cases and by reducing the local susceptible population, and subsequently also reducing the contact rate between infectious and susceptible animals. EU missions to Greece during the epidemic found that the EEC council directives for

implementation of control measures were not being correctly enforced (P Kitching, Pers. comm.). This evidence suggests that the influence of control measures in restricting spread was minimal. Analysis of FMD epidemics has clearly demonstrated that the most effective control measure is the rapid slaughter of all animals within an affected holding (Haydon *et al.*, 1997, Howard & Donnelly, 2000). Failure to perform this action can increase the size of the epidemic dramatically. In this instance, the lack of rigorously enforced slaughter policies does not seem to have increased the size of the epizootic, suggesting that the characteristics of this epidemic were not classical.

The contribution of virological factors to the inability of this strain of FMDV to successfully infect successive generations is unclear. This analysis shows that the demographics of susceptible populations in part influence transmission of this strain between sheep flocks. Conditions on Lesbos (high density, large number of flocks) ensured that levels of virus were maintained and perhaps amplified to levels capable of infecting a number of prefectures on the mainland. Once FMDV reached the mainland, conditions were unfavourable for maintenance and there followed a gradual decline in the degree of transmission within an affected flock. There is considerable variation in the attack rates of FMDV on mainland Greece and the reasons for this variation are not apparent from epidemiological analysis alone. In an attempt to understand the relationship between pathogen and host a number of experiments have been performed to assess virological influence on transmission between sheep. Dose-dependent infection with FMDV may have influenced the

transmissibility of this isolate on the mainland. The infectiousness of a shipment of sheep may vary drastically and the subsequent success of FMDV in invading the susceptible population will depend on the force of infection provided from infectious animals at the time contact with the new susceptible population begins. The six prefectures that received shipments of infected sheep from Lesbos had varying attack rates. These attack rates varied from 7.8% (Kastoria) to 0.4% (Kilkis). A virus with an inability to successfully maintain itself within groups of susceptible hosts would result in a force of infection that, beyond a certain point, would continuously decline. Effective contact with susceptibles beyond this point would be less and less probable with time. On an infected flock to susceptible flock basis this would relate to a gradual reduction in attack rate. Unfortunately, we are unaware of the chronological order at which shipments arrived on the mainland from Lesbos. The lack of virus isolates from every stage of this epizootic also restricts the use of molecular epidemiology to investigate this problem. Transmission from sheep inoculated with varying doses of this isolate of FMDV is assessed in chapter 4. The inability of an exposed flock to amplify or maintain the per flock challenge dose has been investigated in chapter 5. The characteristics of this epizootic may have been as a result of the introduction of a non-sheep-adapted virus into a large sheep population. Only the ideal conditions on Lesbos enabled this virus to maintain itself for such a relatively long period of time.

# CHAPTER 4: DOSE DEPENDENT TRANSMISSION OF FOOT-AND-MOUTH DISEASE VIRUS IN SHEEP

## 4.0 Introduction

The minimum dose required to infect sheep with foot-and-mouth disease virus (FMDV) has previously been reported as 10 $TCID_{50}$ from experiments where sheep were individually exposed to aerosols from infected pigs (Gibson & Donaldson, 1986). The experiments described in this chapter have not been performed in an attempt to refine or supplement this value but to assess the effect of dose on the characteristics of infection for inoculated and in-contact sheep and to assess the effects of dose on the infectiousness of inoculated sheep. During the outbreak of foot-and-mouth disease (FMD) in Greece, from which the virus isolate used in these experiments was taken, there was a reduction in transmission after passage through a number of sheep flocks (Mackay *et al.*, 1995). A continuous decline in the infectious dose may explain the reduction in transmission seen during this epidemic.

## 4.1 Experimental design

Five identical experiments were performed using a range of inoculum dose ($10^3$, $10^{3.5}$, $10^4$, $10^5$ and $10^6$ fifty percent tissue culture infective dose ($TCID_{50}$/ml), herein referred to as experiments 1-5 respectively. Sheep were inoculated

intranasally with the required dose in 2 ml of M25 phosphate buffer using a 2-inch length of sterile rubber tubing. For each experiment a total of 15 sheep were used; five animals were inoculated (Group 1; G1) with the required dose and 24 hrs later 10 in-contact animals were introduced (Group 2; G2). All experiments were performed in identical boxes within high security isolation units. Monitoring was continued until all infected animals were deemed no longer infectious (7 days after the cessation of viraemia) and sufficient time had been allowed for incubating susceptibles (10 days).

### 4.1.1 Inoculum preparation

FMDV type O Greece 23/94 (originally isolated from ovine epithelium using primary calf thyroid cells; BTY) was passed again in BTY cells before titration as described in section 2.3. This stock virus was aliquoted and stored at -70°C. The required dilutions for inoculation were calculated and virus stock diluted immediately prior to administration with M25 phosphate buffer. All inoculum solutions were re-titrated immediately upon return to the laboratory to confirm the given dose.

### 4.1.2 Sampling protocol and methods

On each day of the experiment, 5 ml of peripheral blood and 5 ml of heparinised peripheral blood were taken for serology and virus isolation respectively. Virus

isolation was performed as section 2.2. Plasma samples positive by virus isolation were titrated in BTY cells as described in section 2.2. Total serum antibody responses were measured as section 2.5. Throat swabs were taken daily using a cotton tipped polypropylene swab (Technical service consultants Ltd.). Tips were cut off immediately into 1 ml of TRIzol (Gibco, BRL) for RNA extraction and subsequent reverse transcription nested polymerase chain reaction (RT-nPCR; as described in section 2.9). All sheep were examined daily for clinical signs of FMD in the mouth and on all four feet. Rectal temperatures were recorded daily. Oesophageal-pharyngeal fluid (OPF) was taken from every animal on the final day of the experiment and processed as section 2.6. Interferon-gamma (IFN-γ) levels in plasma of G1 sheep were measured using the BOVIGAM™ enzyme immunoassay (CSL Veterinary, Australia) as described in section 2.11.

## 4.2 Results

An overview of each experiment is shown in figures 4.1-4.4. Experiment 1 is not included as no positive samples were found.

### 4.2.1 Experiment 1

Inoculation of sheep with the lowest dose ($10^3$ $TCID_{50}$) was insufficient to cause clinical or subclinical infection. There was no evidence of any form of transmission between G1 and G2. No live virus was isolated from OPF samples taken 10 days post-inoculation.

### 4.2.2 Experiment 2

Inoculation of sheep with $10^{3.5}$ $TCID_{50}$ was insufficient to cause clinical infection. A short term, low-level serum antibody response was detectable in one member of both G1 and G2. No live virus was isolated from OPF samples taken 18 days post-inoculation.

### 4.2.3 Experiment 3

All G1 sheep inoculated with $10^4$ $TCID_{50}$ developed viraemia and 8 of G2. One member of G1 did not develop vesicular lesions. The remaining two members of

G2 developed subclinical FMD. Virus was recovered from all OPF samples taken (22 days post inoculation) from G1 sheep and from 4 samples taken from G2 animals.

### 4.2.4 Experiment 4

All G1 sheep inoculated with $10^5$ $TCID_{50}$ developed viraemia and 6 of G2. One member of G1 did not develop vesicular lesions. Of the 4 remaining G2 sheep, 1 developed subclinical infection. Virus was recovered from 3 OPF samples taken (24 days post inoculation) from G1 sheep and from 3 samples taken from G2. The time that the last two G2 sheep developed viraemia was 4 and 6 days after the last viraemic day of G1. This suggests that transmission to these two sheep was from contact with infectious members of G2 and not from G1.

### 4.2.5 Experiment 5

Inoculation with $10^6$ $TCID_{50}$ resulted in clinical disease in 4 G1 sheep and subclinical infection in the fifth. There was no infection in any G2 sheep. Live virus could not be recovered from OPF samples taken 19 days post-inoculation from G2 sheep. Virus was recovered from four of the five OPF samples taken from G1 sheep, including the sample from the animal with subclinical infection. To test the susceptibility of uninfected G2 sheep, all 10 were challenged 21 days

post-contact with $10^5$ $TCID_{50}$ of stock virus by intranasal instillation. All G2 sheep developed viraemia (detectable by virus isolation) by 3 days post-challenge.

### 4.2.6 Transmission

The final outcome for each experiment is shown in table 4.1. The proportion of sheep in each infection class for each experiment is shown graphically in figure 4.5.

| Expt | Dose ($TCID_{50}$) | Final infection status (numbers infected) | | | | | | | | Probang results[1] | | Throat swabs[2] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Subclinical | | Inapparent | | Clinical | | Total | | | | | |
| | | G1 | G2 | G1 | G2 | G1 | G2 | G1 | G2 | G1 | G2 | G1 | G2 |
| 1 | $10^{3.0}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| 2 | $10^{3.5}$ | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0.02 | 0.06 |
| 3 | $10^{4.0}$ | 0 | 2 | 1 | 0 | 4 | 8 | 5 | 10 | 4 | 4 | 0.42 | 0.36 |
| 4 | $10^{5.0}$ | 0 | 1 | 1 | 1 | 4 | 5[3] | 5 | 7 | 4 | 4 | 0.53 | 0.11 |
| 5 | $10^{6.0}$ | 1 | 0 | 0 | 0 | 4 | 0 | 5 | 0 | 4 | 0 | 0.09 | 0.02 |

**Table 4.1. Final infection status of sheep and indicators of transmission from dose-dependent transmission experiments.** [1]Probang results show numbers of samples positive by virus isolation. [2]Throat swab results show the mean proportion of positive swabs (by RT-nPCR) per sheep per day. [3]Infection of the last two cases of clinical FMD in G2 from experiment 4 were probably due to contact with infectious members of G2 rather than G1 (see section 4.2.4).

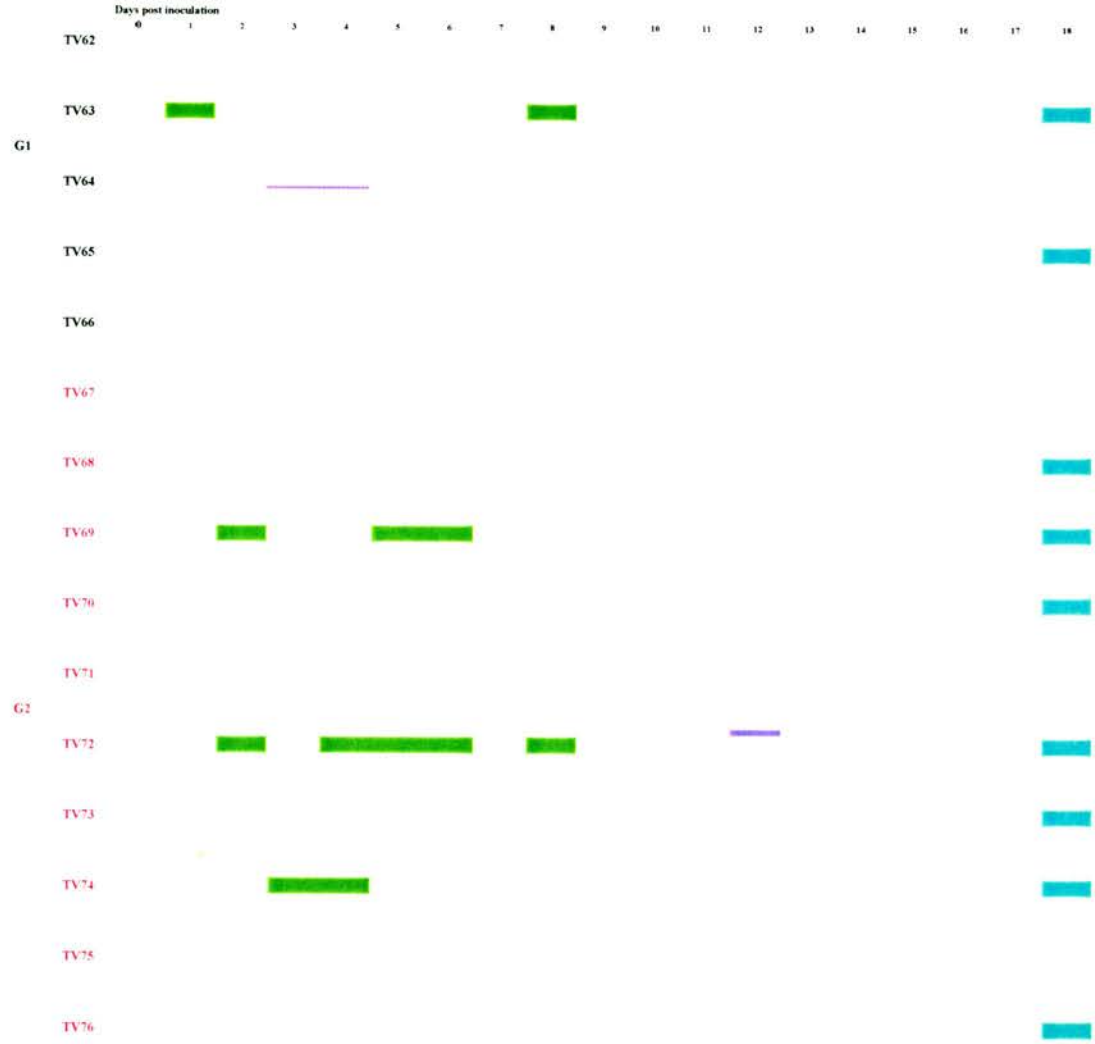


**Figure 4 1. Course of experiment 2 ($10^{3.5}$ $^{-}$$CID_{50}$).** Green bars indicate throat swabs positive by RT-nPCR, light blue bars indicate probang samples positive by RT-nPCR. Purple blocks show total serum antibody titres.

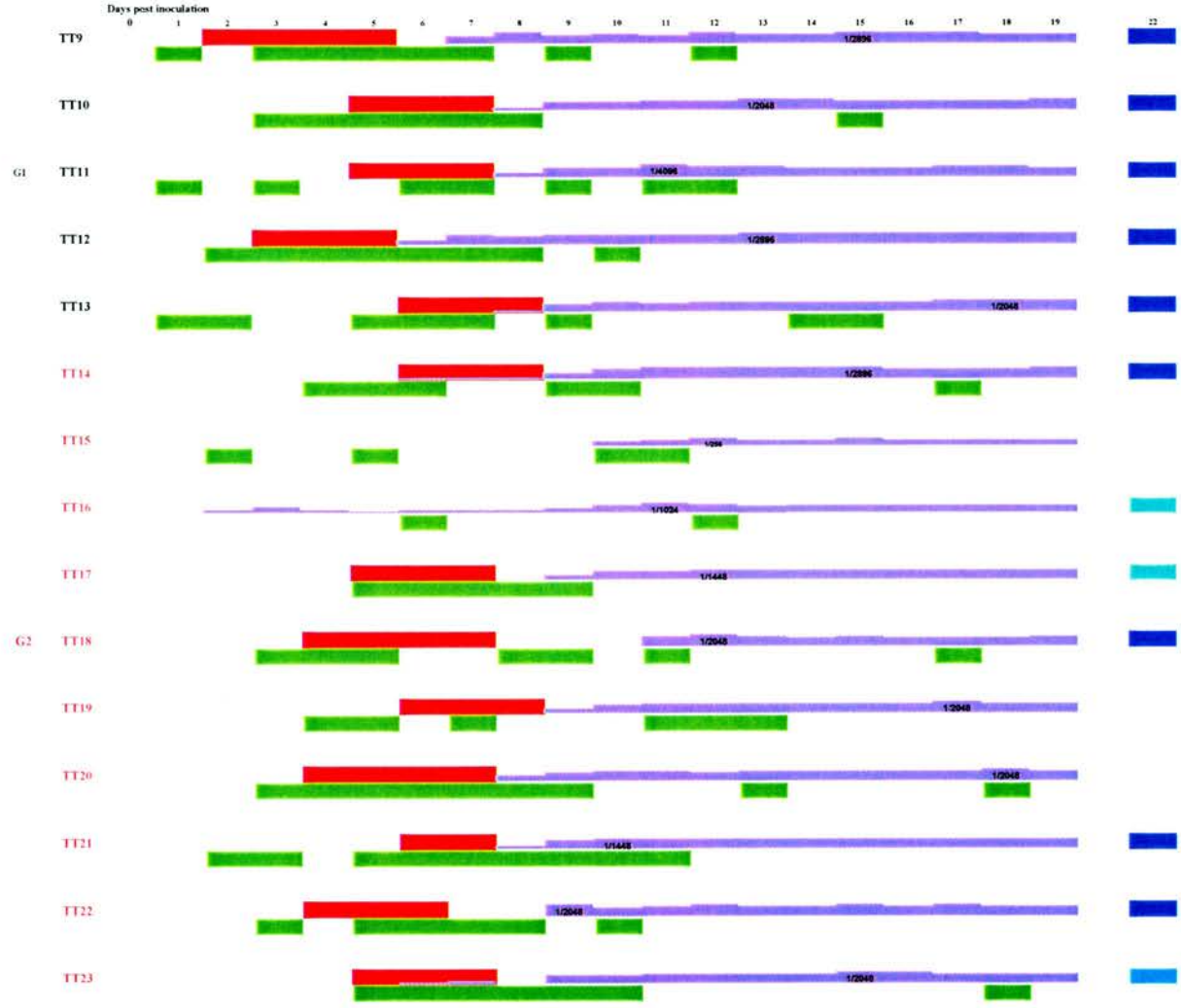


**Figure 4.2. Course of experiment 3 ($10^4$ $TCID_{50}$).** Red bars indicate viraemic periods. Green bars indicate throat swabs positive by RT-nPCR. Blue bars ndicate probang samples positive by virus isolation. Light blue bars show probang samples positive by RT-nPCR. Probang samples positive by virus isolation were not tested by RT-nPCR.Purple blocks show total serum antibody titres with peak values superimposed.

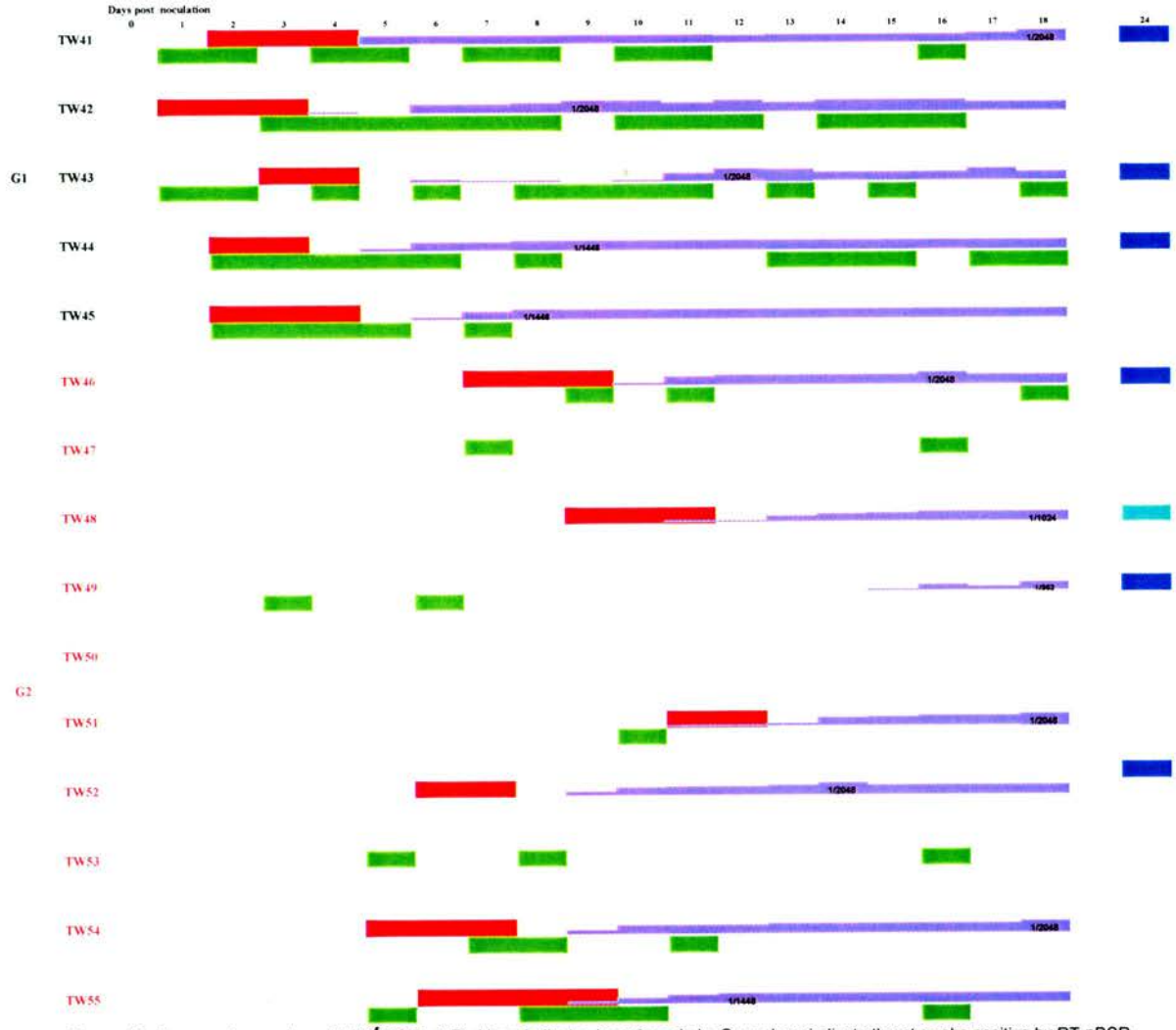


**Figure 4.3. Course of experiment 4 ($10^5$ $TCID_{50}$).** Red bars indicate viraemic periods. Green bars indicate throat swabs positive by RT-nPCR. Blue bars indicate probang samples positive by virus isolation. Light blue bars show probang samples positive by RT-nPCR. Probang samples positive by virus isolation were not tested by RT-nPCR.Purple blocks show total serum antibody titres with peak values superimposed.

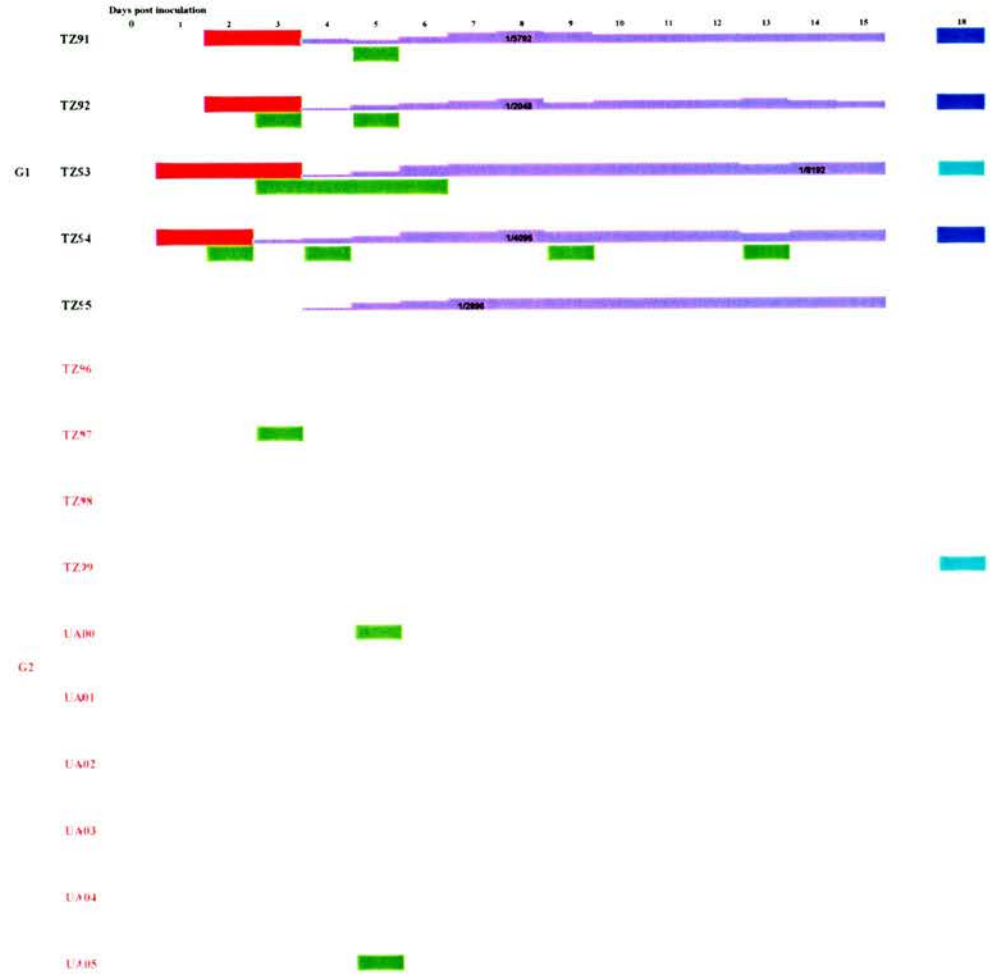


**Figure 4.4. Course of experiment 5 ($10^6$ $TCID_{50}$).** Red bars indicate viraemic periods. Green bars indicate throat swabs positive by RT-nPCR.Blue bars indicate probang samples positive by virus isolation. Light blue bars show probang samples positive by RT-nPCR. Probang samples positive by virus isolation were not tested by RT-nPCR.Purple blocks show total serum antibody titres with peak values superimposed.

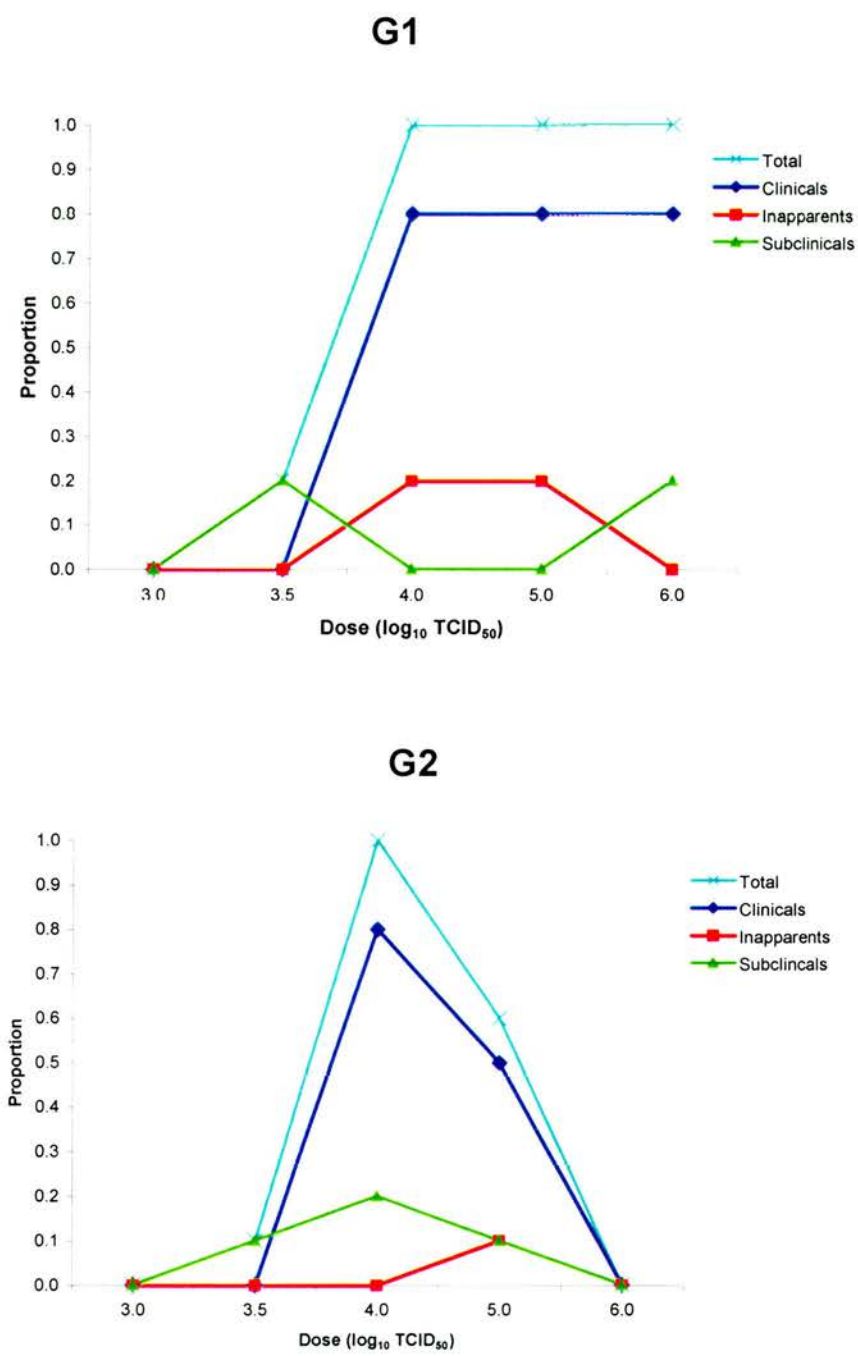


**Figure 4.5. Infection status of sheep following inoculation and contact from dose-dependent transmission experiments.** Proportions are calculated from the total group size (n=5 for G1; n=10 for G2).

### 4.2.6.1 Throat swabs

For each experiment, the mean number of positive throat swabs (determined by RT-nPCR) per sheep per day was calculated (table 4.1). For each group, day zero samples were not included in calculation of the mean as these were negative controls. Values were calculated irrespective of infection status within the same time period for every experiment. The period used was the longest possible i.e. the duration of the shortest experiment, which corresponded to the 18 days of experiment 2. From this, means were calculated from 18 samples for G1 sheep and 17 samples for G2 sheep. Throat swab results are shown graphically in figure 4.6. To assess whether the occurrence of positive throat swabs was clustered (corresponding with the infectious period) runs test was performed on throat swab data to test the randomness of the occurrence of positive results (table 4.2). The results of a runs test signify whether events (in this case the occurrence of a positive throat swab) occur in a random order or appear as a function of a previous event (in this case a proceeding positive throat swab). Significant values ($p<0.01$) indicate that positive throat swabs occurred in a non-random fashion. Figure 4.7 shows the number of positive throat swabs per day taken throughout the course of the experiments. Figure 4.8 shows the number of positive throat swabs and the frequency of new infections for experiments 3 and 4 ($10^4$ and $10^5$ $TCID_{50}$).

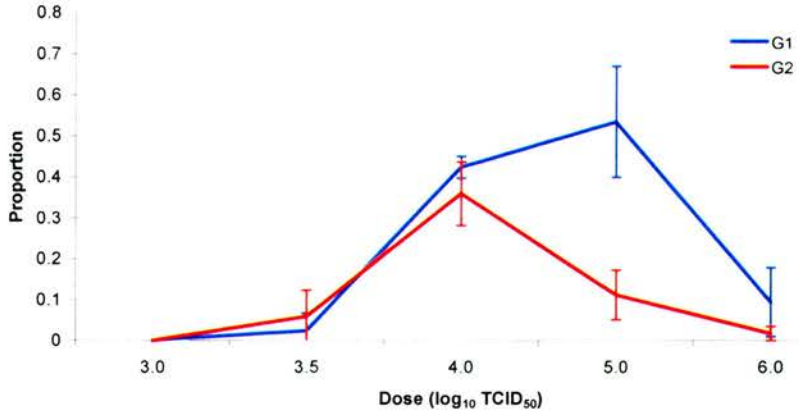


**Figure 4.6. Mean proportion of positive throat swabs (by RT-nPCR) taken from sheep during dose-dependent transmission experiments.** Error bars shows 95% confidence limits of the mean.

| Expt. | Dose ($TCID_{50}$) | Group | Sheep | No. of runs | Z | p |
|---|---|---|---|---|---|---|
| 3 | $10^4$ | 1 | TT9 | 8 | -0.684 | 0.494 |
| | | | TT10 | 5 | -1.928 | 0.054 |
| | | | TT11 | 10 | 0.000 | 1.000 |
| | | | TT12 | 5 | -2.161 | 0.031 |
| | | | TT13 | 8 | -0.684 | 0.494 |
| | | 2 | TT14 | 7 | -0.698 | 0.485 |
| | | | TT17 | 3 | -2.788 | **0.005** |
| | | | TT18 | 9 | 0.000 | 1.000 |
| | | | TT19 | 7 | -0.698 | 0.485 |
| | | | TT20 | 6 | -1.494 | 0.135 |
| | | | TT21 | 4 | -2.499 | 0.012 |
| | | | TT22 | 7 | -0.698 | 0.485 |
| | | | TT23 | 4 | -2.454 | 0.014 |
| 4 | $10^5$ | 1 | TW41 | 10 | 0.000 | 1.000 |
| | | | TW42 | 7 | -0.826 | 0.409 |
| | | | TW43 | 13 | 1.510 | 0.131 |
| | | | TW44 | 8 | -0.541 | 0.588 |
| | | | TW45 | 5 | -1.674 | 0.094 |
| | | 2 | TW46 | 6 | 0.000 | 1.000 |
| | | | TW48 | 1 | - | -[a] |
| | | | TW51 | 3 | 0.000 | 1.000 |
| | | | TW52 | 1 | - | - |
| | | | TW54 | 5 | -0.400 | 0.689 |
| | | | TW55 | 7 | -0.342 | 0.733 |
| 5 | $10^6$ | 1 | TZ91 | 3 | 0.000 | 1.000 |
| | | | TZ92 | 5 | 0.000 | 1.000 |
| | | | TZ93 | 3 | -2.692 | **0.007** |
| | | | TZ94 | 9 | 0.924 | 0.355 |

**Table 4.2. Runs test for the occurrence of positive throat swabs taken from clinically infected sheep during dose-dependent transmission experiments.** [a]When only one run occurred the runs Test could not be performed. p values in bold indicate significant results ($p<0.01$).

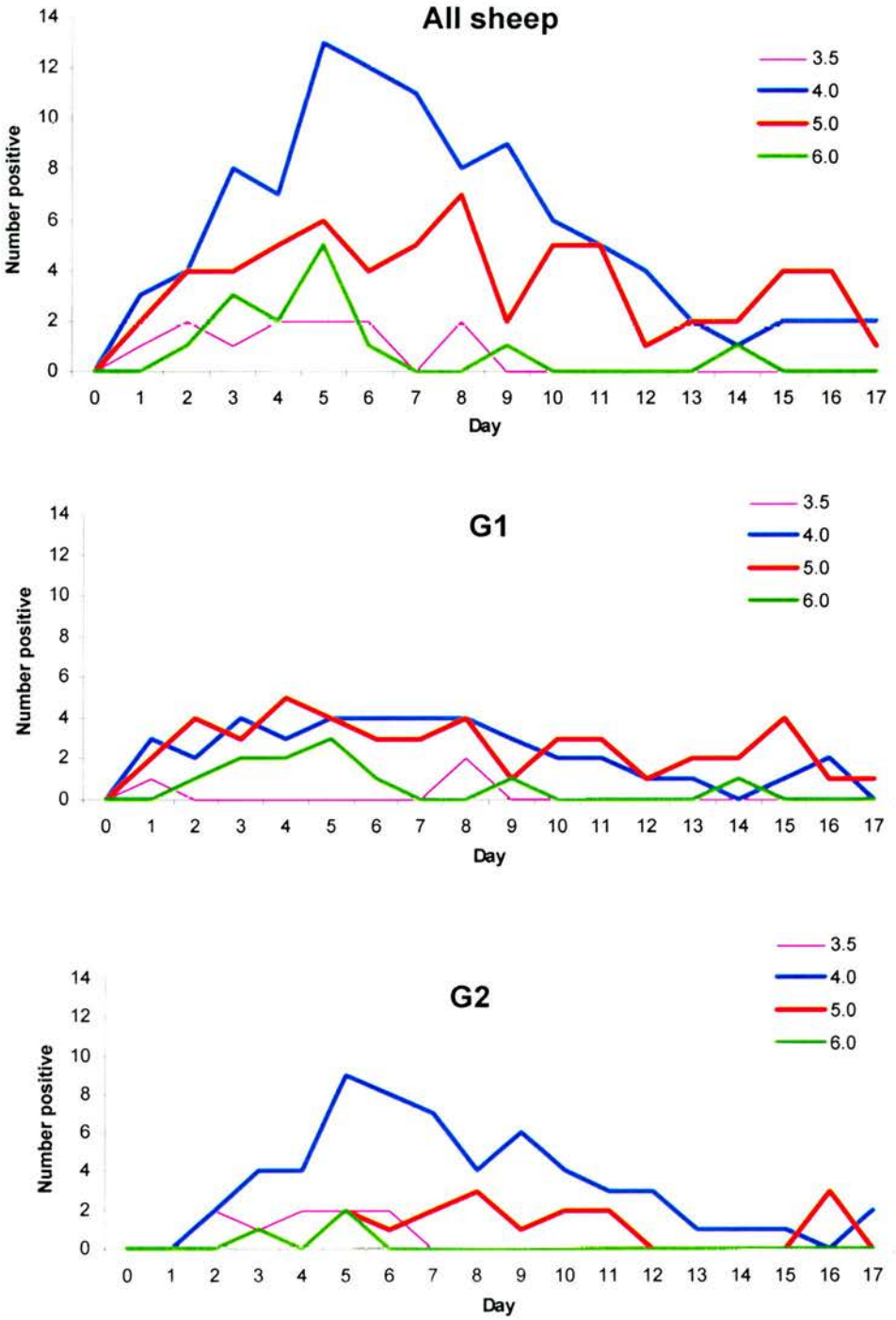


**Figure 4.7. Numbers of positive throat swabs per day taken from sheep during dose-dependent transmission experiments.** Throat swabs were tested by RT-nPCR for the presence of viral RNA. Values in the legend indicate dose ($log_{10}$ $TCID_{50}$).

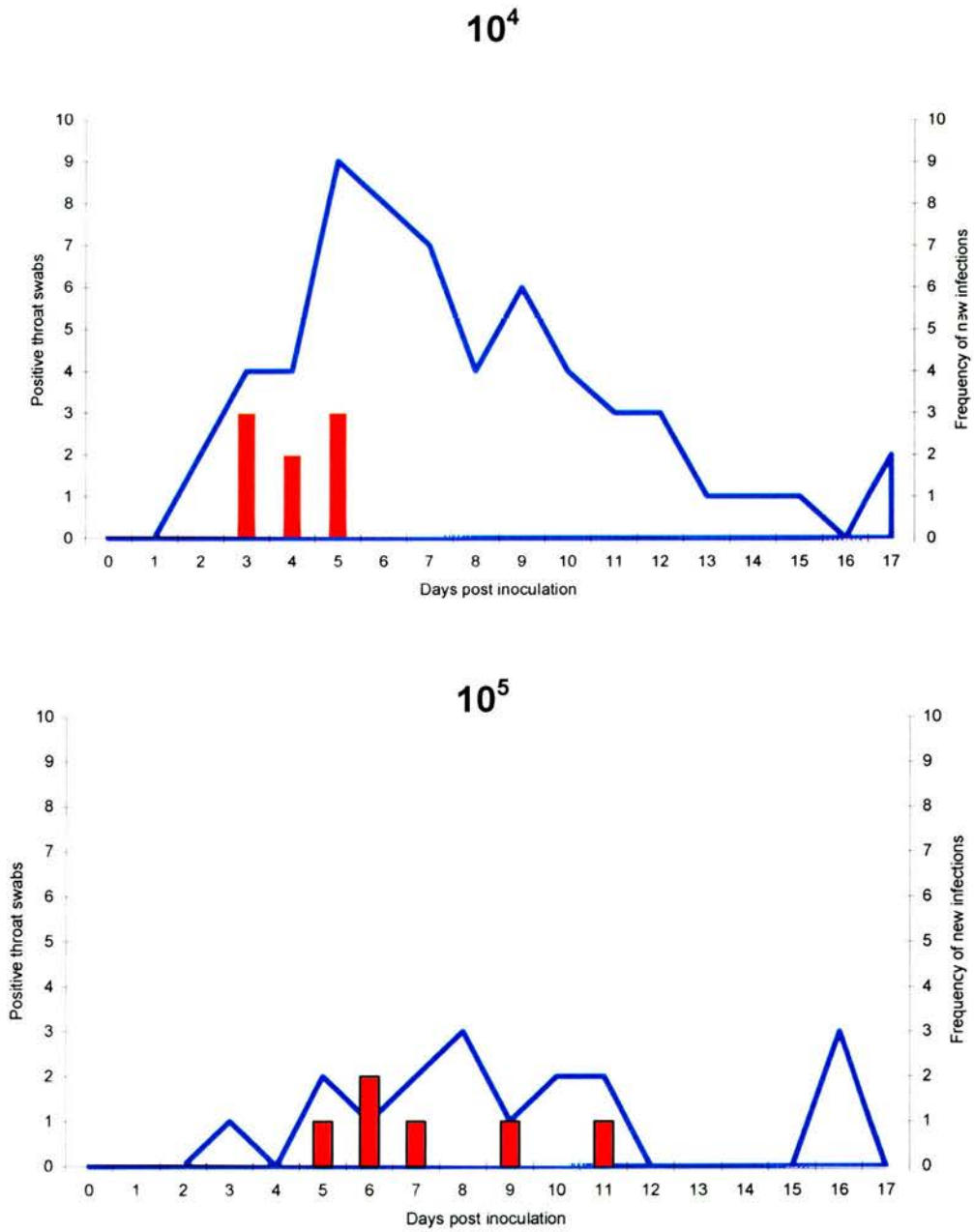


**Figure 4.8. Numbers of positive throat swabs and frequency of contact infections for dose-dependent transmission experiments 3 and 4.** Numbers of positive throat swabs are from G2 sheep only and are shown by the blue line. Red bars show the number of new G2 viraemias occurring on each day of the experiment post inoculation of G1. The figures show that the levels of virus circulating in the box following inoculation with $10^4$ $TCID_{50}$ were higher than with inoculation of $10^5$ $TCID_{50}$.

### 4.2.7 Dose-dependent responses to infection

#### 4.2.7.1 Clinical foot-and-mouth disease

Mean incubation periods for G1 sheep were calculated using the time between inoculation and the occurrence of a detectable viraemia as individual values (table 4.3). Table 4.4 shows the mean number of lesions per clinically infected sheep and the mean number of sites affected by FMD for clinically infected sheep. A site was classified as affected by FMD if vesicular lesions were found. Lameness was not considered a definitive sign of FMD for in a number of cases lameness was caused by factors other than FMDV infection, such as foot rot. In determining the number of sites affected, 5 possible sites were considered: each foot and the mouth. One-way analysis of variance (ANOVA) using the Tukey method for multiple comparisons (where applicable) was performed for incubation period data and for both measures of clinical FMD. Data showed no significant departures from normality ($p>0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p>0.05$).

| Expt. | Dose ($TCID_{50}$) | Mean incubation period |
|---|---|---|
| | | G1 |
| 3 | $10^4$ | 3.4 (5.1-1.7)[a] |
| 4 | $10^5$ | 1.2 (1.8-0.6)[b] |
| 5 | $10^6$ | 1.5 (2.4-0.6)[b] |

**Table 4.3. Incubation periods for G1 viraemic sheep from dose-dependent transmission experiments.** Numbers in brackets show 95% confidence limits of the mean. ANOVA (with multiple comparisons using the Tukey method) showed significant differences between values marked [a] and [b] ($p<0.05$).

| Expt. | Dose ($TCID_{50}$) | Mean number of lesions per clinically infected sheep | | Mean number of sites affected[1] | |
|---|---|---|---|---|---|
| | | G1 | G2 | G1 | G2 |
| 3 | $10^4$ | 3.8 (6.4-1.2) | 4.3 (5.2-3.3) | 2.4 (3.7-1.1) | 2.4 (3.0-1.7) |
| 4 | $10^5$ | 1.8 (3.8-0.2) | 4.0 (5.9-2.1) | 1.6 (2.8-0.4) | 3.0 (4.2-1.8) |
| 5 | $10^6$ | 2.5 (4.4-0.6) | - | 1.8 (2.7-0.8) | - |

**Table 4.4. Severity of clinical disease for sheep from dose-dependent transmission experiments.** Numbers in brackets show 95% confidence limits of the mean. [1]A total of 5 sites were included in calculation of the number of sites affected: four feet and the mouth. ANOVA (with multiple comparisons using the Tukey method for G1) showed no significant differences between any groups ($p>0.05$).

### 4.2.7.2 Viraemia

Characteristics of viraemia are shown in tables 4.5 and 4.6. The time interval between the onset of viraemia and the start of seroconversion ($T_{V-S}$) has been calculated as a possible indicator of the duration of the infectious period. Figure 4.9 shows the relationship between the peak viral titre and the total viral load.

Parametric correlation analysis shows that 96% of the variance in the sum of the viral load is due to the peak viral load. Mean peak viral loads of infected animals are shown graphically in figure 4.10. ANOVA using the Tukey method for multiple comparisons (where applicable) was performed on all data for characteristics of viraemia. All data showed no significant departures from normality ($p>0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p>0.05$). Figure 4.11 shows the relationship between peak viraemia and clinical signs. Clinical signs data showed no significant departure from normality using the Kolmogorov-Smirnov test ($p>0.05$) and were consistent with homogeneity of variance using the Levene statistic ($p>0.05$). Parametric analysis shows that 26% of the variance in the number of lesions was due to variance in the peak viral load. Analysis of covariance showed no significant difference in slopes for G1 and G2 ($F=1.7$; $df=1,2$; $p>0.05$).

| Expt | Dose ($TCID_{50}$) | Mean length | | $T_{V-S}$[1] | |
|---|---|---|---|---|---|
| | | G1 | G2 | G1 | G2 |
| 3 | $10^4$ | 3.2 (3.6-2.8)[a] | 3.1 (3.7-2.6) | 3.0 (3.8-2.2)[a] | 3.6 (4.2-2.9) |
| 4 | $10^5$ | 2.6 (3.1-2.1) | 2.8 (3.5-2.2) | 4.0 (4.8-3.2)[a] | 3.3 (4.0-2.6) |
| 5 | $10^6$ | 2.0 (3.6-1.4)[b] | - | 1.8 (2.2-1.3)[b] | - |

**Table 4.5. Duration of viraemia and relationship with seroconversion for infected sheep from dose-dependent transmission experiments.** [1]Mean time in days between the onset of viraemia and seroconversion (titres≥1/45). ANOVA (with multiple comparisons using the Tukey method for G1) showed significant differences ($p<0.05$) between means marked [a] and [b]. Numbers in brackets show 95% confidence limits of the mean.

| Expt. | Dose ($TCID_{50}$) | $Log_{10}$ mean peak titre | | Mean $Log_{10}$ total viral load[1] | |
|---|---|---|---|---|---|
| | | G1 | G2 | G1 | G2 |
| 3 | $10^4$ | 5.3 (6.4-4.1)[a] | 5.0 (5.7-4.3)[a] | 5.2 (6.3-4.2)[a] | 4.8 (5.4-4.2)[a] |
| 4 | $10^5$ | 3.8 (5.1-2.5) | 2.6 (3.9-1.2)[b] | 3.7 (5.2-2.2) | 2.6 (4.0-1.2)[b] |
| 5 | $10^6$ | 3.0 (5.1-1.0)[b] | - | 2.9 (5.0-0.6)[b] | - |

**Table 4.6. Characteristics of viraemia for infected sheep from dose-dependent transmission experiments.** [1]An estimation of the total viral load was calculated by summing the area of trapezia under the viraemic curve. Means are arithmetic means of logarithmic titres. ANOVA (with multiple comparisons using the Tukey method for G1) showed significant differences ($p<0.05$) between means marked [a] and [b]. Numbers in brackets show 95% confidence limits of the mean.

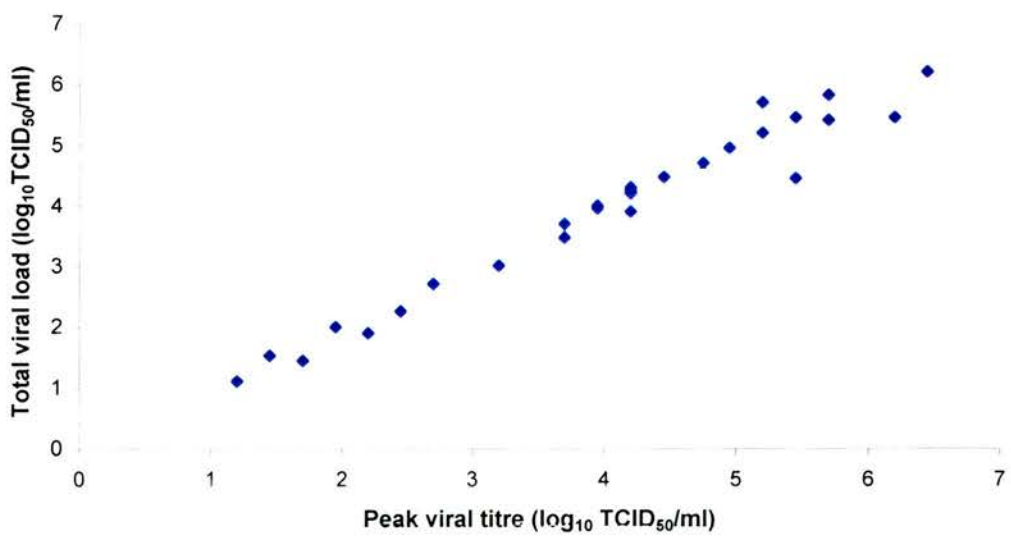


**Figure 4.9. Relationship between total viral load and the peak viraemic titre of sheep from dose-dependent transmission experiments.** Total viral load was estimated by summing the area of trapezia under the viraemic curve. Data represents all viraemic sheep from all experiments. Parametric correlation analysis (Pearson's) $R^2=0.964$; N=28; $p<0.0001$.

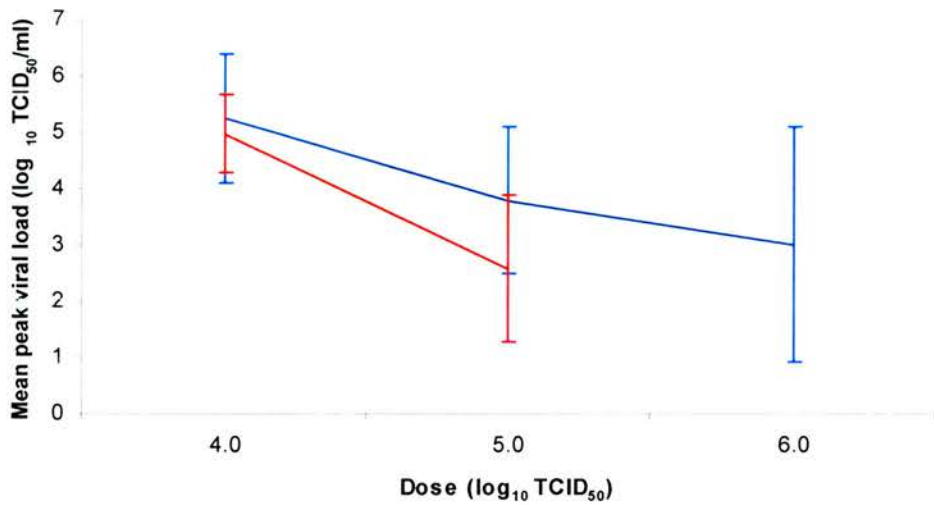


**Figure 4.10. Mean peak viral loads of viraemic sheep from dose-dependent transmission experiments.** The blue line shows the mean for inoculated (G1) sheep, the red line for in-contact (G2) sheep. Error bars show 95% confidence limits of the mean.

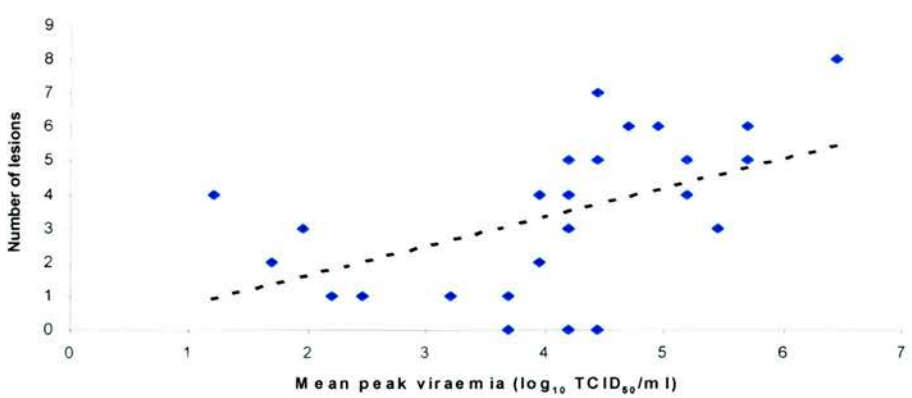


**Figure 4.11. Relationship between peak viraemia and severity of clinical disease for clinically infected sheep from dose-dependent transmission experiments.** Parametric correlation analysis (Pearson's) $R^2$=0.263; N=28; p<0.005.

### 4.2.7.3 Antibody responses

An analysis of antibody responses is shown in table 4.7. The time interval between inoculation and the start of seroconversion ($T_{I-S}$) has been calculated as an estimate of the speed of the humoral immune response and is shown in table 4.7. This interval consists of the number of clear days between inoculation and seroconversion (titre≥1:45). For G2 sheep the exact point of infection is unknown but infection has been assumed to have taken place 2 days prior to the onset of viraemia. This is a conservative estimate of the data from Gibson *et al.* (1986). $T_{I-S}$ for G2 equals the corresponding value of $T_{V-S}$ plus 2 days. Mean peak antibody titres for groups are shown graphically in figure 4.12. ANOVA using the Tukey method for multiple comparisons (where applicable) was performed on all data for characteristics of antibody responses. Data showed no significant departures from normality ($p>0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p>0.05$). Figure 4.13 shows the relationship between antibody responses and peak viraemic titres. Spearman's rank correlation coefficient ($r_s$) shows no significant association between peak antibody levels and the level of peak viraemia ($r_s=0.130$, $N=28$, $p=0.505$) and $T_{I-S}$ and the level of peak viraemia ($r_s=-0.013$, $N=28$, $p=0.946$).

| Expt | Dose ($TCID_{50}$) | Mean peak[1] | | $T_{I-S}$[2] | |
|---|---|---|---|---|---|
| | | G1 | G2 | G1 | G2[3] |
| 2 | $10^{3.5}$ | 128 | 91 | 5 | - |
| 3 | $10^{4}$ | 2896 (3590-2336) | 1878 (2328-1515) | 5.0 (5.9-4.1)[a] | 5.6 (6.3-4.8) |
| 4 | $10^{5}$ | 1783 (2106-1510)[a] | 1625 (2083-1268) | 5.0 (5.9-4.1)[a] | 5.3 (6.2-4.5) |
| 5 | $10^{6}$ | 4096 (6294-2666)[b] | - | 3.2 (4.0-2.5)[b] | - |

**Table 4.7. Antibody responses of clinically infected sheep from dose-dependent transmission experiments.** [1]Mean values were calculated by back-transformation of $log_2$ coded individual peak antibody titre. [2]$T_{I-S}$ shows the mean number of days between inoculation and the onset of seroconversion (titre≥1:45). [3]For G2 animals the time of infection has been assumed to be 2 days prior to the onset of viraemia and the value in the table adjusted as 2 days greater than the $T_{V-S}$ value in table 4.4. Values in brackets show 95% confidence limits of the mean. ANOVA (with multiple comparisons using the Tukey method for G1) showed significant differences ($p<0.05$) between means marked [a] and [b].

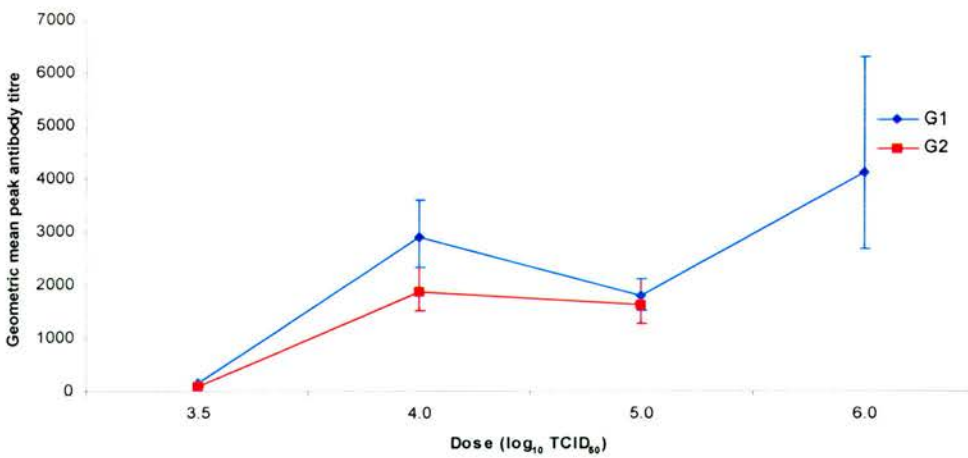


**Figure 4.12. Mean peak antibody titres for sheep from dose-dependent transmission experiments.** Geometric mean values were calculated by back transmission of $log_2$ coded individual antibody titres. Error bars show 95% confidence limits of the mean.

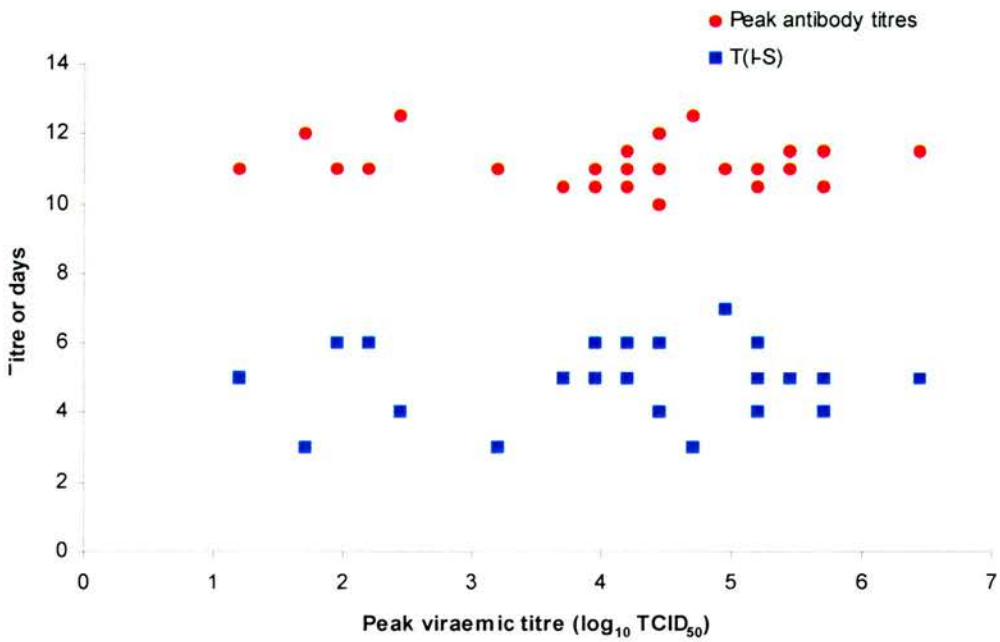


**Figure 4.13. Relationship between antibody responses and peak viraemic titres for sheep during dose-dependent transmission experiments.** $T_{I\text{-}S}$ is the mean number of days between infection and seroconversion. For G2 sheep the point of infection has been assumed to be 2 days prior to the onset of viraemia. Antibody titres are $\log_2$ coded.

### 4.2.7.4 Plasma interferon-gamma levels

Plasma samples from G1 sheep were assayed in duplicate using the BOVIGAM$^{TM}$ interferon-gamma (IFN-γ) enzyme immunoassay (as described in section 2.10). The mean optical density for each sample was normalised against a determined cut off value (mean of the negative control wells plus two standard deviations). OD values for G1 sheep are shown in figure 4.14. Table 4.8 and figure 4.15 show the mean peak IFN-γ level, mean duration of the detectable IFN-γ response and the interval between inoculation and peak IFN-γ production ($T_{I-I}$) for G1 sheep. ANOVA using the Tukey method for multiple comparisons (where applicable) was performed on all data for characteristics of IFN-γ responses. Data showed no significant departure from normality ($p>0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p>0.05$).

Non-parametric correlation analysis (Spearman's rank correlation coefficient, $r_s$) of the relationship between peak IFN-γ production and peak viraemia shows no significant correlation between the two variables when data are combined for all three experiments ($r_s=0.057$, $N=15$, $p=0.839$) and when each experiment is analysed separately ($10^4$: $r_s=-0.200$, $N=5$, $p=0.747$; $10^5$: $r_s=0.252$, $N=5$, $p=0.741$; $10^6$: $r_s=0.200$, $N=5$, $p=0.747$). Non-parametric correlation analysis of the relationship between $T_{I-I}$ and peak viraemia shows no significant correlation between the two variables when data is combined for all three experiments ($r_s=0.286$, $N=14$, $p=0.322$) and when each experiment is analysed separately ($10^4$:

$r_s$=-0.820, N=5, p=0.089; $10^5$: $r_s$=-0.833, N=4, p=0.167; $10^6$: $r_s$=-0.053, N=5, p=0.933). Figure 4.16 shows the relationship between $T_{I-I}$ and $T_{I-S}$. This figure demonstrates that there is a significant positive correlation between the occurrence of peak IFN-γ levels and the onset of seroconversion.

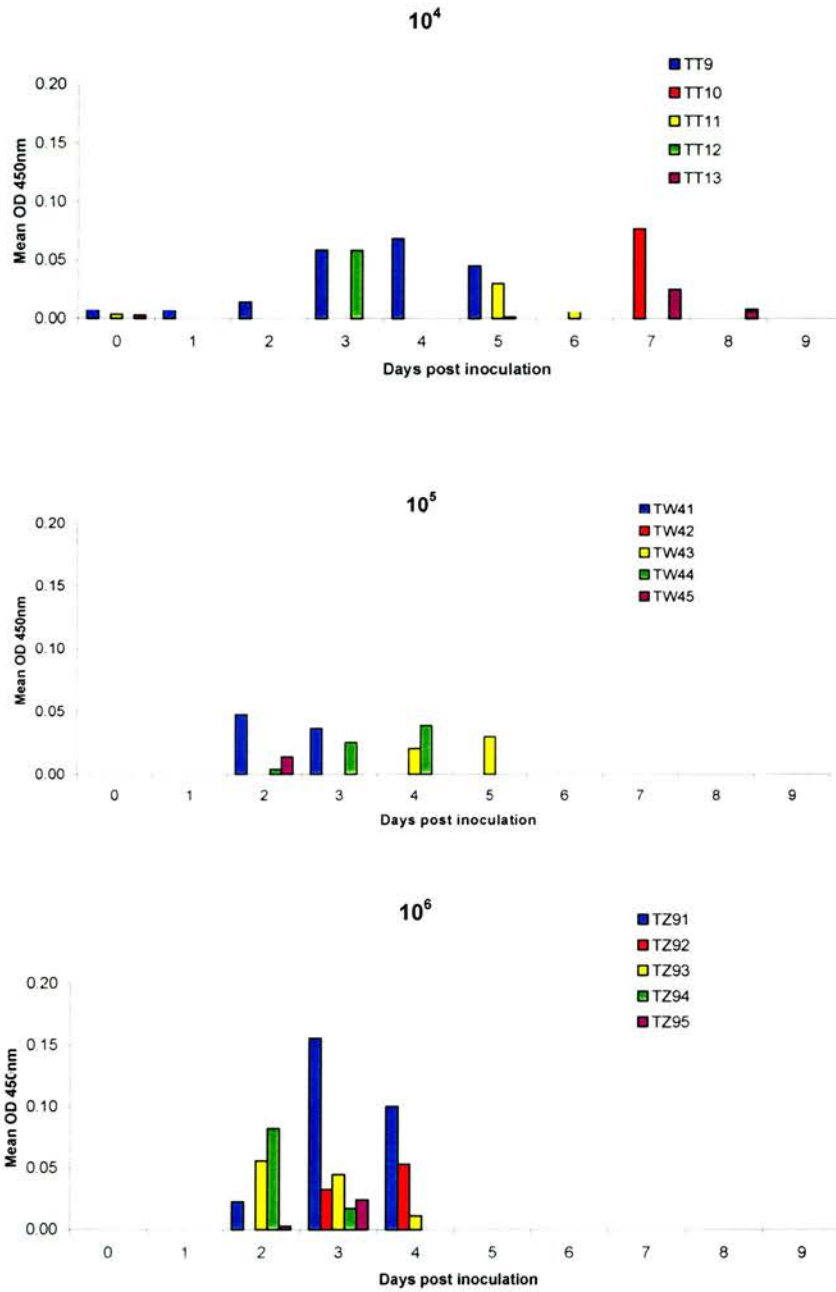


**Figure 4.14. Plasma IFN-γ levels from G1 sheep during dose-dependent transmission experiments.** Levels of IFN-γ were assayed as described in section 2.11 and normalised against a determined cut-off as described in section 3.2.8.

| Expt. | Dose ($TCID_{50}$) | Mean peak IFN-γ level (OD 450 nm) | Mean duration of detectable IFN-γ response (days) | $T_{I-I}$ (days)[1] |
|---|---|---|---|---|
| 3 | $10^4$ | 0.052 (0.081-0.023) | 2.2 (4.2-0.2) | 6.2 (8.4-4.0)[a] |
| 4 | $10^5$ | 0.026 (0.050-0.002) | 1.6 (3.0-0.2) | 3.3 (5.6-0.9)[b] |
| 5 | $10^6$ | 0.074 (0.136-0.012) | 2.0 (3.5-0.5) | 2.8 (3.8-1.8)[b] |

**Table 4.8. Characteristics of IFN-γ response for inoculated sheep from dose-dependent transmission experiments.** [1]$T_{I-I}$ is the time interval between inoculation and peak plasma IFN-γ production. In calculation of $T_{I-I}$ the day of peak occurrence was included in the interval. Values in brackets show 95% confidence limits of the mean ANOVA (with multiple comparisons using the Tukey method for G1) showed significant differences ($p<0.05$) between means marked [a] and [b].

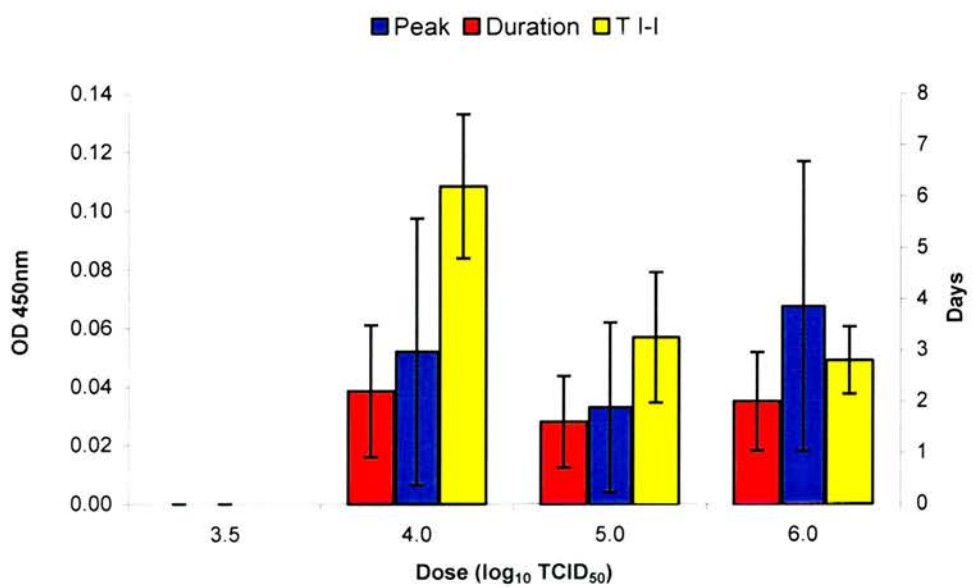


**Figure 4.15. Characteristics of IFN-γ production following inoculation of G1 sheep during dose-dependent transmission experiments.** $T_{I-I}$ is the interval in days between inoculation and production of peak IFN-γ, including the day of peak production. Error bars show 95% confidence limits of the mean.

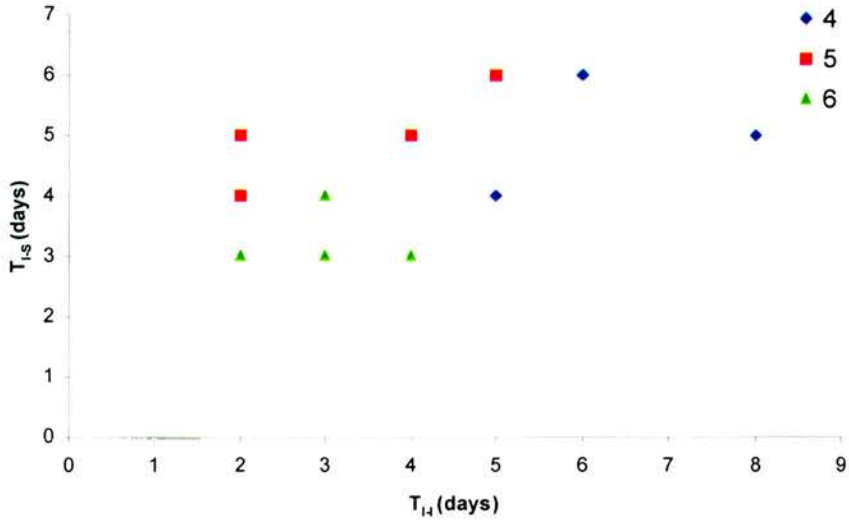


**Figure 4.16. Relationship between $T_{I-I}$ and $T_{I-S}$ for inoculated (G1) sheep during dose-dependent transmission experiments.** Numbers in the legend indicate dose ($log_{10}$ $TCID_{50}$). $T_{I-I}$ is the interval in days between inoculation and the occurrence of peak IFN-γ levels. $T_{I-S}$ is the interval in days between inoculation and the onset of seroconversion (titre≥1:45). Spearman's rank correlation coefficient (using combined data) $r_s$=0.60, N=14, p=0.024.

### 4.2.8 Principal component analysis

To analyse dose-dependent responses to infection of inoculated (G1) sheep, principal component analysis (PCA) of infection variables was performed. Thirteen sheep were included in the analysis, 5 from experiment 3 and 4 each from experiments 4 and 5. PCA analysis is unable to tolerate missing data and as a consequence the non-viraemic sheep (experiment 5) and the sheep that did not have a detectable plasma IFN-γ response (experiment 4) had to be excluded. PCA was not performed for in-contact (G2) sheep as the number of variables was inadequate (incubation periods and IFN-γ data unavailable). This type of model studies the correlations among a number of interrelated quantitative variables in an attempt to resolve the complex relationships between the interaction of various factors. Linear combinations of the variables are used to characterise the variation of each dimension in multivariate space and are assessed for their contributions to the total variance in the data. From this, successive components will explain smaller amounts of the total variance.

The correlation matrix used in this analysis is shown in table 4.9. Variables used were those that could contribute to the level of infection within a sheep irrespective of dose (time interval between inoculation and peak plasma IFN-γ production, incubation period, peak serum antibody levels, peak plasma IFN-γ levels, peak viraemia, and the time interval between the onset of viraemia and seroconversion). Lesion scores were not included as these occur in response to the level of infection. Analysis of these variables using PCA with varimax rotation (to

allow ease of interpretation) resulted in a two-component solution that explained 71.5% of the variance. Eigenvalues for both components and the corresponding variance explained are shown in table 4.10. Eigenvalues are characteristic roots of the variance and describe the variance between closely correlated variables. Components with an eigenvalue of less than 1 were excluded from analysis. Table 4.11 shows the component matrix with coefficients (loadings) that relate the variables (factors) to the components. Loadings were sorted by size and for calculation of factor scores for individual animals, values <0.4 were excluded. Factor scores of individual sheep for each component are shown in figure 4.17. A summary of the PCA analysis for G1 groups is shown in table 4.12.

| | $T_{I-I}$ | $T_{I-V}$ | Ab | IFN-γ | Vir | $T_{V-S}$ |
|---|---|---|---|---|---|---|
| $T_{I-I}$ | 1.000 | - | - | - | - | - |
| $T_{I-V}$ | 0.919 | 1.000 | - | - | - | - |
| Ab | -0.152 | 0.201 | 1.000 | - | - | - |
| IFN-γ | -0.172 | -0.109 | 0.672 | 1.000 | - | - |
| Vir | 0.275 | 0.279 | -0.036 | -0.294 | 1.000 | - |
| $T_{V-S}$ | 0.217 | 0.193 | -0.648 | -0.449 | 0.104 | 1.000 |

**Table 4.9. Correlation matrix of variables used in the principal component analysis of data for inoculated sheep from dose-dependent transmission experiments.** $T_{I-I}$ = time interval between inoculation and peak plasma IFN- γ levels. $I_{I-V}$ = incubation period. Ab = peak antibody level. IFN-γ = peak plasma IFN-γ levels. Vir = peak viraemic titre. $T_{V-S}$ = time interval between the start of viraemia and the onset of seroconversion.

| Component | Eigenvalue | % of variance | Cumulative % of variance |
|---|---|---|---|
| 1 | 2.48 | 41.3 | 41.3 |
| 2 | 1.81 | 30.2 | 71.5 |

**Table 4.10. Eigenvalues and percentages of variance explained for components of principal component analysis of data for inoculated sheep from dose-dependent transmission experiments.** Components with an eigenvalue <1 were excluded.

| Variable | Component 1 | Component 2 |
|---|---|---|
| Ab | **0.912** | -0.010 |
| IFN-$\gamma$ | **0.818** | -0.170 |
| $T_{V-S}$ | **-0.808** | 0.084 |
| $T_{I-V}$ | 0.072 | **0.961** |
| $T_{I-I}$ | -0.104 | **0.946** |
| Vir | -0.171 | **0.487** |

**Table 4.11. Component matrix for principal components of data from inoculated animals from dose-dependent transmission experiments (following varimax rotation).** Values show the loadings that relate variables to the two components. $T_{I-I}$ = time interval between inoculation and peak plasma IFN-$\gamma$ levels. $T_{I-V}$ = incubation period. Ab = peak antibody level. IFN-$\gamma$ = peak plasma IFN-$\gamma$ levels. Vir = peak viraemic titre. $T_{V-S}$ = time interval between the start of viraemia and the onset of seroconversion.

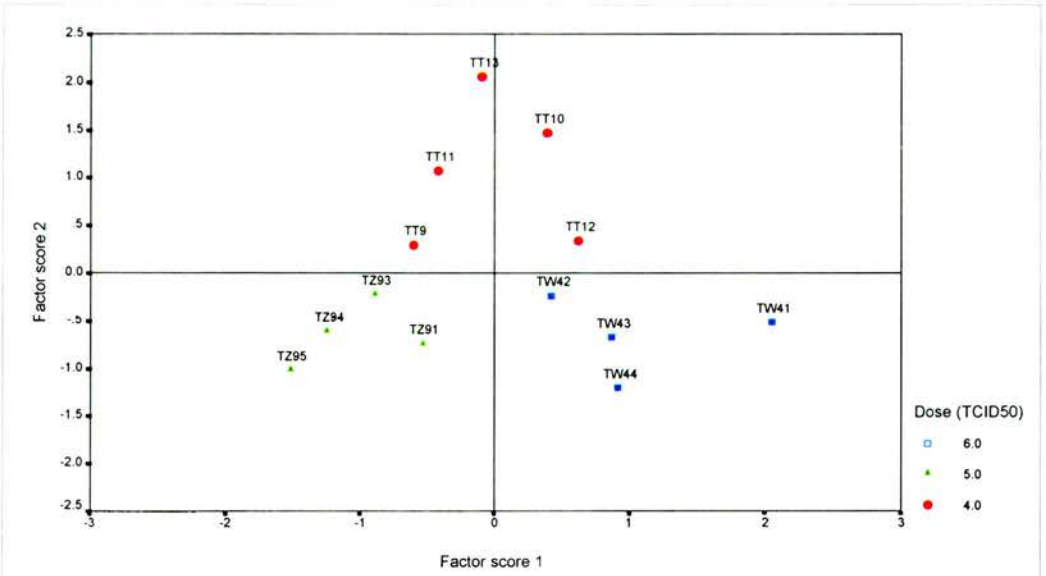


**Figure 4.17. Factor scores plot for components of principal component analysis for inoculated sheep from dose-dependent transmission experiments.**

| Dose ($TCID_{50}$) | Component 1 | Component 2 |
|---|---|---|
| 4.0 | Variable serum antibody titre<br>Low IFN-γ levels<br>Slow seroconversion | Long incubation period<br>Slow IFN-γ production<br>High viraemia |
| 5.0 | Low serum antibody titre<br>Low IFN-γ levels<br>Slow seroconversion | Short incubation period<br>Fast IFN-γ production<br>Low viraemia |
| 6.0 | High serum antibody titre<br>High IFN-γ levels<br>Fast seroconversion | Short incubation period<br>Fast IFN-γ production<br>Low viraemia |

**Table 4.12. Summary of principal component model for G1 sheep from dose-dependent transmission experiments.** This model summary has been inferred from table 4.11.

## 4.3 Discussion

A summary of the results of these experiments is given in table 4.13.

| Response | Dose ($TCID_{50}$) | | |
|---|---|---|---|
| | $10^4$ | $10^5$ | $10^6$ |
| Transmission to in-contact sheep | 80% | 40-60%[1] | 0% |
| Numbers of positive throat swabs | High | High | Low |
| Incubation period* | Long[a] | Short[b] | Short[b] |
| Length of viraemia* | Long[a] | Average | Short[b] |
| Size of viraemia* | High[a] | Low | Low[b] |
| Seroconversion* | Late[a] | Late[a] | Early[b] |
| IFN-γ response* | Slow[a] | Fast[b] | Fast[b] |

**Table 4.13. Summary of responses to infection of G1 sheep from dose-dependent transmission experiments.** *Indicates statistically significant differences in the means marked [a] and [b] ($p<0.05$). [1]Due to the late viraemias of two G2 sheep, the numbers of G2 sheep infected due to contact with G1 could only be 4.

The results of these experiments show there is a complex relationship between the dose of virus, severity of infection and resulting infectiousness of sheep infected with this isolate of FMDV. The lowest dose capable of causing clinical FMD from this study is $10^4$ $TCID_{50}$. Previous studies have estimated that $10^2$ $TCID_{50}$ of a type O FMDV was the minimum dose that would cause clinical infection in all sheep exposed directly to aerosols produced by infected pigs (Gibson & Donaldson, 1986). Although the magnitude of the minimum infectious dose differs greatly between this study and that previously described, there are considerable variations in the experimental approach used including the strain of virus and method of

exposure. Field observations suggest that the isolate used for this study was not adapted for infection of sheep as it had a low morbidity for sheep in the field (Mackay *et al.*, 1995). This may partially explain the higher minimum infectious dose. The experiments described here indicate that above the threshold dose required for infection, clinical FMD occurs for almost all intranasally inoculated animals. Below the threshold dose there is very limited subclinical infection with no transmission to in-contact sheep. The optimum dose for transmission from these experiments was also $10^4$ $TCID_{50}$. The two doses used below this did not result in clinical disease for G1 sheep and inoculation with doses higher than this led to a gradual reduction in transmission from inoculated animals. For experiment 3 ($10^5$ $TCID_{50}$), although six G2 sheep developed viraemia, the point at which the last two sheep developed viraemia was 4 and 6 days after the last viraemic day of G1. Transmission to these last two sheep probably occurred from contact with infectious members of G2. Therefore, the number of clinically infected G2 sheep arising due to transmission from G1 could be as little as four. Further reduction in the infectiousness with dose was seen when sheep were inoculated with the highest dose. No transmission occurred from G1 sheep inoculated with $10^6$ $TCID_{50}$ to in-contact susceptibles.

The nature of FMD in sheep is such that considerable variation in the spectrum of clinical disease is seen for infected animals (those with a detectable viraemia). Within the confines of this study the variation in the number of lesions seen within individual groups is great. Although the severity of clinical FMD does not vary

significantly with dose for these experiments, results show that there is a significant positive correlation between peak viral loads and the severity of clinical FMD. Parametric correlation analysis indicates that 26% of variance in clinical signs score can be attributed to variation in the peak viral load. The sensitivity of these experiments to detect significant variations in the severity of clinical FMD with dose may be limited by the group size (and consequently statistical power). Variation in clinical signs with dose was also not seen in a previous study (Gibson & Donaldson, 1986). Lesions of FMD seem not to be essential for transmission and in the main occur when sheep are no longer infectious. Interestingly, for these experiments, when optimum transmission of FMD occurred (dose of $10^4$ $TCID_{50}$) 79% (15/19) of vesicular lesions occurred prior to the onset of seroconversion where as for doses where transmission was compromised (doses of $10^5$ and $10^6$ $TCID_{50}$) no lesions occurred before seroconversion (0/10 and 0/11 respectively). A more reliable indicator of the severity of FMD infection in relation to infectiousness is perhaps the level of viral load. The length and peak viral titre of the viraemic period measured here differs significantly for sheep inoculated with $10^6$ $TCID_{50}$ compared to sheep inoculated with $10^4$ $TCID_{50}$. The level of infection and ability of FMDV to replicate is reduced with higher dose. The reduced length of the viraemic period for sheep inoculated with $10^6$ $TCID_{50}$ was due to seroconversion occurring significantly earlier for these animals. The onset of seroconversion is known to prevent excretion of FMDV by neutralisation of virus in circulation and at portals of exit (Donaldson & Sellers, 1998). Studies show that cessation of excretion of FMDV

by inoculated sheep corresponds well with the onset of seroconversion (Donaldson *et al.*, 1970; Sellers *et al.*, 1977). A reduction in the time between inoculation and seroconversion would shorten the window of opportunity for transmission and reduce the amount of virus excreted. The results of throat swabs taken from G2 sheep over the course of the experiments suggest that the levels of virus circulating within the animal box decreased with dose from $10^4$ to $10^6$ $TCID_{50}$. The number of positive throat swabs taken from in-contact sheep declines above the optimum dose for transmission, with the mean proportion positive per in-contact sheep per day being lower at $10^6$ $TCID_{50}$ than at $10^{3.5}$ $TCID_{50}$. Runs test analysis of throat swab occurrence implies that swabs taken from inoculated sheep are not an effective indicator of individual viral excretion.

The dose-dependent effects on transmission and responses of inoculated sheep are supplemented by dose-dependent responses in the level of infection for in-contact sheep. Viral loads are significantly higher for G2 sheep exposed to sheep inoculated with $10^4$ $TCID_{50}$ than for G2 sheep exposed to sheep inoculated with $10^5$ $TCID_{50}$. The size of the infecting dose influences not only the degree of infection of inoculated animals (viral load, infectiousness) but also of those infected through contact with these animals. Animals infected through contact with infected sheep exhibit a level of FMDV infection (assessed by viral load) related to the level of infection seen in the donor animals. The results of this study suggest that the level of infection of contact infected sheep will always be less that

that of the donor animals. Similar observations have been found for sheep infected with other isolates of FMDV (Burrows, 1968).

The highest dose of virus used in this study may have induced rapid and strong innate immune responses. Induction of such responses would restrict the spread of virus from local primary sites of replication (nasal/pharyngeal epithelial tissues following intranasal inoculation) resulting in the reduced viraemias seen with sheep inoculated with a high dose of virus. There is a significant reduction in the incubation period for sheep inoculated with $10^5$ and $10^6$ $TCID_{50}$ when compared to animals inoculated with $10^4$ $TCID_{50}$ suggesting that immune stimulation beyond local sites would occur earlier than with lower dose. The amount of virus that escapes local immune surveillance and enters the blood stream induces antigen dependent differentiation of FMDV specific B-cells leading to the production of large amounts of serum neutralizing antibody and eventual clearance of virus from circulation. Early clearance of virus from circulation and sites responsible for the release of virus into the bloodstream may be mediated by cytotoxic T-cell activity induced by early innate immune mechanisms. There are many components of the immune system that may restrict the spread of FMDV from sites of primary replication. Non-specific inflammatory responses and associated virucidal components (NK cells, interferons (IFNs) and phagocytic elements) can block replication of viruses at mucosal surfaces. In the absence of pre-existing specific antibody (as is the case with susceptible animals), local

immune responses (IFN-γ, sIgA and macrophages) at mucosal surfaces rapidly develop and prevent the spread of virus.

Levels of plasma IFN-γ for G1 sheep were measured for these experiments. Plasma IFN-γ is not a measure of local responses that may have reduced the viraemias seen with higher dose, but they do reflect the level of cell-mediated reactivity and T cell immunity of the animal induced by the infectious dose (Jones *et al.*, 1992). Levels of plasma IFN-γ reflect the relative stimulation of local immune mechanisms by antigen. Statistically there is no significant difference (although the highest mean levels are seen with the highest dose) in the mean peak IFN-γ production of inoculated sheep between experiments. There is a statistically significant reduction however in the interval between inoculation and peak IFN-γ production for sheep inoculated with $10^5$ and $10^6$ $TCID_{50}$ compared to those inoculated with $10^4$ $TCID_{50}$. This suggests that cell-mediated immune mechanisms have responded rapidly to the high antigenic dose, stimulated to such a degree that viral replication is inhibited more so than with lower doses of virus.

There is considerable evidence of a role for IFNs in immunity to FMDV and other viral infections. Type I IFNs (as well as TNF-α and IL-12) induce the antiviral state around infected cells shortly after challenge and have recently been implicated in restricting the local spread of FMDV within experimentally infected cattle (Brown *et al.*, 2000) and reducing the degree of cytopathic effects and yield from FMDV infected cells *in vitro* (Chinsangaram *et al.*, 1999). Type I IFNs

activate natural killer (NK) cells which in turn produce large amounts of type II interferon (IFN-γ). Type I IFN induction of IFN-γ production from NK cells has been shown to correlate with resistance to encephalomyocarditis virus in mice (Curiel *et al.*, 1998) and with restriction of spread of Theiler's virus in mice (Monteyne *et al.*, 1997); both of which are picornaviruses. Dose dependent IFN responses to FMDV infection do depend on the strain of virus used but increased doses of virus have been shown to result in increased levels of IFN production *in vitro* (Sellers *et al.*, 1968). IFN-γ causes upregulation of MHC class I and class II antigen presentation, activation of macrophages and differentiation of B-cells and subsequent IgG2a synthesis. These mechanisms lead to clearance of virus from sites of infection (through MHC class I presentation and CTL activity) and from circulation (macrophages) prior to the appearance of serum neutralising antibody. A reduction in viral load has been shown to correlate well with IFN-γ production for other viruses such as hepatitis C virus (Cramp *et al.*, 1999) and simian immunodeficiency virus (Khatissian *et al.*, 1996) and may explain the reduced viral loads seen in this study with high dose. There are many components of the cell-mediated immune system that contribute to local responses to virus infection. IFN-γ has been measured here as an indicator of the level of T-cell immunity. IFN-γ itself may not be directly responsible for cell-mediated restriction, as its role in host cell defense is largely immunomodulatory. For the purpose of this study, plasma IFN-γ levels serve as a useful marker for the speed and magnitude of the cell-mediated immune response.

Principal component analysis (PCA) of data from these experiments assists interpretation of the complicated interactions of a number of infection parameters. When dose is removed as a contributory variable, the responses of inoculated sheep are clearly defined by the results of the PCA. The two principal components extracted from the multivariate analysis of six infection variables provides distinction between sheep infected with different doses of this isolate of FMDV. The two components of the model are distinct in that none of the variables are loaded highly for both components. The first component describes the size of the immune response (humoral and cell-mediated) whilst the second component describes the effect of delayed immune responses.

The first component of the PCA model distinguishes between sheep inoculated with $10^5$ $TCID_{50}$ and those inoculated with $10^6$ $TCID_{50}$. All parameters of the first component have been shown to contribute towards a reduction in transmission during these experiments. The first compartment of the PCA seems to describe the force by which the host immune response counters initial challenge and then restricts dissemination and infectiousness by destruction of virus at portals of exit. The difference in infection with $10^4$ and $10^5$ $TCID_{50}$ is perhaps too slight for all individual animals to be distinguished by component 1, although sheep inoculated with $10^4$ $TCID_{50}$ are on average loaded higher for this component than sheep inoculated with $10^5$ $TCID_{50}$.

Sheep inoculated with the optimum dose of virus for transmission ($10^4$ $TCID_{50}$) are distinguishable by PCA from sheep inoculated with higher doses by the second component. The variables responsible for this component are incubation period, time interval to peak plasma IFN-γ production and level of peak viraemia (minor contribution). The time allowed for viral replication (prior to interference by immune mechanisms) has been shown to influence the level of FMDV infection in this study and the results of this PCA confirm this finding. It is surprising that peak viraemia is not loaded more strongly for this component but it has been shown from this study that when $T_{V\text{-}S}$ is shorter viraemia is also shorter. The strong loading of $T_{V\text{-}S}$ for the first component may explain the absence of a strong loading for viraemia on the second component. The components of the PCA described here define groups of sheep in distinct clusters related by the dose they received. Although dose was excluded from PCA analysis, the influence of the size of the infectious dose on variables is clearly seen in results of this multivariate analysis. Such a system, shown to work well for these experiments, could be used to estimate the dose of virus received by a naturally infected sheep if enough parameters were measured and available for PCA.

# CHAPTER 5: SERIAL PASSAGE OF FOOT-AND-MOUTH DISEASE VIRUS IN SHEEP

## 5.0 Introduction

The outbreak of foot-and-mouth disease (FMD) in Greece during 1994 suggested that certain strains of foot-and-mouth disease virus (FMDV), under certain conditions, were maintained poorly in sheep populations. The experiments described in this chapter attempted to assess transmission and infection of sequential groups of sheep in a well defined experimental situation. Crucial to the design of these experiments was to ensure a consistent exposure period for sequential groups of sheep. Preliminary experiments (results not shown) were performed using only one animal box within the isolation unit. During these experiments, new susceptible groups of sheep were introduced after the earliest definitive sign of FMD. Analysis of these experiments found that due to the early nature of excretion of FMDV by infected sheep, a considerable proportion of viral excretion was lost before the introduction of the next sheep group. For these reasons, it is difficult to expose recipient groups of sheep to the total viral excretion of the donor group and ensure that the total virus excretion from these animals is in the presence of their recipient group. To counter these problems the experiment was designed so that each recipient group was exposed to the same proportion of the total virus excreted by the donor group. Other preliminary experiments (results not shown) demonstrated that a 12 hour exposure period was

insufficient to allow transmission to occur beyond the second group. The exposure period was therefore set at 24 hours for these experiments. All contact groups of sheep were to receive virus for 24 hours by moving "up-stream" and following this period, spend 24 hours donating virus. Staggering of the inoculation of G1 was performed to make sure that some sheep in G1 had reached peak infectivity when G2 was brought into contact.

## 5.1 Experimental design

Two identical experiments were performed within a high security isolation unit using four groups of eight sheep, termed groups 1-4 (G1-4). Groups were housed in individual boxes for three days prior to the start of the experiment. Inoculation of G1 was staggered to ensure an equal distribution of peak infectiousness. For this purpose, half of G1 (termed G1b) were moved in to a separate box on day 0 while the first half were inoculated (termed G1a). All sheep were inoculated with $10^5$ $TCID_{50}$ of FMDV type O Greece 23/94 in 2 ml of M25 buffer (see appendix) intranasally using a 2 inch-length of sterile rubber tubing. The movements of groups between boxes are shown in figure 5.1. Each experiment was continued until all infected animals were no longer infectious (7 days after the cessation of viraemia) and sufficient time had been allowed for incubating susceptibles to become apparent (10 days). All uninfected animals were challenged at the end of each experiment by intranasal inoculation of $10^5$ $TCID_{50}$ of FMDV type O Greece 23/94 in 2 ml of M25 buffer. All sheep movements took place at the same time

each morning. Sheep groups were moved along a "dirty" corridor, starting with the highest group number. The corridor was disinfected thoroughly after each movement. Following movement of sheep all animals were sampled. Sampling started with the lowest group. All staff were disinfected thoroughly before moving to a different box.

### 5.1.1 Inoculum preparation

FMDV type O Greece 23/94 (originally isolated from ovine epithelium using primary calf thyroid cells; BTY) was passed again in BTY cells before titration as described in section 2.3. This stock virus was aliquoted and stored at -70°C. The required dilutions for inoculation were calculated and virus stock diluted immediately prior to administration with the required volumes of M25 phosphate buffer. All inoculum solutions were re-titrated immediately upon return to the laboratory to confirm the given dose.

### 5.1.2 Sampling protocol

On each day of the experiments, 5 ml of peripheral blood and 5 ml of peripheral blood mixed with EDTA were taken for serology and virus isolation respectively. Negative controls were taken on day –3 and day 0. Total serum antibody responses were measured as described in section 2.5. Virus isolation from plasma was performed as described in section 2.2. In the laboratory, 250 µl of whole blood

was added directly to 750 µl of TRIzol LS (Gibco, BRL) for RNA extraction and subsequent quantitative RT-PCR for viral RNA as described in section 2.10. Four additional 250 µl aliquots of whole blood were stored at -70°C in cryotubes. All sheep were examined daily for clinical signs of FMD in the mouth and on all four feet. Rectal temperatures were recorded daily. Oesophageal-pharyngeal fluid (OPF) was taken from every animal on days estimated as 30 and 33 days post-infection for the first experiment and 35 and 38 days post-infection for the second experiment. For G2-4, where the exact point of infection was unknown, the point of infection was assumed to be 48 hours prior to the onset of viraemia. This is a conservative estimate of the interval reported by Gibson *et al.* (1986). For uninfected and subclinically infected animals, sampling was performed at the same time as the last member of the same group. OPF samples were processed as described in section 2.6. OPF samples positive by virus isolation were not tested by reverse transcription nested polymerase chain reaction (RT-nPCR).

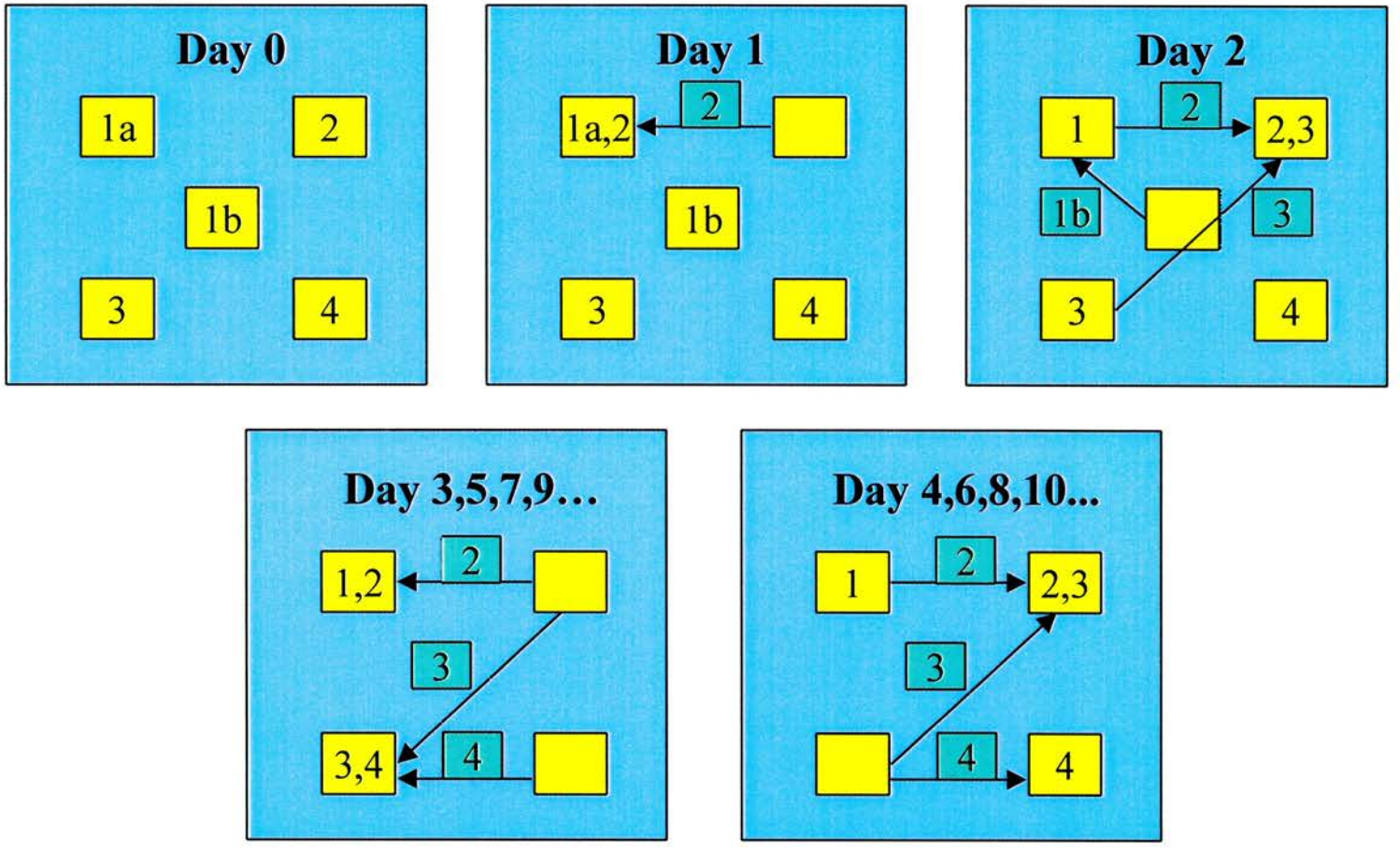


**Figure 5.1. Movement of sheep groups during serial passage experiments.** Yellow boxes indicate boxes within the isolation unit and the groups of sheep within for the 24 hour contact period. Blue boxes indicate the sheep groups moved prior to commencement of the exposure period. The experiment continued in 2 day cycles from day 3 onwards.

## 5.2 Results

### 5.2.1 Transmission

Experimental animals have been classified throughout this analysis according to the levels of infection defined in section 2.12. Susceptible animals were challenged at the end of each experiment to confirm susceptibility to FMDV. A summary of the infection status of sheep from both experiments is shown in table 5.1.

| | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G1 | G2 | G3 | G4 |
| Clinical | 7 | 4 | 7 | 5 | 7 | 3 | 6 | 3 |
| Inapparent | 0 | 2 | 1 | 2 | 1 | 2 | 0 | 1 |
| Subclinical | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 3 |
| Susceptible | 0 | 1 | 0 | 0 | 0 | 2 | 1 | 1 |
| Total infections | 8 | 7 | 8 | 8 | 8 | 6 | 7 | 7 |

**Table 5.1. Numbers of sheep in each infection class following serial passage experiments.** Clinical infections were defined as those where clinical signs of FMD occurred. Inapparent infections were classified as those where viraemia occurred but without the occurrence of clinical signs. Subclinical infections were defined as those where seroconversion was the sole indicator of FMDV infection. Susceptible animals were challenged at the end of each experiment to confirm susceptibility to FMDV.

#### 5.2.1.1 Experiment 1

The viraemic periods and probang results for all groups are shown in figure 5.2. Of the eight G1 sheep, 7 developed clinical FMD. The other member (UB54) developed subclinical FMD, seroconverting 5 days post inoculation. Two in-contact animals developed subclinical FMD, UB66 from G2 and UB76 from G4. Only one sheep remained susceptible throughout the course of the experiment,

UB60 from G2. UB60 was challenged at the end of the experiment and developed clinical FMD within 4 days of inoculation.

#### 5.2.1.2 Experiment 2

The viraemic periods and probang results for all groups are shown in figure 5.3. All 8 G1 sheep developed viraemia. One did not develop lesions. A total of 5 sheep developed subclinical FMD; one each from G2 and G3 (UF95 and UF88 respectively) and 3 from G4 (UF78, UF77 and UF76). Four sheep were still susceptible at the end of the experiment; UF92 and UF93 from G2, UF85 from G3 and UF80 from G4. All susceptible sheep were challenged at the end of the experiment and all developed clinical FMD within 4 days of inoculation.

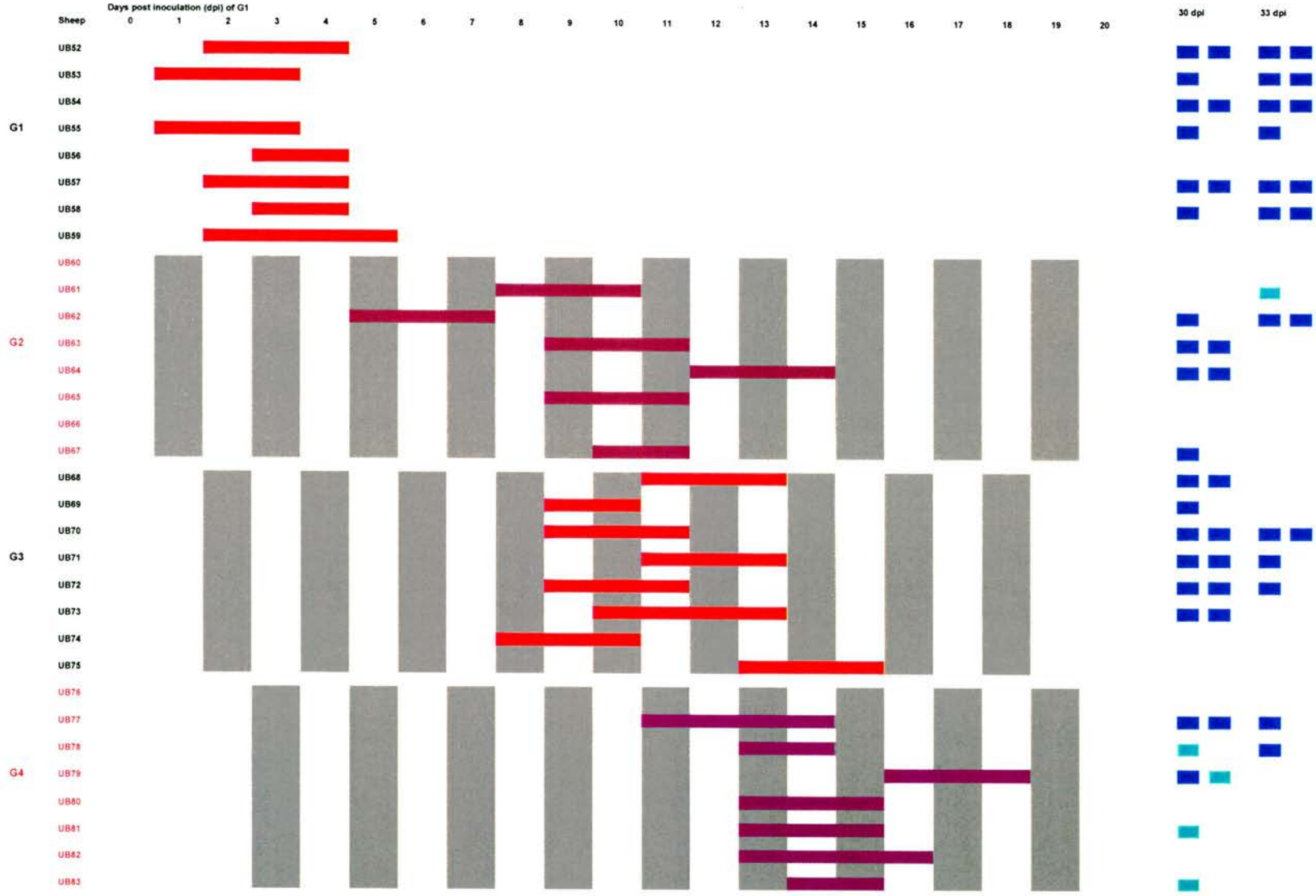


**Figure 5.2 Viraemic periods and probang results for sheep from serial passage experiment 1.** Red and purple blocks indicate viraemic periods. Dark blue blocks incicate OPF samples positive by virus isolation. Light blue blocks indicate OPF samples positive by RT-nPCR. OPF samples positive by virus isolation were not tested by RT-nPCR. Grey regions show periods of exposure to the previous group.

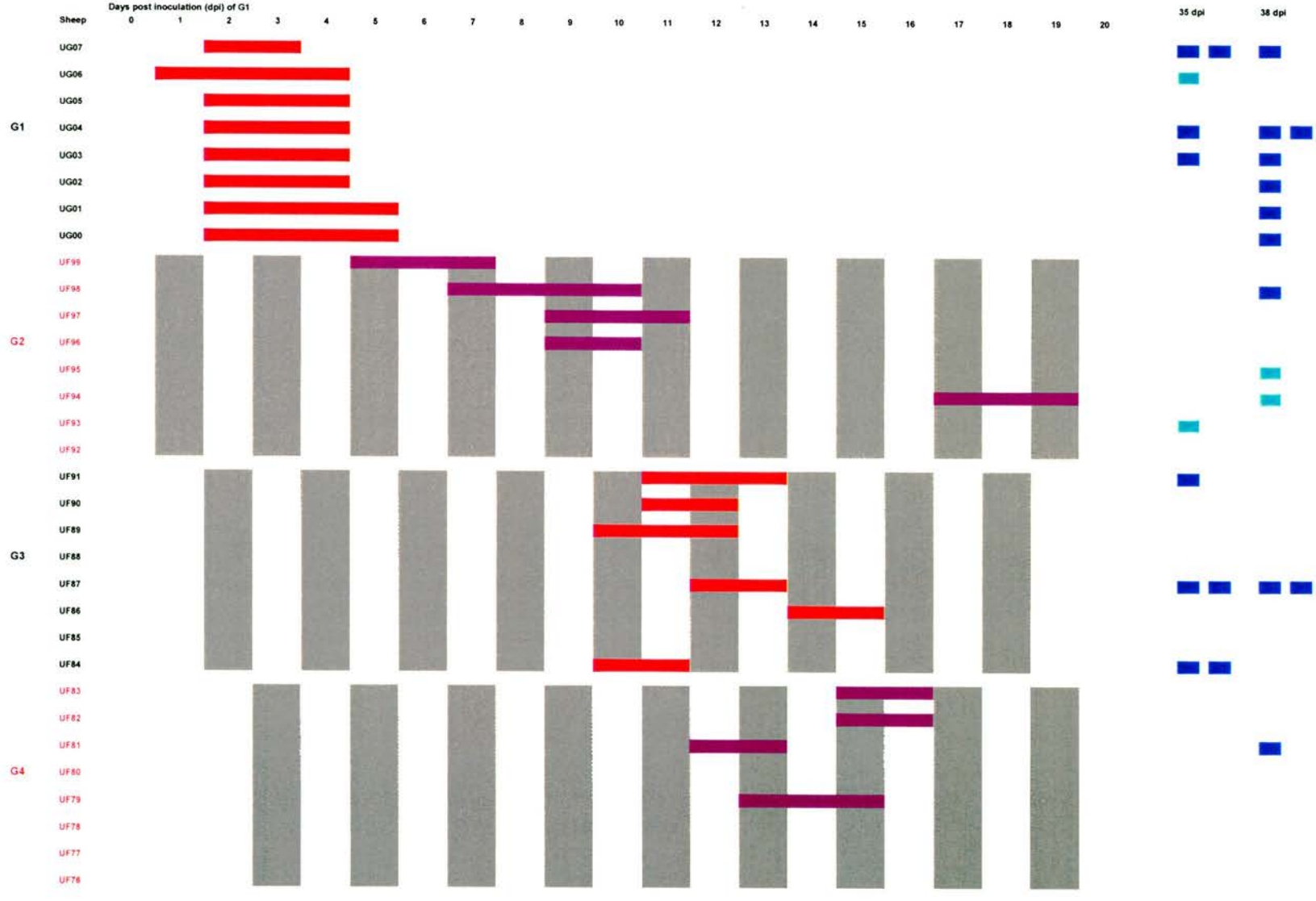


**Figure 5.3 Viraemic periods and probang results for sheep from serial passage experiment 2.** Red and purple blocks indicate viraemic periods. Dark blue blocks incicate OPF samples positive by virus isolation. Light blue blocks indicate OPF samples positive by RT-nPCR. OPF samples positive by virus isolation were not tested by RT-nPCR. Grey regions show periods of exposure to the previous group.

### 5.2.2. Indicators of infection

#### 5.2.2.1 Clinical foot-and-mouth disease

To assess the level of clinical FMD, both the mean number of vesicular lesions and the mean number of sites on which lesions occurred were calculated for groups of sheep from both experiments. Five possible sites were used in calculating the mean number of sites affected (all four feet and the mouth). The mean number of vesicular lesions for clinically infected sheep is shown in table 5.2. The mean numbers of sites affected are shown in table 5.3. One-way analysis of variance (ANOVA) using the least significant difference (LSD) method for multiple comparisons was performed for lesions data (numbers and sites affected) for viraemic sheep, for both experiments. All data showed no significant departure from normality ($p > 0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p > 0.05$). There were no significant differences ($p > 0.05$) between any group means for both experiments, for both measures of clinical FMD.

| | Mean number of vesicular lesions | | | |
|---|---|---|---|---|
| Group | Experiment 1 | N | Experiment 2 | N |
| 1 | 2.0 (0.1-3.9) | 7 | 2.8 (0.9-4.6) | 8 |
| 2 | 1.7 (0.1-3.2) | 6 | 1.8 (0.4-3.2) | 5 |
| 3 | 2.4 (0.5-4.3) | 8 | 1.3 (0.5-2.2) | 6 |
| 4 | 1.8 (0.4-3.6) | 7 | 1.3 (0.3-2.3) | 4 |

**Table 5.2. Mean number of vesicular lesions per viraemic sheep during serial passage.** Numbers in brackets show 95% confidence limits of the mean. ANOVA with multiple comparisons using the LSD method showed no significant differences ($p>0.05$) between groups for experiment 1 ($df=3,24$; $F=0.09$; $p=0.966$) and for experiment 2 ($df=3,19$; $F=1.06$; $p=0.389$).

| | Mean number of sites affected | | | |
|---|---|---|---|---|
| Group | Experiment 1 | N | Experiment 2 | n |
| 1 | 1.4 (0.9-1.9) | 7 | 2.4 (0.9-3.9) | 8 |
| 2 | 1.4 (0.1-2.2) | 6 | 1.6 (0.1-3.4) | 5 |
| 3 | 1.5 (0.5-2.5) | 8 | 1.3 (0.5-2.2) | 6 |
| 4 | 1.4 (0.4-2.5) | 7 | 1.3 (0.0-3.3) | 4 |

**Table 5.3. Mean number of sites affected by FMD per viraemic sheep during serial passage.** Numbers in brackets show 95% confidence limits of the mean. Only five sites were included for analysis (four feet and the mouth). ANOVA with multiple comparisons using the LSD method showed no significant differences ($p>0.05$) between groups for experiment 1 ($df=3,24$; $F=0.14$; $p=0.937$) and for experiment 2 ($df=3,19$; $F=0.85$; $p=0.482$).

### 5.2.2.2 Antibody responses

The mean peak total serum antibody levels for each group are shown in table 5.4. The time interval in days between the onset of viraemia and the start of seroconversion ($T_{V-S}$) has been calculated as a possible indicator of

the duration of the infectious period. Mean values of $T_{V\text{-}S}$ for both groups are given in table 5.5. ANOVA using the LSD method for multiple comparisons were performed on $\log_2$ transformed data for peak antibody production and $T_{V\text{-}S}$ for both experiments. There were no significant differences ($p > 0.05$) between any group means for both measures and both experiments. Humoral immune response data showed no significant departures from normality ($p > 0.05$) using the Kolmogorov-Smirnov test and were consistent with homogeneity of variance using the Levene test ($p > 0.05$).

| Group | Mean peak antibody titre[1] | | | |
|---|---|---|---|---|
| | Experiment 1 | N | Experiment 2 | N |
| 1 | 5405 (7132-4096) | 7 | 7132 (10809-4705) | 8 |
| 2 | 6208 (8192-4390) | 6 | 9410 (17510-5043) | 5 |
| 3 | 4390 (6654-3104) | 8 | 8192 (16384-4096) | 6 |
| 4 | 4390 (7643-2702) | 7 | 7643 (10086-5792) | 4 |

**Table 5.4. Mean peak antibody levels for viraemic sheep during serial passage.** [1]Mean values and standard errors of the mean were calculated by back-transformation of $\log_2$ coded individual peak antibody titres. Values in brackets show 95% confidence limits of the mean. ANOVA of $\log_2$ transformed raw data (with multiple comparisons using the LSD method) showed no significant differences ($p>0.05$) between groups for experiment 1 ($df=3,24$; $F=0.94$; $p=0.438$) and experiment 2 ($df=3,19$; $F=0.32$; $p=0.812$).

| | $T_{V-S}$ | | | |
|---|---|---|---|---|
| Group | Experiment 1 | N | Experiment 2 | N |
| 1 | 2.9 (3.7-2.0) | 7 | 2.6 (3.1-2.2) | 8 |
| 2 | 2.8 (3.3-2.4) | 6 | 2.6 (3.3-1.9) | 5 |
| 3 | 3.0 (3.0-3.0) | 8 | 2.2 (2.6-1.7) | 6 |
| 4 | 2.9 (3.5-2.2) | 7 | 2.5 (3.4-1.8) | 4 |

**Table 5.5. Time interval in days between the onset of viraemia and the start of seroconversion ($T_{V-S}$) for groups from serial passage experiments.** Values in brackets show 95% confidence limits of the mean. ANOVA of $\log_2$ transformed raw data (with multiple comparisons using the LSD method) showed no significant differences ($p>0.05$) between groups for both experiment 1 ($df=3,24$; $F=0.12$; $p=0.946$) and experiment 2 ($df=3,19$; $F=1.07$; $p=0.385$).

### 5.2.2.3 Viraemia

#### 5.2.2.3.1 Length of viraemia

The mean length of viraemia for groups from both experiments is shown in table 5.6. Non-parametric analysis of data using the Mann-Whitney U test showed no significant difference between the distributions of any groups ($p>0.05$).

| Group | Mean length of viraemia | | | |
|---|---|---|---|---|
| | Experiment 1 | N | Experiment 2 | N |
| 1 | 2.9 (2.2-3.5) | 7 | 3.3 (2.7-3.8) | 8 |
| 2 | 2.8 (2.4-3.3) | 6 | 3.0 (2.1-3.9) | 5 |
| 3 | 3.0 (2.6-3.4) | 8 | 2.3 (1.8-2.9) | 6 |
| 4 | 3.0 (2.2-3.8) | 4 | 2.3 (1.5-3.0) | 4 |

**Table 5.6. Mean length of viraemia for sheep during serial passage experiments.** Values in brackets show 95% confidence limits of the mean. Non-parametric Mann-Whitney U test analysis showed no significant difference in the distributions between any groups ($p>0.05$).

#### 5.2.2.3.2 Quantification of viral loads

Levels of viral RNA in blood were measured by quantitative RT-PCR (as described in section 2.10). Initially, all blood samples positive by virus isolation were quantified to determine the point of peak viraemia. Peak samples were then assayed a further two times and the mean value determined. Individual mean peak titres and arithmetic group means are shown in table 5.7. For the purpose of this analysis, UF94 from experiment 2 (G2) has been placed in G4. UF94 was viraemic 5 days after the other 4 sheep in G2 had developed viraemia. It is unlikely that either UF94 had a very long incubation period or that the other 4 members of G2 had excessively long infectious periods. UF94 was therefore most likely infected by back transmission from G3.

Raw data for peak viral loads were transformed to achieve no significant departures from normality ($p>0.05$) using the Kolmogorov-Smirnov test and consistency with

homogeneity of variance using the Levene test ($p > 0.05$). For this purpose, experiment 1 data was square-root transformed and experiment 2 data natural log transformed. ANOVA using the LSD for multiple comparisons of group means was performed on transformed data for both experiments (tables 5.8 and 5.9). Results of the ANOVA showed a significant ($p < 0.05$) effect of group on mean peak viral RNA titre for experiment 1 ($F=3.48$; $df=3,24$; $p=0.03$) but not for experiment 2 ($F=1.31$; $df=3,19$; $p=0.10$). Post-hoc multiple comparisons revealed significant differences ($p < 0.05$) between G2 and G4 for both experiments and between G1 and G2 for experiment 1.

| Group | Mean individual peak titres[1] | N | Arithmetic $log_{10}$ mean titre[2] |
|---|---|---|---|
| Experiment 1 | | | |
| 1 | 4025, 8862, 2663, 3845, 2861, 1977, 15862 | 7 | 3.69 (4.05-3.32) |
| 2 | 3236, 17155, 20120, 14047, 6368, 25038 | 6 | 4.02 (4.55-3.49) |
| 3 | 15941, 9539, 9301, 17041, 6886, 8863, 181, 133 | 8 | 3.60 (4.38-2.82) |
| 4 | 3605, 3417, 5415, 3394, 1054, 4107, 885 | 7 | 3.40 (3.69-3.11) |
| Experiment 2 | | | |
| 1 | 717, 6109, 6162, 374, 3709, 4412, 10216, 6230 | 8 | 3.50 (3.93-3.08) |
| 2 | 30169, 13238, 6048, 602, | 5/4[3] | 3.81 (4.52-3.07) |
| 3 | 37397, 6355, 6066, 388, 1081, 4718, | 6 | 3.58 (4.30-2.85) |
| 4 | 378, 674, 1537, 1313, 493[3] | 4/5[3] | 2.88 (3.37-2.48) |

**Table 5.7. Peak viral RNA loads from blood measured by quantitative RT-PCR for sheep from serial passage experiments.** [1]Peak viral loads were determined by assaying every blood sample positive by virus isolation. Peak samples were assayed three times and the mean calculated. Values are in $TCID_{50}$/ml estimated from a standard curve of known titre from assay in tissue culture. [2]Arithmetic mean for groups has been calculated as the mean from $log_{10}$ transformed individual peak titres. [3]Infection of UF94 occurred as a result of back-transmission from G3 and so for the purpose of analysis has been placed in G4. Numbers in brackets shown 95% confidence limits of the mean.

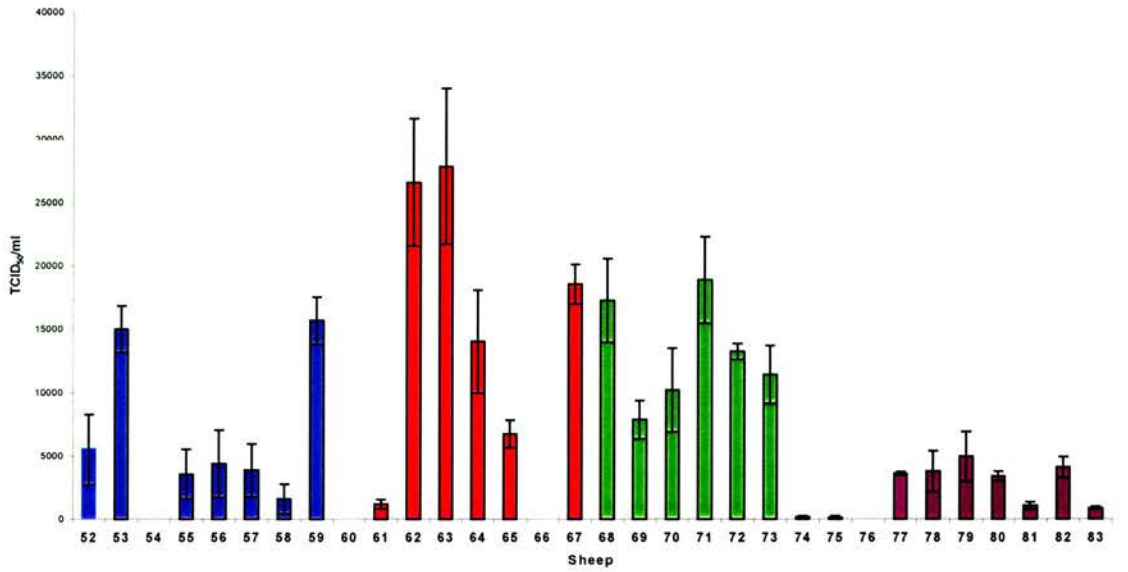


**Figure 5.4. Mean peak viral loads determined by quantitative RT-PCR for experiment 1.** Error bars show 95% confidence limits of the mean. Blue, red, green and purple bars show G1-4 respectively.

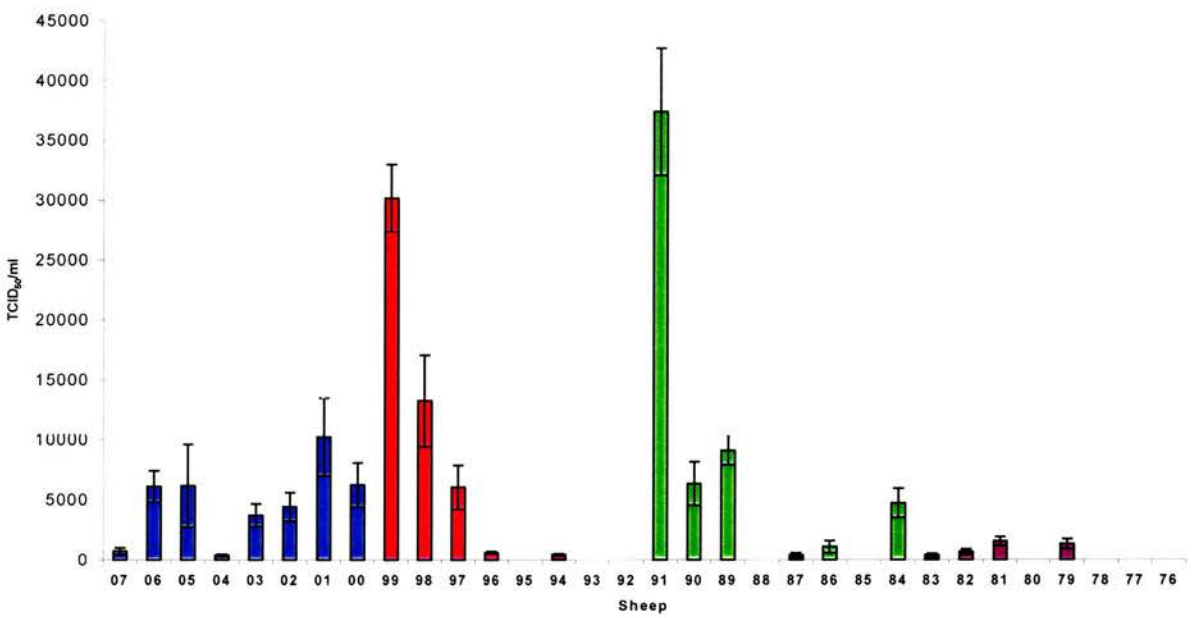


**Figure 5.5. Mean peak viral loads determined by quantitative RT-PCR for experiment 2.** Error bars show 95% confidence limits of the mean. Blue, red, green and purple bars show G1-4 respectively. For the purpose of analysis UF94 has been placed in G4 as detailed in section 5.2.2.3.

| Expt 1 | Analysis of variance | | | |
|---|---|---|---|---|
| Group | 1 | 2 | 3 | 4 |
| 1 | - | **0.032** | 0.544 | 0.372 |
| 2 | - | - | 0.092 | **0.004** |
| 3 | - | - | - | 0.133 |

**Table 5.8. Results of ANOVA with multiple comparisons using LSD of square-root transformed peak viral load data from experiment 1.** Numbers indicate p values. Bold values indicate significant differences ($p<0.05$).

| Expt 2 | Analysis of variance | | | |
|---|---|---|---|---|
| Group | 1 | 2 | 3 | 4 |
| 1 | - | 0.416 | 0.813 | 0.065 |
| 2 | - | - | 0.563 | **0.026** |
| 3 | - | - | - | 0.054 |

**Table 5.9. Results of ANOVA with multiple comparisons using LSD of natural log transformed peak viral load data from experiment 2.** Numbers indicate p values. Bold values indicate significant differences ($p<0.05$).

### 5.2.3. Carrier status

The results of probang samples taken are shown in table 5.10. Each sheep was sampled on two separate days. To assess whether the occurrence of a positive sample (by virus isolation) on the second day was independent of the occurrence of a positive sample on the first day, the effect of repeated sampling on the

occurrence of positive results by virus isolation was tested using the McNemar change test (Sokal & Rohlf, 1995). This test assesses whether previous testing (in this case the first probang samples) significantly associates with the results of subsequent tests (in this case the second probang samples) for related samples. Analysis of data by this method indicates there is no significant association ($p>0.05$) between the results of repeated sampling ($G=1.425$, $n=26$; G values approximate the $\chi^2$ distribution with one degree of freedom). Therefore, the results of samples from each of the two dates from an animal are independent of each other. The results of this analysis suggest that either the presence of virus in OPF is intermittent during the recovery phase or that the sampling technique lacks the necessary repeatability. An animal from which virus could be isolated from OPF on the first date of probang sampling but was then negative on the second date of probang sampling was included as a carrier in further analysis. The weakness of probang sampling does not allow us to make clear inferences about clearance of persistent FMDV infection. Therefore, a positive carrier has been defined as an animal from which virus has been isolated from at least one probang sample.

| Group | Number of carrier animals | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 1 | | | Experiment 2 | | |
| | 30dpi[1] | 33dpi | Total | 35dpi | 38dpi | Total |
| 1 | 6 | 6 | 6 | 3 | 6 | 6 |
| 2 | 4 | 1 | 4 | 0 | 1 | 1 |
| 3 | 6 | 3 | 6 | 3 | 1 | 3 |
| 4 | 2 | 2 | 3 | 0 | 1 | 1 |

**Table 5.10. Number of carrier animals in groups from serial passage experiments.** A carrier animal has been defined as any sheep where virus was isolated from OPF.[1]days post infection; the point of infection for in-contact groups was estimated as described in section 5.1.2.

### 5.2.3.1 Markers of carrier status

#### 5.2.3.1.1 Infection status

The results of probang sampling stratified by final infection class are shown in table 5.11. The one sheep that developed carrier status following subclinical FMD was from G1. The persistence of FMDV in this animal may be linked to the route of inoculation and not as a result of the natural infection process.

| Infection class | N | No. positive carriers | Proportion positive |
|---|---|---|---|
| Clinical | 42 | 24 | 0.57 (0.72-0.42) |
| Inapparent | 9 | 5 | 0.56 (0.88-0.24) |
| Subclinical | 8 | 1 | 0.13 (0.36-0.00) |
| Susceptible | 5 | 0 | 0.00 |

**Table 5.11. Proportions of carrier animals by infection class for serial passage experiments.** Data for both experiments has been combined. A carrier animal has been defined as any sheep where virus was isolated from OPF. Numbers in brackets show 95% confidence limits of the mean.

#### 5.2.3.1.2 Peak viral load

For the purpose of presentation, peak viral loads have been categorised according to magnitude. The proportion of sheep developing carrier status and the corresponding peak viral loads are shown in table 5.12. To assess the association between viral load and the development of carrier status, logistic regression was performed on uncategorised continuous peak viral load data against categorical carrier status (1=carrier, 2=non-carrier). This analysis shows that the level of peak viral load has a highly significant association with the development of carrier status (df=1; Wald statistic=5.980; p=0.009).

| Peak viral load ($TCID_{50}$/ml) | N | Positive carriers | Proportion positive |
|---|---|---|---|
| 4.0-4.9 | 16 | 13 | 0.81 (1.00-0.62) |
| 3.0-3.9 | 25 | 12 | 0.48 (0.68-0.28) |
| 2.0-2.9 | 10 | 3 | 0.30 (0.02-0.58) |

**Table 5.12. Proportions of carrier animals and corresponding viral loads following serial passage experiments.** Data for both experiments has been combined. A carrier animal has been defined as any sheep where virus was isolated from OPF. Numbers in brackets show 95% confidence limits of the mean. Logistic regression analysis shows peak viral load to have a highly significant relationship with the development of carrier status ($p<0.01$).

#### 5.2.3.1.3 Clinical FMD

Table 5.13 shows the number of vesicular lesions and sites affected by clinical FMD with the corresponding number of carrier animals for both experiments combined. For the purpose of this analysis, data from sheep with greater than two lesions or sites affected has been combined. Only viraemic animals were included in the analysis. To assess the association between both measures of the severity of clinical disease and the development of carrier status, logistic regression was performed on clinical signs data against categorical carrier status (1=carrier, 2=non-carrier). This analysis shows that the numbers of lesions (df=1; Wald statistic=0.003; p=0.955) and the number of sites affected (df=1; Wald statistic=0.552; p=0.458) have no significant association with the development of carrier status.

| Number of lesions or sites | Lesions | | | Sites | | |
|---|---|---|---|---|---|---|
| | N | Carriers | Proportion positive | N | Carriers | Proportion positive |
| 0 | 13 | 6 | 0.46 (0.73-0.19) | 9 | 5 | 0.56 (0.88-0.24) |
| 1 | 14 | 8 | 0.57 (0.83-0.24) | 17 | 11 | 0.65 (0.88-0.42) |
| 2 | 14 | 9 | 0.64 (0.89-0.39) | 15 | 9 | 0.60 (0.96-0.24) |
| ≥3 | 10 | 6 | 0.60 (0.90-0.30) | 7 | 4 | 0.40 (0.70-0.10) |

**Table 5.13. Proportions of carrier animals with number of vesicular lesions and sites affected by clinical FMD following serial passage experiments.** Data for both experiments has been combined. A carrier animal has been defined as any sheep where virus was isolated from OPF. Numbers in brackets show 95% confidence limits of the mean.

#### 5.2.3.1.4 Serum antibody response

Table 5.14 shows the peak serum antibody responses of sheep and the corresponding number of carrier animals for both experiments combined. To assess the association between the peak serum antibody titre reached and the development of carrier status, logistic regression was performed on $\log_2$ transformed peak antibody titre data against categorical carrier status (1=carrier, 2=non-carrier). This analysis shows that the peak antibody titre reached had no significant association with the development of carrier status (df=1, Wald statistic =1.25, p=0.268).

| Peak antibody titre | N | Carriers | Proportion positive |
|---|---|---|---|
| 1:2048 | 1 | 1 | 1.00 |
| 1:2896 | 6 | 4 | 0.67 |
| 1:4096 | 7 | 3 | 0.43 |
| 1:5792 | 14 | 10 | 0.71 |
| 1:8192 | 17 | 8 | 0.47 |
| 1:11586 | 4 | 3 | 0.75 |
| 1:16384 | 1 | 0 | 0.00 |
| 1:23186 | 1 | 0 | 0.00 |

**Table 5.14. Peak antibody titres and corresponding numbers of carrier animals for viraemic sheep from serial passage experiments.** Data for both experiments has been combined. A carrier animal has been defined as any sheep where virus was isolated from OPF.

## 5.3. Force of infection

To estimate the force of infection ($\lambda$) for individual groups the rate of transfer of animals from susceptible to viraemic status with time was examined (Anderson & May, 1991). For calculation of $\lambda$ the rate of decline of susceptible animals at time $t$, $S_{(t)}$, from 8 at $t=0$ to $S_{(t)}$ was modelled as

$$\frac{dS}{dt} = -\beta IS \qquad (1)$$

where $\beta$ is the transmission rate and $I$ is the number of infectious animals. $\lambda$ is defined as the rate at which susceptible animals develop viraemia and is given by

$$\lambda = \beta I \qquad (2)$$

thus

$$\frac{dS}{dt} = -\lambda S \qquad (3).$$

Solving equation (3) gives

$$S_t = S_0 e^{-\lambda t} \qquad (4).$$

Taking natural logarithms of each side of equation (4) gives a linear regression line with form

$$\ln S_t = \ln S_0 - \lambda t \qquad (5).$$

Figure 5.6 shows the number of animals developing viraemia with the sum of viral loads for animals viraemic on each day of the experiment. Each individual graph shows the sum of viral loads for sheep within that group and for those in-contact. For G2 and G3 this includes sheep groups both upstream and downstream. Estimates of $\lambda$ were obtained for G2-3, from both experiments, using equation (5) from linear regression analysis of natural log transformed numbers of sheep that have not developed viraemia with time (table 5.15). $\lambda$ is an estimate of the rate of

new infections (clinical and inapparent) and is based on the overall infectiousness within the box, not of individual sheep. Variation in the force of infection, $\lambda$, would be attributed to differences in the number of infectious animals but estimates of $\lambda$ do not account for variation in the infectiousness of individual sheep. The period over which $\lambda$ was estimated was the time between the first point of exposure (contact with viraemic sheep) and the last point of exposure (or the time the last sheep developed viraemia if all did so within the exposure period) and are given in table 5.15.

The relationship between $\lambda$ and exposure to viraemic animals is shown in figure 5.7. Four measures of exposure were used in testing this relationship, all calculated over the chosen time period: (1) the average daily sum of all viraemias to which susceptibles were exposed (irrespective of group), (2) the average daily sum of viraemias from other groups to which susceptibles were exposed, (3) the average daily sum of viraemias within the group to which susceptibles were exposed, and (4) the average number of viraemias irrespective of group (qualitative) to which susceptibles were exposed. The first three measures of exposure rely on the assumption that the level of viraemia is directly proportional to the infectiousness of the animal at that time. All measures of exposure are positively correlated with $\lambda$. There is a significant positive correlation between $\lambda$ and the average number of sheep viraemic on each day of the exposure period. Figure 5.8 shows $\lambda$ for each contact group during serial passage and the corresponding mean peak viral load for both experiments.

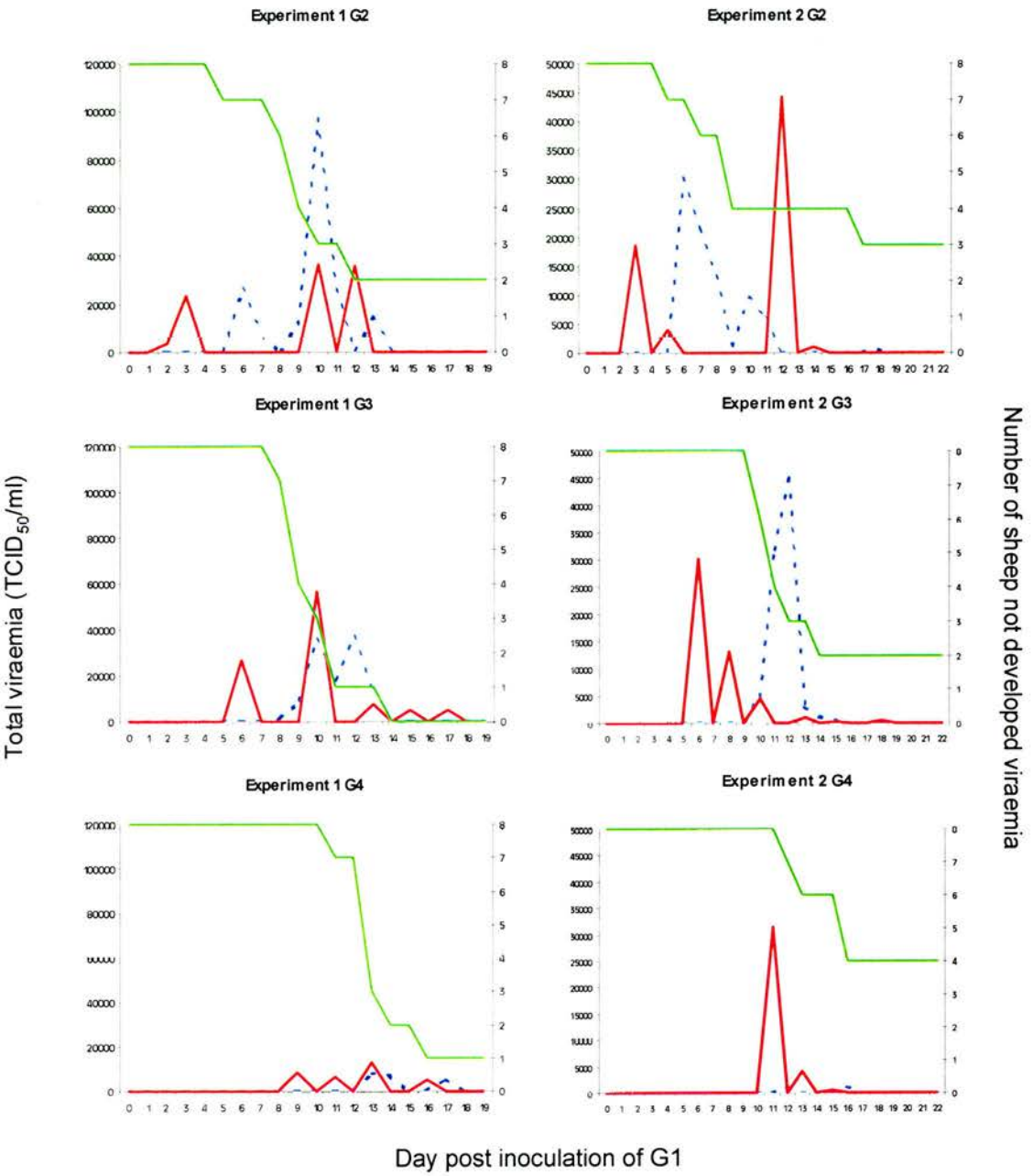


**Figure 5.6. Transmission and exposure for individual contact groups from serial passage experiments 1 and 2.** Individual graphs show the number of sheep who have not developed viraemia (green line) and the sum of viraemias each group was exposed to. The sum of viraemias is shown in terms of the group in question (broken line) and that of in-contact groups (red line). Viral loads were determined by quantitative RT-PCR as described in section 2.10. Viral loads either side of the peak were only assayed once.

| Expt. | Group | Period used (days)[1] | $\lambda$ | $R^2$ |
|---|---|---|---|---|
| 1 | 2 | 1-14 | 0.084 (0.111-0.058) | 0.747 |
| | 3 | 6-14 | 0.120 (0.215-0.026) | 0.470 |
| | 4 | 9-18 | 0.085 (0.140-0.080) | 0.448 |
| 2 | 2 | 1-19 | 0.052 (0.061-0.041) | 0.876 |
| | 3 | 6-18 | 0.071 (0.100-0.041) | 0.624 |
| | 4 | 11-16 | 0.022 (0.075-(-)0.030) | 0.283 |

**Table 5.15. Estimated force of infection ($\lambda$) for contact infected sheep groups from serial passage experiments.** $\lambda$ was estimated for each group by linear regression analysis. Numbers in brackets show 95% confidence limits. [1]The time period used for calculating $\lambda$ was the time between the first point of exposure (contact with first viraemic sheep) and the last point of exposure (last point of contact with a viraemic sheep).

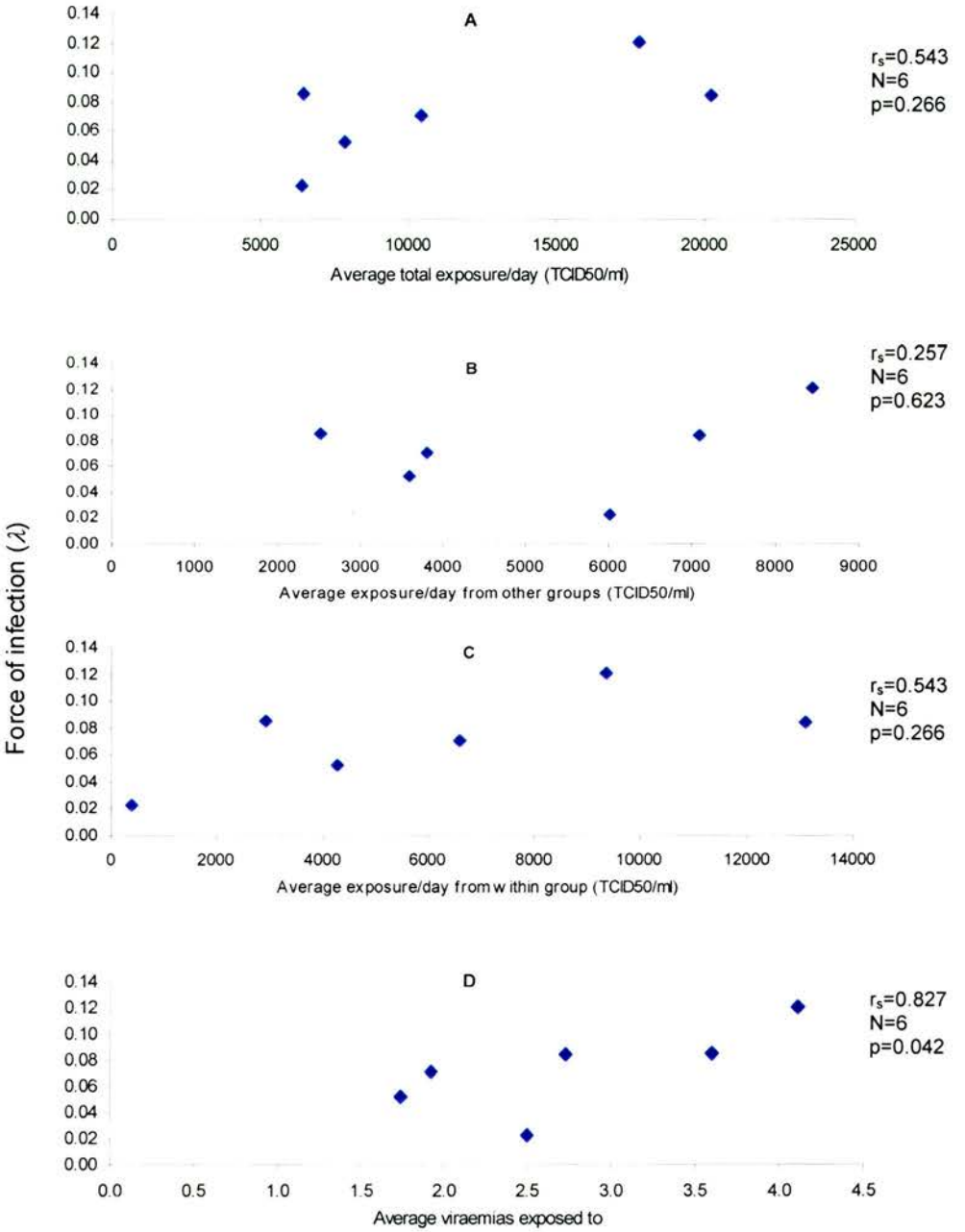


**Figure 5.7. Relationship between force of infection (λ) and measures of exposure for contact infected sheep groups from serial passage experiments.** Graph A shows the average sum of viraemias for all sheep present. Graph B shows the average sum of viraemias of sheep from other groups. Graph C shows the average sum of viraemias of sheep from within the group. Graph D shows the average number of viraemic animals present from any group. Each data point represents a contact group (3 groups from each experiment). Spearman's rank correlation coefficient ($r_s$) has been calculated for individual graphs.

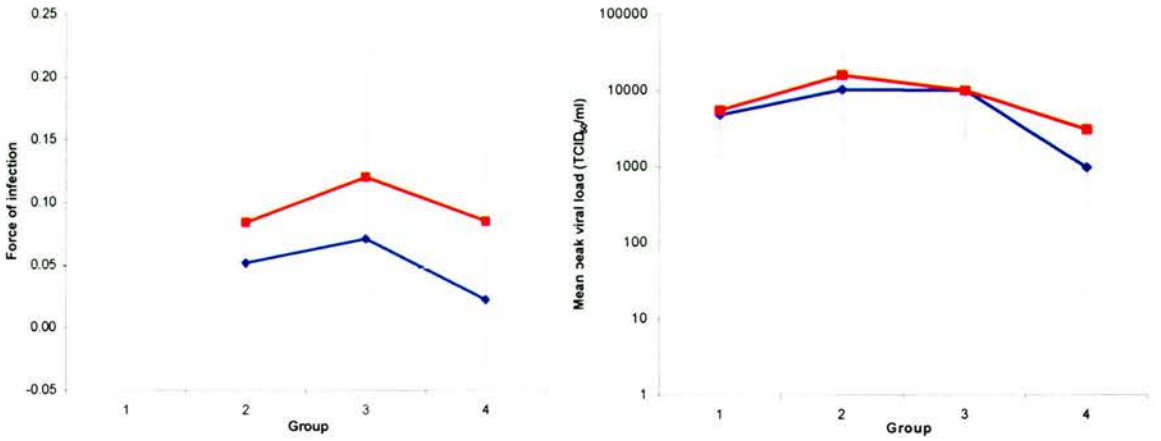


**Figure 5.8. Estimated force of infection ($\lambda$) and mean peak viral load for sheep groups from serial passage experiments.** The red line shows results from experiment 1. The blue line shows results from experiment 2. Error bars show 95% confidence limits of the mean.

## 5.4 Discussion

The experiments described here have attempted to simulate group-to-group transmission of FMDV. Through passage of virus through four groups of sheep, an attempt has been made to detect a reduction in the infectivity of FMDV and the level of infection with passage through sheep groups. The isolate used in this study was taken from the height of the 1994 epizootic of FMDV on mainland Greece. These experiments have attempted to quantify transmission and infection parameters that may have contributed to the epidemiological characteristics of the epizootic. Genetic selection of a less transmissible mutant of this virus is unlikely to have occurred during serial passage either in the field or during these experiments. Such a virus would have a lower fitness and by definition viral evolution does not favour such a process, although bottlenecks during transmission in small populations may result in stochastic loss of fitness. The more probable hypothesis is that an infected sheep flock is unable to amplify, or in fact maintain, the levels of virus received from donor animals per sheep. This would lead to a gradual reduction in the force of infection ($\lambda$) with flock-to-flock transmission. Estimation of $\lambda$ from these experiments shows there was a marked decline in the value for G4 when compared to that estimated for the preceding group (G3). Conversely, the estimate of $\lambda$ for G2 is less than that estimated for G3 but this is likely due to the route of infection for G1. The characteristics of infection for G1 should perhaps not be related to those of the other groups in the experimental analysis. These animals were directly inoculated and the route of infection is

known to influence the degree of infection and transmission from these animals. For both of the experiments described here, G1 had lower levels of viraemia than the first contact infected group (G2) and the estimated value of $\lambda$ is less than for the contact-to-contact transmission steps (G2 to G3 & G3 to G4). This evidence suggests that the transmission step from G1 to G2 is an experimental anomaly and cannot be directly related to later events. Despite the low $\lambda$ estimated for G2, the mean peak viral load for this group was for both experiments higher than for G3. This may be an experimental "artefact" and not a naturally occurring event. The high viraemias seen for G2 sheep that became infected early may be due to effective contact with G1 sheep excreting large amounts of virus, as is known to occur for directly inoculated sheep (Donaldson *et al.*, 1970). For such an excretion profile, where the vast majority of virus is excreted in a 24 hour period, effective contacts must be made during this peak. When such an event occurs the contact animal would receive a very large dose of virus. Of the 11 G2 sheep that developed clinical FMD it is likely that only 3 were infected through contact with G1 (those that became viraemic less than 72 hours after exposure to viraemic G1 sheep). All of these sheep had levels of peak viraemia above the mean for their group. The only published data for FMDV excretion from contact infected sheep shows that the excretion profile is more protracted than for directly inoculated sheep (Sellers *et al.*, 1977). Such excretion profiles increase the probability of infection but reduce the infectious dose. The first two passages in these experiments seem to follow this pattern.

Initially there are less transmission events (G1 to G2) but with higher viraemias. This is followed by a period of higher transmission rate but with a lower level of infection. There is a higher rate of movement of sheep from susceptible to viraemic ($\lambda$) but a lower level of infection (assessed by the level of viraemia) for later transmission steps. There is then a reduction in the force of infection with transmission from G3 to G4 and the level of infection is reduced further still, such that the mean peak viraemia for G4 is significantly less than for G2. The first passage through contact-infected sheep (G2) corresponds with an increase in the force of infection but subsequent transmission fails to enhance the transmissibility and replicative ability of this virus in sheep.

Estimation of $\lambda$ for the three contact groups of sheep from both experiments shows a statistically significant association between $\lambda$ and the average number of viraemic sheep present on each day of the exposure period. No significant association can be found between $\lambda$ and any measure of quantified viral loads although all measures are positively correlated with $\lambda$. The small number of data points (N=6) has restricted the statistical power of testing this relationship. Despite limitations due to the data size, the result of correlation testing suggests that the infectiousness of a sheep infected with this isolate of FMDV may not be directly related to the level of viraemia at that time. The relationship between the level of viral load and the level of excretion is unknown although studies have shown that excretion of FMDV by sheep following infection can occur up to 48 hours prior to the onset of clinical signs (Donaldson *et al.*, 1970; Sellers & Parker, 1969), a time point that coincides

well with the onset of viraemia. Results from the serial passage experiments described here show that the onset of vesicular disease (if present) occurs at a mean of 1.2 days after the start of viraemia (range -1 to 4 days). Studies on the excretion of FMDV from infected sheep have not determined the time of onset of viraemia so assumed excretion profiles can only be placed according to clinical signs. For these experiments the number of viraemic sheep that fail to develop vesicular lesions means such a method is inappropriate for this analysis. Published data of the excretion of FMDV from infected sheep has been collated for use in a computer model to predict the airborne spread of FMDV (Sorenson *et al.*, 2000). The excretion profile used in the model assumes that 97.2% of the total virus excreted by FMDV infected sheep occurs over a three-day period, a period found from these experiments to be the modal length of the viraemic period. The shape of the excretion profile has been shown to be strain-specific (Donaldson *et al.*, 1970) with considerable variation in the amounts of virus excreted, in terms of both peak values and total amount. It is therefore speculative to make any specific assumptions on the levels of this isolate excreted by infected sheep during the course of these experiments.

The route of infection for FMDV does suggest it is unlikely that viraemia is essential for infectiousness. Epithelial cells of the oro-nasopharynx are known to be the primary sites of replication for FMDV following inhalation of aerosol (Burrows *et al.*, 1981) and replication in the mucosa of the upper respiratory tract (Terpstra, 1972). Virus has been detected in OPF and nasal fluids prior to the onset of

viraemia for both intranasally and contact infected sheep (McVicar & Sutmoller, 1972; Sharma, 1978; Sharma & Murty, 1981). These facts suggest that viral excretion is, in the early stages of infection, independent of viraemia, although excretion that occurs at the height of viraemia may be augmented by high levels of circulating virus reaching portals of exit. This evidence suggests it is unlikely that the levels of virus in blood will reflect the relative amounts of virus excreted at that time. Perhaps this is best reflected in the relationship between $\lambda$ and measures of viral load. The best correlation is between $\lambda$ and viraemic days. Quantitative measures of viraemia show less association with the force of infection than qualitative measures.

Figure 5.8 shows clearly how the level of infection (assessed by the level of peak viral load following infection) has decreased with passage group. Reasons for the increase in viral load for G2 compared to G1 have been explained earlier. For these reasons G2 can in a number of respects be classed as the first group in the serial passage. The level of infection at the end of the chain (G4) is significantly different to that at the start (G2) for both experiments. There has been a significant reduction in the level of FMDV infection after passage through only 2 groups of sheep. The transmission characteristics for FMDV from the two experiments described here agree with observations on the natural behaviour of this isolate in the field. These experiments show a reduction in both the force of infection and the level of infection after passage of virus through 2 contact groups of fully susceptible sheep.

A statistically significant association between the level of viraemia and the number of carrier animals has been found from these experiments. The lower viraemias seen with later passage groups would explain the lack of carrier animals found during the field epidemic. At the height of the epidemic when large numbers of clinical cases of FMD were reported, potential carrier animals were destroyed. Later in the epidemic, when clinical FMD was not reported, $\lambda$ and the level of infection may have been much reduced and the probability of developing carrier status would have declined. It has been suggested that the development of carrier status may occur independently of viraemia (McVicar *et al.*, 1970). The results of these experiments suggest that for sheep infected with this isolate of FMDV this is not the case and that the probability of an infected animal developing persistent FMD increases with the level of viral load, and experimentally with low group number. Development of carrier status in sheep would seem to be a secondary event that follows acute infection and not a separate development following infection of primary sites of replication. The observation that vaccinated cattle can develop into carriers following contact with live virus, and in the absence of viraemia, does suggest that the process may be more complex.

# CHAPTER 6: GENERAL DISCUSSION

This research has been performed in an attempt to understand the dynamics and biology of the transmission of an isolate of foot-and-mouth disease virus (FMDV) in sheep populations. FMDV is regarded as one of the most infectious animal viruses but the outbreak of foot-and-mouth disease (FMD) in Greece during 1994 suggested that under certain circumstances transmission might not be so efficient. Analysis of field data from the epidemic has been combined with dose-dependent and serial passage experiments to assess their effects on the transmission of FMDV. It is clear from this work that the behaviour of this isolate of FMDV in sheep populations is different to that classically assumed for FMDV. It would be of great interest to determine whether other isolates of FMDV behaved in this manner in sheep, particularly those isolates that showed high morbidity for sheep in the field. The role of sheep in the recent outbreak of FMD in the UK shows how important it is to understand the behaviour of FMDV in sheep.

In the field, this isolate of FMDV exhibited behaviour such that the outbreak was termed "self-limiting". The Greek island of Lesbos served as an ideal environment for transmission of FMDV and enabled the initial amplification of the force of infection to occur. Without this initial kick-start it seems unlikely that the spread of FMD on the mainland would have been as severe. It has recently been suggested that sheep should be vaccinated in areas of high density but not in areas of low density (Donaldson, 2000). The observations from the outbreak in Greece during

1994 suggest that areas of high sheep density may provide the ideal environment for transmission and maintenance of FMDV whilst areas of low sheep flock density may not. The isolate of FMDV responsible for the 1994 epidemic in Greece was not sheep adapted. Perhaps the self-limiting nature of the outbreak in Greece is due to a combination of the predilection of the strain and the large proportion of FMDV susceptible livestock being sheep.

Upon reaching the mainland the force of infection began to fall. Results of serial passage experiments performed here show this process beginning to occur in a well defined transmission biased system. Estimated measures of the force of infection for sequential stages of the experiments show a fall with natural transmission steps. The level of infection for individual animals (assessed by peak viral load) has also been reduced significantly. It is difficult to assess whether the estimated force of infection would have continued to decline with passage beyond the three groups of naturally infected sheep used in the experiment. The field evidence, coupled with the experimental data, does suggest that sheep would have been unable to amplify the levels of virus to a level necessary to increase the force of infection and so further reductions would seem likely. If the level of viraemia in the field were to fall as seen during serial passage experiments, eventually it would reach levels such that infection would be described as subclinical. At some point, viraemia would fall below the level detectable in the laboratory and below that capable of causing generalised lesion formation. Sheep with a low level of infection may not be

infectious. There is only anecdotal evidence to suggest that subclinically infected sheep are capable of transmission.

It is improbable that the reduction in the level of infection seen here is due to genetic alteration. The evolutionary force required to induce attenuation or adaptation of FMDV to a particular host species is likely to take place over a long period of time. It seems unlikely that genetic alteration would contribute to a "self-limiting" virus, as this would require selection of a less transmissible virus over a relatively short time period. The pig adapted virus in Taiwan (1997) adapted to a niche environment to enhance the reproduction ratio of the virus. This was not the case for the outbreak in Greece during 1994. Viruses isolated from the course of the serial passage experiments described here will be subjected to genetic analysis at a later date to confirm this inference.

The dose-dependent experiments described here demonstrate the complex relationship between virus and host. Early immune responses seem to control the infectiousness of the host and subsequent viraemia. The very high doses of FMDV used here, although capable of infecting inoculated animals and causing clinical FMD, resulted in no transmission to in-contact susceptible animals. The optimum dose of FMDV was just above the minimum infectious dose. Immunologically, sheep respond well to this isolate. High doses seem to stimulate innate immune mechanisms to such an extent that replication and the resultant infectiousness are compromised. Dose-dependent experiment also show that the level of infection of

in-contact animals is less than that of the donors. From dose-dependent experiments the mean level of viraemia of in-contact infected sheep was in both cases less than that of the donor animals. For the analogous transmission step from serial passage experiments (G1 to G2) the level of viral load was higher for the in-contact infected sheep. In these experiments the ratio of inoculated animals to in-contact animals was higher (1:1) compared to the dose-dependent experiments (1:2). This would have led to a greater contact rate between infecteds and susceptibles and the contributory effects of multiple contacts may have led to the increased level of infection for in-contact sheep. This is further evidence to suggest that sheep held at high density are a greater risk of disseminating FMD.

The results of these experiments suggest that sheep may not act well as amplifiers of certain strains of FMDV. If the predilection of the strain is established it may be feasible to, as long as they are kept separate from pigs and cattle, to allow FMDV infection in sheep to run its course in epidemic environments. The fact that lambs are highly susceptible to FMDV must be considered at the time of lambing. However, in an epidemic environment where the presence of seropositive animals in the national flock is not acceptable, sheep will have to be slaughtered. As has been demonstrated with the recent outbreak of FMD in the UK, the nature of the disease in sheep is such that they may serve as asymptomatic disseminators of FMDV. It should be taken into account that this work has only been carried out with one isolate of FMDV. Other strains, particularly sheep adapted strains, may behave differently.

The behavior of FMDV in sheep populations is very different to its behavior in cattle and pig populations. In groups of cattle and pigs there is considerable early spread of infection throughout the susceptible population and the nature of clinical disease makes detection more likely. Rapid spread through the population results in a large number of animals becoming infected early and subsequently a large amount of concentrated virus is excreted. Creation of plumes of virus in this way is of great importance for viral dissemination and enhances the risk of airborne spread over long distances. Sheep, on the other hand, do not transmit infection well within a flock and at best seem capable of viral "turn over" rather than amplification as is seen with cattle and pigs.

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# APPENDIX

## Solutions and buffers

### Phosphate buffered saline (PBS), pH 7.2-7.6, Dulbecco's

| | |
|---|---|
| NaCl | 8.0 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.2 g |
| $KH_2PO_4$ | 0.2 g |
| $CaCl_2$ (anhydrous) | 0.1 g |
| $MgCl_2.6H_2O$ | 0.1 g |
| Distilled $H_2O$ | To 1 litre |

### Eagle's medium

| | |
|---|---|
| NaCl | 640 g |
| KCl | 40 g |
| $MgSO_4.7H_2O$ | 20 g |
| Glucose | 450 g |
| $Fe(NO_3)_3.9H_2O$ | 10 mg |
| $NaH_2PO_4.2H_2O$ | 14 g |
| Glutamine | 29 g |
| $CaCl_2$ | 20 g |
| $NaHCO_3$ | 200 g |
| Phenol Red (0.1%) | 1.5 litres |
| Stock aminoacids | 5.0 litres |
| Vitamin concentrate | 400 ml |
| Penicillin | 100 IU/ml final conc. |
| Streptomycin | 100 IU/ml final conc. |
| Neomycin | 100 IU/ml final conc. |
| Polymixin | 100 IU/ml final conc. |
| Distilled $H_2O$ | To 100 litres |

### Eagle's-HEPES, pH 7.2-7.4

| | |
|---|---|
| Eagle's medium | 1 litre |
| HEPES | 5.0 g |

Antibiotics added to double final concentration of Eagle's medium

**M25**

| | |
|---|---|
| $Na_2HPO_4.2H_2O$ | 6.11 g |
| $KH_2PO_4$ | 0.78 g |
| Distilled $H_2O$ | To 1 litre |

**Antigen detection ELISA blocking buffer, pH 7.2-7.4**

| | |
|---|---|
| PBS | 500 ml |
| Tween 20 | 0.25 ml |
| Skimmed milk | 25 g |

**Phosphate buffered saline-Tween (PBST)**

| | |
|---|---|
| PBS | 500 ml |
| Tween 20 | 0.25 ml |
| Phenol Red Solution (5%) | 10 ml |