Epidemiology of Foot and Mouth Disease in Cattle In Pahang, Malaysia.

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This thesis is presented for the Research Masters (with training)
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Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any other tertiary educational institution.

Jamaliah Binti Senawi
Abstract

Foot-and-mouth disease (FMD) is one of the most contagious diseases of domestic and wild cloven-hoofed animals. The disease has a significant negative impact on the economy of affected countries through reduced livestock productivity and loss of markets. In Pahang, Malaysia a severe outbreak of FMD started in December 2003, after approximately 18 years of freedom from the disease. The FMDV strain O was identified as the cause of this outbreak. The study reported in this thesis focused on three areas of epidemiology of FMD and was designed to: determine the temporal and spatial distribution and pattern of outbreaks of FMD in Pahang; identify the risk factors associated with the occurrence of FMD in Pahang; and determine the antibody response in local cattle following vaccination against FMD.

Although vaccination is adopted as a control measure for FMD in Pahang, the findings of this study indicated that only half (56%) of the respondents believed in vaccination as a preventive measure for FMD with only 37.3% of respondents correctly explaining how the disease spreads. Unfortunately only 29% of the respondents knew that the vaccine needed to be given at six monthly intervals and no one knew that a second priming dose was required to be administered one month after the primary dose. Antibody conferred after vaccination was significantly higher in animals which had been multiply vaccinated than in animals which received their first vaccination. There was evidence that vaccination stimulated a serological immunity; however the immunity, in many cases, was not sufficient to protect
against natural infection. In addition the NSP test indicated 7 animals (5 cows and 2 calves) were positive during the eleven month study period, although no clinical evidence of FMD had ever been seen on the farm.

Three variables (factors) were found to be associated with FMD in Pahang after a multivariable logistic regression analysis. The most strongly associated factor was retaining seropositive animals in the herd ($P = 0.006; \text{OR}=3.62; 95\% \text{ CI } 1.44, 9.11$). Cattle farmers who kept other livestock were more likely ($P=0.003; \text{OR} 3.2; 95\% \text{ CI } 1.47, 7.07$) to have an infected FMD herd than owners who didn’t keep other species of livestock. Farmers which allowed the entry of unauthorised vehicles onto their farmland were also more likely to have an infected herd ($P=0.05; \text{OR} = 2.2; 95\% \text{ CI } 1.0, 4.82$).

The spatio-temporal distribution of FMD outbreaks in Pahang during the period from the 16th December 2003 to the 26th August 2006 was assessed using a Space – Time permutation model. This indicated there were five significant distinctive clusters with no geographical overlap in the secondary clusters for the whole study period. Clusters were identified in the east, west and middle of Pahang with the observed to expected ratio of FMD outbreaks within the spatial temporal clusters being between 2.39 and 17.78. The temporal pattern of the FMD outbreaks in Pahang appeared to be seasonal occurring during the rainy season which coincided with “Hari Raya Korban” when many live cattle are moved throughout the country. The present study provided valuable information for the development of an effective control and eradication program for FMD in the state of Pahang, Malaysia.
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### Abbreviations

<table>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AVO</td>
<td>Assistant Veterinary Officer</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<td>DPV</td>
<td>Days post vaccination</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>FMD</td>
<td>Foot and Mouth Disease</td>
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<td>FMDV</td>
<td>Foot and Mouth Disease Virus</td>
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<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>ID</td>
<td>Identification</td>
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<td>LPBE</td>
<td>Liquid Phase Blocking ELISA</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>NSP</td>
<td>Non Structural Protein</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OIE</td>
<td>Office International des Epizooties</td>
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<tr>
<td>OPD tablets</td>
<td>Ortho-Phenylenediamine</td>
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<tr>
<td>PI</td>
<td>Percentage of Inhibition</td>
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<tr>
<td>PTH</td>
<td>Pusat Ternakan Haiwan</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Abbreviation</td>
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<tr>
<td>RVL</td>
<td>Regional Veterinary Laboratory</td>
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<tr>
<td>SPSS</td>
<td>Statistical package for the Social Sciences</td>
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<tr>
<td>μL</td>
<td>Microlitre</td>
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<tr>
<td>nm</td>
<td>Nanometre</td>
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<tr>
<td>WRL</td>
<td>World reference laboratory</td>
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<tr>
<td>w/v</td>
<td>Weight in volume</td>
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<tr>
<td>v/v</td>
<td>Volume in volume</td>
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<tr>
<td>VIF</td>
<td>Variable Inflation factor</td>
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<td>$\chi^2$</td>
<td>Chi square</td>
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CHAPTER 1

INTRODUCTION, BACKGROUND INFORMATION AND LITERATURE REVIEW

1.1 Introduction

Malaysia is a small country located in South East Asia which has a multiracial population with unique cultural and social practices. Pahang is one of the 13 states in Peninsular Malaysia, and the project outlined in this thesis relates to disease studies undertaken in this state.

Although there are unique ruminant animal husbandry practices in Malaysia, the livestock industry only makes a small contribution to the economy and currently produces far less product (meat) than is required by the population (DVS, 2009b). This situation is exacerbated by the presence of specific diseases, in particular Foot and Mouth Disease (FMD). Foot and mouth disease is considered one of the most significant threats to the livestock industry because of its impact on production, interference with access to international markets and effect on the economy (Kitching, 1998; James and Rushton, 2002). Currently FMD is endemic in Pahang, although there are on-going prevention and control measures against the disease undertaken in the state, as well as in the whole country. The control measures adopted include vaccination, movement control and physical examination of livestock prior to movement. Despite these efforts, numerous outbreaks occur each year. The epidemiology of FMD has been explored extensively in developed countries; however the situation is different for developing countries, such as Malaysia. There is little information available on the epidemiology of FMD in the
livestock population in Malaysia, in particular in the state of Pahang, and the study outlined in this thesis was undertaken to help address this deficiency.

1.2 Background Information

1.2.1 Malaysia and the history of Foot and Mouth Disease

Malaysia is comprised of two main regions, Peninsular Malaysia and Malaysian Borneo (Sabah and Sarawak), which are separated by the South China Sea. Neighbouring countries to Malaysia include Indonesia, Brunei, Thailand and Singapore. Malaysia consists of 13 states and three federation territories. Pahang, the largest state in Peninsular Malaysia, is situated on the east coast of the country (Figure 1.1).

Figure 1.1: Location of Malaysia and its FMD status
Foot and mouth disease has never been reported in Malaysian Borneo (Sabah and Sarawak). In contrast there has been a long history of FMD in Peninsular Malaysia. The first reported occurrence of FMD in Malaysia was in the 1860’s, however there is little detail of the early outbreaks until an outbreak occurred in cattle in July 1909 in the districts of Kulim and Kuala Muda in the state of Kedah. In 1936 a total of 551 animals were affected by FMD in the states of Perak and Selangor (Wallace, 1936). Towards the end of 1938 two outbreaks involving 238 cattle were recorded in Perak (Wallace, 1939). The disease was linked to the movement of animals from neighbouring countries, and resulted in the development of policies to control the movement of livestock in the four northern states (Perlis, Perak, Kedah and Kelantan). After an outbreak in Perlis in 1973, Malaysia adopted an eradication campaign for FMD involving a slaughter policy coupled with strict sanitary procedures. The virus responsible for the 1973 outbreak was confirmed as FMD virus Subtype A22 by the FMD World Reference Laboratory (WRL) at Pirbright, United Kingdom. This outbreak was quickly controlled and no further outbreaks were reported for approximately five years (Chong, 1979).

In October 1978 another outbreak occurred in Rantau Panjang (a border town adjacent to Golok on the Malaysian – Thai border) in the State of Kelantan and the disease spread via the normal trading route to the district of Muar in Johor and to the district of Ipoh in Perak. Late in 1978 another outbreak was reported in the district of Tumpat in Kelantan and the disease subsequently spread to Perlis and Kedah. The virus responsible for this outbreak was identified as Foot and Mouth Disease Virus (FMDV) Subtype O1. A stamping out policy was adopted in all affected areas and
18,117 animals were slaughtered of which 7,511 (41.5%) were cattle (Thuraisingham, 1977).

In January 1979 Malaysia decided to change from a stamping out policy to vaccination in the Northern states (bordering Thailand) as social, political and religious factors were hampering eradication (Chong, 1979). However the vaccination policy was also unsuccessful in controlling the disease as indicated by outbreaks in August 1980, caused by FMDV serotype O1, in the states of Kedah and Perlis which then spread to Penang. Subsequently in July 1981 major outbreaks were reported in three different abattoirs in the states of Selangor, Johor and Perak and in a feedlot in Johor (Babjee, 1994).

As the incidence of FMD was increasing in the early 1980’s it was apparent that the policy of only vaccinating livestock in the border states was not effective. Consequently in December 1982 a policy to vaccinate all animals on mainland Malaysia was implemented. The effectiveness of this policy was also questioned as more outbreaks were reported in June 1984 in Penang and Perak due to FMDV serotype O and in Kelantan and Terengganu in June 1985 due to serotype Asia 1. Since 1989 the policy of only vaccinating animals in the northern states of Malaysia has been reinstated. Intensive vaccination campaigns were instigated with a bivalent vaccine containing serotypes O and Asia 1, along with adoption of other sanitary measures (DVS, 1996). In December 1990 an outbreak occurred in a quarantine station and 29 cattle from Thailand were affected and subsequently destroyed (DVS, 1995). Since 1992 FMD has been seen every year in some states in Malaysia,
particularly in the northern states. Despite efforts to control FMD (Naheed, 2007), the disease is currently endemic in all of Peninsular Malaysia and it is one of the biggest factors hindering livestock production and the growth of the livestock market.

1.2.2 History of Foot and Mouth Disease in Pahang

Clinical signs of FMD have been recognised in Pahang since at least 1917 and were first observed in a herd owned by the Royalty (Azmie, 2006). Following the report in 1929 of FMD in the District of Raub, no cases were documented for the next 56 years. In 1986 FMD was again reported in a herd of cattle near the railway line in the district of Jerantut (Azmie, 2006).

After 18 years of apparent disease freedom, clinical signs of FMD were again reported in 58 cattle on the 22nd December 2003 in the district of Pekan. The disease was confirmed by the Kota Bahru Regional Veterinary Laboratory as FMD Serotype O. The disease spread very rapidly due to the managerial system adopted which allowed cattle to roam freely around villages, making disease control difficult. This outbreak was very large and involved many neighbouring districts in the state of Pahang. Over a two month period to the 12th February 2004, 5,070 animals were recorded as showing clinical signs (4,555 cattle). The worst affected district was Pekan, contributing 77.3% of the cases. Investigation by the Department of Veterinary Service (DVS) indicated there was illegal movement of infected buffalo from the district of Kemaman Terengganu to Pekan. The disease then spread from
Pekan to Rompin, Kuantan and Jerantut. The 2004 outbreak was the largest ever reported in Malaysia and since then the disease has become endemic in mainland Malaysia.

1.2.3 Cattle Management in Pahang

Foot and mouth disease in Pahang is believed to be influenced by the livestock production system practiced. There are three main types of beef cattle production practiced in Pahang: firstly an integrated system where cattle are reared under oil palm plantations; secondly the traditional system where a small number of cattle and other livestock are reared in villages. These animals usually roam freely in the villages and co-mingle with other farmer’s animals and with different species of animals. The animals graze freely unsupervised during the day and return to the owner’s place in the evening. Thirdly is a feed-lot cattle management system.

Commercial large-scale ruminant production is rare in Malaysia due to an absence of natural grasslands, and a limit on the areas of improved pastures (Joseph, 1991). This situation arises because of a national policy preventing the conversion of tropical rainforest into grazing pastures for animals because of both economical and environmental points of view. In contrast, young oil palm plantations provide a favourable environment for cattle production by providing shade and good quality forage with a high metabolisable energy and crude protein content (Rosli and Mokh Nasir, 1997). At the same time the cattle act as a biological control for weeds and their manure acts as an organic fertilizer for the oil palm trees (Ahmad, 2001) and
this form of raising cattle has been encouraged (Harun and Chen, 1995) (Figure 1.2). This integrated system is practiced mainly by Federal Land Development Authority (FELDA) settlers and oil palm plantation companies. There are 3 main types of grazing practiced under this system; 1. The cattle are free to graze anywhere in the oil palm plantation. This practice is similar to the traditional cattle rearing in villages. 2. The cattle are allowed to graze in certain areas, normally defined by fencing in the oil palm plantation. Farmers who practice this system normally have a limited number of cattle and this number is influenced by the area of oil palm plantation owned. 3. Rotational grazing where the cattle are restricted by electric fencing to an area with the cattle being moved daily between areas under the supervision of a herdsman. This system is commonly practiced by large oil palm plantation companies and by groups of FELDA settlers who combine their oil palm plantation areas and cattle.

Figure 1.2: Cattle in an oil palm plantation
1.3 Foot and Mouth Disease

1.3.1 Aetiology

Foot and Mouth Disease is caused by an *Aphthovirus* belonging to the family *Picornaviridae* (Bablanian and Grubman, 1993). It is an acid labile virus which is unstable below a pH of 6.8, and is inactivated at a pH of less than 6.0 or more than 9.0 (OIE, 2002). The virus is a non-enveloped single-stranded positive-sense genome RNA molecule of approximately 8500 nucleotides of positive polarity and is surrounded by an icosahedral capsid made up of four structural proteins (Mahy, 2005). The viral RNA consists of a single open reading frame (ORF), flanked by two highly structured non-coding regions (NCRs) that contain *cis*-acting structural elements involved in viral replication and gene expression (Sáiz et al., 2002). The viral particles are comprised of 60 copies of each of the four virus encoded capsid proteins, VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A) (Belsham and Martinez-Salas, 2004). These proteins are required for viral RNA replication and consequently are essential for infection. The capsid protein of FMD virus regulates viral replication, protein processing and modification of host cells. Foot and mouth disease virus, like other RNA viruses, are prone to errors during replication which leads to the development of mutant strains (Domingo et al., 2004). These errors lead to the virus having the ability to adapt to different factors resulting in genetic heterogeneity resulting in difficulty in the control and eradication of the disease (Domingo et al., 2004).

There are seven FMDV serotypes (A, O, C, Asia 1 and South African Territories (SAT) 1, 2 and 3) with no cross protection between the serotypes. Similar to other
Southeast Asian countries, the serotypes isolated in Malaysia are O, A and Asia 1, with Serotype O being the most frequently isolated type in Pahang (DVS, 2008).

1.3.2 Epidemiology

Infection with FMDV produces an acute, systemic vesicular disease. The epidemiology of FMD is a complex interaction between host, viral and environmental factors (Pereira, 1981; Donaldson, 2007). The viral factors that influence disease include virulence which affects the severity of lesions, the amount and duration of virus release, particle stability in different microenvironments and long-term persistence. In naturally acquired infections the most common route of virus dispersion is by direct contact with infected animals (Alexandersen and Donaldson, 2002). The clinical outcome of the disease varies between species and is dependent upon the infecting virus strain. It is a highly transmissible disease, and a limited number of infective particles can initiate host infection in susceptible animals (Sellers, 1971). Contaminated animal products, agricultural tools, vehicles (fomites) and humans can help contribute to the mechanical dissemination of the virus. Virus multiplication and spread also depends upon the host species and their nutritional and immunological status and their population density (Donaldson, 1987; Amass et al., 2004). Animal movements and contact between domestic and wild animals also contributes to the existence and maintenance of the disease. Geographical location can act as a barrier to the dissemination of virus or, alternatively, can promote virus transmission when appropriate atmospheric conditions prevail (Leforban and Gerbier, 2002).
1.3.2.1 Host species

Foot and Mouth Disease is a highly contagious viral disease of cloven-hoofed animals including domesticated ruminants, pigs and more than 70 wildlife species (Coetzer et al., 1994). The disease is characterized by fever, lameness and vesicular lesions on the tongue, feet, muzzle/snout, and teats (Kitching, 2002a). The severity of the disease can vary from mild or inapparent in some species to more severe in others. Pigs and cattle suffer more severe lesions compared to sheep and goats which develop only mild clinical signs and superficial lesions and consequently the disease can be difficult to distinguish from other common conditions in these species (Donaldson and Sellers, 2000). Although FMD does not result in high mortality in adults, the disease has debilitating effects including weight loss, decreased milk production and reduced draught power resulting in a loss of productivity for a considerable time. Mortality, however, can be high in young animals, where the virus can affect the cardiac muscle (Alexandersen et al., 2003a). Other domesticated animals such as water buffalo (*Bubalus bubalis*) can become infected and may also transmit the disease to other species and persistent infection in this species has important epidemiological significance (Samara and Pinto, 1983; Barros et al., 2007; Maroudam et al., 2008). Camelids can be infected experimentally by direct contact, even though they are not very susceptible and do not transmit the disease to other species (Wernery and Kaaden, 2004).

Studies in the African continent have indicated that wildlife species play a major role in maintaining and spreading the disease to susceptible domestic animals and wild ungulates (Hedger et al., 1980; Vosloo et al., 1996). In particular the African buffalo
(Syncerus caffer) is able to maintain infection for a long period of time and disseminate the virus (Vosloo et al., 2002). Occasionally FMD can be fatal to wildlife, as apparently occurred in South Africa in the late 19th century when large numbers of impala (Aepyceros melampus) succumbed to the disease. A recent study in South Africa indicated that subclinical infection of impala still occurred, especially in an area heavily populated with African Buffalo (Vosloo et al., 2009). Antibodies against FMD virus have been found in a range of wildlife in Africa including buffalo, eland (Taurotragus oryx), Grant's gazelle (Nanger granti), Thomson's gazelle (Eudorcas thomsoni), wildebeest (Connochaetes gnou), impala, waterbuck (Kobus ellipsiprymnus), sable (Martes zibellina) and topi (Damaliscus korrigum) (Anderson, 1981; Anderson et al., 1993). Feral pigs and wild white tail deer (Odocoileus virginianus) are also susceptible to FMD (Ward et al., 2007). Within the Asian region wildlife involved in FMD can include elephants, Sambar deer (Cervus unicolour), Spotted deer/Chital (Axis axis) and Barking deer (Muntiacus muntjak) (Barman et al., 1999). Serotype O was confirmed as affecting a group of 30 elephants after direct contact with infected cattle and buffalo in Kathmandu, Nepal (Pyakural et al., 1976), however serological studies in elephants from Africa have failed to detect an antibody response to the virus (Hedger et al., 1972).

1.3.2.2 Transmission

Transmission of FMDV is primarily from the infected animal itself, especially during the early febrile stage (Brown, 2004), with the typical spread in a herd or flock being 2 to 6 days for all species (Alexandersen et al., 2003c). Transmission can include direct contact between infected and susceptible animals, air borne spread of
virus either through inhalation or open wounds, indirect virus carriage by personnel and fomites and consumption of infected animal products by susceptible animals either through water or feed (Alexandersen et al., 2003c; Schijven et al., 2005).

Direct contact is the most efficient route of transmission for most livestock. However in cattle, air borne transmission is the most common route, unlike for pigs which need higher doses (Donaldson et al., 1987) and are relatively resistant to air-borne infection when compared with cattle and sheep (Alexandersen and Donaldson, 2002). Exposed humans can contribute to the transmission of FMDV through exhalation of viral particles (Amass et al., 2003a; Amass et al., 2004) which can persist in the human nasal passages for up to 28 hours after exposure (Sellers et al., 1970). However the most important role for transmission by humans is through contaminated clothing or fomites.

In pigs, goats and sheep, direct contact is considered to be the most common route of infection (Aggarwal et al., 2002). Although pigs are less susceptible to aerosol infection than cattle, they shed more virus per day in acute infections compared with cattle and sheep (Donaldson et al., 1970; Sellers et al., 1970; Kitching et al., 2005). Even though the virus has been detected in the semen of boars, the risk of sexual transmission is considered low (Guerin and Pozzi, 2005).

During the clinical phase of FMD, the virus is present in all secretions and excretions, and it may be excreted intermittently thereafter (Donaldson et al., 1987). Spread of FMDV from sheep and goats to other susceptible species is more significant in clinical or sub-clinical stages than those of carrier stages, even though they can also act as a carrier, such as with cattle (Barnett and Cox, 1999). The virus can also be recovered from the respiratory tract of infected animals for
approximately two days before clinical signs appear (Donaldson et al., 2001; Kitching, 2002a; Orsel et al., 2009) and can persist on wool for up to 14 days (Cottral, 1969). The movement of infected animals is considered to be the most important factor in the spread of FMD (Robinson and Christley, 2007) particularly with animals showing slight or no clinical signs of disease (Mansley et al., 2003), however the excretion pattern of virus differs in different host species and between vaccinated and non-vaccinated animals (Orsel et al., 2009).

1.3.2.3 Incubation period

The fact that the virus can be excreted during the incubation period is important in the epidemiology of the disease (Brown, 2004). The incubation period can be highly variable depending on host, agent and environmental factors including husbandry management factors (Hugh-Jones, 1976). Host factors include the species of animal and their health status; agent factors include strain, dose and route of infection; and the environmental factors include stocking density, ventilation system, transportation and stress due to handling (Hughes et al., 2002; Alexandersen et al., 2003b). The incubation period within a farm is 2 to 14 days (Hugh-Jones, 1976; Alexandersen et al., 2003c), however it can be as short as 24 hours in pigs challenged with a high dose (Alexandersen and Donaldson, 2002; Alexandersen et al., 2003c). In a recent study the virus was shown to be excreted 1 to 2 days after challenge in non-vaccinated dairy cattle and piglets in contrast to 3 to 3.5 days in non-vaccinated lambs (Orsel et al. 2009). These findings conflicted with earlier findings by Alexandersen and Donaldson (2002) who reported that pigs and other animals, such as sheep, normally had a shorter incubation period.
1.3.2.4 Survival on fomites

Foot and Mouth Disease virus is considered to be a moderately stable virus. At a pH between 7.0 and 8.5 most strains are stable, especially at lower temperatures (Bachrach, 1968). Unlike other picorna viruses the FMDV capsid dissociates at a pH of 6.5 or below. It is stable at a humidity above 55 to 60% but is sensitive to heat and desiccation (Bachrach, 1968). In contrast Bedson et al. (1927) observed a longer period of survival on dry rather than damp hay or bran. The survival of FMDV is also influenced by the nature of the materials as a high concentration of organic material helps the survival of the virus (Donaldson et al., 1987).

The virus can be recovered from the blood, pharynx, vagina and rectum up to 97 hours prior to the onset of vesicular lesions. It can also persist in mammary tissue for 3 to 7 weeks after infection (Burrows et al., 1971). The virus can survive outside the host, and potential sources of virus include excretions and secretions of infected livestock such as saliva, semen, milk, faeces, urine, and vaginal secretions (Bedson et al., 1927; Brown, 2004). The virus can also survive in skim milk, cream and the pelleted cellular debris components of milk obtained from FMD infected cows after the milk had been pasteurised at 72°C for 0.25 minutes and in cream components after heat treatment at 93°C for 0.25 minutes (Blackwell and Hyde, 1976). The FMDV can also survive for long periods in meat and animal products including frozen bone marrow, lymph nodes and offal (Henderson and Brooksby, 1948; Mahy, 2005). The average period of survival of FMDV on wool at 4°C is approximately 2 months with the period of survival decreasing considerably as the temperature increases to 18°C (McColl et al., 1995). The maximum estimated survival period of FMDV outside the host is approximately three months in regions with daily temperatures greater than 20°C (Bartley et al., 2002).
1.3.2.5 Carrier state

A carrier state of FMD is defined as an animal from which FMDV can be recovered more than 28 days after infection (McVicar and Sutmoller, 1969; Woodbury, 1995b; Alexandersen, 2002). Carrier animals are reported to be common in FMD-endemic areas (Salt, 2004), however the role of carriers is not well understood and there is controversy over their role in disease transmission.

A long-term asymptomatic persistent infection can follow the acute phase of infection and can include vaccinated animals which have been infected and exposed to the virus (Salt, 1993; Alexandersen, 2002; Salt, 2004). Persistent infection can last for a variable time in different animals and can be up to 3.5 years in cattle, 9 months in sheep, 4 months in goats but does not appear to occur in pigs (Alexandersen, 2002). The African buffalo is the only wildlife species known to be able to carry the virus and it can do this for periods up to 5 years (Bastos et al., 2000).

The development of a carrier stage in an individual animal is not correlated with the pre-existing antibody level. However host mechanisms, such as humoral antibody at protective levels which restricts viral replication in the oropharyngeal tissue, may be important in the persistence of the virus (Woodbury, 1995a). The prevalence of carriers in a population depends on the animal species present and the immune status of the population (Alexandersen, 2002). Consequently vaccinating animals to ensure a high proportion of animals are immune is considered an important strategy to control the disease when accompanied with intensive clinical surveillance for disease in susceptible animals (Arnold et al., 2008; Schley et al., 2009). However there is no
clear experimental evidence indicating that vaccination can either reduce the establishment or duration of a carrier stage in animals (Hedger, 1970).

1.3.3 Diagnosis

The rapid spread of FMD after the virus is introduced highlights the need for a quick and accurate diagnostic technique. The disease is often initially diagnosed based on clinical signs and therefore requires vigilance by the farming community and veterinary profession and the infrastructure to allow early reporting of disease. The OIE recommends confirming a diagnosis of FMD by either the isolation of the virus or by the detection of antigen and virus-specific antibodies. The determination of the serotype resulting in the infection is essential in order to administer emergency vaccination with an appropriate vaccine to control the disease. However serological diagnosis also has great importance, particularly for epidemiological purposes such as screening for antibody before animals are moved in a prevention and control program (Adam and Marquardt, 2002; Remond et al., 2002).

Numerous diagnostic techniques have been developed for FMD with the aim of developing a rapid, sensitive, specific and reliable method to effectively diagnose the disease. The diagnosis of FMD can be divided into 3 categories based on the detection of: clinical signs, virus, or antibody to the virus (Figure 1.3).
Figure 1.3: Different types of FMD diagnosis
1.3.3.1 Clinical Diagnosis

Cattle and pigs infected with FMDV usually show distinctive clinical signs after an incubation period of 2 to 8 days depending on the virulence of the virus. The disease is characterized by fever, anorexia and the appearance of vesicles on the mucous membranes of the mouth including the tongue, dental pad, gums and lips. On the feet, lesions are most prominent on the bulbs of the heel, along the interdigital cleft and to a lesser extent along the coronary bands. Lesions may also be present in the nares and on the muzzle, udder and the teats. In milking cows, there is a sudden drop in milk production associated with infection. Rumen and heart lesions are frequently found at necropsy, especially in animals prior to weaning. In sheep, goats and deer, lesions are usually not obvious and may go unnoticed making these species a potentially dangerous source of infection (Donaldson and Sellers, 2000).

However the clinical signs of FMD cannot be distinguished from other vesicular diseases such as vesicular stomatitis and swine vesicular disease. Furthermore in cattle, diseases including bovine papular stomatitis, bovine mucosal disease, infectious bovine rhinotracheitis, bovine herpes mammillitis, malignant catarrhal fever and rinderpest induce similar clinical signs and consequently laboratory diagnosis is essential for confirmation of the disease (Mowat et al., 1972; Anon, 2000).

1.3.3.2 Virological Diagnosis

Primary viral diagnosis of FMD is carried out on epithelial tissue or vesicular fluid from clinical samples using specific laboratory diagnostic techniques such as
Complement fixation tests (CFT), Enzyme link immunosorbent assays (ELISA) or Polymerase chain reactions (PCR) as recommended by the OIE.

1.3.3.2.1 Viral isolation

Cell culture is required to detect the presence of live virus in suitable samples. This procedure is recommended in the OIE manual (OIE, 2006) and is widely used by many FMD diagnostic laboratories. There are many types of cell lines used including calf thyroid cells (De Clercq, 2003b), 2 - 7 day old suckling mice cells, IBR – S2 cells, Lamb kidney cells (Snowdon, 1966), Pig cells (Bourma et al., 2001) and baby hamster kidney (BHK 21) (Clarke and Spier, 1980), each which have advantages and disadvantages.

1.3.3.2.2 Immunological methods

1.3.3.3.2.1 Complement Fixation Test (CFT) and Antigen Capture ELISA

The CFT has been the test of choice for the detection and typing of the FMDV in epithelial samples from the field and has been used in many laboratories over the last decade (De Clercq, 2003a). It is still recommended by the OIE but has gradually been replaced by the antigen capture ELISA due to the CFT’s disadvantages which are its relatively low sensitivity and the fact that it is prone to difficulty in interpretation due to both pro- and anti- complementary activity of samples. The sensitivity and specificity of the test is also dependent upon the animal species tested and is not sufficiently sensitive to detect infection (antigen) in pigs (Westbury et al., 1988c). Furthermore the CFT has more cross-reactions compared to the ELISA and the sensitivity of the test depends on the quality and type of samples collected (Hamblin et al., 1984). The advantages of the ELISAs are that they are easier to
perform compared to the CFT, and their sensitivities are generally higher than the CFT (Caballero et al., 1997; Smitsaart et al., 1997).

1.3.3.2.2 Nucleic acid recognition

The nucleic acid recognition methods are comprised of two different techniques (nucleic acid hybridization and PCR assays) which are dependent upon detection of the viral genome. The nucleic acid hybridization assay is no longer used as a diagnostic tool for FMDV as it is hampered by low specificity and sensitivity compared with the PCR, but it did prove very useful in elucidating the cellular basis of persistent infection (Zhang and Kitching, 2001).

Detection of viral RNA by a PCR is the diagnostic method of choice and several tests have been developed for FMDV. The PCRs are very sensitive as they require only a small quantity of sample. The fundamental requirements for PCRs are selection of the primers and an efficient extraction method. The primers selected should specifically target all FMDV types and subtypes without reacting with other unrelated viruses of the same family. Specific primers have been designed to distinguish between each of the seven serotypes (OIE, 2004). Efficient extraction of the FMDV RNA from field samples is a prerequisite for the successful amplification to prepare genetic material for sequencing. Among the techniques, the fluorogenic Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is rapid, with a higher sensitivity and specificity compared with the conventional RT-PCR. This test is more sensitive to Types O, A, C, and Asia1 than to SAT 1 and 2 (Reid et al., 2002).
The RT-PCR can also be used to amplify genome fragments of FMDV in samples other than epithelium or ruptured vesicles, including milk, serum, vesicular fluid and oesophageal–pharyngeal (OP) samples. Reverse transcription, combined with real-time PCR, has a better sensitivity and is quicker when compared to virus isolation (Huang et al., 2009).

1.3.3.2.3 Field tests

Field tests have been developed to accommodate the need for a rapid diagnosis which is necessary for the effective control of FMD to allow prompt action to control an outbreak (Reid et al., 2001) and at the same time avoiding the time taken to transport samples to the laboratory. Over the years field tests have developed from agglutination of particles linked to a specific antibody when antigen is present, to detection of the virus by using a portable real-time PCR instrument (King et al., 2008). However effective preparation of the template RNA is an important consideration for the use of this technology in the field, since the presence of tissue-derived factors may inhibit the RT-PCR (Hearps et al., 2002). The development and use of a rapid chromatographic strip test or pen side lateral flow device (LFD) have been evaluated. These use simple homogenizers that have been demonstrated to be suitable for preparing epithelial suspensions under field conditions (Ferris et al., 2009).

1.3.3.3 Serological Diagnosis

Serological diagnosis is another important method to confirm FMD. These techniques work by detecting an antibody response specific to FMD. They are very
useful for disease surveillance following an outbreak and to identify subclinical infection in species such as sheep and goats which have inapparent clinical signs. There are two different methods in serological diagnoses: those that detect antibody to structural proteins and those that detect antibody to non-structural proteins of the FMDV.

1.3.3.3.1 Testing antibody to structural proteins

1.3.3.3.1.1 Virus neutralization test (VNT)

The Virus Neutralization Test (VNT) is a serotype specific test which is recognised as a gold standard for the diagnosis of FMD (Clavijo et al., 2004; Kitching, 2004) and is recommended by the OIE as the prescribed test for trade (OIE, 2008a). However it requires cell culture facilities and takes two to three days to provide results. It is very laborious and is not a reliable test due to false positive reactions. The VNT is also not capable of differentiating antibodies that arise from natural infection and those induced by vaccination (Moonen et al., 2004).

1.3.3.3.1.2 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) were developed to overcome the disadvantages shown by the VNT and the Liquid phase blocking ELISA (LPBE) has been adopted by many laboratories worldwide for the routine screening of FMDV. These tests are easier to perform and correlate well with the VNT (Hamblin et al., 1986a). The ELISAs are also considered to be more reliable than the VNT and are useful for evaluating the immunological response of animals following infection and
vaccination (Hamblin et al., 1987). However they have some disadvantages including low specificity and the lack of stability of inactivated antigens (Clavijo et al., 2004). There are three types of ELISA: LPBE; Solid Phase Blocking ELISA (SPBE) which has been used on cattle for the detection of Type O virus antibodies and has been validated as a screening test and for the detection of antibody titre (Chenard et al., 2003); and Solid Phase Competitive ELISA (SPCE) which is more sensitive than the VNT in samples from sheep (Paiba et al., 2004). The SPCE is also more serotype specific than the VNT or LPBE (Mackay et al., 2001). The solid-phase ELISAs have better specificity (SPCE: 99.1% - 99.3% compared with LPBE - CEDITEST : 92.2%) and reproducibility than the LPBE and VNT for large scale serological testing (Niedbalski, 2004), however they are not able to differentiate immunity induced from natural infection with that from vaccination.

1.3.3.3.2 Testing antibody to non-structural proteins

Serological diagnosis through the testing of antibody to non-structural proteins (NSP) is valuable in the control of FMD, particularly in endemic areas and in areas which are free of FMD but where vaccination against the disease is practiced. Antibody to NSP produced by infected animals can be used to differentiate natural infection from a reaction to vaccination (Diego et al., 1997; Mackay et al., 1998; Sørensen et al., 1998; Clavijo et al., 2004; Moonen et al., 2004).

1.3.3.3.2.1 Agar gel Immunodiffusion test
The agar gel immunodiffusion test is a useful tool for epidemiological surveys of livestock, however the interpretation of results in animals that have been repeatedly vaccinated can be confusing (McVicar and Sutmoller, 1970).

1.3.3.3.2.2 Latex agglutination

Latex Agglutination Tests (LAT) or Latex Immunoassays involve a simple slide test and are inexpensive, when compared with other techniques (Bangs, 1988). Furthermore there is close agreement between the CFT and the LAT (Ozdural et al., 2002).

1.3.3.3.2.3 Immunoelectro transfer blot analysis

The Immunoelectro transfer blot analysis, along with ELISAs, can detect antibodies against recombinant 3AB1 proteins to differentiate between infected and vaccinated cattle (Clavijo et al., 2004). The application of the enzyme linked immunoelectro transfer blot assay has been upgraded from defining the antibody profile of infection to confirming suspected or positive samples (Bergmann et al., 2000). Western blot assays using NSP 2C and 3D as antigen are useful methods for the differentiation of infected from vaccinated pigs (Fukai et al., 2008).

1.3.3.3.4 Non-Structural Protein (NSP) ELISA

Presence of antibody against NSP 2C, 3A, 3B, 3C, 3AB and 3ABC are indicative of infection. Among these, the polypeptide 3ABC is recognized as the most appropriate antigen which has high immunogenicity (Bergmann et al., 2000; Robiolo et al., 2006). Previous studies have also indicated that serum antibody specific for NSP
3ABC is a reliable marker of FMDV replication in vaccinated cattle (Mackay et al., 1998; Sørensen et al., 1998). This is of great relevance for serological surveillance purposes following an outbreak to help substantiate freedom from FMD (Paton et al., 2006) and to distinguish between animals infected with FMD from those with immunity arising from vaccination against FMD to allow the safe movement of animals (Kitching, 2002b; Kweon et al., 2003). The NSP test is also used to differentiate vaccinated from convalescent animals and to determine the carrier status of animals (Niedbalski and Haas, 2003a; Clavijo et al., 2004). Many diagnostic methods to differentiate infection from vaccinated animals using NSP tests have been developed, with some being used for screening purposes and others as confirmatory tests. The ELISA-based diagnostic techniques for NSP antibody detection provide many advantages, such as objectivity compared with gel diffusion tests for NSP, high sensitivity and specificity, and the capability for large-scale screening. The NSP ELISA tests have been recommended by the OIE to be used for serological surveillance in regions or countries that practice vaccination against FMD and for monitoring virus circulation in the field (OIE, 2004b). The NS Panaftosa ELISA and Western Blot technique developed by Bergmann et al. (2000) has been accepted by the OIE as the standard test for discriminating infected from vaccinated groups of animals.

There are many commercial ELISAs available for the NSP. Six commercially available ELISA (NCPanaftosa-screening from PANAFTOSA; 3ABC trapping-ELISA from IZS-Brescia; Ceditest® FMDV-NS from Cedi Diagnostics B.V., Lelystad, The Netherlands; SVANOVIRM FMDV 3ABC-Ab ELISA from Svanova, Upsala, Sweden; CHEKIT-FMD-3ABC from Bommeli Diagnostics, Bern,
Switzerland; and the UBI® FMDV NS ELISA from United Biomedical Inc., New York, USA) have been compared (Brocchi et al., 2006). Five of these tests detect antibodies to the viral non-structural polypeptide 3ABC, expressed as recombinant antigen in different expression systems, while the UBI kit recognises antibody to a 3B synthetic peptide. The study showed that the Brescia ELISA and the Ceditest ELISA were more sensitive in non-vaccinated cattle than vaccinated cattle within 100 days of infection, with a sensitivity approaching 100%. Whereas in vaccinated cattle the sensitivity varied from 93.9% (Panaftosa ELISA) to 68.2% (Chekit ELISA). In contrast the specificities for all six ELISAs in non-vaccinated and vaccinated cattle were not significantly different (all being more than 98%) (Brocchi et al., 2006). However receiver-operator characteristic (ROC) curve analysis of the six NSP ELISAs showed the IZS Brescia and Ceditest had specificities of 99% and 99.5% respectively which were higher than the other tests in exposed cattle (Dekker et al., 2008). A study undertaken in New Zealand (Kittelberger et al., 2008) with the Ceditest FMDV NS Blocking ELISA and the Bommeli/IDEXX Checkit FMD 3 ABC indirect ELISA reported similar specificities (Brocchi et al., 2006) in cattle, sheep and pigs. The diagnostic specificity for Bommeli/IDEXX for cattle, sheep and pigs were 99.9, 99.7 and 99.6% respectively and for the Ceditest FMD NS 99.5, 99.7 and 99.6% respectively. The repeatability of the Ceditest FMD NS was reported to be better than the Bommeli/IDEXX test (Kittelberger et al., 2008).

Comparative evaluations, including the above NSP ELISA kits, were conducted by a consortium of European and American FMD reference laboratories in 2006 using large panels of sera from cattle that had been vaccinated or vaccinated-and-infected with different serotypes of FMDV. However in that study there were insufficient
samples from pigs and sheep to evaluate the tests for these species (Brocchi et al., 2006). The use of commercial NSP ELISAs is very useful for evaluating the status of a herd but not so for individual animals (Clavijo et al., 2004). Repeated vaccination may also result in positive reactions in cattle due to impurities in the FMD vaccine (Lee et al., 2006).

1.3.4 Control of FMD

Foot and mouth disease has occurred in almost two thirds of the OIE member countries. Recent epidemics in countries such as the United Kingdom and Taiwan have resulted in significant economic losses, however the disease remains endemic in many developing nations (Rweyemamu and Astudillo, 2002).

Social, economic and political factors in some countries in Asia can impact on the control of FMD (Anonymous, 2008). Although the economic status of a country has a significant impact on the ability of a country to control FMD, other factors also play key roles in the control of the disease. Geographical barriers can play an important role in the control of the disease, as does implementation of legislation on animal health and movement, the availability of expertise (both field and laboratory staff) and adequate diagnostic laboratory facilities. Since FMD is a transboundary animal disease, the control of it requires cooperation between neighbours at the local, national and regional level (Lubroth et al., 2007). Based on the status of FMD, OIE member countries are divided into FMD free countries and FMD endemic countries. The FMD free countries are further subdivided into three categories: FMD free without vaccination; FMD free with vaccination; and FMD free zone within the country (OIE, 2008b).
1.3.4.1 Vaccination

Vaccination is one of the tools used in the control of FMD and has been studied extensively. Vaccination has been demonstrated to limit transmission of FMDV to in-contact susceptible animals (Doel et al., 1994; Salt et al., 1998; Cox et al., 1999). Moreover some outbreaks of FMD that have been controlled by vaccination have not recurred (Barteling, 2002; Leforban and Gerbier, 2002). The ability of a vaccine to protect against the disease and prevent the spread of the virus is dependent upon the vaccine’s potency (Barnett and Carabin, 2002). However recent studies have indicated that protection is also affected by the serotype, as well as the type of adjuvant used in the vaccine (Jamal Syed et al., 2008).

Although culling has been reported to be more effective than vaccination, combination of both practices can allow for the more rapid control of the disease (Ferguson et al., 2001a). In countries which were previously free from FMD, the use of emergency vaccination and culling of animals suspected to be infected or originating from high risk areas, and restriction on animal and product movements can result in disease eradication (Orsel et al., 2005). Other studies have demonstrated that emergency vaccination, when implemented swiftly, will significantly reduce clinical disease and subclinical virus replication and shedding, particularly in the early stages of infection (Cox et al., 2007). However the significance of carrier animals in the epidemiology of FMD is still not clearly understood (Kitching et al., 2007) and there is a potential risk that they could subsequently lead to new outbreaks. Some studies have demonstrated that whether an animal becomes persistently infected is influenced by vaccination, where vaccination is able to reduce viral replication, persistence and excretion compared to unvaccinated and clinically
infected sheep (Barnett et al., 2001; Parida et al., 2008). However a model constructed by Schley et al. (2009) indicated that, although there was a potential to increase the number of undetected carrier animals following vaccination, vaccination offered significant benefits if administered prior to exposure to the virus. This finding was in contrast to that of others who consider the risk of persistently infected animals transmitting the disease is low (Cox et al., 2005).

In countries where FMD is endemic, vaccination can be used as a primary tool to suppress the disease. Vaccination is applied to induce herd immunity leading to a reduction in clinical infection and viral transmission (Orsel et al., 2007) and systematic vaccination has been shown to be an effective way of controlling and eliminating FMD from certain regions of the world, such as western Europe and south America (Leforban and Gerbier, 2002; Saraiva, 2004). A study by Anderson et al. (1974) indicated that vaccination was a useful tool for the eradication of FMD from an endemic area. The crucial part of a vaccination programme is conferring immunity to a sufficiently high proportion (>80%) of the population to prevent the infection spreading to susceptible animals. One study indicated that goats also need to be included in vaccination control programmes using the same schedule as for cattle (Madhanmohan et al., 2009). Susceptible animals also must be prevented from entering the vaccinated population. However implementation of a proper, systematic vaccination programme as a control measure can be difficult since vaccines are expensive, have a narrow antigenic spectrum, provide only short term immunity and are very fragile. This results in a significant economic cost for most developing countries where the disease is endemic (Kitching et al., 2007).
1.3.4.2 Movement Restrictions

Movement of infected animals is one of the most important factors in the spread of FMD, especially in an endemic region (Cleland et al., 1996; Rweyemamu et al., 2008b). Control of livestock movement, together with vaccination, can be effective in the control and eradication of FMD (Anderson et al., 1974). Therefore movement restrictions combined with effective ring vaccination, proper quarantine procedures and establishment of a buffer zone can be applied in certain predefined areas in an endemic country or region to create an FMD free zone. A zoning approach has been effectively applied in many parts of the world to help control the disease and this approach has been predicted to have a high level of success in Southeast Asia (Edwards, 2004). However it has also been suggested that proper quarantine procedures with vaccination, without movement restrictions, is more appropriate in Southeast Asia because of cultural and lifestyle reasons (Sasaki, 1994a).

1.3.4.3 Stamping out

Stamping-out involves the slaughter and destruction of all infected animals and their immediate susceptible contacts, followed by thorough cleaning and disinfection of the affected premises. It was first adopted in the United Kingdom in 1892 and has been used widely since (Fogedby, 1958). A stamping out policy is frequently implemented in previously FMD free countries to ensure the disease is completely eradicated in the event of an outbreak. This involves culling of all susceptible animals on infected or high risk premises and strict movement restrictions (Scudamore and Harris, 2002). Culling is considered a crucial step to regain FMD free status from the OIE, especially for economic reasons. The decision for radial
culling or vaccination is normally made based on a mathematical model developed for the local infected area such as in the 2001 outbreak in the UK (Ferguson et al., 2001a; Keeling et al., 2001).

Some countries believe stamping out by slaughter or culling is the most effective way to eradicate or control FMD. It is also often regarded as the cheapest and fastest way to regain the desired freedom status. However it gives rise to ethical issues, as well as the potential for environmental pollution and the negative psychological effects it can have on farmers (Woods, 2004). Consequently alternative methods have been investigated extensively. A combination of culling with emergency vaccination to provide early protective immunity (Doel et al., 1994; Cox et al., 1999) and reduced virus replication and excretion to limit transmission of the disease to susceptible animals has been recommended (Cox et al., 1999; Orsel et al., 2007; Parida et al., 2008). Furthermore the ability to detect infection in vaccinated animals by using the NSP antibody test further supports the use of emergency vaccination as a useful tool (Bruderer et al., 2004; El-Hakim, 2005). Therefore an optimal reactive and responsive vaccination programme, combined with efficient culling and restrictions on animal movements, could be effective control measures in FMD outbreaks (Tildesley et al., 2006). Other researchers believe that establishment of a quarantine-barrier to prevent the introduction of disease, and a veterinary and laboratory capability to rapidly detect the disease are important (Boyle et al., 2004). However the effectiveness of FMD control is also greatly influenced by the geographical situation. For example a stamping out policy was successful in the British Isles, Ireland, Scandinavia, and North America due to their favourable geographical isolation (Sutmoller et al., 2003). The greatest disadvantage of a stamping out policy is the extreme demand for resources including veterinarians,
slaughter teams, disinfection teams, heavy machinery and the permanent lost of genetic potential (Pinto, 2004).

1.3.4.4 Application of Models

The evaluation and creation of simulation models for FMD has advanced rapidly and models can be used to aid in the choice of control policies during an outbreak (Morris et al., 2002). However the creation of a reliable model requires the availability of good data. Models should be as simple as possible, transparent and easily adapted or extended with limitations and strengths clearly identified (Thornley and France, 2009). Some researchers consider that the strength of models lies in their ability to evaluate a wide variety of epidemiological parameters and control options (Tildesley et al., 2006). Many simulation models have been developed to formulate effective control strategies, however most are based on data from FMD free countries and optimal control policies are not always easily transferred between countries (Tildesley and Keeling, 2008). Some simulation models have been designed to help in the management of future outbreaks and to identify aspects which are critical for the rapid control and eradication of the disease (Yoon et al., 2006) while others have been used to predict the role of risk factors such as wild or feral animals (Ward et al., 2007), distribution of airborne particles (Gloster et al., 2007) and wind direction and velocity on airborne spread of virus between farms (Gabier et al., 2002).

Geographical Information Systems (GIS) have been used in spatial and temporal models. Much work has been done utilising existing historical local data (Ferguson et al., 2001b; Keeling et al., 2001; Picado et al., 2007). Some studies have only looked at disease occurrence patterns (Gallego et al., 2007) while others have
incorporated other factors, such as disease dynamics, host demography and logistical constraints, to formulate optimal disease control (Jacquez, 2008; Shiilegdamba et al., 2008; AlKhamis et al., 2009).

A model developed to estimate the prevalence of carriers of FMD after vaccination indicated that the prevalence of carrier-containing herds was likely to be very low (Arnold et al., 2008). In contrast other models have indicated an increase in the number of carrier animals (Schley et al., 2009). More advanced models integrate genetic data with the relative timing of infection events to compute the likely transmission of infection between farms (Cottam et al., 2008).

1.3.5 Disease Distribution

Foot and mouth disease is a global animal disease which is considered to be a major constraint to the international trade of livestock and animal products in infected countries. Outbreaks of FMD have occurred in every livestock-containing region of the world with the exception of New Zealand, and the disease is currently endemic in all continents except for Australia, Antartica and North America (Grubman and Baxt, 2004). Even though there are many countries free from FMD, the virus is widely distributed in the world. In contrast the distribution of the serotypes is not even throughout the infected regions and countries. Six of the 7 serotypes are present in Africa (O, A, C, SAT 1, 2, 3), 4 in Asia (O, A, C and Asia 1) and only 3 in South America (A, O, and C). Periodically infections with SAT 1 and SAT 2 occur in the Middle East originating from Africa (Rweyemamu et al., 2008b).
Foot and Mouth Disease is currently endemic in 7 Southeast Asian countries: Cambodia, Laos, Malaysia, Myanmar, parts of The Philippines, Thailand and Vietnam. Singapore, Brunei, Indonesia, East Malaysia and the Islands of Mindanao, Visayas, Palawan and Masbate in the Philippines are considered free without vaccination (OIE, 2009). The most common serotype present in this region is O with occasional outbreaks of serotype A (Sabirovic et al., 2009). The Southeast Asian topotype is considered to be the indigenous strain and two other topotypes present are the Pan-Asia and Cathay strains. Topotype Cathay is a pig adapted strain. The Cathay and Pan Asia O topotypes originated from South China and crossed into Vietnam and then spread westward into Cambodia, Laos and eventually Thailand. Asia 1 followed the movement of livestock from Bangladesh (which imports up to 2 million head of cattle each year from India) into Myanmar and finally followed the route of livestock movement down to Malaysia (Rweyemamu et al., 2008b). The movement of livestock resulted in the spread of the disease from the southern part of Thailand to the northern part of Malaysia. Clustering of outbreaks occur during the months of October to December when there is a high demand for live cattle because of the holding of local festivals in Malaysia (Sasaki, 1994b). A similar pattern of outbreaks following the movement of cattle has also been recognised in the southern regions of Cambodia and Vietnam (Sasaki, 1994b). Other than cattle movement, contact with infected cattle at grazing/watering areas has been recognised as important in Myanmar (Kyaw Naing Oo, personal communication 2010) and in the Philippines spread has been reported to occur mainly through contaminated fomites (Abila and Foreman, 2006).
1.4 Overview of the current study

Although FMD is endemic in peninsula Malaysia, including the state of Pahang, there is a deficiency of knowledge about the epidemiology of the disease in Pahang. Consequently the work reported in this thesis was developed to address this lack of knowledge. The specific objectives of the study reported in this thesis were:

1. To determine the temporal and spatial distribution and pattern of historical FMD outbreaks in Pahang, Malaysia
2. To identify the risk factors associated with the occurrence of FMD in Pahang
3. To study the antibody response in local cattle following vaccination against FMD.

The information gained from this study will help further the understanding of the epidemiology of FMD in Pahang which, in turn, will help in the development of suitable methods for controlling the disease.

In the following chapter the materials and methods adopted in this thesis are summarised.
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Serological tests for measurement of antibodies against FMD

Whole blood from cattle was collected from the jugular vein in plain blood tubes (BD Franklin Lakes USA). After collection the tubes were stored at a 45° angle at room temperature for approximately one hour and then placed in a refrigerator at 4°C for 12 hours. The tubes were then centrifuged at 2000 rpm for 10 minutes and the sera decanted. The sera were stored at -20°C until used in the assay for FMD.

2.1.1 Test for antibody against non-structural proteins

A commercially available NSP ELISA (Ceditest FMDV-NS ELISA manufactured by Cedi Diagnostics, B.V., Lelystad, The Netherlands) was used to test for antibodies to NSP’s (Sørensen et al., 1998). The Ceditest is a blocking ELISA which can be used for the detection of antibodies against FMDV in serum from cattle, sheep, goats and pigs. It was used to detect antibody directed against the non-structural 3ABC protein of FMDV as described in Appendix 1.

2.1.2 Post-vaccination evaluation test

A liquid phase blocking ELISA for detection of FMDV (Serotypes O, A and Asia 1) antibodies was ordered from the FMD world reference laboratory (WRL), Pirbright, United Kingdom. The test is based upon specific blocking of FMDV antigen in liquid phase by antibodies in the test serum sample (Hamblin et al., 1986b, a). The
test procedure adopted was obtained from the FMD WRL, Pirbright as described in Appendix 2.

2.2 Statistical and descriptive analysis

All recorded data in this study, such as the questionnaire data in Chapter 3 and the percentage of inhibition data for the antibody response towards FMDV vaccination as described in Chapter 4, were entered into a spreadsheet in Microsoft excel 2007 and exported into a statistical package (SPSS version 17 Statistics GradPack for Windows) for analysis. Descriptive analyses were performed using the frequency and percentage function. Percentages and their 95% confidence intervals using the exact binomial method were calculated. Before any statistical tests were performed, all recorded information was carefully compared with the paper questionnaires to identify any typographical or transcription errors.

All hypothesized risk factors (categorical and continuous variables) were individually screened for association with a history of FMD clinical infection. Initially univariable analyses were conducted with a Chi-Square test for independence or Fisher’s exact test to investigate possible associations between categorical variables and a clinical history of FMD. Results were considered to be significant if $P < 0.05$. Odds ratios (OR) and their 95% confidence intervals were also calculated (Pallant, 2004; Thrusfield, 2005). Factors with a significance level of $P < 0.2$ and/or which were considered to be biologically important were offered to the multivariable binary logistic regression model.
Continuous variables were tested for normality by performing a Kolmogorov-Smirnov and Shapiro-Wilk test for normality. Skewness, kurtosis and P-P plot were also examined. The data were considered to be normally distributed if the significance for the Shapiro-Wilk was > 0.05, skewness was zero or close to zero; the Kurtosis was zero or very near zero and all data points were symmetrical and perpendicular to the diagonal line of the P-P plot (Field, 2009). The linearity of continuous variables was also examined. The mean values of normally distributed continuous variables were analysed for significant difference with an ANOVA. Continuous variables which were not normally distributed and were not linear were categorised prior to analysis. The log odds ratios for each group for these variables were plotted against the midpoint for that group prior to inclusion in the multivariable logistic regression model. A correlation coefficient of 0.7 or higher was used to support the assumption of linearity (Hill and Ward, 2008). Continuous variables with a $P < 0.2$ and/or which were considered to be biologically important were then subsequently included in the multivariable binary logistic regression model.

Potential multi-collinearity between predictor variables (close linear relationship between two or more variables) was assessed by checking the collinearity parameters between each independent variable through calculating a Variable Inflation Factor (VIF). Variables were considered to have a high level of multicollinearity if the collinearity value was greater than 0.8 and the VIF greater than 1 (Field, 2009). The correlation between variables was assessed by calculating Pearson’s or Spearman’s
correlation coefficients, where appropriate. If the variables were not mutually exclusive and consequently highly correlated, only one of the related variables (selection was based on predictor variables that were more highly related to the infection) was included in the initial model. The decision to include/exclude variables was made on the basis of distribution and biological plausibility (Dohoo et al., 2003).

A binary logistic regression model was built using a manual backwards elimination process as described by Dohoo et al. (2003), with a significance level of $P > 0.05$ as the criterion for removal of a variable from the model. Variables with high collinearity ($r > 0.8$) were excluded from the analysis. The analysis commenced with a full or saturated model and variables were eliminated from the model in an iterative process. The level of significance for a factor to remain in the final model was set at 5%. The suitability of the final model was assessed based on the Hosmer and Lemeshow, Cox and Snell R Square and Nagelkerke R Square values (Field, 2009).

The percentage of inhibition (PI) data were analysed using the repeated measures analysis of variance (Repeated-Measures ANOVA) to describe the within random samples variance rather than the between group variances. In this experiment the sample members were matched according to the age of the cattle (calf and cow groups) which had been vaccinated once or twice. The analysis for this study is outlined in more detail in Chapter 4.
2.3 Questionnaire

A standardized questionnaire containing open and closed questions was developed in English and then translated into Bahasa Melayu. It was also translated back into English to correct any misunderstanding in the use of language before the final version was produced (Lee et al., 1999). The questionnaire (Appendix 3) was then pretested in Pahang on ten Assistant Veterinary Officers (AVO) and ten cattle owners. The use of the questionnaire was approved by the Murdoch University Human Ethics Committee. The questionnaire was designed to collect information about the management, husbandry and health of cattle and to determine the association between these factors and the presence of FMD infection. Questions were included on the importation or purchase of cattle for breeding purposes, the practice of exchanging cattle with other farmers, the vaccination program adopted, the feed supplements provided to cattle, source of water, practices adopted if cattle with positive serology were detected, farm biosecurity practices adopted, presence of other animals on the farm, distance to the nearest farms with livestock, and the farmer’s knowledge about the clinical signs of FMD and the treatment and control of the disease (Appendix 3).
CHAPTER 3

RISK FACTOR ANALYSIS ASSOCIATED WITH FOOT AND MOUTH DISEASE IN THE STATE OF PAHANG, MALAYSIA.

3.1 Introduction

Pahang is one of 13 states in Malaysia and is the third largest state after Sabah and Sarawak. It is situated in the eastern coastal region of Peninsular Malaysia and has an area of 3,596,585 hectares and contains 11 districts and 71 subdistricts. The physical geography can be broken down into 3 sections: highlands, rainforest and coastal areas with rainforest accounting for approximately 2/3 of the land area (Anonymous, 2007). The major economic activity is agriculture, primarily oil palm cultivation and rubber plantations (Pahang, 2007). In 2009 the ruminant population in Pahang was: 156,753 cattle, 19,884 buffalo, 37,712 goats and 11,395 sheep (DVS, 2009c). Cattle are commonly raised to supplement household income and for slaughter during religious and other special occasions. This situation makes the control of disease more challenging because of the low priority farmers place on their animals. Many cattle owners also keep small numbers of sheep and goats (Figure 3.1); however they do not raise pigs (DVS, 2009c). Although pig farming is not encouraged by the state government, importation of live pigs from other states is allowed. These pigs are transported on lorries to the local pig slaughter houses located in the 4 districts of Kuantan, Raub, Bera and Temerloh.

In late December 2003 until 2004, Pahang experienced the largest epidemic of FMD in the state’s history after 18 years of freedom, as described in Chapter 1. The epidemic was caused by FMD virus serotype O and was suspected to have been
introduced through the illegal movement of infected cattle from Terengganu (the neighbouring state). This epidemic spread rapidly in the state involving many of the districts; however no detailed epidemiological studies were undertaken to evaluate the relative importance of potential risk factors associated with the outbreak.

Currently there is a control program for FMD in Pahang and mass vaccination was one of the options considered in this control program, however due to the cost of mass vaccination the program was revised. A trivalent vaccine (0, A and Asia 1) is administered by the DVS twice a year to give at least 80% coverage in the ruminant population across the state (DVS, 2002). The policy to control local outbreaks involves ring vaccination with the trivalent vaccine, together with the control of animal movements. Despite this program, the disease is endemic in the state. There are many possible reasons for a considerable number of outbreaks occurring each year in the face of vaccination. However a thorough understanding of the epidemiology in the state is required to design ecologically and culturally acceptable and appropriate control measures for this important trans-boundary animal disease. The aim of the research outlined in this chapter was to identify management and husbandry factors adopted in Pahang that facilitate the distribution of FMD within the state and factors which were protective against the disease.
3.2 Materials and methods

3.2.1 Study Area

The study was conducted from August 2008 to June 2009 and involved ten of the 11 districts in Pahang. The temperature is relatively uniform throughout the year (range of 21°C to 32°C) and the humidity is approximately 80% throughout the year. During the months of January to April, the weather is generally dry and warm. Pahang has an average annual rainfall of 2,000 to 2,500 mm with May to December being the wettest months. The ten districts included in this study were selected based on their cattle rearing activity. Cameron Highland was the only district not included due to the fact that no cattle rearing activity is undertaken in the district. No prior sampling frame existed; therefore this study was constructed using the DVS Pahang Cattle Rearing Activity Record and historical reported cases of FMD.
3.2.2 Study design

A case-control study was conducted based on the clinical history of FMD recorded by the DVS together with data from cattle rearing activity records. The cattle rearing activity record was collected from the DVS in Pahang and compared with data on the occurrence of FMD from the DVS Pahang records. A stratified multistage random sample of cattle herds involving 200 cattle owners (participants) was formulated. Twenty cattle owners who owned more than 15 cattle from each of the 10 districts were chosen (total 200 cattle owners). Ten of the owners selected from each district had a clinical history of FMD in their farms (case group) and 10 owners had not had a clinical history of FMD in their farms (control group). The cattle owners were notified of the survey by telephone and a letter explaining the purpose of the research, together with a consent form and information about the confidentiality of the farmer’s identity was then sent to the participants. The questionnaires had been approved by the Human Ethics Committee at Murdoch University (Human Ethics Permit 2008/194). One Assistant Veterinary Officer from each district was chosen to distribute and collect the questionnaire from the selected participants. Each cattle owner was given a letter explaining the study, confidentiality of the data and participants, the studies objectives and the participant’s ability to withdraw from the survey at any time.

3.2.3 Questionnaire

The development of the questionnaire was outlined in Chapter 2.
3.2.4 Data analysis

The questionnaires were collected and analysed as described in Chapter 2.

3.3 Results

3.3.1 Descriptive analysis

Of the 200 farmers selected, 176 completed questionnaires but only 171 could be used as 5 respondents had less than the minimum number of 15 cattle (all these 5 respondents were from the districts of Kuala Lipis). Completed surveys were obtained from 93 controls and 78 cases. No questionnaires were obtained from the district of Pekan as the key person could not be contacted. Twenty surveys were obtained from each of the other districts except for Temerloh where four farmers did not participate.

The result of the study indicated that there was active movement of cattle within the state, with 98% of respondents trading cattle in the year preceding the survey. All these respondents traded animals with other farmers in Pahang. In contrast few respondents imported cattle from other countries (1.8%) or from other Malaysian states (2.4%). Some (14%) respondents exchanged animals with other farmers and 25% introduced bulls from outside their herd for breeding purposes.

Approximately half (53%) of the respondents allowed their cattle herds to graze freely and 51% of herds were grazed close (within 2 km) to other herds. People, other than the family or workers, were allowed to enter the land where the cattle were grazed by over half (58%) of the respondents.
Some of the questions in the questionnaire were designed to assess the farmer’s knowledge regarding the clinical signs, spread, prevention and control measures implemented for FMD. Approximately 87% of all participants (89.6% of the case group and 87.5% of the control group - \( OR = 1.23 (0.48, 3.18) \)) were able to describe the clinical signs of FMD. However only 36.9% of all participants (51.6% case group, 40.4% control group - \( OR = 1.57 (0.84, 2.93) \)) were able to explain the way the disease spread while a larger proportion (overall 59.5%, with 54.5% and 63.5% in the case and control groups respectively - \( OR = 0.69 (0.37, 1.27) \)) of participants admitted that they did not have any knowledge on how the disease spread. However there were no significant differences between control and case groups for these factors (95% CI for OR included the value 1.0). Just over half (56%) of the participants identified vaccination as one of the measures to control the disease, however only 29% understood that vaccination of animals was required every 6 months. Approximately one third (32.7%) believed it (vaccination) should be administered only once a year, 1% replied three times a year and 37% did not know the required frequency of vaccination. The respondents also gave a wide range of the age at which vaccination should be commenced, starting from immediately after birth to more than one year of age, however 60% of the respondents did not know the age cattle should be when they received their first vaccination.

### 3.3.2 Univariable analysis

In Tables 3.1 and 3.2 the potential risk factors for the disease and their association with case and control farms is displayed. A total of 18 from 31 potential risk factors
met the criteria for inclusion into the multivariable logistic regression analysis (indicated by asterisks in the tables). Most of these were factors relating to biosecurity. The factors that increased the likelihood of FMD were owning other livestock, owning goats, exchanging stock, serological reactors to FMD remaining in the herd after testing, free grazing of pasture all the time, purchasing of new cattle from other farms, allowing unauthorised vehicles to enter the farm, not giving feed supplements, not practicing quarantine procedures, not fencing the area where the herd grazed, not disinfecting tyres, vehicles or footwear of visitors prior to entering the herd, animals not tested for FMD prior to entering the herd or before leaving the herd and separating cattle according to their age group.
Table 3.1 Univariable analyses of putative risk factors for foot and mouth disease in cattle in Pahang

<table>
<thead>
<tr>
<th>Factor</th>
<th>% herds positive for FMD</th>
<th>Odds Ratio (OR)</th>
<th>95% confidence intervals</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other livestock owned</td>
<td>56.7</td>
<td>2.29</td>
<td>1.22, 4.32</td>
<td>0.01*</td>
</tr>
<tr>
<td>No other livestock owned</td>
<td>36.4</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goats owned</td>
<td>59.2</td>
<td>2.30</td>
<td>1.17, 4.55</td>
<td>0.015*</td>
</tr>
<tr>
<td>Goats not owned</td>
<td>38.7</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffalo owned</td>
<td>53.3</td>
<td>1.53</td>
<td>0.69, 3.38</td>
<td>0.291</td>
</tr>
<tr>
<td>Buffalo not owned</td>
<td>38.7</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deer owned</td>
<td>100.0</td>
<td>0.44</td>
<td>0.37, 0.53</td>
<td>0.264</td>
</tr>
<tr>
<td>Deer not owned</td>
<td>44.3</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palm Kernel Cake given as a feed supplement</td>
<td>37.2</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palm Kernel Cake not given</td>
<td>52.4</td>
<td>1.86</td>
<td>3.45, 1.01</td>
<td>0.047*</td>
</tr>
<tr>
<td>Tap water used for drinking water</td>
<td>38.5</td>
<td>0.75</td>
<td>0.32, 1.78</td>
<td>0.513</td>
</tr>
<tr>
<td>Tap water not used for drinking water</td>
<td>45.4</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>River water used for drinking water</td>
<td>45.4</td>
<td>1.22</td>
<td>0.58, 2.56</td>
<td>0.601</td>
</tr>
<tr>
<td>River water not used for drinking water</td>
<td>40.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Different age groups separated</td>
<td>9.1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Different age groups not separated</td>
<td>50.0</td>
<td>1.0</td>
<td>43.48, 2.26</td>
<td>0.000*</td>
</tr>
<tr>
<td>Quarantine procedures practiced</td>
<td>32.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quarantine procedures not practiced</td>
<td>51.3</td>
<td>2.23</td>
<td>4.51, 1.12</td>
<td>0.022*</td>
</tr>
<tr>
<td>Herd area fenced</td>
<td>40.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd area not fenced</td>
<td>58.1</td>
<td>2.08</td>
<td>4.22, 1.03</td>
<td>0.039*</td>
</tr>
<tr>
<td>Factors</td>
<td>% herds positive for FMD</td>
<td>Odds Ratio (OR)</td>
<td>95% Confidence interval</td>
<td>P</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>--------------------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Disinfectant used at the farm entrance</td>
<td>27.6</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disinfectant not used at the farm entrance</td>
<td>48.9</td>
<td>2.51</td>
<td>6.06 , 1.04</td>
<td>0.036*</td>
</tr>
<tr>
<td>Unauthorised person allowed to enter the herd/farm</td>
<td>47.4</td>
<td>1.28</td>
<td>0.69 , 2.38</td>
<td>0.442</td>
</tr>
<tr>
<td>Unauthorised person not allowed to enter herd/farm</td>
<td>41.4</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unauthorised vehicles allowed to enter the herd/farm</td>
<td>50.0</td>
<td>1.94</td>
<td>0.99 , 3.83</td>
<td>0.052*</td>
</tr>
<tr>
<td>Unauthorised vehicles not allowed to enter herd/farm</td>
<td>34.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm registered with DVS Pahang</td>
<td>47.0</td>
<td>1.55</td>
<td>0.71 , 3.41</td>
<td>0.271</td>
</tr>
<tr>
<td>Farm not registered with DVS Pahang</td>
<td>36.4</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle kept in a barn and fed</td>
<td>58.8</td>
<td>1.89</td>
<td>0.68 , 5.23</td>
<td>0.215</td>
</tr>
<tr>
<td>Cattle not kept in a barn and fed</td>
<td>43.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free grazing allowed for a period of time</td>
<td>41.3</td>
<td>0.80</td>
<td>0.43 , 1.51</td>
<td>0.496</td>
</tr>
<tr>
<td>Free grazing not allowed</td>
<td>46.7</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free grazing practiced all the time</td>
<td>50.0</td>
<td>1.75</td>
<td>0.93 , 3.30</td>
<td>0.082*</td>
</tr>
<tr>
<td>Free grazing not practiced all the time</td>
<td>36.4</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd vaccinated against FMD</td>
<td>42.2</td>
<td>0.66</td>
<td>0.32 , 1.35</td>
<td>0.252</td>
</tr>
<tr>
<td>Herd not vaccinated against FMD</td>
<td>52.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exchange livestock with other farmers</td>
<td>65.2</td>
<td>2.57</td>
<td>1.03 , 6.47</td>
<td>0.039*</td>
</tr>
<tr>
<td>Don’t exchange livestock</td>
<td>42.1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>% herds positive for FMD</td>
<td>Odds Ratio (OR)</td>
<td>95% Confidence interval</td>
<td>P</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>New stock brought into the herd</td>
<td>38.9</td>
<td>0.77</td>
<td>0.36, 1.63</td>
<td>0.493</td>
</tr>
<tr>
<td>New stock not brought into the herd</td>
<td>45.3</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New cattle purchased from friends</td>
<td>53.1</td>
<td>3.02</td>
<td>0.68, 13.51</td>
<td>0.138</td>
</tr>
<tr>
<td>New cattle not purchased from friends</td>
<td>27.3</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New cattle introduced into the herd</td>
<td>52.0</td>
<td>1.53</td>
<td>0.78, 2.97</td>
<td>0.212</td>
</tr>
<tr>
<td>New cattle not introduced into the herd</td>
<td>41.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outside bull introduced into the herd</td>
<td>41.7</td>
<td>0.86</td>
<td>0.41, 1.81</td>
<td>0.685</td>
</tr>
<tr>
<td>Outside bull not introduced into the herd</td>
<td>45.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical inspection by veterinarian practiced before buying new animals</td>
<td>48.7</td>
<td>0.82</td>
<td>0.45, 1.57</td>
<td>0.547</td>
</tr>
<tr>
<td>Physical inspection by veterinarian not practiced before buying new animals.</td>
<td>53.7</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood test performed on cattle before entering herd</td>
<td>26.53</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood test not performed before cattle enter the herd</td>
<td>51.33</td>
<td>2.92</td>
<td>6.10, 1.40</td>
<td>0.003*</td>
</tr>
<tr>
<td>Blood test performed on cattle before leaving the herd</td>
<td>28.07</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood test not performed before cattle leave the herd</td>
<td>51.42</td>
<td>2.71</td>
<td>5.41, 1.36</td>
<td>0.004*</td>
</tr>
<tr>
<td>FMD seropositive animals remain in the herd after testing</td>
<td>70.3</td>
<td>3.94</td>
<td>1.79, 8.68</td>
<td>0.000*</td>
</tr>
<tr>
<td>Seropositive animals removed from the herd</td>
<td>37.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>% herds positive for FMD</td>
<td>Odd Ratios (OR)</td>
<td>95% confidence interval</td>
<td>P</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Seropositive animals slaughtered</td>
<td>42.5</td>
<td>0.76</td>
<td>0.40, 1.45</td>
<td>0.407</td>
</tr>
<tr>
<td>Seropositive animals not slaughtered</td>
<td>49.2</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seropositive animals isolated on the same farm</td>
<td>43.8</td>
<td>0.95</td>
<td>0.44, 2.06</td>
<td>0.889</td>
</tr>
<tr>
<td>Seropositive animals not isolated on the same farm</td>
<td>45.1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Univariable analyses of potential continuous risk factors for FMD in cattle in the state of Pahang.

<table>
<thead>
<tr>
<th>Variable name</th>
<th>Mean positive</th>
<th>Mean Negative</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of herds</td>
<td>2.45</td>
<td>1.51</td>
<td>14.899</td>
<td>0.000*</td>
</tr>
<tr>
<td>Number of cattle</td>
<td>123.65</td>
<td>125.98</td>
<td>0.014</td>
<td>0.905</td>
</tr>
<tr>
<td>Number of new cattle introduced</td>
<td>7.84</td>
<td>9.68</td>
<td>0.177</td>
<td>0.675</td>
</tr>
<tr>
<td>Number of new cattle introduced in a year</td>
<td>2.05</td>
<td>0.76</td>
<td>3.414</td>
<td>0.066</td>
</tr>
<tr>
<td>Number of cattle sold in a year</td>
<td>34.6</td>
<td>25.67</td>
<td>0.334</td>
<td>0.564</td>
</tr>
<tr>
<td>Number of cattle vaccinated recently</td>
<td>84.95</td>
<td>106.24</td>
<td>0.749</td>
<td>0.389</td>
</tr>
<tr>
<td>Number of vaccinations used in a year</td>
<td>1.44</td>
<td>1.52</td>
<td>0.577</td>
<td>0.449</td>
</tr>
</tbody>
</table>

*: Factors included in the Logistic Regression Model

3.3.3 Multivariable analysis
The final model was produced using backward conditional binary logistic regression and the results are displayed in Table 3.3. Five variables (factors) were retained in the final model, of which three were associated with the disease and two were protective. The most strongly associated factor with FMD was retaining seropositive animals in the herd ($P = 0.006; \text{OR}=3.62; 95\% \text{ CI 1.44, 9.11}$). Cattle farmers who kept other livestock were more likely ($P=0.003; \text{OR} 3.2; 95\% \text{ CI 1.47, 7.07}$) to have an infected FMD herd than owners who didn’t keep other species of livestock. Farmers which allowed the entry of unauthorised vehicles onto their farmland were also more likely to have an infected herd ($P=0.05; \text{OR} = 2.2; 95\% \text{ CI 1.0, 4.82}$).

Farmers who owned only one herd were less likely ($P=0.001; \text{OR} = 0.27; 95\% \text{ CI 0.12, 0.60}$) to have an infected herd than farmers with more than one herd. Separating a herd into different age groups also provided some protection against the disease ($P=0.02; \text{OR} = 0.15; 95\% \text{ CI 0.03, 0.72}$).

The final model had a good fit with a chi square value for the Hosmer and Lemeshow Test of 3.48 ($P = 0.9$). The Nagelkerke R square was 0.344 suggesting that 34.4% of the variability could be explained by the final set of variables.
Table 3.3: Multivariable analysis of potential risk factors for foot and mouth disease in cattle in Pahang.

<table>
<thead>
<tr>
<th>Variable name</th>
<th>B</th>
<th>P</th>
<th>OR</th>
<th>95% CI for OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Own only one herd</td>
<td>-1.300</td>
<td>0.001</td>
<td>0.273</td>
<td>0.124</td>
</tr>
<tr>
<td>Keep other livestock</td>
<td>1.171</td>
<td>0.003</td>
<td>3.224</td>
<td>1.471</td>
</tr>
<tr>
<td>Cattle separated according to age groups</td>
<td>-1.926</td>
<td>0.018</td>
<td>0.146</td>
<td>0.029</td>
</tr>
<tr>
<td>Positive NSP animals remain in the herd</td>
<td>1.285</td>
<td>0.006</td>
<td>3.615</td>
<td>1.435</td>
</tr>
<tr>
<td>Allow unauthorised vehicles to enter the herd area (farm land)</td>
<td>0.774</td>
<td>0.057</td>
<td>2.168</td>
<td>0.976</td>
</tr>
<tr>
<td>Constant</td>
<td>-0.419</td>
<td>0.343</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**3.4 Discussion**

The final model contained three factors associated with infection and two protective factors. Not surprisingly a history of positive serology to FMD was strongly associated with clinical disease. Studies have shown that FMDV can be transmitted from infected to susceptible animals by a variety of mechanisms including air borne spread (Hyslop, 1965a; Alexandersen et al., 2003b; Schijven et al., 2005) with pigs excreting the most virus followed by cattle and sheep (Donaldson et al., 1970). However direct contact is the most common route of transmission (Donaldson et al., 1987) and importantly the virus can be excreted during the incubation period.
(Alexandersen, 2002; Brown, 2004; Orsel et al., 2009) and animals incubating the disease play an important role in the dissemination of the virus during this period (Sutmoller and Olascoaga, 2002). Therefore while a shedder remains in a herd the risk of virus transmission to susceptible animals remains. Based on the finding that herds that retain seropositive animals were 3.6 times more likely to be infected, it is advisable to cull/dispose of FMD seropositive reactors as soon as possible after identification. However in vaccinated herds, detection of NSP positive results do not necessarily mean animals are infected and further testing is required before the culling or disposal of seropositive animals can be recommended (Clavijo et al., 2004; Scudamore, 2004). Cattle owners are reluctant to cull/dispose of NSP seropositive animals probably as a result of not having a clear understanding about the disease, particularly with respect to its spread and control. This was confirmed in the descriptive analysis where only 37.3% of respondents were able to correctly explain how FMD spread. Therefore further extension and education programs, to increase the cattle owner’s knowledge on FMD, are essential in order to help them understand the disease and to appreciate the implementation of more suitable and effective control programs. In Pahang there is no compensation for the culling of clinically affected or NSP seropositive animals and consequently there is little financial incentive to support culling (Rossides, 2002; Cassagne, 2002).

Respondents who also owned other livestock (predominantly small ruminants - sheep and goats and buffalo - *Bubalus bubalis*) had increased odds of having an infected cattle herd. Others have also highlighted the role of owning other livestock in increasing the risk of FMD infection in a herd (Al-Majali et al., 2008). Another study in Thailand reported that co-grazing of cattle and buffalo with other livestock
on a farm was commonly linked with FMD outbreaks (Cleland et al., 1995). Foot and mouth disease can infect all cloven-hoofed animals, including domestic and wild ruminants and pigs. The virus usually induces milder clinical signs in adult sheep when compared with cattle or pigs, and these signs are often subtle enough to go undetected (Alexandersen et al., 2003c). Furthermore studies have demonstrated that the virus can be detected in the respiratory tract of sheep 1 to 2 days prior to the appearance of clinical signs (Sellers and Parker, 1969; Donaldson et al., 2001; Kitching, 2002a; Orsel et al., 2009). The fact that some animal species, such as sheep and goats, may not exhibit obvious clinical signs makes the potential for spread of infection considerable (Barnett and Cox, 1999; Alexandersen et al., 2003c). Sheep and goats are considered to be important sources of FMDV during the first seven days of clinical or subclinical infection (Barnett and Cox, 1999) with the intensity of contact between animals important determinants of disease (Quan et al., 2009). Other studies have also identified that buffalo (Bubalus bubalis) can act as a source of infection for cattle and other animals (Gomes et al., 1997; Maroudam et al., 2008). As with cattle, buffalo can excrete the virus prior to the development of clinical signs. As different animal species have different incubation periods (Hugh-Jones and Tinline, 1976; Orsel et al., 2009), the ability of the virus to be transmitted during the incubation period increases the risk of the disease being transmitted between different animal species (Brown, 2004).

Allowing unauthorised vehicles to enter a herd without any restrictions or precautions, such as using tyre washers or sprays at the entrance to the farm, was another factor associated with diseased herds in this study. It is well documented that biosecurity measures are very important in minimising the entry and spread of the
disease (Amass et al., 2003b). The virus is known for its ability to survive outside reservoir hosts on fomites (Bedson et al., 1927; Hyslop, 1965b; Brown, 2004), especially those with a high organic matter (Donaldson and Ferris, 1975). Consequently soil on vehicles’ tyres has the potential to spread the virus from an infected to a susceptible herd. Furthermore contaminated boots and clothing may also transmit FMDV (Amass et al., 2003b; Amass et al., 2004).

The multivariable analysis showed owning only one herd was protective for infection. Cattle owners could act as mechanical carriers for FMDV by sharing equipment between herds, or by the movement of cattle from one herd to another. Movement of infected animals is considered the biggest threat of entry of the disease into a free area or herd (Abila and Foreman, 2006).

Foot and mouth disease can infect cattle at any age, but, as with other diseases, many factors influence when disease will occur, including the immune status and level of stress of animals and the virulence of the virus. In this study keeping different age groups separated was shown to be protective. This finding was supported by a study in Thailand which indicated that adult beef cattle were at higher risk of FMD infection compared to calves less than one year old (Cleland et al., 1995) and adult cattle and buffalo were approximately three times more likely to become a case than were working cattle (Cleland et al., 1995). Management of livestock classes by separating different age groups will reduce the risk of the herd from contracting the disease from the higher risk age group.
Although vaccination is adopted as a control measure for FMD in Pahang, the
descriptive analysis indicated that only half (56%) of the respondents believed in
vaccination as a preventive measure for FMD. Unfortunately only 29% of the
respondents knew that the vaccine needed to be given at six monthly intervals and no
one knew that a second priming dose was required to be administered one month
after the primary dose.

Ideally the case and control herds should be differentiated by the NSP serological
test to confirm their FMD status (Bruderer et al., 2004; Clavijo et al., 2004) due to
the potential presence of carriers with the disease (Burrows, 1966; Burrows et al.,
1971; Salt, 2004). The presence of carriers allows the continued circulation of
FMDV in the cattle population even after vaccination (Alexandersen, 2002; Schley
et al., 2009), however the role of carriers in transmitting infection to susceptible
animals is not clearly understood (Woodbury, 1995b; Salt, 2004). It was not possible
to undertake serology because of time and cost constraints, therefore the status of a
herd in the current study was based solely on a history of clinical disease. Given that
the clinical signs of FMD are easily recognisable in cattle it was considered that
using the presence of clinical signs to classify case and control farms was acceptable.

Others (Barteling, 2002; Cox et al., 2007; Parida et al., 2008; Schley et al., 2009)
have highlighted the role that vaccination plays in the development of immunity and
prevention of disease outbreaks. Given the low knowledge of the benefits of
vaccination against FMD and the lack of awareness of the recommended vaccination protocol in Pahang a study was developed to determine the serological response to vaccination of Nallore cattle on a farm in Pahang. The results of this vaccination study are reported in the following chapter.
CHAPTER 4

ANTIBODY RESPONSE FOLLOWING VACCINATION AGAINST FOOT AND MOUTH DISEASE

4.1 Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease which results in significant economic impacts due to: reduced productivity in affected animals; the cost of implementing disease control practices; and the losses resulting from restrictions placed on the trade of livestock and their products (Kitching, 1998). Based on benefit cost analysis a stamping out policy has generally been the preferred control policy in countries that have previously been free of the disease (Ferguson et al., 2001b; Keeling et al., 2001; Bates et al., 2003; Ward et al., 2009), while vaccination and restriction of livestock movements are commonly adopted in areas where the disease is endemic (Anderson et al., 1974; Cleland et al., 1996; Rweyemamu et al., 2008a). In that situation vaccination is adopted to induce herd immunity leading to a reduction in clinical infection and viral transmission (Orsel et al., 2007). Vaccination, in combination with culling of clinically affected and repeated NSP seropositive animals, has also been successfully adopted to control and eradicate FMD in countries in western Europe and South America (Saraiva, 2004; Lubroth et al., 2007). Although numerous studies have demonstrated the positive impacts of implementing vaccination to control and eradicate FMD, many local factors also need to be identified and considered before the practice can be adopted in an area. In particular a benefit cost analysis is required, as is a thorough understanding of the local epidemiological factors of the disease (Hutber et al., 2010).
Vaccination has been used for many years to control FMD in Malaysia (Babjee, 1994; Hiong, 1994). The use of vaccination was initially restricted to the northern border states of peninsular Malaysia until an outbreak in late December 2003 when it was adopted in Pahang. Vaccination, along with movement restrictions are now key elements in the control of FMD in Malaysia.

Following vaccination antibodies to the structural proteins of FMDV are produced which impair the ability to differentiate vaccinated animals from those naturally infected (Doel et al., 1994). Differentiating animals that have been naturally infected from those vaccinated is of considerable importance because it is well established that infected cattle and sheep frequently become carriers (Clavijo et al., 2004) and consequently may be the source of new disease outbreaks (Sutmoller and Olascoaga, 2002). Although the infectivity of persistently infected ruminants to naïve animals is believed to be low under most circumstances, it is not negligible. These factors lead many parts of the world to control the disease without vaccination, for example vaccination against FMD has been banned in the European Union since 31st December 1991, and outbreaks have been controlled solely by stamping out. However, the need for emergency vaccination in future outbreaks, as an adjunct to stamping out, cannot be ruled out due to the huge economic losses incurred, adverse psychological effects on farmers and negative effects on the environment resulting from the slaughter and disposal of livestock.

Diagnostic techniques have been developed in an attempt to differentiate vaccinated animals from those that are convalescent and from those that have been vaccinated
and subsequently become carriers (Sorensen et al., 1992; Bergmann et al., 2000; Clavijo et al., 2004). Antibody to the NSP of FMDV, which is detected only in naturally infected animals, has been used to differentiate between vaccinated and infected cattle (Diego et al., 1997; Mackay et al., 1998; Sørensen et al., 1998; Clavijo et al., 2004; Moonen et al., 2004). Vaccines which consist of partly purified inactivated virus preparations induce antibodies principally to the structural proteins of the virus, whereas infected animals produce antibodies to both structural and non-structural proteins. Therefore in the current study the 3 ABC ELISA was utilised to study the serological response to cattle vaccinated against FMD. The detection of antibody to the polyprotein 3ABC in the 3 ABC ELISA is reported to be the most reliable diagnostic test (Berger et al., 1990; Lubroth and Brown, 1995) because of its high immunogenicity and its relative low concentration in infected cell lysates (Doel et al., 1994; Bergmann et al., 2000). The ELISA, based on the detection of antibody to NSP, has been recommended by the OIE for serological surveillance in regions or countries that practice FMD vaccination and for monitoring the circulation of virus in the field (OIE, 2004). On the other hand the liquid phase blocking ELISA (LP Blocking ELISA) relies on the detection of antibodies to the structural, capsid VP1 t-VP4 proteins of FMDV (Hamblin et al., 1986a). Antibodies to structural proteins are induced by both vaccination and infection. The LP Blocking ELISA has been used in endemic areas to indicate the antibody response produced after vaccination in conjunction with the NSP ELISA (Periolo et al., 1993a; Robiolo et al., 2006).

The study outlined in this chapter describes and compares the antibody response following vaccination against FMD. The serological response in a group of cows which had been vaccinated yearly for more than 3 years and a group of calves which received their initial vaccination were compared. In this study a 3ABC-ELISA and
an LP Blocking ELISA were used to monitor the level of protection (herd immunity) in the population.

4.2 Materials and methods

4.2.1 Vaccination and sample collection

The study outlined in this chapter involved animals from a cow and calf unit in Pusat Ternakan Haiwan (PTH) Ulu Lepar, Kuantan, Pahang, which is a government owned herd of Nellore beef cattle. The farm had no clinical history of FMD. Forty-five cows and forty-five calves were selected based on systematic random sampling (Thrusfield, 2005) and were tested for antibody to NSP with a commercially available NSP ELISA assay (Ceditest FMDV-NS ELISA - Cedi Diagnostics, B.V., Lelystad, The Netherlands). All the cows had been vaccinated annually since March 2004 and the calves were all vaccinated for the first time on the 12th February 2008 (during this study). At the time of their first vaccination the calves were 4 to 6 months of age. The vaccine used in this study was an inactivated purified trivalent vaccine (O, A and Asia 1) (Aftovaxpur® Merial Animal Health Ltd). Blood samples were collected from the coccygeal vein into a plain 10 ml vacutainer (BD Franklin Lakes USA) at 0, 14, 57, 93, 183, 212, 254 and 317 days post vaccination (DPV). These samples included ones collected on the days of vaccination (days 0 and 183).

At 183 DPV, twenty eight cows and twenty eight calves were randomly selected from the larger group for testing with the LP Blocking ELISA to monitor the antibody response to vaccination. Antibody response was expressed as Percentage of Inhibition (PI). Values greater than 50% were classified as seropositive (Naheed,
Fourteen cows and 14 calves from this smaller group were revaccinated at 183 DPV with the same trivalent vaccine, with the remaining 14 cows and 14 calves not being revaccinated. Sera were then collected at 183, 212, 254 and 317 DPV from all animals. During the study all 90 cows and calves, including the 56 cows and calves whose sera were tested with the LPBE test, were run in one paddock along with other (untested) cows and calves.

After collection blood samples were centrifuged, sera decanted and stored at -20°C at the Regional Veterinary Laboratory, Kota Bahru (RVL KB) until tested.

This study had been approved by the Murdoch University Animal Ethics Committee (R2191/08).

4.2.2 Measurement of antibody to NSPs

The antibody response to FMDV NSPs was measured with a commercially available ELISA (Cedi FMDV-NS for the NSP 3 ABC peptides, Cedi-Diagnostics B.V., the Netherlands). This blocking ELISA measures the competition between the test sera and a NSP specific monoclonal antibody for the binding to the 3ABC NSP of FMDV (Sørensen et al., 1998; Sorensen et al., 2005). The assay was performed according to the manufacturer’s protocol. The optical density (OD) result obtained from the assay was expressed as PI of the monoclonal antibody binding. The PI cut off value between a positive and negative was set at 50%. Animals with an NSP PI value more than 50% were then not included in the analysis to monitor the LPBE level of antibody produced from vaccination (herd immunity). The detailed NSP test procedures are summarised in Appendix 1.
4.2.3 Measurement of antibody on the Liquid phase blocking ELISA

The LPB ELISA is based upon the specific blocking of FMDV antigen in a liquid phase by antibodies present in the test serum sample (Hamblin et al., 1986b, a). The LPB ELISA was performed according to the procedure adopted by the FMD WRL, Pirbright, UK (Appendix 2). It was carried out to detect antibodies directed against structural proteins of FMDV. Antibodies were expressed as PI values and a value greater than 80% was considered protective (Westbury et al., 1988b).

4.2.4 Statistical analysis

Data were managed, collated and analysed using SPSS (SPSS version 17 Statistics GradPack for Windows). One way repeated measures ANOVAs (analysis of variance) were carried out to identify significant differences in the mean PI of the LPBE for the serially collected sera. The results of the LPBE (PI) were computed and transformed to arcsine data. The data were then explored to check for normality. The calf group data were normally distributed, therefore the data were analysed with a one-way repeated ANOVA. The multiple comparison Bonferroni test was used as a post hoc test to compare differences between groups (vaccinated once and revaccinated). The difference in the antibody level produced between the revaccinated group and the group vaccinated only once for both cows and calves were also determined through the use of repeated measures ANOVAs. As the cow group data were not normally distributed, the analyses were continued using a non-parametric test (Friedman’s ANOVA). The mean PI values for all DPVs were ranked and the asymptomatic Friedman’s analysis carried out for all DPVs values.
The odd ratios (OR) were also calculated to identify differences within and between groups. If a $2 \times 2$ table contained cells with the value 0 then 1 was added to all cells in that table to enable calculation of OR. The OR value was considered significant if the value of the 95% confidence intervals did not include the value 1.

### 4.3 Results

#### 4.3.1 Detection of antibodies against FMDV NS-Proteins

Over the total period of the study 7 animals were identified as positive to the NSP assay (7.8%: 95% CI; 2.2%, 13.3%) (5 cows and 2 calves) (Table 4.1). There was no significant difference in the number of NSP positive animals between the cow and calf groups (OR 2.7; 95% CI 0.5 – 14.6).

Table 4.1 Number of cows and calves positive to the NSP test throughout the study

<table>
<thead>
<tr>
<th>Group</th>
<th>NSP + (%: 95% CI)</th>
<th>NSP-</th>
<th>Odds ratios (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>5 (11.1: 1.9, 20.3)</td>
<td>40 (88.9%)</td>
<td>2.69 (0.49 - 14.64)</td>
</tr>
<tr>
<td>Calf</td>
<td>2 (4.4: 0.0, 10.5)</td>
<td>43 (93.3%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total</td>
<td>7 (7.8: 2.2, 13.3)</td>
<td>83 (91.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Both cow and calf groups were subsequently divided into two groups at 183 DPV, with one group receiving another dose of FMD vaccine, while the second group did not. Results for the comparison between the groups are summarised in Tables 4.2, 4.3 and 4.4. Again there were no significant differences in the number of NSP
reactors in both cow and calf groups after day 183 (OR 5.2, 95% CI 0.5 – 54.1; and OR 3.5, 95% CI 0.3, 37.5, respectively) (Table 4.4).

Overall 7 individual animals were positive at some point during the study (one cow was positive 7 times, three animals (2 cows and 1 calf) 2 times and three animals (2 cows and 1 calf) were positive at only one point in time (Tables 4.5 and 4.6). There were no NSP positive animals at the start of the study. Two cows became positive at 14 DPV, 1 at 93 DPV, 1 at 183 DPV and 1 at 212 DPV (Tables 4.2 and 4.5). In comparison the first NSP positive calf occurred at 93 DPV and the second at day 212 (Tables 4.3 and 4.6).

Table 4.2 Number of NSP positive samples* in the non-revaccinated and revaccinated cows

<table>
<thead>
<tr>
<th>DPV</th>
<th>Number of NSP positive samples (non-revaccinated)</th>
<th>Number of NSP positive samples (revaccinated)</th>
<th>Total number of NSP positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>57</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>93</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>183</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>212</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>254</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>317</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

*Represents 5 positive animals as the same animals were positive at multiple times
Table 4.3 Number of NSP positive animals* in the non-revaccinated and revaccinated calves

<table>
<thead>
<tr>
<th>DPV</th>
<th>Number of NSP positive samples (non-revaccinated)</th>
<th>Number of NSP positive samples (revaccinated)</th>
<th>Total number of NSP positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>57</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>93</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>183</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>212</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>254</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>317</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*Represents 2 positive animals as one calf was positive on two occasions

Table 4.4 Comparison between cow and calf groups after animals were divided at 183 DPV into a revaccinated and a non-revaccinated group.

<table>
<thead>
<tr>
<th></th>
<th>NSP +</th>
<th>NSP -</th>
<th>Odds ratios (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cows</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not revaccinated</td>
<td>4 (28.6%)</td>
<td>10 (71.4%)</td>
<td>5.2 (0.50 – 54.05)</td>
</tr>
<tr>
<td>Revaccinated</td>
<td>1 (7.1%)</td>
<td>13 (92.9%)</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total cows</strong></td>
<td>5 (17.9%)</td>
<td>23 (82.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Calves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not revaccinated</td>
<td>2 (14.3%)</td>
<td>12 (85.7%)</td>
<td>3.5 (0.32 – 37.48)*</td>
</tr>
<tr>
<td>Revaccinated</td>
<td>0 (0.0%)</td>
<td>14 (100.0%)</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total Calves</strong></td>
<td>2 (7.1%)</td>
<td>26 (92.9%)</td>
<td></td>
</tr>
</tbody>
</table>

* 1 added to all cells to enable OR calculation
Table 4.5 NSP results for individual cows during the study

<table>
<thead>
<tr>
<th>Days of study</th>
<th>0</th>
<th>14</th>
<th>57</th>
<th>93</th>
<th>183</th>
<th>212</th>
<th>254</th>
<th>317</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tag ID</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Cows vaccinated at day 0 but NOT revaccinated at day 183</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>UN 8166</td>
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4.3.2 Liquid phase blocking ELISA

Although 28 cows and 28 calves were selected for the LPB ELISA analysis, only 21 cows (10 from the non-revaccinated group and 11 from the revaccinated group) and 20 calves (10 from the non-revaccinated group and 10 from the revaccinated group) were included in the final analysis because these animals had been consistently tested for FMD infection and had negative NSP results at all samplings and some of the animals (2 cows and 6 calves) were not included because they were not sampled at all times throughout the one year study. The objective of performing the LPB ELISA test was to identify whether the antibody produced by the animals following vaccination was sufficient to protect the host against FMDV infection. For the purpose of this study a cut-off point of 80% was used to determine the protective immunity level (Westbury et al., 1988b). The mean PI values for all serotypes in the cow group were above the LPB ELISA cut-off point at all sampling times, irrespective of whether the animals had been revaccinated or not. In Figures 4.1, 4.2 and 4.3 the mean PI is displayed for the three serotypes (O, A and Asia1 respectively) in animals revaccinated and those that weren’t. The descriptive analysis for the cow groups showed that the mean PI values were not normally distributed for all DPVs (skewness and median values ranging from -1.0552 to 1.168 and -0.709 to -2.340, respectively). Therefore the non-parametric Friedman ANOVA was used to demonstrate that the PI values for cows after revaccination was significantly higher than the value prior to vaccination ($p = 0.000$ and $\chi^2 = 55.009$, df 7).
For Serotype O the mean PI value was already above the mean PI cut off point (80%) at the time of the initial vaccination and subsequently at all sampling points. After revaccination at 183 DPV the PI values in both groups increased significantly to 212 DPV before decreasing ($\chi^2 = 20.985$ and $P < 0.05$).
The pattern for the Serotype A (Figure 4.2) was different to that of the other serotypes (Figures 4.1 and 4.3). The mean PI value increased significantly after vaccination at 0 DPV; however it decreased again at 57 DPV before it increased gradually with significantly higher PI values until 212 DPV ($\chi^2 = 27.015$ and $P < 0.05$). In both groups the mean PI values increased significantly after the time of revaccination at 183 DPV with the non-revaccinated group showing a similar trend as the revaccinated group but with higher PI values from 212 DPV.
The mean PI values to serotype Asia 1 increased significantly after the vaccination at 0 DPV ($\chi^2 = 57.038; P < 0.05$) (Figure 4.3). Again surprisingly the PI values in both groups increased when the revaccinated group received its second dose before they decreased significantly at 212 DPV ($\chi^2 = 29.183$ and $P < 0.05$). The response of cows to serotype Asia 1 was similar in both groups. After revaccination the PI values were similar until 254 DPV. At this point, the mean PI value for the non-revaccinated group dropped dramatically, while the revaccinated group showed only
a small reduction. However, the mean PI values for both groups were above the protective cut off value at all times throughout the study.

For the calf groups, the mean PI value was highest at DPV 212 in the revaccinated group for the Serotype Asia 1 (90.2%) as shown Figure 4.6. The PI data were normally distributed at all sampling times with skewness values between; -0.030 to -1.151 and mean values between 0.5983 and 0.9029 (data not shown). The significance value (0.00) for the Mauchly’s Test of Sphericity indicated that the assumption of the sphericity had been violated with an approximate $\chi^2$ value of 94.523. Therefore a Greenhouse-Geisser correction value was chosen (0.699). The closer the Greenhouse-Geisser correction is to 1, the more homogenous the variance of difference is, and hence the closer the data are to being spherical (Field, 2009). The multivariate test statistic was also used due to the violation of sphericity. In this analysis there was a significant change ($P < 0.001$) in the mean calf PI over time. However the level of antibody was not protective at most DPVs, except for days 183 and 212 for Serotypes O and Asia 1. This indicated that the calves were susceptible to FMD for most of the time throughout the study.
Figure 4.4. LPB ELISA results for serotype O in revaccinated and non-revaccinated calves.

The mean PI values for serotype O (Figure 4.4) in the two groups of calves followed similar patterns. Following the initial vaccination the PI increased significantly to 14 DPV and again in both groups at 183 DPV when only the revaccinated group received a booster vaccination. However the revaccinated group had a similar mean PI value to that of the non-revaccinated group, indicating there was no significant effect of revaccination in the revaccinated calf group with \( F = 3.190; P = 0.091 \). Moreover, most of the PI values were below the 80% protective level throughout the study.
Figure 4.5 LPB ELISA results for serotype A in revaccinated and non-revaccinated calves.

The mean PI value for Serotype A (Figure 4.5) followed a different trend compared to the other two serotypes. At only one point in time (212 DPV) did some revaccinated calves (n = 4) have protective immunity to Serotype A. At 14 DPV there was a significant reduction in the PI value. Subsequent to DPV 183 (time of revaccination) the revaccinated group had a significantly higher PI value than the non-revaccinated group until the end of the trial at day 317 when the titres were similar. The PI value in the revaccinated group was significantly different to the non-revaccinated group ($F = 10.229; P = 0.005$). However, most of the PI values were below the 80% protective level throughout the study.
Figure 4.6 LPB ELISA results for serotype Asia 1 in revaccinated and non-revaccinated calves.

The mean PI value for serotype Asia 1 increased sharply and significantly after the first vaccination (Figure 4.6), however neither group failed to generate a mean protective level until the revaccinated group received their second dose of vaccine. Generally the two groups were not significantly different ($F = 0.298; P = 0.592$), with most of the PI values below the 80% protective level throughout the study.

4.4 Discussion

The presence of NSP antibodies has been shown to be a reliable indicator of infection with FMDV in cattle (Diego et al., 1997; Mackay et al., 1998; Niedbalski and Haas, 2003b) although the detection of persistently infected cattle is not perfect.
The persistence of neutralizing antibody to FMDV in cattle has previously been shown to be short and cattle are immune to re-infection for only approximately 4 to 6 months (Hunter, 1996). Therefore it is important to follow the manufacturer’s protocol for vaccination to prevent infection. This is especially important for the initial vaccination when calves should receive their first dose around the age of 4 months (P1). This should be followed with a second dose (P2) three to five weeks later to boost the antibody response. Subsequently a third dose (P3) should be administered 4 to 6 months later. Booster vaccinations should ideally then be given every 4 to 6 months. This protocol is recommended by manufacturers to ensure the vaccinated animals have titres above the minimum protective level to reduce the risk of infection. However this frequent vaccination schedule is expensive and labour intensive and therefore is rarely adopted in the field. Consequently this also results in many countries trying to eradicate FMD without the use of vaccination. Furthermore a country which is classified as FMD free with vaccination has restrictions placed on the trade of animals and animal-products, therefore most exporting countries prefer to have the status of FMD free without vaccination. However in South East Asia, where FMD is generally endemic, eradication is difficult and often not practical. Therefore zoning and compartmentalization are often adopted (Scott et al., 2005) along with vaccination to control the disease. Consequently diagnostic tools are required to differentiate infected from vaccinated animals. In Malaysia the Ceditest 3 ABC ELISA is used routinely to differentiate infected from vaccinated animals. This test has a reported specificity of 99.8% (Sørensen et al., 1998). Because of this high specificity, in the current study the Ceditest 3 ABC ELISA was chosen to detect infection along with the LPBE to evaluate the immune response following vaccination.
The LPBE demonstrated a similar pattern in both the revaccinated and the non-revaccinated cow groups. A single dose of vaccine was adequate to confer good immunity (mean PI value above 80% cut off value) and revaccinating cows at six monthly intervals produced no significant advantage over the group that only received one annual vaccination. At the beginning of the study all 90 cattle were vaccinated at 0 DPV. As a result, it is not surprising that the mean PI value increased at 14 DPV. The number of cattle that were positive to the NSP increased at 93 DPV when the cow group recorded 2 NSP positive animals and the calf group 1 NSP positive animal. This could be the reason why the mean PI of the LPB ELISA increased at 93 DPV. The NSP positive animals could be indicative of subclinical infection as all animals were reared in the same area for the whole study period. Furthermore the NSP positive cattle were repeatedly positive. However since the number of animals in this study was small and the NSP ELISA used was not able to identify the specific serotype present and moreover no further tests were performed in this study, conclusions cannot be drawn on the cause of the immunity conferred pattern by the animals involved in this study. The NSP ELISA detected positive animals in both the cow and calf groups even though this farm had never had a clinical history of FMD (Tables 4.5 and 4.6). More NSP positive animals were observed in the non-revaccinated cow group followed by the revaccinated cow group. The revaccinated calf group on the other hand, showed the least number (0) of positive NSP animals. The effect of repeated vaccination has been studied elsewhere, and some researchers have shown that the reactivity to NSP in a revaccinated population approaches the necessary level to confer protection after several vaccinations (Espinoza et al., 2004; Lee et al., 2006). A study undertaken by Lee et al. (2006) in Taiwan found 3.9% of field samples positive with the Cedi test kit,
indicating that the specificity was lower than that reported previously. Some researchers also have demonstrated that cattle which received multiple vaccinations over several years were positive to NSP (Mackay et al., 1998). In contrast other researchers (Niedbalski and Haas, 2003b) reported that multiple vaccinations did not affect the number positive to NSP. However no similar studies have previously been undertaken in Pahang. In addition to that, the district of Kuantan, Pahang was severely affected during the 2004 FMD outbreak and the disease has been considered endemic in the district since that date. Therefore the percentage of NSP positive animals cannot be determined based on the use of only a single test, as it is unclear if reactors were true positive animals from natural infection, or were false-positives including those resulting from use of a vaccine contaminated with NSP. Thus, additional methods for conformation and epidemiological investigations are necessary to differentiate naturally infected cattle from vaccinated animals. Furthermore the NSP reactors could also be influenced by other factors such as the subclinical circulation of FMDV in the cattle population. It was possible that the positive NSP reactors observed in this study were caused by natural, subclinical infection with FMDV based on the endemic nature of FMD in the area. The NSP positive animals identified in this study could potentially be carriers of FMDV. Such carriers are considered to be sources for the spread of disease to susceptible animals as the protective immunity does not block FMD infection completely and some level of viral replication can still occur in vaccinated cattle upon exposure to the field virus (Alexandersen et al., 2003c). In addition Doel et al. (1994) and Mackay et al. (1998) pointed out that cattle protected by vaccination can become transient FMD virus carriers, with or without clinical signs, and they can also become persistently infected without displaying any clinical signs of FMD. Therefore it is crucial for
farms to undertake testing with an NSP test, to implement a proper disease control program which is tailored to the local FMD situation and to consider the culling of NSP positive animals as soon as possible after their identification.

Historically the schedule of vaccination practiced on this farm was not carried out in accordance with the manufacturer’s recommendations (where P1 FMD vaccine is followed by P2 one month later and subsequently animals are revaccinated every 6 months). However reports of the successful control of FMD by vaccination in other countries, which also have not followed the manufacturer’s recommended protocol, have been made (Doel, 1999). Vaccination programs are often altered to suit the local epidemiological features of the disease, although emphasis has traditionally relied more on the vaccination coverage of the population (at least 80% of the population). As for this particular farm during this study the mean PI values on the LPB ELISA were, in general, above the seropositive cut off point (PI value : 80%) (Westbury et al., 1988a; Robiolo et al., 2010) for the cow groups.

The LPBE is a reliable technique for the evaluation of a protective response (Hamblin et al., 1987; Periolo et al., 1993a; Robiolo et al., 2006). Although vaccination was commenced on this farm in 2004 it was only administered annually for both adult and young cattle. The reason for this was not confirmed and this situation requires further research since this study indicated only the cows had good LPB ELISA values (above the protective level of 80% at all-time throughout the study). Furthermore, the mean PI on the LPBE for the calf group was lower than that for the cows and similar trends were followed for all serotypes except for serotype A. Unlike for other serotypes, the mean PI values for serotype A in calves decreased...
following the initial vaccination but the levels did increase after the animals were revaccinated. The mean PI value was generally lower in the calf groups for serotypes O and Asia 1 than for the cow groups. This probably was a result of the cows receiving multiple vaccinations over their lifetime. Moreover no second priming vaccination dose was given to calves a month following the first vaccination, as is recommended by the manufacturer. This would likely contribute to the low mean PI values for the calf groups, which were less than the protective level. Although there were significant differences in the LPBE PI at different sampling times (DPVs) (albeit below the protective levels), there was no significant difference between the revaccinated and non-revaccinated groups. The failure to detect a higher titre in the revaccinated calf group was surprising and could be due to several factors including: 1. Inappropriate vaccination protocol which omitted P2, leading to insufficient antibody production; 2. In this study calves received their first vaccination at the age of at least four months. At the start of the study all calves already had some LPBE PI although no NSP positive animals were detected. The initial LPBE PI values could be due to the presence of maternal antibody as a result of residual passive immunity transferred from multiply immunized cows which may have a neutralising effect towards the vaccination given (Periolo et al., 1993b). These two factors require further study.

The protective cut off point for the LPBE is normally calculated on the titre rather than the mean PI value (Robiolo et al., 2006), however due to time and monetary constraints the protective cut off point was set at 80% mean PI value following the results of a study in Thailand (Westbury et al., 1988a). Based on the 80% protective mean LPBE cut off value, the cows appeared to be protected against FMD.
throughout the study, in contrast calves were at risk of infection at various times through the study. Although there were significant differences over the study in the PI for calves, there was no significant difference between calves that were revaccinated at 6 months of age and those that were not revaccinated. All calves in both groups were susceptible to the disease at almost all sampling times except for the revaccinated calf group at 212 and 254 DPV and only for serotypes O and Asia 1. None of the Serotype A mean PI values of the calf groups were above the protective level at any sampling time. These findings indicate that the calves were at a greater risk of infection than cows throughout the study.

Surprisingly the mean PI LPBE increased in both the revaccinated and non-revaccinated cow groups for all serotypes after some animals were revaccinated at 183 DPV. This may have arisen from subclinical infection in the herd.

An important finding of the present study was that the antibody level conferred after vaccination was not the same for animals which had been multiply vaccinated and animals which received their first vaccination. Therefore it is suggested that the vaccination practices should be tailored to the local situations based on the existing local epidemiological factors. It is also important to adopt a vaccination monitoring system for different FMD situations and to monitor the vaccination status of a herd.

In conclusion it was evident that vaccination stimulated serological immunity, however the immunity in many cases was not sufficient to protect against natural infection. Additional research is required to further understand the titre induced and a challenge study is required to confidently conclude that the immunity induced by vaccination is protective.
CHAPTER 5


5.1 Introduction

Foot and mouth disease is a highly contagious febrile vesicular disease of cloven-hoofed domesticated and wildlife species (Alexandersen et al., 2003c). The disease results in severe economic impact because of reduced animal production and restrictions to local and international trade (James and Rushton, 2002). Historically, the state of Pahang has experienced several incursions of FMD caused by Serotype O. During the initial 2003/2004 outbreak in Pahang, ring vaccination and movement control were implemented to control the spread of the disease. Animals were vaccinated within a 5 km radius of the outbreak and disease surveillance was conducted within a 10 km radius of the outbreak. In order to minimise the spread of the disease at that time, a mass vaccination campaign of the whole state was also conducted. This campaign commenced in March 2004 using a trivalent vaccine containing the three serotypes O, A and Asia 1. The vaccine was administrated to all cattle, buffalo, goats and sheep older than 4 months of age. A booster vaccination was given six months after the initial vaccination. However, despite these control measures, further outbreaks were recorded in subsequent years (DVS, 2007).

The highly contagious nature of FMD results in clustering of cases in time and space (AlKhamis et al., 2009). Assessment of outbreaks with respect to spatial clustering
and their temporal pattern is useful when planning specific prevention, surveillance and control strategies against the disease (Shiilegdamba et al., 2008). Spatial cluster analysis plays an important role in identifying the geographical patterns of a disease (Jacquez, 2008). Cluster analysis can address the: (1) rapid identification of epidemic clusters; (2) identification of confounders; and (3) generation of research hypotheses (Carpenter, 2001). Many methods can be used to quantify disease outbreaks including the one-dimensional scan statistic which has long been used in purely temporal disease surveillance (Wallenstein, 1980) and spatial scan statistic which has been used for detecting clusters in a multi-dimensional point process in a geographical region (Kulldorff, 1997). Spatial autocorrelation methods look for the presence of systematic patterns such as the clustering effect in the spatial distribution of a variable (Lai et al., 2009), while temporal statistical techniques are used in many well-defined geographical areas for monitoring and detecting sudden temporal increases in the risk of disease, particularly for rare diseases (Bjerkedal and Bakketeig, 1975; Radaelli, 1996). For the purpose of this study a space-time permutation model was used to analyse the space and time of the outbreak in one model using only outbreak data to identify the geographical areas and period of time in which the disease is more likely to occur.

The objective of the current study was to describe the spatio-temporal patterns of outbreaks of FMD in the state of Pahang. Findings from this study could then be used to direct future research into the epidemiology of FMD and could serve as a starting point for developing more effective control programs in Pahang.

5.2 Materials and methods
5.2.1 Study area and study period

The study area involved all eleven districts in the state of Pahang. The period of interest in this study was from 16 December 2003 to 26 August 2006.

5.2.2 Data source and case definition

The outbreak data were obtained from the retrospective disease reporting database for the State of Pahang Department of Veterinary Services (DVS) for the period of study. The outbreaks were defined according to the Office International des Epizooties (OIE) as epidemiological units (herds, farms, feedlots or where premises could not be precisely delimited, areas with free-grazing animals) in which at least one FMD infected animal had been detected (OIE, 2008b). When a case of FMD is suspected, a report is issued from the District Veterinary Office to the State Veterinary Officer. The State Veterinary Officer then organised an inspection of the outbreak site searching for FMD-like clinical signs in livestock. The estimated starting date is predicted based on the age of the clinical lesions. Details of the outbreak, including the number of animals affected, the breed, species, farm address and geographical location (latitude and longitude of the case), were recorded. Fresh epithelial samples were collected into phosphate buffered saline (PBS) from vesicular lesions in the oral cavity or inter-digital skin of affected animals. These samples were then submitted to the Regional Veterinary Laboratory in Kota Bahru, Kelantan for confirmation by the antigen detection test. Isolated serotypes were then sent to the FMD World Reference Laboratory in Pirbright, United Kingdom for identification.
The historical vaccination and surveillance data were also examined and incorporated into the analysis. The recorded date of an outbreak used in the analysis was based on the earliest date the cattle showed clinical signs of FMD in a population.

5.2.3 Spatiotemporal analysis

A map of administrative units and boundaries was obtained from the DVS. Location data were downloaded from the Global Positioning System (GPS) receiver. The latitude and longitude data were then calculated for each case using Excel and saved as a tab-delimited text file. The prepared text files were then imported into Quantum GIS software version 1.0.2 – Kore (www.qgis.org/) to visualise the location of cases on a map. The same text file was then used as the coordinate file for analysis with spatial scan statistics.

The total number of cases for each month from 16 December 2003 to 26 August 2006 were recorded and plotted on a graph. The duration of vaccination campaigns against FMD were superimposed on the plots to identify potential links between cases and vaccination.

The spatio-temporal clusters for the FMD outbreaks were identified by computing the Space-Time Permutation model of the scan statistic test using SaTScan Version 8.0 of the retrospective data of 342 locations with a total of 5335 cases in the study period from 3rd December to 26th August (Kulldorff et al., 2005; Kulldorff and Inc., 2009). The technique utilised a large number of overlapping cylinders to define the
scanning window, each of which was a possible candidate for an outbreak at the geospatial coordinate of the location. The base of the cylinder represents the geographical area of the potential outbreak (spatial) while the height represents the number of days (temporal) with the last date always included together with a variable number of preceding days up to the maximum number of defined days. Scanning for clusters was done with high rates and time aggregation by day. The maximum spatial cluster size was set to 50% of the population at risk using circular spatial windows and the maximum cluster size was also set at 50% of the study period (Kulldorff and Nagarwalla, 1995; Kulldorff, 1997; Kulldorff et al., 2005). The statistical significance was evaluated using 999 Monte-Carlo simulations in combination with the scan test to detect significant differences between the observed and expected results. The cut-off value for the identification of significant clusters was set at $P < 0.05$.

5.3 Results

The largest number of outbreaks occurred between 16th December 2003 and 15th January 2004. The number of outbreaks reported decreased dramatically after the ring-vaccination program was implemented in early January 2004 (Figure 5.1). The number of reported cases decreased further in April 2004. However they increased gradually from early May until the middle of July 2004. Surprisingly there were no cases reported after the middle of July 2004 until early September 2005 and after then the number of cases fluctuated until the end of the study period.
Figure 5.1. Number of cases of FMD recorded between 16th December 2003 to 26th August 2006.
There were 342 outbreak locations with 5335 FMD cases recorded in the whole state of Pahang for the period of approximately 2.75 years (16\textsuperscript{th} December 2003 to 26\textsuperscript{th} August 2006). Cases of FMD were detected in all districts in the state of Pahang except for the Cameron Highlands (Figure 5.2).

Figure 5.2 Geographical distribution of outbreaks of FMD in the state of Pahang that were reported to the DVS during the period 16\textsuperscript{th} December 2003 to 26\textsuperscript{th} August 2006. Each red dot represents a reported outbreak.

The Space – Time permutation model indicated there were five significant clusters with no geographical overlap in the secondary clusters for the whole study period. Clusters were identified in the east, west and middle of Pahang with the observed to
expected ratio of FMD outbreaks within the spatial temporal clusters between 2.39 and 17.78.

There were 44 location identification sites included in the primary cluster (Cluster E) (Table 5.1). This cluster involved an area with a 50.31 km radius and occurred between the 20th January 2006 and the 24th August 2006 (218 days). There were 1150 cases ($P = 0.001$) in this cluster. Four secondary clusters were recorded; Cluster A involved a smaller area (35.94 km radius) within a shorter time frame of 16th December 2003 to 25th February 2004 (72 days). There were 187 location identification sites in this cluster which involved 1847 cases ($P = 0.001$). Cluster B involved a smaller area compared to the primary cluster and Cluster A and included an area with a radius of 25.09 km. It involved the longest time frame compared to the other clusters identified (15th July 2004 to 21st September 2005 - 433 days). There were 592 cases included in this cluster ($P = 0.001$). Cluster D involved only one GPS point representing one farm, resulting in a cluster size of radius 0 km with the shortest time frame (17th January 2006 to 17th January 2006 - 1 day). It included only one location identification site with 220 cases ($P = 0.001$). The cluster size is determined by the GPS point of location. If it is only one point it is indicated as having a radius of 0 km because the statscan software recognises it as only a dot (GPS point), where as if the clusters containing 2 or more points the cluster size is determined by the distance from one point to others. There was no information on the farm size to input into the program for analysis. Finally secondary Cluster C included seven location identification sites within an area with a radius of 28.62 km. It involved 185 cases within a time frame of 25th September 2005 – 19th January 2006 (117 days) ($P = 0.001$).
### Table 5.1 Spatio-temporal clusters detected by the spatial scan permutation model for 342 FMD outbreak locations in the state of Pahang from 16 December 2003 to 26 August 2006.

<table>
<thead>
<tr>
<th>Clusters</th>
<th>Centre Coordinate</th>
<th>District</th>
<th>Cluster size km (radius)</th>
<th>Time frame</th>
<th>Cluster period (days)</th>
<th>No of outbreaks</th>
<th>No of FMD cases</th>
<th>No of observed to expected ratio of FMD outbreaks</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.535583N, 103.454083E</td>
<td>Pekan Kuantan</td>
<td>35.94</td>
<td>16/12/2003 - 25/02/2004</td>
<td>72</td>
<td>140</td>
<td>1847</td>
<td>2.39</td>
<td>0.001</td>
</tr>
<tr>
<td>B</td>
<td>3.890367N, 102.593417E</td>
<td>Maran Kuantan Jerantut</td>
<td>25.09</td>
<td>15/07/2004 - 21/09/2005</td>
<td>433</td>
<td>25</td>
<td>592</td>
<td>6.41</td>
<td>0.001</td>
</tr>
<tr>
<td>D</td>
<td>4.014583N, 103.156750E</td>
<td>Kuantan</td>
<td>0</td>
<td>17/01/2006 - 17/01/2006</td>
<td>1</td>
<td>1</td>
<td>220</td>
<td>17.78</td>
<td>0.001</td>
</tr>
<tr>
<td>E</td>
<td>3.719767N, 101.941917E</td>
<td>Raub Temerloh Jerantut Lipis</td>
<td>50.31</td>
<td>20/01/2006 - 24/08/2006</td>
<td>218</td>
<td>44</td>
<td>1150</td>
<td>3.75</td>
<td>0.001</td>
</tr>
</tbody>
</table>
5.4 Discussion

This study presents the findings of an assessment of the spatio-temporal distribution of FMD outbreaks in the state of Pahang during the period from the 16th December 2003 to the 26th August 2006. The space-time permutation result showed that the FMD outbreaks were clustered into five distinctive clusters with no geographical overlap between the secondary clusters in space and time within the study period (Table 5.1).
The results indicated that the size of the clusters (spatial) were not uniform. They varied from one which only encompassed a single outbreak location (Cluster D) to one with a radius of 50.31 km (Cluster E). The largest cluster was identified as the primary cluster and involved four districts (Raub, Temerloh, Jerantut and Lipis). However the temporal analysis indicated that this cluster was detected at the end of the period (20\textsuperscript{th} January 2006 to 24\textsuperscript{th} August 2006). Although it involved the largest area it included the smaller number of outbreak locations and cases compared to cluster A which occurred at the start of the outbreak (16\textsuperscript{th} December 2003 to 25\textsuperscript{th} February 2004). Cluster A included 140 FMD outbreak locations with 1847 cases but affected only two districts (Kuantan and Pekan). As the radius of the largest cluster detected was more than 50 km it would suggest that a buffering zone larger than 50 km is required to control future epidemics in the state. The control measures should include ring vaccination with two doses of vaccine, restriction of animal movement and pre-emptive quarantine of susceptible animals within the buffer zone as has been suggested by others (Donaldson and Wood 2004, Yadin et al 2007). As four of the five clusters detected in this analysis had radii wider than the surveillance zone practiced in Pahang (10 km radius), it is likely that this surveillance zone is insufficient to contain an outbreak of FMD. However to impose a control activity with a radius greater than 50 km may be logistically difficult and challenging and imposing movement control can be difficult due to the animal production systems practiced in this state.
Similar to that reported in other countries the temporal pattern of the FMD outbreaks in Pahang appeared to be seasonal. Three of the five clusters commenced in December and January which is when school holidays end as well as in the rainy season in the east coast of Peninsular Malaysia. “Hari Raya Korban” is celebrated in Zulhijjah in the Muslim/Islamic calendar, and normally falls in the month of November or December in the Gregorian calendar. During this celebration many live cattle are moved throughout the whole country, as well as in Pahang. The majority of people in Pahang are Muslim and the Muslims believe in sacrificing specific food animals on this day. The fresh meat from the sacrificed animals is then distributed to the poor. Furthermore the wedding season in Pahang, as in other parts of Malaysia, coincides with these school holidays. Both situations are associated with a substantial increase in the number of cattle slaughtered in Pahang. One of the clusters started on the 25th September 2005 and also included the months of December and January. This cluster also coincided with another big festival in the Muslim community (“Aidil Fitri” celebration) which usually falls in the month of October. It is a celebration after one month of fasting and there are many parties held within the month of Syawal in the Muslim calendar (after the fasting month). During this month Muslims are encouraged to visit their families, relatives and friends. Consequently there also is a tremendous amount of human movement during that month. Furthermore when visiting relatives and friends it is traditional to exchange food, and therefore there is an increase in the movement of animals for slaughter for these purposes as well as increased movement of fresh meat and processed products all of which have been shown by others to increase the risk of transmission of FMD (Kitching, 2002a; Moutou, 2002; Mansley et al., 2003; Mahy, 2005; Wongsathapornchai et al., 2008; Lin et al., 2009; Bressel et al., 2010).
There was a decrease in the number of cases of FMD after the mass-vaccination program was implemented in March 2004 (Figure 5.1). The absence of reported cases from August 2004 to July 2005 might reflect the effect of this mass vaccination campaign. However the mass vaccination program was not able to be continued after the initial round due to the high cost of vaccine administration because of the animal husbandry system adopted where many farmers did not have proper holding yards or facilities for the effective implementation of a herd health program. As a result the government had to cover the cost of purchasing and administering the vaccine as well as providing portable (transportable) holding facilities (yards and crush) to allow the animals to be handled and vaccinated safely and efficiently. The lack of adequate husbandry facilities makes achieving an 80% vaccine coverage challenging. Consequently the disease was not able to be eradicated completely and clinical cases were reported again in the month of August 2005 until the end of the study. Furthermore local spread is an important factor in the distribution of the disease and it is important to rapidly identify outbreaks and implement control or stamping out practices (Gibbens and Wilesmith, 2002). However the control program adopted by the state of Pahang does not involve the culling of infected animals, but only the application of movement restrictions and vaccination. Although vaccination can reduce the incidence of disease it has been found to be effective only when combined with selective culling of clinically infected animals and NSP positive animals (McVicar and Sutmoller, 1969; Woodbury, 1995b; Alexandersen, 2002; Saraiva, 2004; Lubroth et al., 2007).
This analysis can be considered the first step towards understanding the spatial aspects of outbreaks of FMD in Pahang and highlights the need for further statistical and empirical analyses to be conducted to ensure suitable control procedures are implemented and subsequently evaluated. It is suggested that cluster identification tests should be conducted with directional tests to measure the significance of the mean directional spread of an outbreak and phylogenetic investigations undertaken in every cluster. These will increase the local information gathered to allow planning of a more complete and suitable control program for Pahang.
CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The overall aim of the research reported in this thesis was to improve the understanding of the epidemiology of FMD in Pahang. Specifically the temporal and spatial distribution of outbreaks of FMD in Pahang during the period 2003 to 2006 were described; risk factors associated with the occurrence of FMD in Pahang were identified; and the antibody response in Nallore cattle following vaccination against FMDV was reported. The information gathered can be used to develop more effective control and eradication measures for the state. Other work in Southeast Asia has suggested that eradication of FMD offers significant economic benefits to a country, even when no export of livestock occurs (Perry et al., 1999).

Pahang is one of the 13 states in Malaysia, therefore the findings of this study could be useful in helping to achieve Malaysia’s plan to become an FMD-free country through vaccination in the year 2016 (DVS, 2009a). One of the most important factors which determines the amount of effort provided by affected personnel in a disease control program is the level of awareness about the relevant disease (Garland, 1999; Leforban and Gerbier, 2002). The success of any control and eradication program developed in Pahang is dependent upon the combined efforts of all involved personnel including DVS staff, farmers, butchers and traders. To involve this diverse group requires a clear understanding of the epidemiology of the disease, knowledge about ways to control its spread, cooperation between agencies and suitable educational and awareness campaigns.
The legal and illegal movement of infected animals is considered to be the most important factor involved in the spread of FMD (Mansley et al., 2003; Robinson and Christley, 2007), particularly in the Southeast Asian region (Abila and Foreman, 2006). Geographically Peninsular Malaysia is connected to other Southeast Asian countries which can facilitate the spread of the disease through both international and local movements of livestock. In this study (Chapter 3), a high percentage of cattle trading were undertaken between farms within the state of Pahang (98%) which confirmed the findings of a study on the movement of cattle conducted in Pahang in 2005 (Azmie et al., 2006). Although there is legislation requiring all animal movements in Pahang to be accompanied by an animal movement permit, which includes a veterinary health certificate, there are no clear penalties documented for breaches to this legislation (DVS, 2004). Many studies have demonstrated that the most effective way for the spread of FMD is through direct contact either with clinically infected or subclinically affected animals incubating the disease (Donaldson et al., 1987; Alexandersen, 2002; Brown, 2004; Orsel et al., 2009). Consequently strengthening and enforcing the existing regulations is required.

In Pahang, as in all other states in Malaysia, there are special cultural and religious festivals. For these celebrations there is a strong community preference for the consumption/purchase of fresh meat from ruminants rather than frozen or processed products. This results in a high demand for fresh meat and the requirement for the daily slaughter of ruminant livestock. However the country is not self-sufficient in meat, producing only 26.9% of the beef and 9.6% of the sheep and goat meat required (DVS, 2009c). Consequently live cattle are imported from Thailand and other Southeast Asian countries into Peninsular Malaysia, resulting in increased risk
of the importation of a transboundary animal disease such as FMD (Wongsathapornchai et al., 2008). This situation makes disease control activities challenging. Findings from the temporal analysis in this study (Chapter 5) indicated that significant clusters of disease started just before and during the festival months. During these periods there is a dramatic increase in the movement of ruminants and in slaughter activities. It is crucial to consider this information when developing control measures for FMD. The control measures should be based on the establishment of effective biosecurity, surveillance and disease control programs which suit the local situations, particularly with respect to the festive seasons.

In Chapter 5 it was also found that three of the four clusters of outbreaks progressed for a long time, in contrast to outbreaks in some other countries such as Israel and Palestine where outbreaks have lasted for not more than a month (AlKhamis et al., 2009). The prolonged outbreaks with large clusters are the result of many combined factors including the vaccination regime adopted in Pahang and the absence of a culling policy for clinically affected and NSP positive animals. In other parts of the world it has been demonstrated that vaccination campaigns, combined with other zoo-sanitary control measures, have been successful in controlling the disease (Garland, 1999; Leforban and Gerbier, 2002). The limitations of vaccination must be considered when undertaking a control program. These include the generation of protective immunity only to those serotypes and subtypes included in the vaccine (Lubroth et al., 2007), difficulties in achieving a high coverage and problems with the storage, maintenance and distribution of the vaccine to field locations. At least 80% of animals in a herd need to be vaccinated to induce protective herd immunity (Lombard and Schermbrucker, 1993; Leforban and Gerbier, 2002). Effective
immunity must be considered on a herd basis and it is vital to vaccinate as many
animals as possible in a population to reduce the potential for disease in that
population (Doel, 2003). Failure to achieve an 80% vaccine coverage in a population
and maintaining a regular vaccination schedule, as recommended in Chapter 3, has a
significant negative impact on the development of protective immunity. This was
highlighted in Chapter 4 where it was found that not administering a second priming
(P2) dose to calves four weeks after the primary vaccination failed to induce high
levels of protective immunity. Furthermore an ideal immunization program should
induce both a systemic (serum) and mucosal (secretory) antibody response to protect
against clinical disease and to inhibit local virus replication and consequently virus
persistence (Barnett et al., 2004). Results reported in Chapter 3 indicated that in
Pahang most primary vaccinations were not followed by a P2. This situation was
worsened by the fact that only a few respondents understood the reason behind
vaccination and the importance of the P2 vaccination, as well as subsequent booster
vaccinations, to provide sufficient protection against FMD. The effectiveness of
vaccination in the control and eradication of FMD has been proven in other countries
(Perez et al., 2004; Saraiva, 2004; Lubroth et al., 2007). To achieve the target of
Malaysia being an FMD Free country by 2016 it is essential that there is adoption of
a second priming vaccination and booster vaccinations at 6 monthly intervals and a
high level of vaccination coverage. It is important to not just rely on vaccination but
also to improve diagnostic proficiency and capability, enforce stricter legislation
requirements, establish a surveillance network capable of providing early warning of
disease incursions/outbreaks, implement effective immediate control measures
through the coordination of several agencies, manage livestock transport
mechanisms, improve market inspection and hygiene compliance, and develop
public communication, education and awareness programs (Garland, 1999; McLaws et al., 2009).

In Chapter 4 NSP positive cattle were detected on a farm which had no previous history of clinical disease. This finding is not surprising since the farm was located in an endemic area and FMD is known to have a persistent carrier stage (Sallt, 1993; Alexandersen, 2002; Salt, 2004), moreover the virus can be spread by air (Donaldson, 1987), personnel (Sellers et al., 1970; Amass et al., 2003b; Amass et al., 2004), water, feed (Alexandersen et al., 2003c; Schijven et al., 2005) and fomites (McColl et al., 1995; Bartley et al., 2002). Importantly sub-clinically infected animals may spread FMDV through secretions and excretions (Bedson et al., 1927; Blackwell and Hyde, 1976; Donaldson, 1987; Donaldson et al., 1987; Brown, 2004). Therefore, depending on the objectives of the country, to eradicate the disease it is important to cull clinically infected animals and those with repeatedly positive NSP results. This recommendation is supported by the findings reported in Chapter 3 where it was demonstrated that retaining FMD seropositive animals in a farm had a significant impact on the likelihood of a farm having the disease. The role of virus shredders has been highlighted in other studies along with the potential for the transmission of virus within and between herds by aerosols. Under appropriate environmental conditions virus-laden droplets may travel vast distances whilst maintaining infectivity (Donaldson, 1987; Alexandersen et al., 2003b; Alexandersen et al., 2003c; Grubman and Baxt, 2004; Schijven et al., 2005). The virus may also persist (Sallt, 1993) in the pharyngeal region of infected cattle for up to 2.5 years, in sheep for 9 months and for up to 5 years in African buffalo. In addition, vaccinated
animals may become persistently infected long-term carriers without showing evidence of clinical disease (Burrows, 1966; Alexandersen, 2002; Doel, 2003). However the epidemiological significance of these carriers is controversial (Alexandersen et al., 2003c). Although the amount of persisting virus is low and the ability of carriers to transmit virus to other animals is equivocal, carriers are still considered a potential, but unquantified risk, for the spread of infection (Thomson and Bastos, 2004). The fear that they may occasionally initiate new outbreaks has led to international trade rules requiring either long waiting periods for carriers to recover from infection or the adoption of methods to remove carriers before FMD-free status can be restored to regions that have had outbreaks (OIE, 2008b). Consequently legislation to enable the culling of repeatedly seropositive animals, along with a suitable compensation scheme to ensure support from local farmers, is recommended in Malaysia. These, along with the development of more effective educational and awareness programs to increase farmer’s knowledge about the disease should be encouraged, allowing a quicker and more effective control program to be implemented (McLaws et al., 2009). A study in Australia showed that farmers, in the face of a hypothetical outbreak, were more concerned about losses to their individual animals than they were to losses to the livestock industry as a whole (Chen, 2008). This may lead some farmers not to report disease or to ignore animals with mild lesions and these may even be sold before the final disease diagnosis can be confirmed (Chen, 2008).

In the current study (Chapter 3) most farmers had little concern about the biosecurity of their own farm. This was probably related to the low level of awareness about the importance of good biosecurity measures and its significance in controlling FMD, as
well as other diseases. Unlike in other countries where animals form part of an important industry, cattle rearing in Pahang is mostly undertaken to supplement the farmers’ income. The farmers’ priority is on their primary income activity, which generally involves oil palm plantations. As FMD does not usually cause the death of adult cattle, most farmers are not concerned with the disease or do not appreciate the losses arising from infection. Therefore, efforts to change the farmers’ perceptions through education so they can understand the potential benefits of raising livestock should be considered. This study has identified there is an urgent need to improve the farmers’ knowledge, not only on the disease but also on its economic impact. This situation has also been identified in other countries, such as Thailand and India, where it has been shown that more educated farmers are more likely to cooperate with a disease control program (Saini et al., 1992). A study in the United Kingdom also indicated that education and awareness campaigns were important in the quick control and eradication of the disease (McLaws et al., 2009). In the current study most participants (87%) were able to recognise the clinical signs of the disease. It is important to educate farmers on the importance of early detection and reporting of suspect cases to the DVS. However, for the success of a control and eradication program for FMD in a country, identification of important local epidemiological factors along with a cost benefit analysis should be undertaken (Hutber et al., 2010).

Although many countries have successfully controlled and eradicated FMD through a stamping out or vaccination program, these can be challenging to implement in countries where the disease is endemic (Garland, 1999; Scudamore and Harris, 2002; Sutmoller et al., 2003; Lubroth et al., 2007). Clear Government policies are important to eliminate any source of infection and to restrict the movement of
animals and animal products as soon as possible after disease is detected to minimise the spread of the disease (Garland, 1999; Kahn et al., 2002). This recommendation has been clearly supported by other studies which have concluded that the most effective way for the spread of FMDV in ruminants is through direct contact, particularly through the movement of affected animals. Furthermore the capability of the virus to infect many animal species fosters rapid spread. Several authors have recommended pre-emptive culling in combination with vaccination to control an epidemic of FMD (Woolhouse et al., 2001; Bouma et al., 2003). The current study demonstrated that keeping NSP seropositive animals in a herd increased the likelihood of infection, and therefore it is recommended that clinically infected and repeatedly NSP seropositive animals are culled. However currently there is no legislation on the mandatory slaughter or culling of animals and there is no compensation provided by the Government to encourage the culling of clinically infected or seropositive animals in Malaysia (DVS, 2009a). Movement restrictions and ring vaccination, which were imposed during the initial stages of the FMD epidemic in Pahang, were applied to herds within a 5 km radius of new outbreaks. This did help in controlling the disease for a short period; however subsequent analysis of outbreaks has highlighted the need for wider control zones than those previously implemented. By vaccinating all potentially exposed animals, sufficient immunity should be developed to provide at the very least partial protection against clinical disease (Perez et al., 2004).

The multivariable analysis conducted as part of this study demonstrated that farmers with more than one species of ruminants were at a higher risk of having animals with FMD. This was probably due to the ability of small ruminants to act as a potential
source of infection (Barnett and Cox, 1999; Alexandersen et al., 2003c) and these findings supported the results of other studies (Al-Majali et al., 2008; Maddur et al., 2009). Transmission between cattle and buffalo has been reported, both in naturally occurring outbreaks (Dutta et al., 1983; Samara and Pinto, 1983) and experimentally induced disease (Gomes et al., 1997; Maroudam et al., 2008). Moreover the ability of the buffalo to be persistently infected and to act as a carrier has been confirmed (Barros et al., 2007; Maddur et al., 2009). Small ruminants, such as sheep and goats, can also be infected but not detected because of the absence of obvious clinical signs (McLaws et al., 2009). These animals may result in virus circulation for an extended period of time resulting in the spread of disease due to the common practice of farmers in Pahang of keeping more than one species of ruminants.

Although it is very important to understand the specific reasons behind the duration of the prolonged clusters reported in Chapter 5, this was not possible in this study because of time constraints. This should be investigated in detail in the future. It is important to investigate clusters of disease to identify predisposing factors for the outbreaks so that effective disease control plans can be developed, implemented and evaluated (Carpenter, 2001).

The majority of the important risk factors for FMD identified in Pahang were related to a lack of biosecurity. The role of direct and indirect spread is important and the movement of livestock, animal products and fomites were identified as important risk factors for disease in this study. The spread of the disease through indirect contact with fomites containing infectious viral particles, such as contaminated vehicles, feed, or clothing of livestock personnel has previously been well
documented (McColl et al., 1995; Bartley et al., 2002; Amass et al., 2003b; Amass et al., 2004). Importantly in this study, only 37.3% of farmers were able to correctly explain how FMD is spread. This situation highlights the need for an appropriate education campaign for personnel involved in the livestock industry. Although this study concentrated on farmers, it is likely that similar educational programs are warranted for other groups, including the DVS support staff, traders and butchers (Garland, 1999).

It was suggested that in order to control FMD more effectively in an endemic area, vaccination should be adopted in combination with movement controls together with compulsory culling of clinically affected animals. Emergency vaccination in the face of a severe challenge will help prevent or reduce the replication of virus and thereby dramatically reduce the amount of virus released into the environment in the important post-exposure period. Once vaccinated, these animals present a barrier to the further spread of the disease. While animals exposed to infection and those that are diseased will be eliminated, animals vaccinated for protective purposes could be tested to confirm the absence of viral activity and, if they are free of infection, allowed to live for the term of their productive lives. It is crucial to also get farmers involved in the timely adoption of a vaccination program, with the first and second vaccination given approximately one month apart, followed by booster vaccinations at six monthly intervals. This requires input and commitment from all levels from the DVS to the farmers. The effect of the mass vaccination program conducted immediately after the outbreak in March 2004 was seen in 2005 with no clinical cases reported between 15th July 2004 and the 15th September 2005. However clinical disease subsequently reappeared, indicating the ineffectiveness of the FMD
control and eradication measures adopted in the state of Pahang. Control and eradication of FMD either by stamping out or by vaccination is costly, however studies have shown that vaccination is economically beneficial (James and Rushton, 2002). Stamping out has significant problems from an operational point of view and is often not well accepted by the farming community. Due to the high cost and implementation issues with mass vaccination, such a program needs to be replaced by more cost-effective, strategic and carefully planned vaccination programs which are adequately resourced and effectively administrated and supported with appropriate legislation and penalties. Furthermore in order to achieve more than 80% vaccination coverage in the state, full commitment by the farmers is crucial.

In conclusion this study identified important factors associated with the rapid spread of FMD in Pahang. These included the movement of infected animals, poor biosecurity measures and ineffective vaccination programs. It is recommended that appropriate strategic vaccination, including the use of priming and booster vaccinations, be adopted to control FMD in Pahang. Along with vaccination it is recommended that legislation be developed to allow for the rapid compulsory culling of clinical cases of FMD and suitable broad educational campaigns be developed and implemented. Until such control measures are adopted it is likely that FMD will remain endemic in both Pahang and Peninsular Malaysia.
Appendix 1

Non-structural Protein (NSP) test: Ceditest procedure

The test procedure adopted followed the manufacturer’s test manual as described below:

**Day 1:**

1.0 Incubation with test serum

80 µl ELISA buffer was dispensed into all wells

20 µl of negative control sera were dispensed into wells A1 and B1

20 µl of weak negative control sera were dispensed into wells C1 and D1

20 µl of positive control sera were dispensed into wells E1 and F1

20 µl of each sera to be tested were dispensed into the remaining wells

The test plate(s) were sealed using the enclosed plate sealers

The test plates(s) were gently shaken

The plates were incubated overnight (16-18 hours) at room temperature (20-25°C)

**Day 2:**

Incubation with conjugate and chromogen/substrate solution

The test plate(s) were emptied after the incubation period and the plate(s) were washed 6 times with washing fluid. The plate(s) were tapped firmly after the last washing.
100 µl of the working dilution of the conjugate were dispensed into each of the wells.

The test plate(s) were sealed using the enclosed plate(s) sealers

The plates were incubated for 1 hour at room temperature (20-25°C)

The test plate(s) were emptied after the incubation period and the plate(s) washed 6 times with washing fluid. The plate(s) were tapped firmly after the last washing.

100 µl of chromogen/substrate solution were then dispensed into all wells

The plates were incubated for 20 minutes at room temperature (20-25°C)

100 µl of the stop solution was then added to all wells

The contents of the wells of the test plate(s) were mixed prior to measuring.

NB: The addition of the stop solution was started 20 minutes after the first well was filled with chromogen/substrate solution. The stop solution was added in the same order as the chromogen/substrate solution had been dispensed.

Reading of the test plate(s) and calculating the results

The optical density (OD) of the wells was measured at 450 nm preferably within 15 minutes after colour development had been stopped.

The $OD_{450}$ of wells A1 and B1 (negative control = OD max) was measured

The percentage inhibition (PI) of the control and the test sera were calculated according to the following formula:

$$\frac{OD_{450,\text{test sample}}}{OD_{450,\text{control}}}$$
\[ PI = 100 - \frac{OD_{450}}{OD_{\text{max}}} \times 100 \]

NB; The \( OD_{450} \) values of all samples were expressed as percentage inhibition (PI) relative to the mean OD of the negative control (ODmax).

**Ceditest procedure:**

The test procedure adopted followed the manufacturer’s test manual as described below:

**Day 1:**

Incubation with test serum

80 \( \mu l \) ELISA buffer was dispensed into all wells

20 \( \mu l \) of negative control sera were dispensed into wells A1 and B1

20 \( \mu l \) of weak negative control sera were dispensed into wells C1 and D1

20 \( \mu l \) of positive control sera were dispensed into wells E1 and F1

20 \( \mu l \) of each sera to be tested were dispensed into the remaining wells

The test plate(s) were sealed using the enclosed plate sealers

The test plates(s) were gently shaked
The plates were incubated overnight (16-18 hours) at room temperature (20-25°C)

**Day 2:**

Incubation with conjugate and chromogen/substrate solution

The test plate(s) were emptied after the incubation period and the plate(s) were washed 6 times with washing fluid. The plate(s) were tapped firmly after the last washing.

100 µl of the working dilution of the conjugate were dispensed into each of the wells.

The test plate(s) were sealed using the enclosed plate(s) sealers

The plates were incubated for 1 hour at room temperature (20-25°C)

The test plate(s) were emptied after the incubation period and the plate(s) were washed 6 times with washing fluid. The plate(s) were tapped firmly after the last washing.

100 µl of chromogen/substrate solution were then dispensed into all wells

The plates were incubated for 20 minutes at room temperature (20-25°C)

100 µl of the stop solution was then added to all wells

The contents of the wells of the test plate(s) were mixed prior to measuring.

NB; The addition of stop solution was started 20 minutes after the first well was filled with chromogen/substrate solution. The stop solution was added in the same order as the chromogen/substrate solution had been dispensed.
**Reading of the test plate(s) and calculating the results**

The optical density (OD) of the wells was measured at 450 nm preferably within 15 minutes after colour development had been stopped.

The $\text{OD}_{450}$ of wells A1 and B1 (negative control = OD max) was measured.

The percentage inhibition (PI) of the control and the test sera were calculated according to the formula below:

$$\text{PI} = 100 - \frac{\text{OD}_{450\text{test sample}}}{\text{OD}_{450\text{max}}} \times 100$$

NB: The $\text{OD}_{450}$ values of all samples were expressed as percentage inhibition (PI) relative to the mean OD of the negative control (ODmax).
Appendix 2

Liquid phase blocking ELISA test procedure

Reagent and sample preparation

Trapping Antibody Stocks

The freeze dried contents of a vial of each trapping rabbit antibody (FMDV Serotypes O, A and Asia 1) were reconstituted with 0.5 ml sterile deionized water by gentle agitation.

The stock was stored in the original vials at 1 to 8°C

The sera were further diluted 1000 fold with coating buffer to coat plates.

Only 1 vial was used at a time

Detecting Antibody Stocks

The freeze dried contents of a vial of each detecting guinea pig antibody (FMDV Serotype O, A and Asia 1) were reconstituted with 0.5 ml sterile deionized water by gentle agitation.

The stock was stored in the original vials at 1 to 8°C.

The sera were further diluted 100-fold with diluent Buffer B (phosphate buffered saline) and used in the assay

Only 1 vial was used at a time
Anti-Species Conjugate Stocks

The stock was prepared by reconstitution of the freeze dried contents of a vial with 1 ml of sterile diluent #2 (water plus glycerol, including 0.02% merthiolate) and gently mixed until completely dissolved.

The stock was stored in the original vials at -20°C

The conjugate was further diluted 200-fold with diluent Buffer B (phosphate buffered saline) and used in the assay.

Only 1 vial was used at a time

Antigen Stocks

Working dilution:

O₁ Manisa 1:100

A₂₂ Mahmatli 1:100

Asia 1 Shamir 1:100

The master stock of the control antigen were stored at -80°C

Control Serum Stocks

The freeze dried contents of each positive (C++, strong antibody positive. C+, medium antibody positive) and negative (C-, antibody negative) control were
reconstituted with 0.5 of sterile deionized water including 0.02% (reconstitution diluent #1) by gentle agitation.

The stock was stored in the original vials at 1 to 8°C.

Only 1 vial was used at a time.

**Chromogen Buffer**

0.05M Phosphate-Citrate Buffer, pH 5.0 was obtained by dissolving one tablet in 100 ml of locally produced deionized water.

The Chromogen buffer was stored in 1 to 8°C for no longer than 1 week.

**Chromogen Stock**

Ortho-Phenylenediamine (OPD tablets). 1 tablet (30 mg) was dissolved per 50 ml of 0.05M phosphate-citrate buffered solution at pH 5.0 and stored at room temperature in the dark.

**Substrate Stock**

3% (w/v) H₂O₂ (882mM). 1 hydrogen peroxidise tablet was dissolved in 10 ml of locally produced sterile distilled/deionized water.

The stock was stored at 1 to 8°C in the dark.

**Coating Buffer**
0.05 M Carbonate/bicarbonate, pH 9.6 +/- 0.05. 1 capsule was dissolved in 100 ml of locally produced sterile distilled/deionized water

The stock was stored at 1 to 8°C for no longer than 1 week

**Diluent Buffer A (for antigen and test sera dilutions)**

0.01 M Phosphate Buffered Saline, pH 7.4 +/- 0.02 plus 0.05% (v/v) Tween 20.

5 PBS tablets were dissolved per 1 litre of locally produced distilled/deionized water.

500µl of Tween 20 was added per litre and the solution mixed.

The diluent was stored at 4°C for no longer than 2 weeks.

**Diluent Buffer B (for detecting antibody and conjugate)**

0.01 M Phosphate Buffered Saline, pH 7.4 +/- 0.02 plus 0.05% (v/v) Tween 20 plus 5% (w/v) Skimmed Milk Powder

Diluent Buffer A was prepared:

0.01 M Phosphate Buffered Saline, pH 7.4 +/- 0.02 plus 0.05% (v/v) Tween 20.

500 µl of Tween 20 was added per litre and the solution mixed.
The diluent was stored at 4°C for no longer than 2 weeks.

On the day of testing Skimmed milk powder (5%, w/v) was added to diluent Buffer A to make the diluent Buffer B required.

PBS tablets were dissolved per 1 litre of locally produced distilled/deionized water.

Wash Buffer

Phosphate Buffered Saline, pH 7.4 +/- 0.20

5 PBS tablets were dissolved in 1 litre of locally produced sterile distilled/deionized water.

The wash buffer was transferred to a washing container

The wash buffer was further diluted by the addition of 4 litres of locally produced distilled/deionized water

The wash buffer was stored at room temperature.

Stopping Solution

1.25 M Sulphuric Acid

68 ml of concentrated sulphuric acid (18 M) was slowly added to 932 ml of locally produced distilled/deionized water. The stopping solution was stored at room temperature.
Disinfectant

0.2% (w/v) citric acid solution

2 gm of citric acid was dissolved in 1 litre of locally produced distilled/deionized water

The disinfectant was stored at room temperature.

Assay Procedure

Coating of Microplates

a. Vials containing trapping rapid stock (FMDV serotypes O, A and Asia 1) were gently agitated

b. A working dilution of trapping antibody stock in coating buffer was prepared at a dilution of 1:1000 (Each serotype-specific trapping antibody was diluted separately).

The working dilution was agitated gently to ensure uniform dispersion.

All the plates were aligned correctly (with the letters on the left hand side)

50 µl of working dilution of trapping antibody was immediately dispensed into all 96 wells.

The sides of the microplates were tapped to ensure the trapping antibody was evenly distributed over the bottom of the wells.

The microplates were covered and incubated at 1 to 8°C
The remainder of the aliquot of the FMDV trapping rabbit antibody stock was then returned to 1 to 8°C

**Test and Control Serum Incubation (Liquid phase)**

**Screening Assay**

The test and control sera were gently agitated to ensure homogeneity.

A 1/16 dilution of each control and test sera in suitable dilution tubes were prepared by the procedure below:

15 µl of an undiluted control bovine serum was added to 225 µl of Diluent Buffer A

10 µl of diluted serum was added to 150 µl of Diluent Buffer A for each test serum.

The dilutions were gently agitated to ensure homogeneity.

The test and control sera were added to the wells of the polypropylene U-bottom microplate at a dilution of 1/16

50 µl volume of pre-diluted test and control sera were added to the wells of polypropylene U-bottom microplate according to the plate layout #1 (Appendix 1)

50 µl of Diluent Buffer A was added to the antigen control wells

The details and position of test sera on the ELISA Data Sheet were recorded (Appendix 3)
**Titration Assay**

Two fold dilution range (for evaluation of positive sera)

A 1/16 dilution of the control sera were prepared as in 2.1.1

A 1/8 dilution of test sera in suitable tubes or microplates were prepared by adding 20 µl of diluted serum for each test serum to 140 µl of Diluent Buffer A

For each run and for each FMDV serotype the C++ control was also titrated in the same manner as the test serum.

50 µl of diluents Buffer A was dispensed into columns 3 – 12 for each polypropylene U-bottom microplate.

50 µl volume of pre-diluted control sera were added to the appropriate wells according to Plate layout #2 (Appendix 2)

50 µl of Diluent Buffer A was added to the antigen control (Ca) wells

50 µl volumes of pre-diluted test serum and the C++ control were added to the appropriate wells of row A or E, columns 3 – 12 according to the plate layout #2.

The 100 µl contents in the well were carefully mixed by filling and emptying the pipette tip several times, taking care not to introduce air bubbles.

50 µl of the dilution was transferred to the next row (A to B) and the contents of row B were carefully mixed.

50 µl from row B was transferred to the next row (B to C) and the mixing procedure was repeated.
50 µl from row C was transferred to row D. The dilution was carefully mixed and 50 µl of the dilution was finally discarded. This will result in a test sample dilution series from 1/16 to 1/128 in 50 µl volumes.

The above procedure was repeated for the next test samples for row E through to H using new pipette tips.

Details and position of test sera were recorded on the ELISA Data Sheet.

**Five-fold dilution range (for evaluation of post-vaccination sera)**

A 1/16 dilution of the control sera were prepared as in 2.1.1

20 µl of an undiluted control serum was added to 300 µl of Diluent Buffer A for each control serum

60 µl volume of the pre-diluted control sera were added to the appropriate wells of polypropylene microplates according to Plate Layout #2 (Appendix 2)

60 µl of Diluent Buffer A was added to the antigen control (Ca) wells.

1/5 dilution of each test serum (and the C++ control) were prepared by adding 20 µl of undiluted serum to 80 µl of Diluent Buffer A.

60 µl of Diluent Buffer A was added to all columns 3 -12 in each polypropylene microplate.

15 µl volumes of 1/5 diluted test serum (and the C++ control) were added to the appropriate wells of row A or E, column 3 – 12 according to Plate Layout #2.
The 75 µl contents in the well were carefully mixed by filling and emptying the pipette tip several times, taking care not to introduce air bubbles.

15 µl of the dilution was transferred to the next row (A to B) and the contents of row B were carefully mixed.

15 µl from row B was transferred to the next row (B to C) and the mixing procedure was repeated.

15 µl from row C was transferred to row D. The dilution was carefully mixed and 15 µl of the dilution was finally discarded. This resulted in a test sample dilution series from 1/25 to 1/3125 in 60 µl volumes.

The above procedure was repeated for the next test samples for row E through H using new pipette tips.

Details and positions of test sera were recorded on the ELISA Data Sheet.

**Addition of FMDV antigen**

The antigen stock was removed from the freezer and thawed.

Prior to making the working dilution in Diluent Buffer A the antigen stocks were equilibrated to room temperature.

Working dilution of the FMDV antigen (FMDV serotype O, A and Asia 1) in Diluent Buffer A in a volume sufficient for microplate use was prepared (5 ml per plate plus an additional 1 ml for 5 five-fold dilution range plates).

The suggested working dilutions: O₁ Manisa 1:100, A₂₂ Mahmatli 1:100 and Asia 1 Shamir 1:100.
50 µl of the antigen working dilution were added to all 96 wells of the respective polypropylene U-bottom microplate for evaluation of positive sera. All wells contained 100 µl of a serum dilution and antigen at this stage. This resulted in a final serum dilution of 1/32 to 1/256 in the titration assay.

60 µl of the antigen working dilution was added to all 96 wells of the respective polypropylene U-bottom microplate for evaluation of post vaccinal sera. All wells contained 120 µl total of a serum dilution and antigen at this stage. This resulted in a final serum dilution of 1/50 to 1/6250 in the titration assay.

The microplates were briefly placed on an orbital shaker to ensure thorough mixing.

The polypropylene microplates were incubated at +1°C to 8°C overnight with the seal on.

The remainder of the master stocks of the control antigens were returned to -80°C for future use.

**Transfer of Serum/Antigen Mixture to the ELISA Plate**

The contents of all antibody coated microplates (NUNC Maxisorp) were discharged by inverting the microplates using an abrupt downward hand motion into a sink and the inverted microplates were slapped onto a lint-free absorbent towel to remove all residual contents.

All 96 wells of the microplates were filled with wash buffer using the Handiwash.
The contents were discharged again after filling using the same abrupt downward hand motion into a sink and the inverted microplates were slapped onto a lint-free absorbent towel to remove all residual contents.

The washing procedure was repeated with two more wash cycles of filling and emptying.

50 µl volumes of the serum/antigen mixture were immediately transferred from the polypropylene U-bottom microplates to the appropriate wells of the different polystyrene microplates (NUNC Maxisorp) according to the same layout as being used for the “liquid phase” after three complete wash cycles and ensuring that no residual contents were left in the microplates.

The microplates were sealed and placed in an orbital shaker housed in a incubator at 37°C for 1 hour with continuous shaking.

**Preparation of Diluent Buffer B**

0.01 M Phosphate Buffered Saline, pH 7.4 +/- 0.02 plus 0.05% (v/v) Tween 20 plus 5% (w/v) Skimmed Milk Powder

On the day of testing, the amount of Diluent Buffer B required was calculated and prepared by adding skim milk powder to a final concentration of 5% (w/v) to the required volume of Diluent Buffer A. (100 ml Diluent Buffer B was prepared : 5 gm skim milk powder was added to 100 ml of Diluent Buffer A )

The pH was adjusted to pH 7.4 +/- 0.20 using 0.1 M NaOH.
Addition of Detecting Antibody

A 1:100 working dilution from the homologous detecting antibody stock (anti-FMDV serotype O, A and Asia 1) in Diluent Buffer B in sufficient volume (5 ml of working dilution per plate plus additional 1 ml) for the plates being used were prepared immediately before the end of the serum/antigen mixture incubation for the first plate.

The remainder of the detecting guinea pig antibody stocks were returned to 1°C to 8°C.

The microplates were removed from the incubator after one hour incubation then washed with wash buffer as described previously.

A 50 µl volume of the working dilution of the detecting antibody (FMDV serotype O, A and Asia 1) were added into the 96 wells of the respective microplates immediately after washing.

The sides of the microplates were tapped to ensure that the working dilution was evenly distributed over the bottom of each well.

The microplates were sealed and placed on an orbital plate shaker housed in an incubator at 37°C for 1 hour with continuous shaking.
**Addition of Conjugate**

A 1:200 working dilution of the conjugate in Diluent Buffer B in sufficient volume for all microplates (5 ml of working dilution per plate plus an additional 1 ml) were prepared immediately before the end of the detecting antibody incubation.

Both the conjugate stock and its working dilution were agitated gently and thoroughly.

The remainder of the conjugate stock was returned to -30 to -50°C

The microplates were removed from the incubator after one hour incubation then washed with wash buffer as described previously.

A 50 µl volume of the working dilution of the conjugate was added into each of the 96 wells in the microplates.

The sides of the microplates were tapped to ensure that the conjugate working dilution was evenly distributed over the bottom of each well.

The microplates were sealed and incubated at 35 to 39°C for 1 hour with continuous shaking.

**Addition of Substrate/Chromogen and Stopping Solution**

The substrate/chromogen solution in a volume sufficient for the number of microplates being run was prepared immediately before the end of the conjugate incubation.

The final colourless substrate/chromogen solution was stored in the dark.
A clean microplate (not coated with trapping antibody) was used as the “blanking plate” for the photometric reading.

The microplates were removed from the incubator after one hour incubation then washed with wash buffer as described previously.

All 96 wells of each microplate were completely flooded with wash buffer to eliminate unreacted conjugate.

50 µl volumes of the substrate/chromogen solution were added into the 96 wells of each microplate, starting with the first column of the “blanking plate” followed by all 96 wells of the microplates in the test run immediately after washing.

The microplates were incubated at ambient temperature for 15 minutes without plate shaking immediately after filling the first wells.

50 µl volumes of the stopping solution (1.25 M sulphuric acid) was added immediately starting with the first column of the “blanking plate” followed by all 96 wells of the test run after 15 minutes of substrate/chromogen incubation.

The microplates were placed briefly on the shaker to ensure even mixing. All wells contained 50 µl of substrate/chromogen solution plus 50 µl of stopping solution at this stage.

**Measurement of Substrate Development**

The “blanking plate” was placed in the carriage of the photometer and the blanking sequence was initiated.
The first microplate of the test run was placed in the carriage of the photometer and the reading sequence was initiated.

The step described in 2.2.9.2 was repeated for each microplate.

**Assay performance and interpretation**

**Data Expression**

Microplate reading was used in two data analysis.

Percent Inhibition (PI) values which were used for Quality Assurance (QA) acceptance. These values were calculated as follows:

\[
PI = 100 - \frac{\text{Replicate OD of control \times 100}}{\text{Median OD of Ca}}
\]

Percentage Inhibition (PI) values were used for replicate values for test sera. These values were calculated as follows:

\[
PI = 100 - \frac{\text{Replicate OD of test serum \times 100}}{\text{Median OD of Ca}}
\]
Calculation and acceptance of Control Data

The data expressed in OD values and PI values for the antigen control (Ca) and the data expressed values in three other controls (C++, C+ and C-) were used to determined whether or not the test had been performed within acceptable limits of variability and therefore, whether or not the test sera data may be accepted from any given microplate.
Appendix 3

Survey on the possible risk factors contributing to Foot and Mouth Disease in the state of Pahang, Malaysia

Background to the Survey

This survey is designed to identify the risk factors involved in the occurrence of Foot and Mouth Disease (FMD) in the state of Pahang. It is believed the control of FMD in beef cattle could be optimized by identifying and reducing the risk factors for infection which can help reduce production losses from disease outbreaks. This study is jointly sponsored by the Department of Veterinary Services, Malaysia and Murdoch University, Australia. The confidentiality of any information given by respondents in this study will be maintained throughout the course of the study.

_________________________________________________________

Farm No.: District: Farm Location: Date:

________________________________________________________________________________________

Instructions: Please respond by ticking or circling the appropriate answer or by writing the answer in the allocated area.

MANAGEMENT

1. What is the total number of cattle present on your farm? ....................

2. How many herds are run on your farm? .............................

3. How many animals are there in one herd? .............................

4. Is your farm registered with the State Department of Veterinary Service (DVS)?

   Yes ☐   No ☐
5. What breed(s) of cattle are present on your farm?

Kedah Kelantan

Nellore

Cross breed

Other: Please specify ..................

6. What type of grazing management is practiced in your herd?

Kept in barn and fed

Free grazing for a fixed time

Free grazing all the time

Other: Please Specify ..................

7. If the cattle are able to graze, what type of grazing system(s) are adopted for the herd?

No restriction (free area)

Systematic rotational grazing (the cattle are moved daily to graze, electric fencing is used to confine the cattle)

The cattle are allowed to graze in particular areas restricted by fencing.
8. For each of the following groups are the cattle separated according to their age groups?

- Weaned males
- Heifers
- Female Breeders
- Bulls
- Cows with calves

9. Are the cattle separated according to gender (males, females)?

- Yes □
- No □

10. Are the cattle fed any feed supplement?

- Yes □
- No □

11. If yes, what type of feed supplement is used?

- Palm Kernel Cake (PKC)
- Concentrate
- Other: Please Specify □  ....................

12. If a feed supplement is used, is it specially made or purchased?

- Made □
- Purchased □
- Both □
13. If the feed supplement is purchased where do you buy it from?

- Feed distributor
- Pet shops
- Other: Please specify

14. What is the source of water for the herd?

- Tap water
- River water
- Other: Please specify

15. Approximately how far is your herd from the nearest cattle herd or other livestock, such as buffalo, goats, sheep or pigs?

- <2Km
- 2 – 4Km
- >4Km

16. Did you introduce any new animals to your farm in the last year?

- Yes
- No

17. If yes how many animals did you introduce last year?

- …………….
18. If animals were introduced how many times did you introduce new animals to your farm over the last year?  

19. Over the last year have you introduced any bulls to your herd for breeding purposes?

Yes ☐  No ☐

20. If yes when was the last date of introduction?  

21. Have you purchased new replacement animals in the last year?

Yes ☐  No ☐

22. If you purchased replacement animals, how many did you purchase in the last year?  

23. Where did you buy/obtain your replacement stock from?

From traders ☐

From a known farm ☐

Other: Please Specify ☐

24. If you buy from traders, do you know where the traders get their animals from?  

25. Do you buy animals from other Malaysian states or other countries?

Yes ☐  No ☐

26. If yes, please specify the name of the state/country?
27. Do you exchange livestock with friends, relatives or other people?

   Yes ☐    No ☐

28. Do you examine/check the animals before you buy them?

   Yes ☐    No ☐

29. How many times in the last year have you sold cattle?

   ................................

30. If you sold cattle how many cattle did you sell in the last year?

   ................................

31. Is a blood screening test for Foot and Mouth Disease performed prior to movement of cattle in and out of your herd?

   Yes ☐
   No ☐
   Don’t know ☐

32. If you test animals against FMD, what do you do with any FMD test positive cattle?

   Culled or Slaughtered ☐
   Sent to another farm ☐
   Isolated on the same farm but confined to an isolation area ☐
   Remain in the herd ☐
BIOSECURITY

33. Do you undertake any quarantine procedures before you introduce new cattle to your herd?

Yes ☐  No ☐

34. If yes, please specify ............................................

35. Is the herd confined to an area surrounded by a fence?

Yes ☐  No ☐

36. Can the public or any unauthorized personnel enter the herd area?

Yes ☐  No ☐

37. Is there a dip/spray for disinfectant at the entrance to the farm?

Yes ☐  No ☐

38. If yes, what type of disinfectant is used? ........................

39. Do you allow unauthorized vehicles to enter your farm?

Yes ☐  No ☐

40. Are vehicles sprayed with disinfectant either on entering or leaving your farm?

Yes ☐  No ☐

41. If you use a vehicle dip is it covered from rain and sunlight?

Yes ☐  No ☐
42. Are animals vaccinated against Foot and Mouth Disease (FMD) in your herd?  
   Yes ☐  No ☐

43. If vaccination is used, when was your herd first vaccinated against FMD?  
   Date………………

44. If vaccination is used, when was the most recent vaccine against FMD given?  
   Date………………

45. If vaccination is used, what type (brand) of FMD Vaccine was used most recently in your herd?  ……………………

46. Do you know the strain(s) of the FMD virus in the vaccine?  
   Yes ☐  No ☐

47. If yes, what FMD strain/types are included in the vaccine used ………………

48. How many cattle were vaccinated at the most recent vaccinations ………………

49. How many times your animals are vaccinated each year?  ………………
50. Has your herd previously been infected with FMD?

Yes ☐ No ☐

51. Do you know what causes FMD?

Yes ☐ No ☐

52. If yes please explain. ...........................................................

53. Do you know the clinical signs of FMD?

Yes ☐ No ☐

54. If yes, please describe these signs. ...........................................

55. Do you know how to control FMD?

Yes ☐ No ☐

56. If yes, please explain how you think the disease should be controlled.

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Thank you for taking your time to complete this questionnaire.

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