Epidemiology, pathogenesis and surveillance of the pig adapted strain of foot and mouth disease in Taiwan

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DVM, MSc

This thesis is presented for the degree of Doctor of Philosophy, Murdoch University, 2008.

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

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Abstract

Foot-and-mouth disease (FMD) is one of the most contagious infectious diseases of domestic and wild cloven-hoofed animals, particular in cattle, sheep, pigs, goats and domestic buffalo, as well as wild ruminants such as deer. In Taiwan, there was a severe outbreak of FMD after more than 60 years freedom from the disease. The virus strain, O Taiwan 97 from the March 1997 outbreak of FMD in Taiwan, however, has been shown to have a species-specific adaptation to pigs. Although there are 7 distinct serotypes of FMD found in different regions of the world, this study focuses on the pig-adapted type O strain of FMD.

After the FMD outbreak commenced in Taiwan, the spread of disease was very rapid and the whole of the western parts of Taiwan was affected within a few days after the diagnosis of FMD was confirmed. In some situations airborne transmission of FMD virus was suspected and it was speculated that this was the explanation for such rapid spread in Taiwan. Therefore, studies were conducted to investigate the transmissibility of O Taiwan/97 FMDV to susceptible pigs by direct and indirect spread including airborne spread in an enclosed animal house. This study showed that pigs in direct contact with challenged pigs became infected but none of the close-contact pigs became infected. These experiments clearly demonstrated that the pig adapted strain O Taiwan/97 was only efficiently transmitted by direct contact. This indicates that effective control against future outbreaks of pig adapted FMDV strains could be achieved by restriction of pig movement and stamping out if the outbreak has been detected in the early stages and prior to the

movements of pigs from the infected premises.

The measures used to control the Taiwanese FMD outbreak in 1997 were initially the slaughter of whole herds in the infected premises. However, with the rapid spread and large numbers of cases, the decision was taken to use universal compulsory vaccination of pig herds to control the outbreak when sufficient supply of vaccines was organized. Type O FMD vaccines were imported from a number of major FMD vaccine manufactures from around the world. Initially, vaccine efficacy for the imported vaccines was tested by measurement of neutralizing antibody titers in vaccinated pigs. To establish the relationship between serum neutralizing titers and protection from foot and mouth disease in pigs after vaccination, challenge studies were conducted with O/Taiwan/97 FMD in vaccinated pigs. Additionally, antibody responses to structural (neutralizing antibody) and non-structural proteins (NSP) were evaluated in vaccinated pig herds after primary and secondary vaccination in herds infected before and after vaccination.

In order to be able to monitor the circulation of virus in vaccinated pig populations, valid diagnostic kits based on the detection of antibody against NSP were required. These tests needed to be evaluated against pig sera derived from challenge studies and natural FMD outbreaks. Three commercially available ELISAs (Cedi, UBI and Checkit), which were available to differentiate infected from vaccinated pigs, were tested and results showed that the Cedi test had the optimal sensitivity and specificity for pig adapted type O FMD testing. This test was used to retrospectively evaluate the sera collected from infected and non-infected pig herds collected sequentially in the year after the 1997 FMD outbreak in

Taiwan. These studies also showed that the early vaccines used, stimulated NSP antibody production in swine herds that were vaccinated but not infected. This resulted in the requirement for purified FMD vaccines to be used when monitoring programs for FMD infection by NSP testing were in place. In these studies, it was also demonstrated that the purified FMD vaccines used later in the control program did not induce NSP antibody after multiple double dosage to pigs.

Although clinical FMD appeared to be successfully controlled with vaccination program in Taiwan it was essential for the eradication plan to maintain active surveillance for NSP reactors in the pig population. The UBI and Cedi NSP kits were applied as screening and confirmatory tests, respectively, to pig sera collected in auction markets distributed around Taiwan to monitor for evidence of the circulation of FMD virus. Herds with positive reactors were followed-up by clinical inspection and 15 sera from suspected herds were further sampled. Negative results were obtained from all these investigation. With the absence of clinical outbreaks and the lack of evidence of FMDV circulation in the field from the NSP reactor surveillance, the Taiwanese government has progressed the eradication plan to a progressive cessation of vaccination, commencing with banning of vaccination on one isolated island in December 2006. The absence of outbreaks on that island, paved the way for further cessation of FMD vaccination in Taiwan from July 2008.

Publications

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Chapter 1 - Introduction and literature review

1.1 Background

Foot-and-mouth disease (FMD) is caused by infection with an Aphthovirus belonging to the family Picornaviridae and is one of the most contagious infectious diseases of cloven-hoofed animals, particular in cattle, sheep, pigs, goats and domestic buffalo, as well as wild ruminants such as deer (Sobrino et al., 2001). The onset of disease can be very rapid, especially in pigs. It is characterized by fever and vesicles on the snout, mouth, feet and udder of lactating animals.

The acute phase of the disease lasts for approximately one week then starts to resolve in the face of a mounting immune response of which the antibody response appears to be of particular importance as it is highly efficient in clearing virus from the blood stream. Mortality can occur in young animals, especially sucking piglets, due to the large amount of virus excreted in milk resulting in a viraemia and infection of the heart muscle causing heart failure (Burrows, 1968; Chang et al., 1997; Yang, 2002). The significant suffering and the loss of yield coupled with the highly contagious nature of the disease are the ultimate reasons for controlling this disease (Sutmoller et al., 2003).

There are a number of different serotypes of FMD found in different countries, but in Taiwan only type O FMD viruses have been found. There have been two distinct types of field viral strains, O Taiwan/97 and O Taiwan/99, isolated from outbreaks in 1997 and 1999. The virus strain, O Taiwan/97, however, has been shown to be a virus specifically adapted to infect pigs (Dunn and Donaldson, 1997) and caused overt clinical signs only in pigs in the March 1997 outbreak (Yang et al., 1999). This virus is highly virulent in pigs and field observations in Taiwan and Hong Kong (Dr. Trevor Ellis, personal communication, 2007) has shown that clinical signs can be found within 24 hours of placing pigs in heavily contaminated areas, which is different from pigs or cattle infected with other FMD viral strains. The other type O FMD virus (FMDV) that has been characterized in Taiwan, O/Taiwan/99, is a pan-Asian topotype of type O FMDV that caused recent outbreaks of FMD in the United Kingdom, Netherlands, Ireland, Japan, the Republic of Korea, and South Africa and can readily infect both ruminants and pigs (Huang et al., 2001; Leforban and Gerbier, 2002; Ozawa, et al., 2006; Sakamoto and Yoshida, 2002).

Unlike ruminants, in particular cattle, which can become carriers of live FMDV for a variable period of time after contact with infection, pigs have not been reported to be a carrier after 28 days post infection (Sutmoller et al., 2003).

After the outbreak of FMD in Taiwan, a blanket vaccination program was implemented which led to a large reduction in new outbreaks and quickly brought the outbreak under control in 1997. The disease has been largely eradicated from Taiwan by compulsory vaccination campaigns of susceptible animals. The last reported case was caused by O Taiwan/97 in pigs in a slaughterhouse in 2001 (Yang, 2002). Since then no further outbreaks have been reported. In May 2004 Taiwan was granted FMD free status with vaccination by the OIE (OIE, 2006). By the end of 2007, the Taiwanese Government is planning to pave the way for gaining FMD freedom status without vaccination and will progressively ban vaccination throughout Taiwan starting from 2008. Some islands around Taiwan have already banned vaccination and authorities are testing for evidence of any FMDV circulation in the field. The consideration of a non-vaccination policy was a direct result of the success of the annual vaccination campaigns after the outbreak of FMD in 1997. However the recurrence of FMD in the future is possible from new incursions as more animals become susceptible in Taiwan following the ban of vaccination.

The successful control of FMD with vaccination was reported in Europe in 1991 and Argentina in 2003 and subsequently in Taiwan in 2005. There were major differences in

the epidemiology of the outbreaks in Taiwan in 1997 and the UK in 2001. A massive blanket vaccination program was implemented in Taiwan, in contrast to the complete absence of vaccination in the UK epidemic (Kitching, 2005; Yang et al., 1999). In evaluating and understanding the success of the Taiwanese vaccination program, it is important to study the kinetics of immune responses in pigs with vaccines or natural infection; the level of neutralizing antibodies which is necessary to prevent clinical infection; and FMD vaccine formulations to ensure that optimum vaccines are used for controlling pig adapted strains of type O FMDV, because these behave differently from other type O FMD strains.

In countries which have implemented prophylactic FMD vaccination, such as Taiwan, a major issue is to differentiate infected from vaccinated animals during surveillance programs to detect the circulation of FMDV in the field. Methods available to achieve this include virus isolation in cell cultures, detection of virus by PCR diagnostics applied to field specimens, or by serological means to detect antibody to non-structural proteins of FMDV (OIE, 2004 b). Recently, commercial kits have become available for detecting non-structural protein antibodies in sera from infected animals. These tests have been largely validated in cattle (Brocchi et al., 2006). Further studies on the sensitivity and specificity of these kits are urgently needed with “gold standard” pig

serum samples derived from experimental challenge studies and field outbreaks caused by pig-adapted FMDV as well as other types of FMDV infection in pigs.

Studies need to focus on the differences in the antibody mediated immune responses in pigs vaccinated and/or infected with pig adapted FMDV, including data on the kinetics of antibody development in vaccinated or non-vaccinated pigs subsequently challenged with FMDV and to relate the level of antibody to protection from disease with O Taiwan/97 FMD virus.

Foot and Mouth Disease can spread by different mechanisms including direct contact with infected animals, contaminated animal products, equipment and vehicles, in particular by animal transport vehicles, by people and transmission of virus by the wind (Amass et al., 2004; Donaldson, 1997). Quick recognition of disease, followed by slaughter of infected livestock and the implementation of movement restriction greatly reduces the risk of mechanical spread. However, the potential for airborne spread of virus is feared by farmers and veterinary authorities because it would be less effectively controlled by quarantine and movement control.

The determinants of airborne transmission include species of animals infected, the virus

strain, the environmental conditions, and the location of more susceptible animals, such as cattle, downwind (Alexandersen et al., 2003 b). Pigs are considered to be relatively resistant to infection by the airborne route. From recent studies, the risk for pig to pig transmission by airborne spread is considered relatively low (Alexandersen et al., 2002a; Kitching, 2000). Alexandersen and Donaldson (2002) emphasized the need for more quantitative data on airborne transmission of FMD in pigs. This will be especially important for the pig adapted strain found in Taiwan and will be very important in developing control measures for any future outbreaks in Taiwan.

These studies also aim to test the hypothesis that aerosol transmission of pig adapted type O FMDV was not significant in the spread of pig adapted FMDV in Taiwan. To investigate this, studies were conducted to determine the transmissibility of O Taiwan/97 FMDV to susceptible pigs by direct or indirect spread including airborne spread in an enclosed animal house and chamber.

1.2 Etiology

Foot and Mouth Disease is caused by a virus of the genus Aphthovirus, family Picornaviridae. The virus is a non-enveloped, icosahedral virus, 26 nm in diameter (Figure 1.1) containing a positive sense RNA genome of about 8500 nucleotides which

can be directly translated into a polyprotein and subsequently cleaved to produce the individual viral structural (VP1, VP2, VP3 and VP4) and nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D). The cleavage of the polyprotein is mediated by two proteinases, NSP leader protein and $3C^{pro}$ (Grubman and Baxt, 2004; Murphy et al., 1999)

There are seven serotypes of FMDV: O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1 determined by antigenic tests which include cross-neutralization tests and sera type specific liquid phase blocking ELISAs (OIE, 2004b). There is no cross-immunity between serotypes; immunity to one type does not induce protection against the others. Within the serotypes there are different subtypes, especially within types A and O, which have different levels of immunogenicity and cross-protection (O.I.E., 2004b).

Previous methods used to differentiate variants of FMDV strains included the migration of viral structural proteins in SDS-polyacrylamide gel electrophoresis (Knowles and Sharma, 1990) and using electrofocusing (King et al., 1983). However, now the main technique for characterization of genetic relationships between strains is the nucleotide sequence of viral RNA. The seven serotypes of FMDV were clustered into distinct genetic lineages with 30-50% difference in the VP1 gene (Knowles and Samuel, 2003). Based on VP1 sequence data the most prevalent FMD type O viruses could be further

grouped into eight topotypes in which the differences in nucleotide sequence are up to 15%. The eight topotypes are Cathay, Middle East-South Asia, South–East Asia, Europe-South America, Indonesia-1, Indonesia-2, East Africa and West Africa (Samuel and Knowles, 2001). Indonesia-1 and 2 are considered to be distinct, were not isolated outside Indonesia and have been eradicated. The Cathay topotype, which can not be grown in bovine derived cells, is highly adapted to pigs and it was characterized by a deletion in the C-terminal half of 3A coding region of the viral genome (Reard and Mason, 2000)

Domestic species which are susceptible to FMD include cattle, pigs, sheep, goats, camels and buffalo, while susceptible wildlife include deer, antelope and wild pigs. In experimental challenge studies in rabbits, mice, guinea pigs and white rats, infection with FMDV has been reported (Alexandersen and Mowat, 2005; OIE, 2004b).

1.3 Occurrence

Various serotypes of FMDV can cause disease in all cloven-hoofed animals and is endemic in parts of Africa, Asia and South America. The disease is currently not present in Japan, South Korea, USA, New Zealand and Australia (OIE, 2006). Western Europe is now free from disease but there recently have been several devastating outbreaks and

a more recent limited outbreak in the UK (2007- type O BFS 1860) all caused by FMD type O. In 1967-1968, the outbreak involved the slaughter of 407,341 animals. In 2001, the outbreak lasted for 8 months during which over 4 million animals were slaughtered and there were connected outbreaks in France, Netherlands and Ireland. The source of origin of the virus was not able to be identified (Gloster et al., 2005; Leforban and Gerbier, 2002). In 2007, there were limited outbreaks in the UK, with the source of infection from a nearby high security animal virus research institute and FMD vaccine plant (Anonymous, 2007).

The pig-adapted FMD strain (Type O- Cathay topotype), which only causes overt disease in pigs but not other cloven-hoofed animals, is endemic in parts of Asia namely China, Hong Kong, Philippines and Vietnam (Kitching, 2000). The virus was first found in samples submitted to the world reference laboratory in 1970 from Hong Kong. It has been reported regularly from Hong Kong since then and it is considered that the virus might have evolved in southern China, from where approximately 2 million pigs were imported each year. This virus appeared in the Philippines in 1994, and a country wide control program was commenced. Currently, the Philippines is on track to extend its OIE free zone statues to include Luzon Island (Professor John Edwards, personal communication, 2007), Subsequently, FMD was detected in pigs in Vietnam and Taiwan

in 1997 (Gleeson, 2002; Huang et al., 2000; Kitching, 2000; Samuel and Knowles, 2001). The outbreak in Taiwan in 1997 resulted in over 3 million pigs being slaughtered (Yang, 2002; Yang et al., 1999). It was contained after compulsory vaccination of all pig farms in Taiwan and the last case was reported in a slaughterhouse in 2001. In Vietnam, there were more outbreaks in 2006 and there is a risk that this pig-adapted strain might spread throughout South East Asia in the near future (Professor John Edwards, personal communication, 2007).

In Taiwan, soon after the containment of the large outbreak of FMD in 1997, there were limited outbreaks while the program of compulsory vaccination of pigs was in place. The last sporadic outbreak was found in March 2001. There were several outbreaks from 1997 to 2001 which involved 21 pig farms and three abattoirs. During those sporadic outbreaks 1735 pigs were slaughtered (Yang, 2002).

1.4 Methods of transmission

Foot and mouth disease is transmitted by different mechanisms between herds, countries and continents, including direct contact with infected animals, indirect contact via contaminated animal products, equipment and vehicles, in particular by animal transport vehicles, by people and also by airborne spread (Kitching, 2005).

Transmission of FMDV by direct contact between inoculated and sentinel pigs has been confirmed under experimental conditions (Alexandersen et al., 2002a). Pigs shed larger quantities of virus in their oropharyngeal secretions than other animals (Alexandersen and Donaldson, 2002). Under appropriate weather conditions, aerosolized virus can potentially spread a considerable distance, particularly if the source is a large infected pig herd. Airborne spread over a distance of over 250 km was suspected to be responsible for the transmission of the virus from infected pig herds in Brittany in France to the Island of Jersey (where it infected cattle), and then as far as the Isle of Wight off the coast of southern England (Gloster et al., 1982).

However, recent evidence, from experimental observations and circumstantial evidence from the field, has shown that pigs especially are relatively more resistance to transmission by airborne FMDV. Alexandersen et al. demonstrated that the transmission of O1 Lausanne and O Taiwan/97 strains of FMDV to susceptible pigs could be prevented if physical barriers were installed to prevent infected pigs from making direct physical contact with susceptible pigs and if measures were taken to prevent the mechanical transfer of virus (Alexandersen et al., 2002a). In this study however there was one sentinel pig which was infected and showed clinical signs and some pigs had

no clinical signs but produced antibody to FMD. In the Netherlands, in one outbreak of FMD on two veal-calf farms in 2001 there appeared to be a limited outbreak with minimal clinical signs of FMD in infected calves. A challenge study by Bouma et al. showed that none of the directly or indirectly exposed calves became infected with FMDV, even from affected calves from which the saliva sample contained $10^{4.9}$ p.f.u./ml of FMDV (Bouma et al., 2004). This report showed that virus transmission by aerosol or close contact to individually housed calves with this FMD strain was very inefficient.

Alexandersen et al.demonstrated that pigs challenged with O Taiwan/97 and estimated to be excreting $10^{4.4}$ $TCID_{50}$/24 hrs for 24-48 hours were not able to infect sentinel pigs by the airborne route (Alexandersen et al., 2003a).

1.5 Clinical findings and pathology

The clinical signs of FMD in cattle and pigs are prominent and severe, with the lesions easy to detect grossly. However, the clinical diagnosis of FMD in small ruminants may be difficult to detect due to the mild nature of the lesions (Kitching, 2002; Kitching and Alexandersen, 2002; Kitching and Hughes, 2002; Alexandersen et al, 2003b). Some strains of FMDV may be of low or have no virulence for some species however be extremely virulent for other species (Kitching 2000), for example, O/Taiwan/97 strain

causes severe infection in pigs but not in ruminants (Dunn and Donaldson, 1997; Yang et al, 1999). Other vesicular diseases, such as vesicular stomatitis and swine vesicular disease cannot be easily distinguished from FMD simply based on the clinical findings.

Infection of susceptible animals with FMDV leads to lameness, listlessness and in-appetence. The characteristic lesions of vesicles can be found on the feet, in and around the mouth, and on the mammary glands and teats. In severe infections of the feet, hooves may be shed, especially in pigs. Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs. The severity of clinical signs varies with the strain of virus, the exposure dose, and the species, age, breed and degree of immunity of the animal. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals and myositis may also occur in other sites (Alexandersen and Mowat, 2005; O.I.E., 2004b). The histopathological findings in cornified and stratified squamous epithelium are ballooning degeneration and intercellular edema within the epidermis. This is followed by the necrosis of epidermis and infiltration of neutrophils in the stratum spinosum and the superficial epidermis can easily rupture or be torn off by mechanical forces. Immature myocardial cells in young animals particularly are

susceptible to direct virus damage leading to acute myocarditis (Alexandersen ea al., 2003b; Chang et al., 1997).

In the 1997 Taiwanese outbreaks, clinical signs in the diseased sows were characterized by a febrile response, depression and anorexia with vesicular lesions on the snouts, feet and mammary glands. Vesicles were seen in the tongue, oral cavity, vulva and other skin sites particularly of the feet and coronary band. The shedding of claws and vesicles on pressure points on the knees and hocks were other common lesions. Blood smears on the floor with sloughed claws were found in the pens of severely affected pigs. Painful feet resulted in pigs being reluctant to move and walk which resulted in loss of appetite. Abortion of infected sows was seen during the outbreaks. Large fluid-filled vesicles were often located on the tip of the snouts. Lesions found in nursery and fattening pigs consisted of vesicles on the snouts and feet. Examples of clinical findings are shown in Figure 1.2. Examples of pathological findings in piglets dying of FMD induced myocarditis are shown in Figure 1.3 (Chang et al., 1997).

1.6 Morbidity and mortality rate

The morbidity rate in outbreaks of FMD in susceptible animals can reach over 90%. Mortality rates in adult animals is low, however, in young animals, especially sucking

piglets, mortality can approach 90-100% (Yang, 2002). Dead sucking pigs were apparent in litters where vesicular lesions were prominent in the mammary glands and teats of the sows in the 1997 outbreak in Taiwan (Chang et al., 1997). The sources of FMDV were from milk or directly contracted from the ruptured vesicular fluid on the mammary glands and teats (Burrows, 1968). Farmers were surprised in this outbreak to find whole litters of dead sucking pigs without any apparent clinical signs in the sows other than vesicular lesions on the mammary glands. This was in stark contrast to the situation with swine vesicular disease (SVD) which is seen occasionally in Taiwan (Straw, 2006).

1.7 Economic impact

Foot and mouth disease is one of the most important livestock diseases in the world with the potential to cause a huge economic impact (James and Rushton, 2002). The reasons for this are not only due to the ability of the disease to cause reduced production, but are also related to restrictions on the trade of animals and animal products both locally and internationally (Perry et al, 2002; Randolph et al., 2002). The severe economic damage caused by FMD infection in both UK and Korea highlights the impact of this infection (Ozawa et al., 2006; Thompson et al., 2002). In Taiwan the economic impact of the 1997 FMD outbreak was estimated to be 378.6 million US

dollars (Yang et al, 1999). The cost included compensation of pig producers for culling their pigs (US$187.5 million), loss of market value (US$125 million), cost of 21 million doses of vaccines imported from Argentina (US$13.6 million), carcass disposal (US$24.6 million) and environmental protection and other miscellaneous expenses (US$27.9 million) as well as the loss of export markets for the Taiwanese pig industry.

1.8 Diagnosis

1.8.1 Diagnostic methods

Definitive diagnosis of FMD from other vesicular diseases must depend on laboratory assessments. Immunological methods, viral isolation and PCR are used to identify viral antigen, virus or viral genome respectively. Enzyme linked immunosorbent assay (ELISA) detection of specific FMDV antigens in epithelial tissue samples and cell culture isolation is now used routinely in laboratories to diagnose FMD (OIE, 2004b). Antigen-capture ELISA's use specific high titred guinea pig and rabbit antiserum to detect specific FMD serotype antigens. Different rows in multiple well microtitre plates are coated with rabbit antisera against each of the seven serotypes of FMDV. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Then guinea-pig antisera against each of the respective serotypes of FMDV are added followed by rabbit anti-guinea-pig serum conjugated to an enzyme. After

adding enzyme substrate and chromogen, a color change indicates a positive reaction (Ferris and Dawson, 1988; Hamblin et al., 1984). The test can be applied to detect specific FMD antigen in a specimen and also to confirm the FMD serotype in virus culture supernatants (OIE, 2004b).

Viral isolation is carried out by inoculating suspected specimens into monolayer cell cultures, such as BHK21, IB-RS-2, primary bovine thyroid cells and primary pig, calf or lamb kidney cells. The cytopathic effect of cell cultures should be examined 24 to 48 hours after inoculation (OIE, 2004b).

Reverse transcription polymerase chain reaction (RT-PCR) can be applied to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum, vesicular fluid and oesophageal– pharyngeal (OP) samples. Reverse transcription combined with real-time PCR has a better sensitivity comparable to that of virus isolation and large numbers of small volume samples can be tested by automated procedures. Specific primers have been designed to distinguish between each of the seven serotypes (OIE, 2004b).

1.8.2 Differentiating infected from naïve or vaccinated animals

During FMDV infection the viral polymerase protein and other non-structural proteins are produced inside the infected cells but are not incorporated in the released virions. Therefore, antibody to these proteins will only develop if an animal has been actively infected and not vaccinated with purified virus (Doel, 2005). The non-structural peptides (2A, 2B, 2C, 3A, 3B, 3C, 3D) can be used as target antigens for tests to differentiate infected versus vaccinated animals. Antibody to certain non-structural proteins (NSP) of FMDV has been used to differentiate vaccinated from infected cattle in the field (Clavijo et al., 2004; De Diego et al., 1997; Mackay et al., 1998; Moonen et al., 2004; Sorensen et al., 1998). Recently, a number of ELISA's with high sensitivity and specificity for detection of antibody to NSP in pigs have also been reported as suitable for large scale eradication programs (Chung et al., 2002b; Lee et al., 2004; Shen et al., 1999). However, the numbers of validated samples used in the Lee et al. (2004) study for evaluation of the sensitivity of three assays were below the numbers recommended by the OIE for sensitivity evaluation (OIE, 2004a). The study by Chung et al (2002b) only evaluated an in-house kit based on the 3AB antigen obtained from Dr Sorensen that was not commercially available at that time. Since the ELISA-based methodology for NSP antibody detection provides many advantages, such as objectivity compared with the agar gel immunodiffusion tests for virus infection associated antigen, high sensitivity and specificity, and the capability for large-scale screening, NSP ELISA

tests have been recommended by the OIE to be used for serologic surveillance in regions or countries that practice FMD vaccination and for monitoring virus circulation in the field (OIE, 2004b). Recently, several NSP-ELISA assays have become commercially available for evaluation. A blocking ELISA has been developed (Sorensen et al., 2005) which has shown high sensitivity and specificity when tested with pig sera collected from naïve, infected, and vaccinated pigs. The test specificity for vaccinated pigs is 99% and can be improved to 99.5% by further treatment by filtration and inactivation at 56°C for 30 minutes. For naïve pigs the specificity was 99.8%. Another commercial kit was evaluated by Bruderer et al. (2004) who demonstrated the specificity was 99% for 3600 samples tested including bovine, ovine and porcine species. Using this assay antibodies specific for 3 ABC could be detected as early as 10 days post-infection. A third kit evaluated uses a 3B synthetic peptide based NSP ELISA which can be used to differentiate convalescent animals from vaccinated animals (Shen et al., 1999; Wang et al., 2001). There were no positive results detected in sera from vaccinated animals. However, preliminary field trials in Taiwan with this kit showed only 98.9% specificity in samples collected from vaccinated pigs. Comparative evaluations, including the above NSP ELISA kits, have been conducted by a consortium of European and American FMD reference laboratories in 2006 with large panels of sera from cattle that have been vaccinated or vaccinated-and-infected with different

serotypes of FMDV. Some sheep and pig sera were tested in the study but these authors stressed that insufficient numbers of samples from pigs had been tested in order to complete the evaluation of these tests for use with pigs (Brocchi et al., 2006). As these commercialized NSP ELISA kits will be very important for monitoring pigs for active FMD infection in countries using FMD vaccination programs, further evaluation of these tests are needed under different field conditions in order to determine the diagnostic sensitivity and specificity.

1.9 Control

Control of FMD has the ultimate aim of eradication of the virus from the population. Quick recognition of disease, imposition of quarantine followed by slaughter of infected livestock and implementation of movement restriction to reduce the risk of mechanical spread has been the classical approach to FMD control and this was used in the UK outbreaks in 2001 and 2007. However airborne spread of the virus, which is feared by farmers and veterinary authorities, is not able to be controlled by movement restrictions.

1.9.1 Vaccination

Control of FMD by stamping out depends on having effective veterinary services, funding for compensation, and adequate resources to slaughter and dispose of carcasses

and to enforce movement restrictions. In some countries and situations it is not possible to control the disease using stamping out procedures (Yang et al., 1999). Vaccination is used as an option to control FMD when stamping out is not feasible. The aim is still to control and eradicate the infection but in the short term vaccination is aimed at preventing the disease and reducing the level of virus shedding. When used in an emergency outbreak situation the effectiveness of control of FMD by vaccination will depend on the vaccine strains available. At this time a closely matched strain will be required immediately to formulate a vaccine. Appropriate vaccine strains related to the field outbreak strain must be determined based on the evaluation of r_1 values (Kitching et al., 1989; O.I.E., 2004b). The method used to compare antigenic differences between a vaccine strain and a field isolate involves calculation of an r_1 value, which is determined from the ratio of the neutralization titre of a reference antiserum against the field isolate and against the homologous vaccine strain. If the r_1 value is less than 0.4, it indicates that the field strain has a weak relationship with antisera against a vaccine strain (Doel, 2003)

At the time of the 1997 outbreak in Taiwan before the decision was taken to use control with vaccination, stamping out was carried out until vaccines were available from different countries. The appropriate vaccine strain antigenic comparison studies were

conducted between O Taiwan/ 97 and other type O FMD vaccine strains by the World FMD Reference Laboratory, Pirbright. The r_1 values for O1 Manisa, O 4174 and O1 Campos were 1, 1 and 0.7, respectively (Yang et al., 1999). Other factors have to be considered when using vaccines to control FMD including the antigen mass contained in vaccines, vaccine production procedures and antigen adjuvant interactions (Doel, 2003). During the Taiwanese outbreak, the emergency vaccines approved for use were required to contain a minimum of 3 PD_{50} per dose as recommended by the OIE Code (OIE, 2004b). Some of the vaccines used could generate better immune responses than others (Chen et al, 1998). It is important to make sure the vaccines used in outbreaks of FMD are properly evaluated against field isolates by reference laboratories before accessing FMD vaccine banks.

1.9.2 Vaccine application

Successful application of a FMD vaccination programme also requires other precautions, like an effective cold-chain to maintain the temperature in the vaccines between 3 and 8°C; in large animals, such as cattle, adequate restraints to allow safe application of the vaccine and to reduce the stress the animals are subjected to are required. It is also important to emphasize that only healthy animals should be vaccinated (Doel, 2003).

Experiences gained from the pig FMD vaccination program in Taiwan indicated that the volume of vaccine dose, the type of adjuvant used in the vaccine, temperature of vaccine when administrated, timing for vaccination in the herd and the farmers' acceptance of vaccination recommendation all had important impacts on the effectiveness of vaccination programs.

At the beginning of the vaccination programme, some vaccines were formulated to have a dose of 1 ml. Problems occurred in trying to administer this small volume of vaccine in the farm because of the movement of pigs when given intramuscular injections. Up to one third of the volume of vaccine was potentially leaking from the vaccination site. Subsequently all vaccines were formulated to have a 2 ml dose, which overcame this problem. Vaccines formulated with single oil adjuvant were not favored by workers who carried out the injection due to the high viscosity of the vaccine. Farmers preferred the FMD vaccines formulated with double oil adjuvant which has lower viscosity and is easy to administer. Vaccines were stored at 3-8°C and were allowed to warm to room temperature before use to reduce side effects in vaccinated animals. The recommended age for vaccinating pigs is 8 weeks with a booster at 12 weeks of age (Chung et al, 2003). However, pigs at this age also tend to have other pathogenic infections other than FMDV. Consequently farmers tended to delay the age of vaccination to avoid

vaccinating unhealthy pigs. This resulted in a period of approximately one month when pigs were not protected after the loss of maternally derived immunity and before the development of an active immune response from vaccination.

Because these factors could all have impacted on the effectiveness of vaccinations in the field, it was important to have well validated tools to monitor antibody responses to vaccination and infection. This study in part examines performance of tests for antibodies to structural and non-structural FMD proteins for monitoring vaccination responses and field infections with pig-adapted O/Taiwan/97 FMDV.

1.10 Substantiation of country freedom from FMD

The ultimate objective of FMD control programs is to eradicate the disease and achieve freedom from infection. The OIE expert committees have designated rules or definitions whereby countries can achieve or be considered free from FMD without having ever used vaccination, or the steps required to achieve freedom after they have used vaccination (OIE, 2007).

A country such as Taiwan that has achieved the status with OIE of freedom with vaccination wanting to progress to freedom without vaccination will be considered eligible if they have had no outbreaks of FMD, no evidence of FMDV infection found,

no vaccinated animal being introduced into the zone and no vaccination against FMD during the preceding 12 month period. They should have an active surveillance for both FMD and FMDV following the instructions in Appendix 3.8.7 of the OIE manual (OIE, 2005) and supply evidence showing that FMDV circulation has not been detected during this 12 month waiting period (OIE, 2007).

There is a clear requirement for a substantial level of surveillance in a country that plans to move to FMD freedom without vaccination and this requires a clear understanding of the capabilities of the various diagnostic tests used to successfully plan and complete the required surveillance.

1.11 The aims and hypotheses tested in this study

The potential threat from the rapid spread of FMD via the airborne route needed to be investigated in order to formulate an effective control plan for the pig adapted strain of FMD. For assessment of protection of pigs after vaccination it was necessary to determine the relationship between serum neutralization (SN) titres and protection and then assess whether the SN titre post-vaccination could be used to assess vaccine potency for the pig-adapted strain. Evaluation of NSP ELISA kits for use in pigs vaccinated against FMD had to be studied before this test was applicable for monitoring

of circulation of FMDV in the field. Therefore the specific hypotheses to be tested in this study were:

1. Airborne transmission route may not be a significant route with pig adapted FMDV (O/TAW/97). This was evaluated by experimental transmission studies.
2. Vaccine potency can be determined in pigs based on the relationship between SN antibody titres and protection from FMD disease in vaccinated pigs after challenge. This was evaluated by a series of challenge studies in vaccinated pigs.
3. A clear understanding of the immune responses of structural and non-structural proteins in pigs given commercial FMD emulsion vaccines and/or live virus challenge will permit the use of these tests for the control of FMD in Taiwan and use the NSP ELISA's as a means to assess the purity of vaccines for use in the control program for FMD in Taiwan. This was determined by conducting detailed serological examination of pigs of known status with respect for FMD vaccination and/or infection throughout Taiwan after 1997 FMD outbreak.
4. Optimal FMD NSP ELISA tests in terms of sensitivity and specificity for use in Taiwan can be determined by comparative testing of the three commercial FMDV NSP ELISA kits that are used in Taiwan. This was evaluated by comparative testing of the kits on pig sera collected during the year after 1997 FMD outbreak.

5. NSP ELISA kits can be successfully used as parts of the nationwide FMD surveillance in pigs to substantiate freedom from FMD in Taiwan. The valuable use of the optimal NSP ELISA kits was demonstrated during the final stages of eradication of FMD from Taiwan.

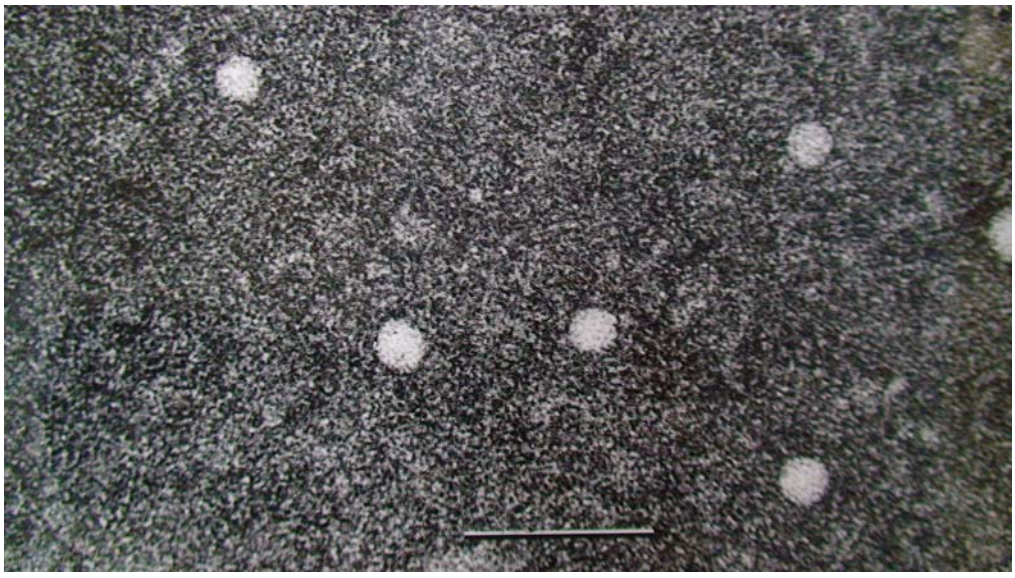


Figure 1.1. FMD virus is a non-enveloped, icosahedral virus, 26 nm in diameter (bar= 100 nm) (S. P. Chen, 1997).

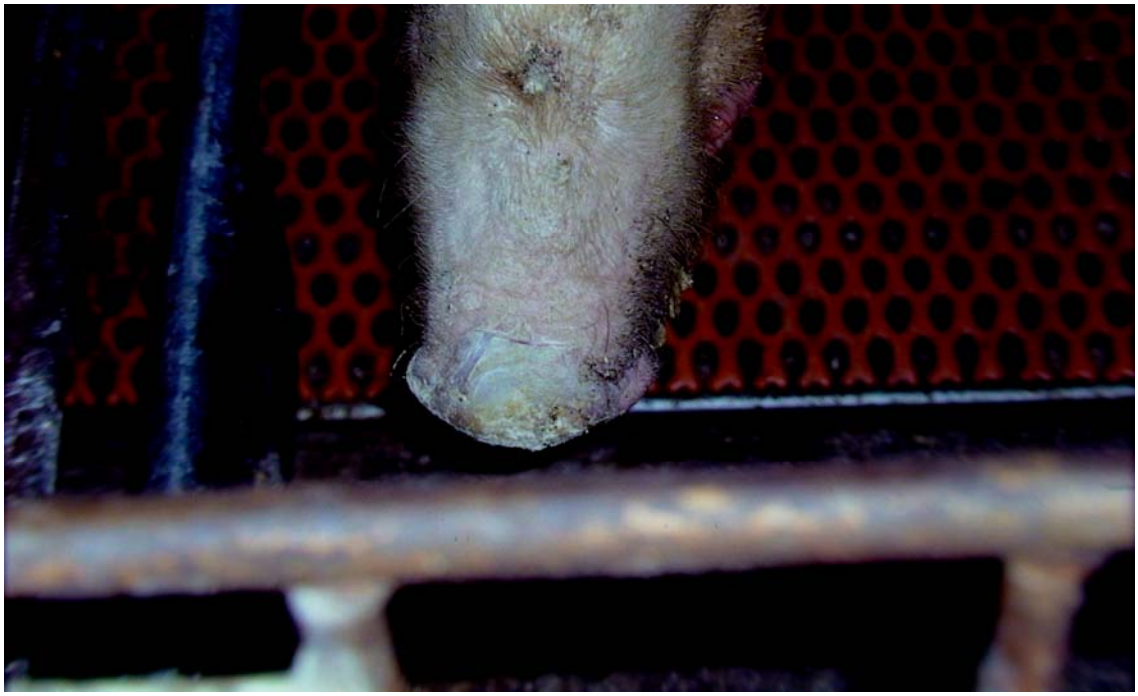
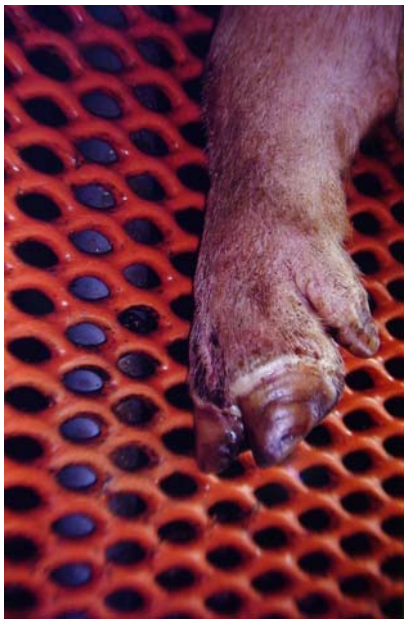
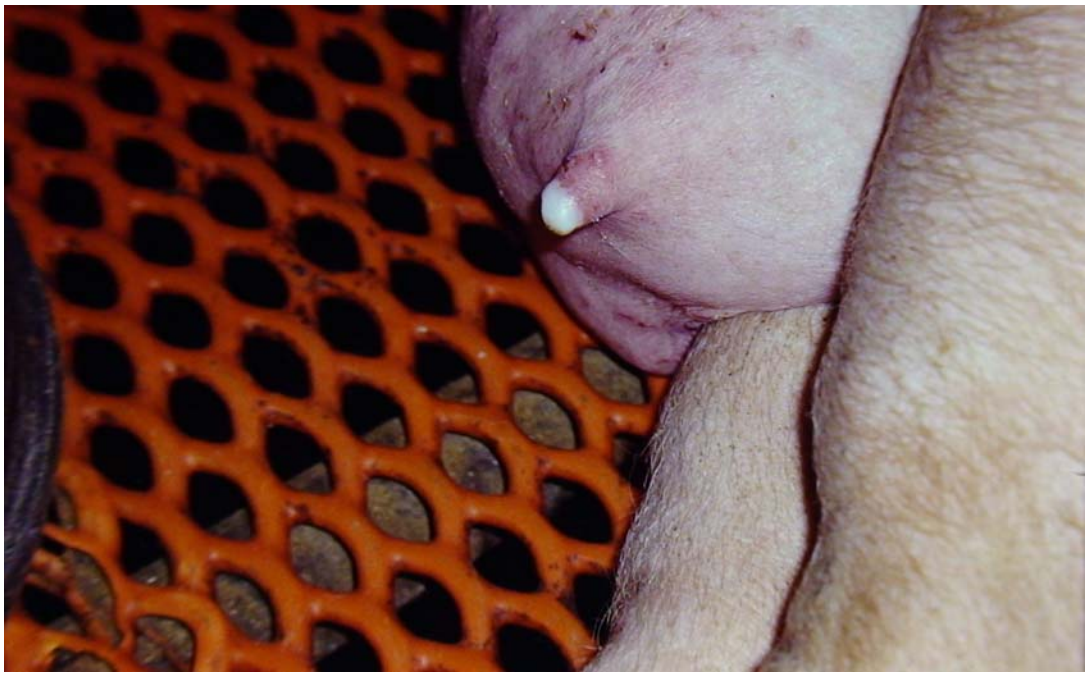


Figure 1.2. FMD lesions in pigs taken on 20 March 1997 in a commercial pig farm with 2500 sows. The early detection of vesicular lesions emphasises the importance of vigillance of pig producers. The infected herds had experienced severe outbreaks with high mortality rates in nursery piglets in the early stage of the outbreak, losses of some pigs due to secondary bacterial infection and severe FMD lesions in pigs of all age groups. In this herd erosive lesions of feet and snouts were still evident 4–6 weeks after the start of the outbreak. (a) Tip of snout nozzle showing large blister in a sow (S. P. Chen, 1997).

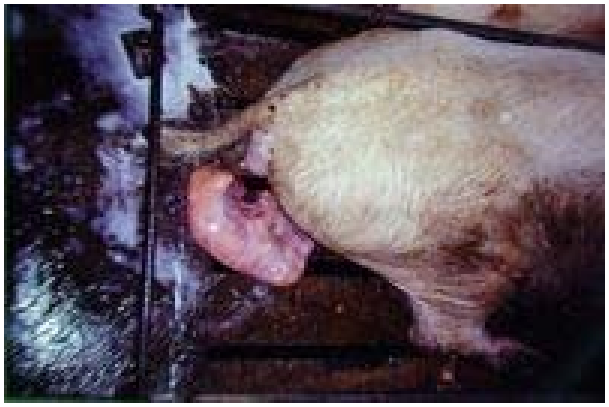


(b) Foot. There is a white band adjacent to the coronary band. This is all one large vesicle (S. P. Chen, 1997).

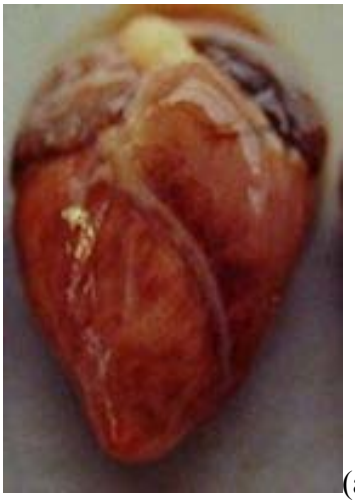


(c) Tip of a nipple covered by a blister in a sow, which contained approximately 10^8 $TCID_{50}$ viral particles as measured with PK-15 monolayer tissue cultures (S. P. Chen, 1997).

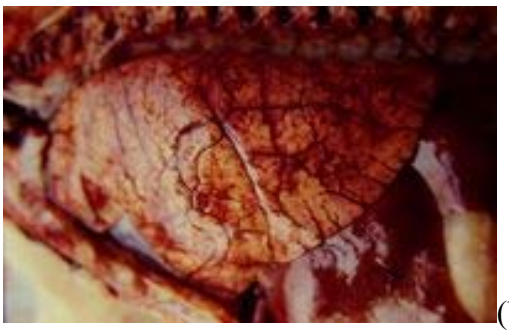
(d) Older lesions. The blisters have burst and ulceration formed with a secondary bacterial infection (S. P. Chen, 1997).



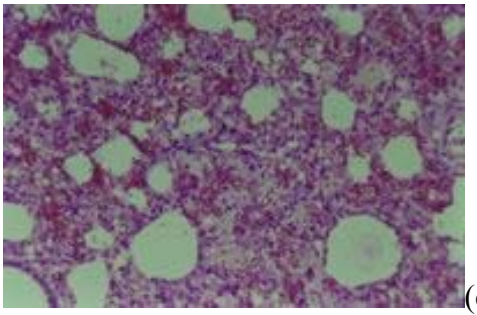
(e) Reproductive failure was manifested by abortion if pregnant sows were infected (S. P. Chen, 1997).



(a)



(b)



(c)

Figures 1. 3. FMD lesions in dead suckling pigs from the same farm as Figure 1.2. (a) Pale streaks on the myocardium of the suckling piglet. (b) Pulmonary oedema was prominent in the same piglet of (a). (c) Histopathological lesions of oedematous lung (S. P. Chen, 1997).

Chapter 2 - Materials and Methods

2.1 Serological tests for measurement of antibodies to FMD

Blood was collected from the jugular vein with blood collection tubes (BD Vacutainer® SST, USA)**.** It was centrifuged at 3000 rpm for 15 minutes prior to collection of the sera. The sera were stored at -20°C until assayed for the presence of antibodies to FMD.

2.1.1 Virus neutralization

The quantitative virus neutralization (VN) microtest for FMD antibody was performed with BHK-21 cells in flat-bottomed tissue-culture plates (CELLSTAR®**,** Germany). The virus stock used for the VN test was O Taiwan/97 and was prepared from a virus isolated from samples of affected pigs in Chu-Pei, Taiwan. This virus had been passaged 4x in BHK-21 cells and titrated in BHK-21 cells before being designated as VN stock virus and stored in 0.5 ml aliquots at -80°C.

The sera were inactivated at 56°C for 30 minutes before testing. The positive control serum was 21-day convalescent or post-vaccination serum with a known titre of antibody to O Taiwan/97 FMDV. The negative serum was from a naïve pig from a pig herd that had not been infected with FMDV. The medium used was Delbecco's Modified

Eagle Medium (DMEM, low glucose with L-glutamine and pyridoxine hydrochloride and sodium pyruvate, GIBCO™, USA) with 5% FBS and antibiotics (O.I.E., 2004b).

2.1.1.1 Test procedure

(1) The test used equal volumes (50 µl) of virus suspension and serum. Serial two fold dilutions of inactivated test serum in cell culture medium were prepared in flat-bottomed tissue-culture plates with the initial dilution of 1:4.

(2) Stock virus was diluted in cell culture medium to prepare a suspension containing approximately 100 $TCID_{50}$ (50% tissue culture infective dose)/50 µl (with an accepted range of 30–300 $TCID_{50}$/50µl) and 50 µl aliquots were added to appropriate wells of the flat-bottomed tissue-culture plates.

(3) Controls included a standard antiserum of known titre (1:256), a negative serum, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test.

(4) Virus and serum mixtures were incubated at 37°C for 1 hour in the covered plates.

(5) A cell suspension of $4x10^5$ cells/ml was prepared in a medium containing 5% bovine serum (FMD antibody negative) for cell growth. A volume of 100 µl of cell suspension was added to each well.

(6) Plates were covered with loosely fitting lids and incubated in an atmosphere of 5%

carbon dioxide at 37°C for two days.

(7) Microscope readings were carried out after 48 hour of incubation.

(8) The results of the test were recorded as positive when the virus had been neutralized and the cell monolayers remained intact without cytopathic effects (CPE). A negative result was recorded if the virus had not been neutralized and the monolayer was destroyed. Titres were expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells were protected (OIE, 2004b*)*. The test was considered to be valid when the amount of virus used per well was in the range 30-300 $TCID_{50}$, and the positive standard serum was within two fold of its expected titre.

2.1.2 Tests for antibody against non structural proteins

Three commercialized NSP ELISAs were used, These were:(1) UBI FMDV NS ELISA (United Biomedical Inc., NewYork, USA. - Shen et al., 1999). (2) Ceditest FMDV-NS (Cedi Diagnostics B.V., Lelystad, The Netherlands - Sorensen et al., 1998). (3) CHEKIT-FMD-3ABC (Bommeli Diagnostics, Bern, Switzerland). Ceditest is a blocking ELISA which can be applied to any animal species and the other two kits are indirect ELISAs, requiring species specific conjugates. Detailed testing procedures for three commercial ELISA kits are shown in Appendix I.

2.2 Identification of the agent

2.2.1 Virus isolation and titration

Generally the samples tested were epithelium and samples of vesicular fluid which were transported in PBS or virus transport medium (Medium 199, Earle's balanced salt solution with L-glutamine, GIBCO™, USA) with 7.5% BSA and antibiotics. A suspension was prepared by grinding the epithelial sample with the handheld rotor-stator homogenizer (QIAGEN™, Germany). Further medium was added until a final volume of nine times that of the epithelial sample had been added, giving a 10% suspension. This was clarified on a bench centrifuge at 2000 g for 10 minutes. Once clarified, the suspensions (epithelial samples or vesicular fluid) were inoculated onto BHK-21 (baby hamster kidney) which has been found to support the growth of all O/Taiwan/97 FMDV strains tested. The cell cultures were examined for cytopathic effect (CPE) at 24-48 hours. Generally, CPE was evident on the first passage if samples were from strongly suspected cases and if no CPE was detected, the cells were frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours.

The virus titre was determined by serial 10-fold dilution (8 wells for each dilution) of the virus suspension in cell culture medium (Medium 199, Earle's balanced salt solution with L-glutamine, GIBCO™ plus 7.5 % foetal calf serum) in 96-well tissue culture plates (CELLSTAR®, Germany). Then 100 µl of a suspension of BHK-21 cells at a concentration of 4 x 10^5 cells/ml was added to each well and the plates were incubated at 37°C in a 5% CO_2 atmosphere and examined for CPE after 36 to 48 hrs. The titre was determined by the method of Reed and Muench (1938).

2.2.2 Nucleic acid recognition methods

The reverse transcription polymerase chain reaction (RT-PCR) test was applied to amplify genome fragments of FMDV in epithelial, serum and vesicular fluid samples (Höfner et al., 1993; OIE, 2004b). The RT-PCR assay consists of the following procedures: (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) Detection of the PCR products by agarose gel electrophoresis.

2.2.2.1 Test procedure

(1) Add TRIzol ® Reagent (InvitrogenTM, USA) to 250 µl of test sample to make a total volume in 1 ml in a sterile tube. Store at –80°C until required for RNA extraction.

(2) Transfer 1 ml of the solution from i) into a fresh sterile tube containing 200µl of chloroform. Vortex mix for approximately 10 to 15 seconds and leave at room temperature for 3 minutes.

(3) Centrifuge for 10 minutes at 12,000 g.

(4) Transfer 500 µl of the aqueous phase into a fresh sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

(5) Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 g.

(6) Discard the supernatant fluid from each tube and add 1 ml of 70% ethanol. Vortex mix for a few seconds.

(7) Centrifuge for 5 minutes at 12,000 g

(8) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

(9) Air dry each tube at room temperature for 2 to 3 minutes.

(10) Re-suspend each pellet by adding 50 µl of RNase -free water to the tube.

(11) Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –80°C

(12) For each sample to be assayed, add 2 µl of each primer (20 µM) in a 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

(13) Add 10 µl of RNA from the extraction procedure described above to give a volume of 14 µl in each tube. Mix by gently pipetting up and down.

(14) Prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

(15) RT reaction buffer, 10× conc. (5 µl); dNTPs (Invitorgen TM, USA), 2.5 mM mixture each of dATP, dCTP, dGTP, dTTP (4 µl); Reverse Transcriptase (AMV Reverse Transcriptase , Promega, Gremany), 40 U/ µl (1 µl); RNase free water (26 µl).

(16) Add 36 µl reaction mix to the 14 µl of primer/RNA mix. Mix by gently pipetting.

(17) Incubate at 37°C for 45 minutes.

(18) Keep the RT products on ice if the PCR amplification step is about to be performed. Otherwise store at –20°C.

(19) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

(i) DNase-free water (26 µl); PCR reaction buffer, 10× conc (5 µl); dNTPs, 2.5 mM mixture each of dATP, dCTP, dGTP, dTTP (4µl); primer 1, 20 µM (2 µl); primer 2, 20 µM (2 µl); Taq Polymerase **(**Invitorgen ^{TM}), 5 units/µl (1 µl). (Primers 1 and 2 are described below)

(ii) Add 40 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 10 µl of the RT product to give a final reaction volume of 50 µl

(iii) Spin the tubes for 10 seconds in a suitable centrifuge to mix the contents of

each well.

(iv) Place the tubes in a thermal cycler PCR machine type (Applied Biosystem, ABI, GeneAmp® PCR system 9700) for PCR amplification and run the following programme:

- 94°C for 5 minutes: 1 cycle;
- 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;
- 72°C for 7 minutes: 1 cycle.

(v) Mix a 10 µl aliquot of each PCR reaction product with 2 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a cDNA band of 402 bp in size corresponding to the targeted FMDV sequence.

Primers were used at a concentration of 20 µM: Primer 1 sequence 5' - AGC TTG TAC CAG GGT TTG GC - 3' (positive strand); Primer 2 sequence 5' - GCT GCC TAC CTC CTT CAA - 3' (negative strand) (Vangrysperre and De Clercq, 1996).

2.3 Recognition and scoring of FMD lesions

The pigs were observed clinically for signs of FMD. Clinical signs were scored as follows: Lesion scores - each foot with lesions, 1 point; lesions in the mouth, 1 point. The highest score per animal was 5; Severity scores - scored from 1+ to 3+ according to

the degree of lameness, dullness, reluctance to stand and reluctance to feed (Figures 2.1 to 2.4).

2.4 Analytic procedures

Chi-square tests and ANOVAs were used to analyse the data statistically. The 95% confidence intervals were determined for proportions or percentages of positive reactors and were shown in graphs. The ANOVA was used to investigate differences in antibody titres between treatments. Chi-square tests for independence were used to compare differences in the number of affected pigs or antibody reactors at different time points (SAS Institute., 1988).

2.5 Follow-up procedure for positive NSP test responders in the surveillance program

NSP ELISA testing was conducted using both UBI and Ceditest on 15 follow-up serum samples collected from all the herds with NSP reactors from the auction market surveillance or other screen testing to investigate whether pigs in follow-up herds show any evidence of infection. In addition, clinical inspection of pigs from all age groups on these farms were undertaken as part of the herd follow-up monitoring and blood sampling program. Particular attention was paid to feet and snouts for evidence of

current FMD lesions as shown in Figures 2.2 to 2.4. Healed lesions in the claws of previously infected pigs were also looked for. These were recognized as linear defects in the claw structure parallel to the coronary band or growth of new claw into areas where damaged claw had partially sloughed (Figures. 2.5-2.6).

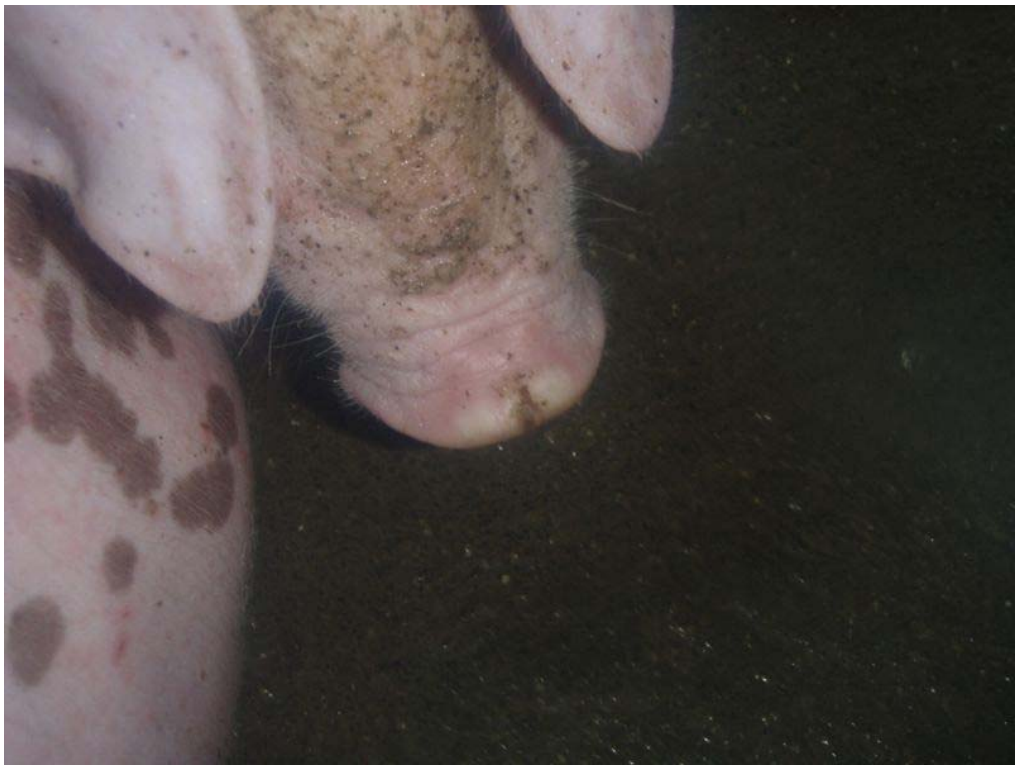


Figure 2.1. Lesion score for vesicle on the nose is one point

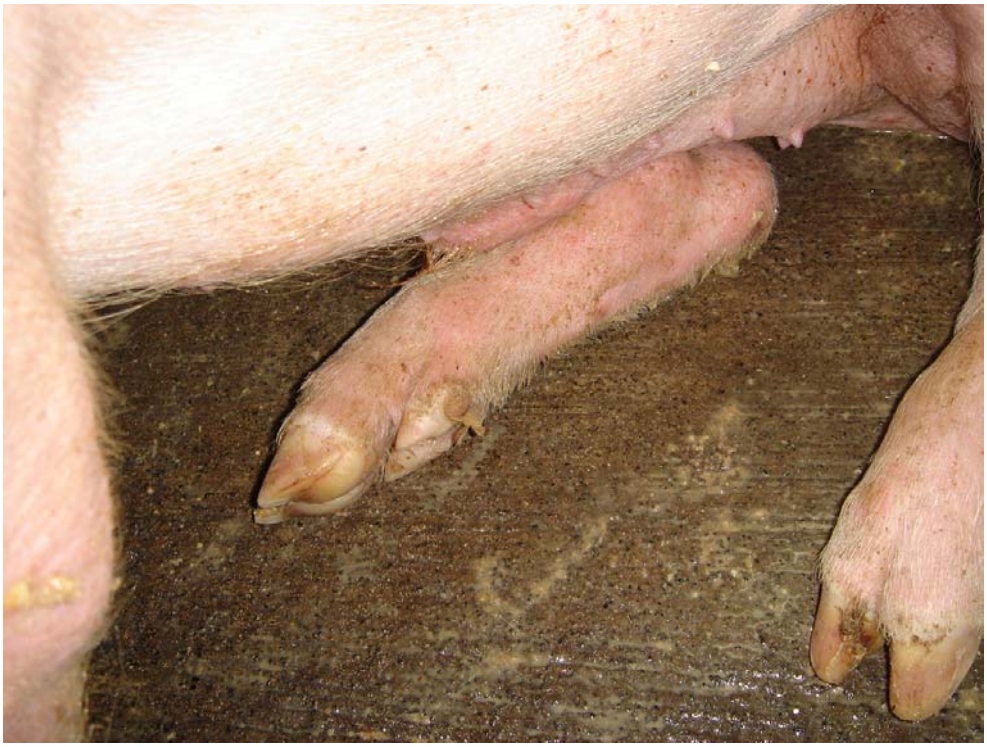


Figure 2.2. Lesion score for each foot with lesions is 1 point

Figure 2.3. Scores for having both hind feet with lesions is 2 points

Figure 2.4. After the challenge with FMDV, the pig at the corner became lame, dull and reluctant to stand resulting in a severity score of 3+ and the other 4 had a severity score of 1+.

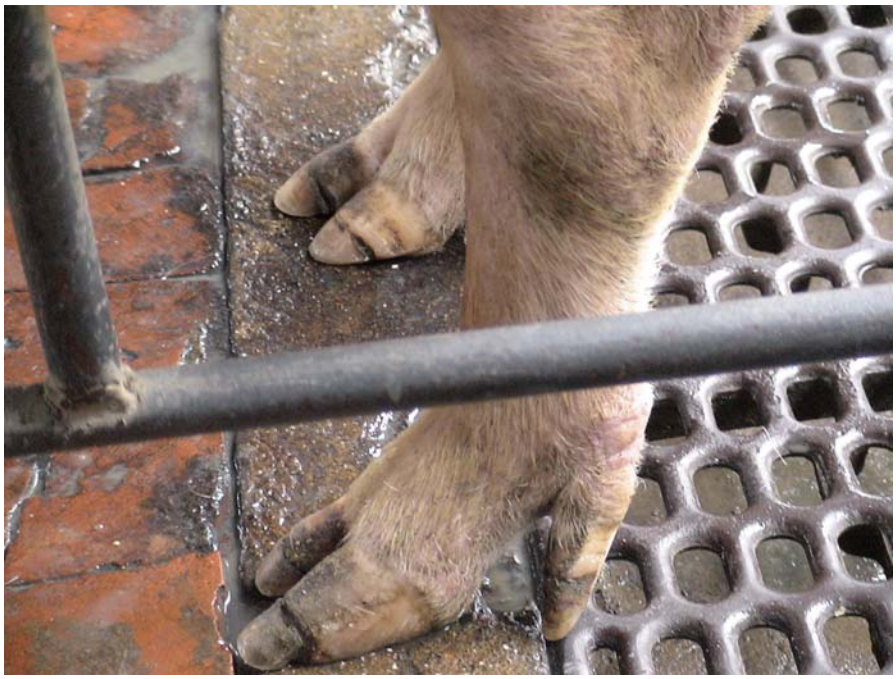


Figure 2.5. Healed lesions in the claws of previously infected pigs were recognized as linear defects in the claw structure parallel to the coronary band.

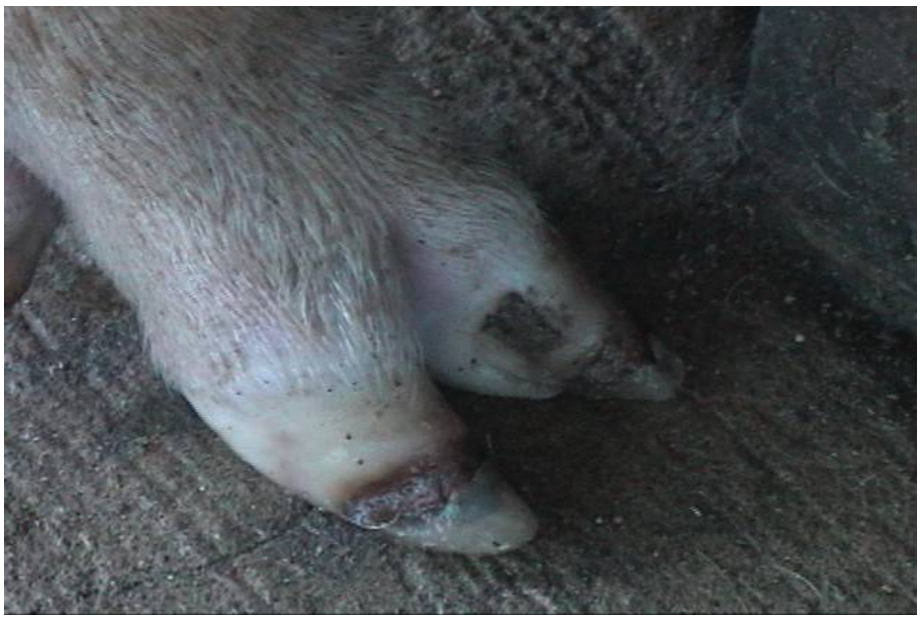


Figure 2.6. Healed lesions in the claws of previously infected pigs were recognized as the growth of new claws into areas where damaged claws had partially sloughed.

Chapter 3 - Assessment of the mode of transmission of the pig adapted foot-and-mouth disease virus (O/TAW/97)

3.1 Introduction

Foot and Mouth Disease can spread by different mechanisms including direct contact with infected animals, indirect contact via contaminated animal products, equipment and vehicles, in particular vehicles used in the transport of animals, by people and also by airborne spread (Kitching, 2005). Quick recognition of disease, followed by slaughter of infected livestock and implementation of movement restrictions greatly reduces the risk of mechanical spread. However airborne spread of the virus, which is feared mostly by farmers and veterinary authorities, is not able to be controlled by such procedures.

Transmission of FMDV by direct contact between inoculated and sentinel pigs has been confirmed under experimental conditions (Alexandersen et al., 2002a; Alexandersen and Donaldson, 2002). The determinants of airborne transmission include the species of animals infected, the virus strain, the environmental conditions, and the location of susceptible animals, such as cattle, downwind (Alexandersen et al., 2003b; Kitching, 2005). Pigs shed larger quantities of virus in their oropharyngeal secretions than other

animals (Alexandersen and Donaldson, 2002). Under the appropriate weather conditions, aerosol virus can potentially spread a considerable distance, particularly if the source is a large infected pig herd. Airborne spread over 250 km was suspected to be responsible for the transmission of the virus from infected pig herds in Brittany in France to the Island of Jersey (where it infected cattle), and then as far as the Isle of Wight off the coast of southern England (Gloster et al., 1982).

After the 1997 FMD outbreak commenced in Taiwan, the spread of disease was very rapid and the whole of the western part of Taiwan was affected within a few days after the diagnosis of FMD was confirmed. In some situations airborne transmission of FMD virus was suspected and it was speculated that this was one of the explanations for such rapid spread in Taiwan (Yang et al., 1999; Yang, 2002)

However, more recent evidence, from experimental observations and circumstantial evidence from the field, has shown that pigs especially are relatively more resistant to transmission by airborne FMDV. Alexandersen and Donaldson (2002) demonstrated that the transmission of O1 Lausanne and O Taiwan/97 strains of FMDV from infected to susceptible pigs could be prevented if physical barriers were installed to prevent infected pigs from making direct physical contact with susceptible pigs and if measures

were taken to prevent the mechanical transfer of virus. However in their study one sentinel pig became infected and showed clinical signs, while others failed to develop clinical signs but produced antibody against FMD. In the Netherlands, in one outbreak of FMD on two veal-calf farms in 2001 there appeared to be a limited outbreak with minimal clinical signs of FMD in infected calves. No directly or indirectly exposed calves became infected with FMDV, even when affected calves had saliva containing $10^{4.9}$ p.f.u./ml of FMDV (Bouma et al., 2004). This report showed that virus transmission by aerosol transmission or close contact to individually housed calves with this FMD strain was very inefficient.

Alexandersen et al.and Donaldson demonstrated that pigs challenged with O Taiwan/97 and estimated to be excreting $10^{4.6}$ $TCID_{50}$/24 hrs for 24 to 48 hours were not able to infect sentinel pigs by the airborne route (Alexandersen et al, 2003a). There are no further publications reporting on airborne transmission with O Taiwan/97 strain of FMDV. Understanding the transmission routes of pig adapted strains of FMDV in Taiwan is important in developing control measures against future outbreaks of pig adapted strains of FMDV. Therefore, this chapter reports on studies conducted to further investigate the transmissibility of O Taiwan/97 FMDV to susceptible pigs by direct and indirect spread including airborne spread in an enclosed animal house.

3.2 Materials and methods

3.2.1 The O Taiwan/97 FMDV stock:

The O Taiwan/97 virus stock, derived from the first pig passage of the O Taiwan/97 pig origin virus from the Taiwanese epidemic in 1997 (Dunn and Donaldson, 1997), was passaged in pigs and the experimental inoculum was prepared as a suspension of vesicular fluid. The titre of the inoculum was 10^5 $TCID_{50}$/ml measured in BHK-21 cells using the method of Reed and Muench (1938). Each pig received a 0.5 ml dose of 10^5 $TCID_{50}$/ml FMDV.

3.2.2 Animals and the challenge procedure:

The experimental challenge rooms were located in National Animal Health Institute Taiwan in their BSL 3 facility. Pigs were housed in the biosecurity rooms maintained at negative pressure with HEPA filtered exit air and with containment and decontamination of waste. The layout of the challenge room is indicated in figure 3.1 and shows the details of the pen size, the location and nature of the partitions and the location of ventilation ducts.

To monitor room temperature and relative humidity throughout the experiments, wet bulb thermometers were placed in each challenge room. Room temperature and relative humidity readings were taken at 24-hour intervals for each day of the experiments.

A series of six challenge studies was conducted using 6-week-old conventionally raised male or female pigs that were seronegative for antibody to FMDV by the SN test. The pigs were obtained from a herd periodically monitored for FMDV and known to be free of the virus. For these challenge experiments, some pigs were challenged by injection into the heel bulb with 0.5ml of 10^5 $TCID_{50}$ FMDV O Taiwan/97 stock virus and the inoculated pigs were placed in direct or indirect contact with naïve pigs in the same challenge room. The pigs were monitored daily for clinical signs of FMD. This included measurement of body temperatures and observation for lameness and vesicular lesions on the snout and coronary bands. Selected specimens from affected pigs were tested to confirm the presence of O Taiwan/97 virus. Blood samples were collected from pigs on the day prior to challenge for measurement of SN titres and again 2 weeks post-challenge. Six separate experiments were conducted as follows:

Experiments 1, 2, and 3 involved investigation of direct or indirect contact on the transmission of virus. These were a set of replicated experiments. For each experiment,

twelve pigs were allocated to three groups in two pens. Three challenged pigs were housed in the one pen with another three sentinel pigs to assess direct contact transmission. An indirect contact transmission group of six pigs was housed in an adjacent pen separated by a solid wooden board to prevent the direct physical contact between the two pens.

Experiments 4 and 5 involved investigation of close contact on the transmission of virus. These were also replicated experiments. Five or six pigs were allocated to three groups in two pens. Two or three challenged pigs were housed in one pen with one sentinel pig to assess direct contact transmission. A close contact transmission group of two pigs was housed in another pen separated by a distance of 30 centimetres to prevent nose to nose contact of these pigs with the challenged pigs.

Experiments 6 involved intramuscular or intranasal challenge. Two groups of three pigs were challenged with O Taiwan/97 stock virus (10^5 $TCID_{50}$ per ml) by different routes. One group was challenged with 0.5 ml inoculated intramuscularly into the neck. The other group was challenged by intranasal inoculation of 0.5 ml stock virus into each nostril.

3.2.3 Measurement of serum neutralizing antibody

Serum neutralizing antibody (SN) titres to FMDV type O Taiwan were measured in a micro-neutralization assay as described in Chapter 2.1.1.

3.2.4 Virus isolation

Isolation of FMDV and titration procedures were conducted as described in Chapter 2.2.1.

3.3 Results

Figure 3.2 summarises the results of clinical examinations and serological testing for the challenged and contact pigs from experiments 1 to 5. Further details of the results from all six experiments are given below.

3.3.1 Experiments 1, 2, and 3: direct or indirect contact

Three challenged pigs developed vesicles on their snouts (Figure 3.3) and coronary bands (Figure 3.4) 1 to 2 days post infection. The pigs were lame and reluctant to move, especially after the vesicles ruptured and progressed to deep ulcers. Body temperatures of the pigs during the experiments are shown in Figure 3.5. The pigs had their highest body temperatures one day before the vesicles were found in the feet and nostrils. The

contact pigs were showing similar lesions as the challenged pigs 1 to 3 days after the challenged pigs developed lesions. The challenge pigs and contact pigs developed neutralizing antibody titres 4 to 5 days after the development of lesions. No lesions or neutralizing antibody titres were found in the indirect contact pigs.

3.3.2 Experiment 4 and 5: direct or close contact

In the challenged pigs typical vesicles were found on the snouts and coronary bands 1-2 days post infection. Body temperatures of the pigs during the experiments are displayed in Figure 3.6. The contact pigs were showing the same lesions as the challenged pigs 1-3 days after the challenged pigs developed lesions of FMD. The challenge pigs and contact pigs produced neutralizing antibody titres by 4-5 days after the development of lesions. No FMD lesions and neutralizing antibody titres were found in the close contact pigs.

3.3.3 Experiment 6: Intramuscular and intranasal challenge

The three pigs challenged intramuscularly developed vesicles on the coronary bands 1 to 2 days post challenge but none of the intranasally challenged pigs developed lesions (Table 3.1). All of the intramuscularly challenged pigs produced neutralizing antibodies within 5 days of challenge. In contrast no neutralizing antibody was detected in pigs

challenged intranasally.

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3.4 Discussion

Although the airborne transmission of FMDV has been documented in cattle, sheep and pigs challenged with different strains (Donaldson et al., 1987; Donaldson et al., 1982; Alexandersen et al., 2002a), it is unclear if pig adapted strains can be transmitted in this manner. This study was conducted to broaden our understanding of the transmission of the pig adapted strain of FMDV (O Taiwan/97). The means of exposure to FMDV in the field includes direct contact, close contact, aerosol contact, indirect contact, and intramuscular injection and intradermal challenges. In a previous pig to pig transmission study with strains of FMD other than the pig adapted strain, sentinel pigs with indirect contact with inoculated pigs became infected, with clinical signs observed in some pigs within 2 days of exposure and others only undergoing subclinical infection and expressing neutralizing antibody titres to FMDV within 14 days of exposure (Alexandersen et al., 2003a).

In experiments 1 to 3 all pigs in direct contact with pigs challenged with O Taiwan/97 FMDV became infected but none of the indirect contact pigs became infected. In experiments 4 and 5, the distance separating the pens (30 cm) was selected to prevent

any direct nose-to-nose contact. These experiments (4 and 5) aimed to determine whether transmission would occur after removing the physical barrier between pens. Again, only the pigs in direct contact with challenged pigs became infected but none of the close-contact pigs became infected. These experiments clearly demonstrated that the pig adapted strain O Taiwan/97 was only effectively transmitted by direct contact.

From the well documented ability of FMDV to spread rapidly between herds and even spread across the English Channel, the lack of transmission of O Taiwan/97 by indirect or close contact in these experiments was unexpected. Substantial amounts of feed, water, urine, faeces, saliva and possibly other body fluids would have been expected to have been spread throughout the challenge rooms by the infected pigs in the close-contact experiments where there was no physical barrier and only separation of pens by a distance of 30 cm. The animal husbandry staff took normal biosecurity measures to reduce the risk of indirect transmission. However, the fomites and/or aerosol droplets at close range were expected to have spread the disease to the sentinel pigs in the nearby pen.

The results of these experiments further questions the role of airborne spread in transmitting the pig adapted strain (O/Taiwan/97) as raised by the findings of

Alexandersen and Donaldson (2002) and Alexandersen et al. (2003a) where O Taiwan/97 did not spread readily to indirect contact groups. In their study, none of the eight indirect contact pigs developed clinical signs, but one developed a transient antibody response. These experiments also demonstrated that transmission of O Taiwan/97 did not occur to close contact pigs when barriers to prevent the possible transmission by faeces, urine and body secretion were removed. They also supported previous findings that pigs are very resistant to infection by airborne FMDV as compared to cattle and sheep (Alexandersen et al., 2002a; Alexandersen and Donaldson, 2002; Alexandersen et al., 2003a). Furthermore from the result of the intranasal challenge experiment, it would be expected that a very high virus dose would be required to infect pigs by the airborne route with the pig-adapted FMDV. Further studies are needed to understand the transmission mode and minimum infectious dose by various routes for pig adapted FMDV strains like O/Taiwan/97, which clearly differs from that of other O type FMDV.

These studies clearly demonstrated that the airborne transmission mode, suspected in some reports (Yang et al., 1999; Yang, 2002), is unlikely to be a cause of the wide spread of disease during the 1997 Taiwanese FMD outbreak. This indicates that effective control against future outbreaks of pig adapted FMDV strains could be

achieved by restriction of pig movement and stamping out if the outbreak has been detected in the early stages and prior to the movements of pigs from the infected premises.

Figure 3.1 The layout of challenge pens in the room and air inlet and outlets. The room is 4.6 Mx 4.2 M x 3 M. The black partition wall shown in the plan is a steal fence covered with hard plastic panels to make it impermeable barriers. The height of partition wall is 120 cm. The pale blue strip panel is a solid steel and concrete fence. The aisle behind pale blue panel is a partitioned area for personnel to observe and care for the experimental pigs. Personnel have to shower in and out of the room as a biosecurity measure.

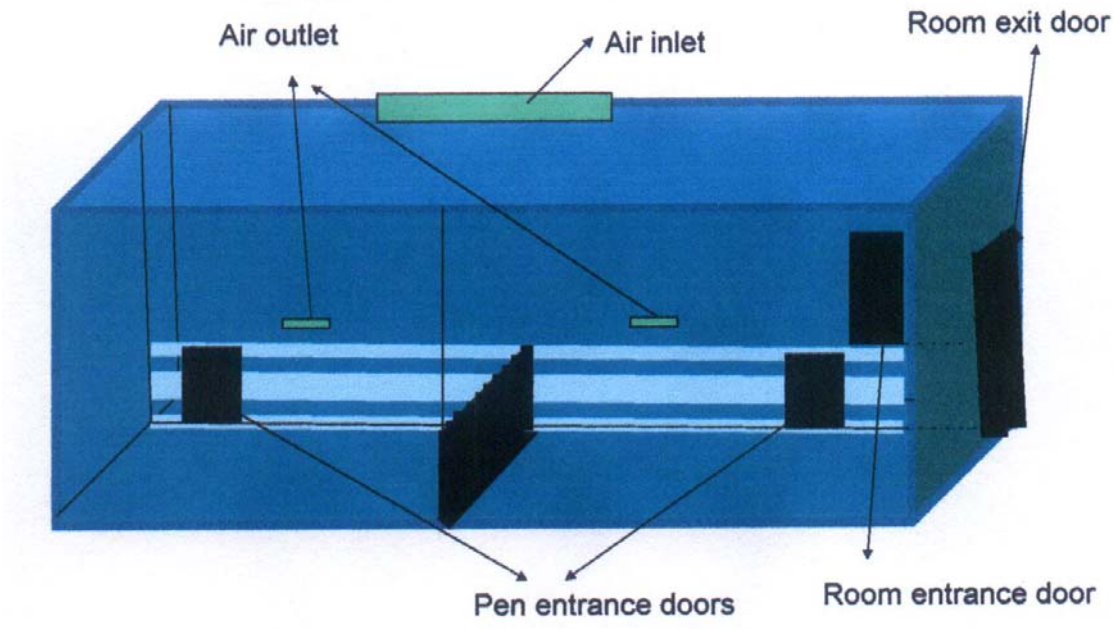


Figure 3.2 Results for the various challenge routes by O/Taiwan/97 FMDV (inoculated challenge, direct contact, indirect contact and close contact) in experiments 1-5.

Route of infection	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Inoculated challenge	● ● ● ● ● ●	● ● ● ● ● ●	● ● ● ● ● ●	● ● ● ● ● ●	● ● ● ●
Direct contact	● ● ● ● ● ●	● ● ● ● ● ●	● ● ● ● ● ●	● ●	● ●
Indirect contact	○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○		
Close contact				○ ○ ○ ○	○ ○ ○ ○
Legend/key	One circle represents one challenged pigs. In each box, the left column represents clinical signs and the right column represents neutralizing antibody post challenge. ○: pigs without clinical signs or negative neutralizing antibody. ●: pigs with clinical signs or positive neutralizing antibody.				

Table 3.1. Results of experiment 6: intramuscular and intranasal challenge of pigs with pig adapted O/Taiwan/97 FMD virus.

Challenge Route	Pig No.	Challenge dose	Infection
Intra-muscular	1	0.5 ml of 10^5	Y
route	2	$TCID_{50}$/ml	Y
	3		Y
Intranasal route	4	1 ml of 10^5	N
	5	$TCID_{50}$/ml	N
	6		N

* Y: infected * N: non-infected

Figure 3.3. Vesicles on the nostril of a challenged pig two days post challenge.

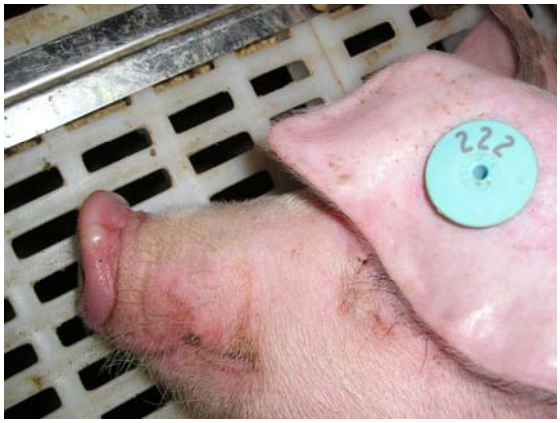


Figure 3.4. Vesicles on the coronary bands two days post challenge.

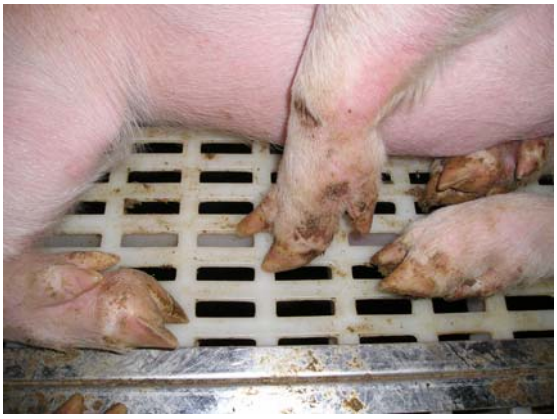


Figure 3.5. Body temperatures of three challenged pigs in experiment 1. The pigs had a peak fever one day before the vesicles were observed in the feet and nostrils.

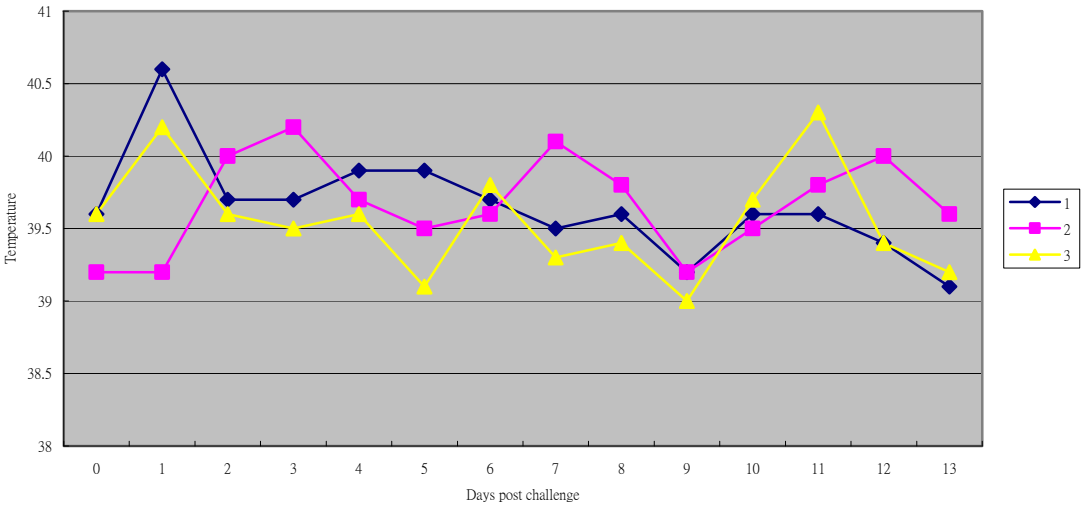
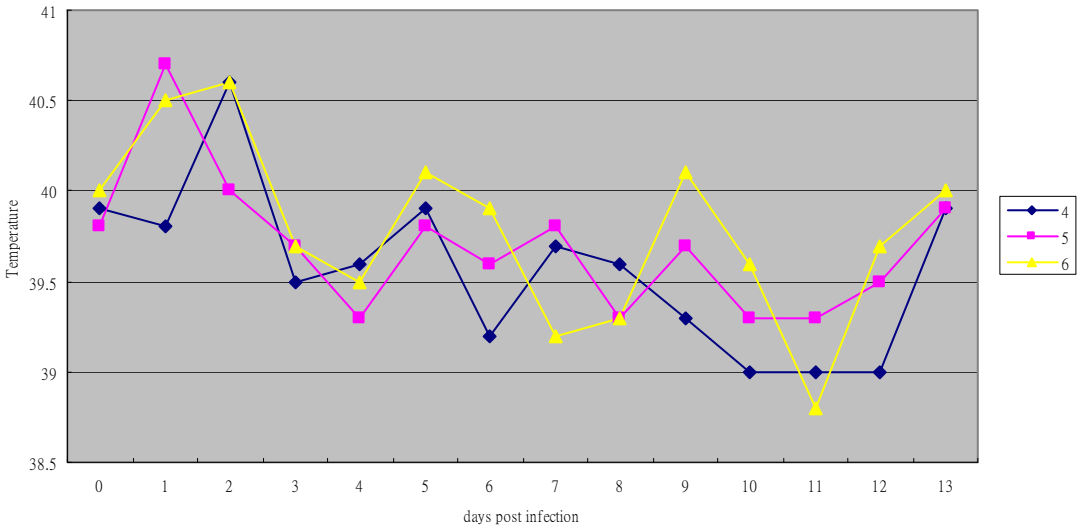


Figure 3.6 Body temperatures of three challenged pigs in Experiment 4. The pigs had a peak fever one day before the vesicles were observed in the feet and nostrils.



Chapter 4 - The relationship between serum neutralizing antibody titres and protection from foot and mouth disease in pigs after vaccination

4.1 Introduction

In cattle, parameters such as serum neutralization titre (Pay and Hingley, 1992) and antibody titre on ELISA (Hamblin et al., 1987; Van Maanen and Terpstra, 1989; Robiolo et al., 1995) have been established and successfully applied to assess the potency of FMD vaccine as *in vitro* correlates of protective immunity (Amadori et al., 1991; Vianna Filho et al., 1993). SN titre in cell culture, ELISA titre, or SN titres by suckling mice challenged after vaccination have been recommended in the OIE manual as indirect method, compared to the challenge test three or four weeks post vaccination used in Europe and South America, to evaluate the vaccine potency in cattle (O.I.E., 2004b). However, McCullough et al (1992) reported that anti-FMDV antibody titres in cattle could be classified into three zones - the white zone where antibody titres were high and challenged animals were likely to be protected; the black zone where antibody titres were low and challenged animals were likely to be susceptible to infection and the grey zone where the titres were intermediate and no interpretation could be made with respect to protection.

In pigs, there has not been a definite cut off value of antibody titres that can be equated with protection as has been proposed in cattle. Although similar studies to those in cattle have shown that pigs with higher neutralizing antibody titres would be most likely to be protected in the challenge test (Black et al., 1984). However, the method used in previous challenge studies focused on using either airborne or intradermal routes of challenge. The airborne route is not suitable for challenge with the pig adapted type O FMD viruses, due to lower susceptibility of pigs when challenged by the airborne route as reported in Chapter 3 and by Alexandersen et al. (2003a). In some challenge studies in pigs, challenge by the intradermal route has been insufficient to even infect pigs with low neutralizing titres (Dr. Mikhalishin, personal communication, 2007). This has also been observed in pig challenge studies with FMDV O Taiwan/97 in Taiwan (Lin et al, 2004). Yet, from field observation with pig adapted strains of FMDV, pigs with low neutralizing antibody were readily infected by direct contact with either infected pigs or heavily contaminated areas in lairages of abattoirs in Taiwan and Hong Kong. It is understandable therefore why an association has been difficult to establish between FMDV antibody titres in pigs and protection against infection in FMDV challenge studies.

Recently, the field analysis of the previous outbreaks of FMD caused by pig adapted type O FMD viruses in Taiwan has shown that heavily contaminated areas and introducing infected pigs from unknown sources to the farms were the most likely transmission routes to cause disease outbreaks. Therefore, it is important to establish what FMD neutralizing antibody titres in vaccinated pigs would be expected to equate with protection in order to evaluate vaccination programs and the risk of recurrence of outbreaks in pigs after vaccination.

As indicated in the previous chapter, contact transmission is the major transmission route for the pig adapted FMD virus. Therefore, in this chapter, the focus is on determining the neutralizing antibody titre that will protect pigs exposed by the contact route to pig-adapted type O FMD virus and the findings might be applied to evaluate vaccine potency in pigs.

4.2 Materials and methods

4.2.1 Animals and the challenge procedure:

A series of five challenge studies was conducted using 6-week-old conventionally raised male or female pigs that were seronegative for FMDV antibody by the SN test (Table 4.1). The pigs were obtained from a herd periodically monitored for FMDV and known

to be free of the virus. For these challenge experiments donor pigs were challenged by injection into the heel bulb of 0.5 ml of a suspension to deliver 10^5 $TCID_{50}$ FMDV O Taiwan/97 stock virus and were placed in direct contact with vaccinated pigs in the same challenge room. The pigs were monitored daily for clinical signs of FMD and lesion scores were recorded based on the degree of lameness and the presence and severity of vesicular lesions on the snout and coronary bands. Details of the five separate experiments are provided below:

Experiments 1 to 4. These were replicated experiments. 14, 15, 12 and 13 pigs were used in experiments 1, 2, 3 and 4, respectively. Pigs were randomly allocated to three groups in the same pen. Two of the pigs would be directly inoculated with virus as the source of the direct challenge, another two were unvaccinated sentinels and the other pigs were vaccinated with oil emulsion FMD vaccine. They were all housed together in the same pen to assess protection in vaccinated pigs exposed to FMDV by direct contact challenge. The relevant pigs were vaccinated at 6 weeks of age and the two challenged pigs were inoculated with virus at 10 weeks of age. Sera of these pigs were tested to measure the neutralizing antibody titres before the challenge. In experiments 2, 3 and 4, in order to induce lower SN titres some pigs were injected with 2 ml which corresponds to the administration of the standard dose of vaccine, a 1/4 dose of vaccine by injecting

0.5 ml, and a 1/16 dose by injecting 0.125 ml as indicated in Tables 4.2 to 4.5.

Experiment 5. Twelve pigs were randomly allocated to three groups in the same pen. Two challenged pigs and another two sentinel pigs were housed with the vaccinated pigs to assess the protection in those vaccinated pigs by direct contact challenge. The pigs were vaccinated at 6 weeks of age and revaccinated at 10 weeks and challenged at 12 weeks of age. Sera of these pigs were tested to measure the neutralizing antibody titres before challenge (Table 4.6).

4.2.2 Measurement of serum neutralizing antibody

Serum neutralizing antibody (SN) titres to FMDV type O Taiwan were measured in a micro-neutralization assay as described in Chapter 2.1.1.

4.2.3 Scores of FMD lesions

Lesions were scored as outlined in Section 2.3.

4.2.4 Statistical analysis

The relationship between SN titres and lesion scores in challenged pigs was evaluated by linear regression and calculation of the correlation coefficients (r) (Microsoft Excel

11).

4.3 Results

In each experiment, the challenged donor pigs developed vesicles on their snouts and/or coronary bands 1 to 2 days after challenge. The in-contact unvaccinated sentinel pigs developed the same lesions 1 to 3 days after the challenged pigs.

Tables 4.2 to 4.6 and Figure 4.1 display the challenge results and neutralizing titres for the pigs in experiments 1 to 5. The pigs with high neutralizing titres were generally protected while poor protection was found in those pigs with very low titres. Of the 16 pigs with SN titres greater than 1:64 four weeks after vaccination, 15 showed complete protection. The one infected pig had low lesion and severity scores. Severe lesions were found in 9 of 10 pigs with SN titres less than 1:8. Only some (18 of 30) of the pigs with titres between 1:8 and 1:64 were not protected. A correlation curve for the data on SN titre and protection from these experiments is shown in Figure 4.2 and the correlation coefficient, r = -0.79

4.4 Discussion

In the present study, it was shown that the absence of FMD SN titre or low titres was

correlated with disease severity after challenge and higher lesion scores if infection was present. Even though the contact challenge method is likely to be much more severe than a single intradermal challenge, there was a strong negative correlation (r =-0.79, r^2=0.6243) between SN titres and lesion scores of infected pigs challenged with a pig adapted type O FMDV strain.

Some of the pigs with low neutralizing titres were protected from challenge. Complete understanding of the protection mechanism against FMD has still to be elucidated, but individual variation in susceptibility (Black et al., 1984) and the protective effects of cytokines, such as IL-6, IL-8 and IL-12, may play a role in protecting pigs when challenged soon after emergency vaccination (Barnett et al., 2002).

The contact challenge used in the studies is substantially greater than the challenge from intradermal inoculation. It provides physical contact with infected excretions or secretions from vesicular fluid or vesicular epithelium. Moreover, the challenged pigs become lame and reluctant to move, especially when they are febrile and the vesicles rupture and progress to deep ulcers. Unchallenged pigs can nudge and lick at lesions on snouts or ruptured vesicles on coronary bands and are in intimate contact with floor, feed troughs and drinkers that have been contaminated with infectious secretions on a

24 hour per day basis. From our observation of outbreaks in Taiwan caused by the pig adapted FMDV it was clearly evident that the major transmission pathway is by exposure of naïve pigs or pigs with low neutralizing titres to infected pigs by direct or indirect exposure. Therefore, in order to block the circulation of pig adapted FMDV and control the disease it is important to ensure that exposed pigs have protective levels of neutralizing antibodies. This study has demonstrated that pigs with neutralizing antibody titres greater than 1:64 are essentially protected from contact challenge. For evaluation of vaccine potency and to have confidence in the protection level in a compulsory vaccinated pig population, it is important to ensure that the vaccinated pigs have titres higher than 1:64 to prevent infection and control outbreaks caused by pig-adapted type O FMDV.

Table 4.1 The details of pig number, vaccination number, vaccine strain used for challenges and dosage of inoculation in each individual experimental design

Experiment	*No of pigs*	*No of vaccinations received*	*Dose, virus strain and route of inoculation of donor pigs*	*Number of inoculated, sentinel and vaccinated pigs per room*
1	14	1	10^5 $TCID_{50}$ FMDV O Taiwan/97, heel bulb	Two inoculated, 2 sentinel and 10 vaccinated pigs
2	15	1	10^5 $TCID_{50}$ FMDV O Taiwan/97, heel bulb	Two inoculated, 2 sentinel and 11 vaccinated pigs
3	12	1	10^5 $TCID_{50}$ FMDV O Taiwan/97, heel bulb	Two inoculated, 2 sentinel and 8 vaccinated pigs
4	13	1	10^5 $TCID_{50}$ FMDV O Taiwan/97, heel bulb	Two inoculated, 2 sentinel and 9 vaccinated pigs
5	12	2	10^5 $TCID_{50}$ FMDV O Taiwan/97, heel bulb	Two inoculated, 2 sentinel and 10 vaccinated pigs

Table 4.2 Protected and non-protected pigs and their SN titre, severity and lesion scores of challenge Experiment 1.

Pig No.	Dosage	SN titre	lesion scores*	Severity*
1	1/16	3	5	+++
2	1/16	3	2	+
3	1/16	3	5	+++
4	1/16	3	5	+++
5	1/16	4	5	+++
6	¼	6	5	+++
7	¼	6	5	+++
8	¼	8	0	-
9	¼	8	5	+++
10	¼	91	0	-

*: lesion scores and severity scores as described in chapter 2.3

-: indicates that pigs show no other clinical signs.

Table 4.3 Protected and non-protected pigs and their SN titre, severity and lesion scores of challenge experiment 2.

Pig No.	Dosage	SN titre	lesion scores*	Severity*
11	1/16	4	0	-
12	1/16	32	3	++
13	1/16	45	0	-
14	1/4	64	0	-
15	1/4	64	2	+
16	1/4	91	0	-
17	1/4	128	0	-
18	1	128	0	-
19	1	181	0	-
20	1	362	0	-
21	1	512	0	-

*: lesion scores and severity scores as described in chapter 2.3

-: indicates that pigs show no other clinical signs.

Table 4.4 Protected and non-protected pigs and their SN titre, severity and lesion scores of challenge experiment 3.

Pig No.	Dosage	SN titre	lesion scores *	Severity *
22	1/16	3	5	+++
23	1/16	4	4	+++
24	1/4	11	5	++
25	1/4	45	5	+++
26	1/4	64	0	-
27	1	64	0	-
28	1	128	0	-
29	1	181	0	-

*: lesion scores and severity scores as described in chapter 2.3

-: indicates that pigs show no other clinical signs.

Table 4.5 Protected and non-protected pigs and their SN titre, severity and lesion scores of challenge experiment 4.

Pig No.	Dosage	SN titre	lesion scores*	Severity*
30	1/16	11	1	+
31	1/16	16	0	-
32	1/16	23	5	+++
33	1/4	45	2	+
34	1/4	64	3	++
35	1/4	64	0	-
36	1	91	0	-
37	1	128	2	+
38	1	724	0	-

*: lesion scores and severity scores as described in chapter 2.3

-: indicates that pigs show no other clinical signs.

Table 4.6 Protected and non-protected pigs and their SN titre, severity and lesion scores of challenge experiment 5.

Pig No.	Dosage	SN titre	lesion scores *	Severity *
39	1	32	0	-
40	1	32	0	-
41	1	45	0	-
42	1	64	0	-
43	1	128	0	-
44	1	362	0	-
45	1	724	0	-
46	1	724	0	-

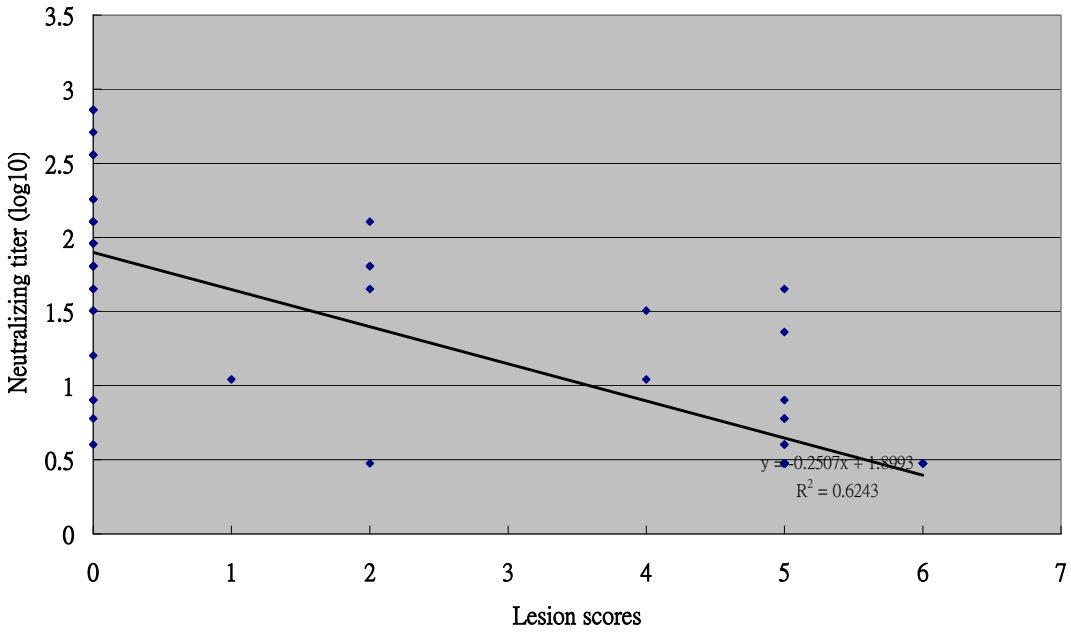
*: lesion scores and severity scores as described in chapter 2.3

-: indicates that pigs show no other clinical signs.

Figure 4.1 Summary of results of challenge tests by O/Taiwan/97 FMDV in experiments 1-5.

SN titre	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
724				○(0)	○(0) ○(0)
512		○(0)			
362		○(0)			○(0)
181		○(0)	○(0)		
128		○(0) ○(0)	○(0)	●(2)	○(0)
91	○(0)	○(0)		○(0)	
64		○(0) ●(2)	○(0) ○(0)	○(0) ●(3)	○(0)
45		○(0)	●(5)	●(2)	○(0)
32		●(3)			○(0) ○(0)
23				●(5)	
16				○(0)	
11			●(4)	●(1)	
8	●(5) ○(0)				
6	●(5) ●(5)				
4	●(5)	○(0)	●(5)		
3	●(5) ●(5) ●(2) ●(5) ●(6) ●(5) ●(5) ●(5)	●(5) ●(5) ●(5) ●(5)	●(5) ●(6) ●(5) ●(5) ●(5)	●(5) ●(5) ●(5) ●(5)	●(6) ●(5) ●(5) ●(6)
Legend/key	One circle represents one challenged pigs. In each box, ○: pigs without clinical signs ●: pigs with clinical signs ( ) :lesion scores of pigs				

Figure 4.2 Relationship between SN titres and lesion scores after contact challenges



Chapter 5 - Comparison of sensitivity and specificity in three commercial foot-and-mouth disease virus non-structural protein ELISA kits with swine sera in Taiwan

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Abstract

Three commercialized ELISA kits for the detection of antibodies to the non-structural proteins (NSPs) of FMD virus were compared, using sera from uninfected, vaccinated, challenged and naturally infected pigs. The kinetics of the antibody response to NSPs was compared on sequential serum samples in swine from challenge studies and outbreaks. The results showed that ELISA A (UBI) and ELISA B (CEDI) had better sensitivity than that of the 3ABC recombinant protein-based ELISA C (Chekit). The peak for detection of antibodies to NSPs in ELISA C was significantly delayed in sera from natural infection and challenged swine as compared to the ELISA A and B. The sensitivity of the three ELISAs gradually declined during the six months post-infection as antibodies to NSP decline. ELISA kits A and B detected NSP antibody in 50% of challenged pigs by the 9-10th days and 7-8th days post challenge, respectively. ELISA B and C had better specificity than ELISA A on sequential serum samples obtained from swine immunized with a type O FMD vaccine commercially available in Taiwan. Antibody to NSPs before vaccination was not detected in swine not exposed to FMD virus, however, antibody to NSPs was found in sera of some pigs after vaccination. All assays had significantly lower specificity when testing sera from repeatedly vaccinated sows and finishers in 1997 that were tested after the 1997 FMD outbreak. However, when testing sera from repeatedly vaccinated sows or finishers in 2004, the specificity

for ELISAs A, B and C were significantly better than those in 1997. This effect was less marked for ELISA A. The ELISA B was the best test in terms of the highest sensitivity and specificity and the lowest reactivity with residual NSP in vaccinates.

Key words: Foot-and-mouth disease; diagnosis; non-structural proteins; ELISA (enzyme linked immunosorbent assay)

5.1 Introduction

Foot-and-mouth disease (FMD) is caused by infection with FMD virus, an Aphthovirus genus of family Picornaviridae and is one of the most contagious infectious diseases of cloven-hoofed animals. The virus strain, O Taiwan/97, however, has been shown to have a species-specific adaptation to pigs (Dunn and Donaldson, 1997) and only caused overt clinical signs in pigs in the March 1997 outbreak of FMD in Taiwan (Shieh, 1997).

FMD causes severe economic losses. The March 1997 FMD outbreak in Taiwan resulted in financial losses in the hundreds of millions of dollars for the Taiwanese pork industry (Chang et al., 1997; Shieh, 1997). The estimated value of the pork export market in Taiwan in 1996 was 1.53 billion US dollars. More than 400 million US

dollars was spent to control the disease and compensate farmers for large numbers of pigs killed and incinerated during the outbreak. In addition to this direct cost were the costly control measures and the lost exports for the pork producers in Taiwan during the period needed to regain FMD free status (Yang et al., 1999).

Vaccination has been used successfully in controlling FMD in Taiwan (Yang et al., 1999) and for eradication in Europe (Leforban and Gerbier, 2002). However, differentiation of vaccinated from convalescent animals is still a major problem challenging those countries, which are working toward the eradication of FMD with compulsory vaccination programs. Therefore there is a great need for reliable approaches to detect infected animals in the vaccinated population.

Antibody to non-structural proteins (NSP) of foot and mouth disease virus, which is produced in infected animals, has been used to differentiate vaccinated from infected cattle in the field (Clavijo et al., 2004; De Diego et al., 1997; Mackay et al., 1998; Moonen et al., 2004; Sorensen et al., 1998). Recently, a number of enzyme-linked immunosorbent assays (ELISA) with high sensitivity and specificity for detection of antibody to NSP in pigs have also been reported as suitable for large scale eradication programs (Chung et al., 2002; Lee et al., 2004; Shen et al., 1999). However, the

numbers of validated samples used in the Lee et al (2003) study for evaluation of the sensitivity of three assays were below the numbers recommended by Office International des Epizooties (O.I.E.) for sensitivity evaluation (O.I.E., 2004a). The study by Chung et al only evaluated an in-house kit based on the 3AB antigen obtained from Dr Sorensen that was not commercially available at that time (Chung et al., 2002). Since the ELISA-based methodology for NSP antibody detection provides many advantages, such as objectivity compared with gel diffusion tests for NSP, high sensitivity and specificity, and the capability for large-scale screening, NSP ELISA has been recommended by the OIE to be used for serologic surveillance in regions or countries that practice FMD vaccination and for monitoring virus circulation in the field (O.I.E., 2004b).

Recently, several NSP-ELISA assays have become commercially available for evaluation. A blocking ELISA has been developed (Sorensen et al., 2005) which has shown high sensitivity and specificity when tested with pig sera collected from naïve, infected, and vaccinated pigs. The test specificity for vaccinated pigs is 99% and can be improved to 99.5% by further treatment by filtration and inactivation at 56℃ for 30 min. For naïve pigs the specificity was 99.8%. Another commercial kit was evaluated by Bruderer et al who demonstrated the specificity was 99% for 3600 samples tested

including bovine, ovine and porcine species. Antibodies specific for 3 ABC could be detected as early as 10 days post-infection (Bruderer et al., 2004). A third kit evaluated uses a 3B synthetic peptide based NSP ELISA which can be used to differentiate convalescent animals from vaccinated animals (Shen et al., 1999; Wang et al., 2001). There were no positive results detected in sera from vaccinated animals. However, preliminary field trials in Taiwan with this kit showed only 98.9% specificity in samples from vaccinated pigs. Comparative evaluations, including the above NSP ELISA kits, have been conducted by a consortium of European and American FMD reference laboratories in 2006 with large panels of sera from cattle that have been vaccinated or vaccinated-and-infected with different serotypes of FMD virus. Some sheep and pigs sera were tested in the study but these authors stressed that insufficient numbers of samples from pigs had been tested in order to complete the evaluation of these tests for use with pigs (Brocchi et al., 2006).

As these commercialized NSP ELISA kits will be very important for monitoring pigs for active FMD infection in countries using FMD vaccination programs, further evaluation of these tests are needed under different field conditions in order to determine the diagnostic sensitivity and specificity. This study evaluated these assays for sensitivity and specificity by testing sera from pigs which had been exposed to O Taiwan/97 in

experimental or field challenge and on sera from pigs in Taiwan that had not been exposed to FMDV.

5.2 Materials and Methods

5.2.1 Pigs challenged without vaccination:

42 non-vaccinated 6-week-old pigs born to unvaccinated sows in Taiwan were used for the study. They were serologically tested, before the experiment, and shown to be negative for antibodies against FMDV structural proteins. The naive pigs were inoculated intradermally in the heel bulb of the right fore foot with approximately 10^5 $TCID_{50}$ of FMDV O/Taiwan/97 strain, prepared from vesicular fluid. Following inoculation, serum samples were obtained daily during the first 2 weeks and then monthly for 6 months. These piglets all showed FMD lesions and had FMD confirmed by virus isolation or RT-PCR when tested. They were all housed in approved bio-containment facilities in Taiwan.

Additionally, sera was collected serially from pigs naturally infected with FMDV type O Taiwan/97, at 1-week intervals for the first 5 weeks and 2-week intervals up to 25 weeks post infection as shown in Figure 5.3. The characteristic vesicular lesions were observed 9 days before the first blood samples were taken and this was designated the

second-week post-outbreak. These serum samples were used to evaluate the sensitivity of the kits and to test the kinetics and the duration of the antibody response to NSPs detected by the three NSP ELISA assays.

5.2.2 Pigs unexposed with or without vaccination:

To evaluate the specificity of the assays, 130 sera from pigs from ordinary pig farms and 40 sera from pigs from SPF pig farms that had been collected from slaughterhouses in 1994 (before the outbreak) were accessed. These pigs were around 6 months of age. Another 144 sera, 80 collected before vaccination and 66 collected one week before slaughtering were collected after the outbreak of FMD in 1997. These pigs had been vaccinated twice at 8 and 12 weeks of age (Chen et al., 1998). Also, 360 sera were collected sequentially in 2003 from 120 pigs vaccinated with commercially available O Taiwan monovalent vaccines (all formulated using O Taiwan/97 type strain) on a closed farm that had no FMDV infection. Vaccinations were given at 8 and 12 weeks of age. Sera were collected before vaccination, four weeks after first immunization, and one week before pigs were sent to slaughterhouses (24 weeks of age). These sera had variable titers of antibody to the structural proteins of FMDV after vaccination.

Additionally, 68 and 127 sows that had received multiple booster vaccinations (up to 10

boosters) with commercial FMDV vaccines in 1997-8 and 2004 respectively, and which had not been exposed to FMDV, were also tested by the NSP ELISAs.

5.2.3 Evaluation of field sera after the 1997 outbreak

To evaluate the herd detection rate using NSP ELISAs, 3 infected farms were chosen. Serum was collected from 10 randomly selected sows on each farm at approximately 60-90 day intervals from 150-400 days after the 1997 FMD outbreak.

5.2.4 The NSP ELISA kits

Three commercially available ELISAs for detection of antibodies directed against FMD non structural proteins (NSP ELISAs) were compared. The assays were ELISA A (UBI FMDV NS EIA Swine; United Biomedical Inc., Nauppauge, USA), ELISA B (CEDI Diagnostics BV, Lelystad, The Netherlands) and ELISA C (Chekit: Bommeli diagnostics, Intervet, Leibefeld-Bern, Switzerland-this kit has subsequently been marketed at IDEXX Laboratories, Maine, USA). ELISAs A and C were designed to detect antibodies to a synthetic peptide of FMDV protein 3B or a recombinant FMDV NSP 3ABC polyprotein, respectively. ELISA B tests for competitive binding of antibody to the 3ABC protein versus a 3ABC specific monoclonal antibody. The assays were conducted according to the manufacturers' instructions.

5.2.5 Interpretation and analysis of results

The results were determined in accordance with the manufacturers' instructions. Sera with absorbance values less than the cut off value were considered negative for FMDV NSP antibodies. Sera with absorbance values greater than the cut off value were considered positive. For comparison of sensitivity and specificity, ambiguous values according to the manufacturers' criteria were scored as negative when calculating the sensitivity and positive when calculating the specificity. The 95% confidence intervals of the tests at different time points were calculated and are shown in the figures. Significance of differences between the ELISA kits and at different points was determined by Chi-square tests.

5.3 Results

5.3.1 Post-infection development of antibodies to NSPs in experimentally infected pigs

The proportion of NSP antibody reactors for the first 13 days after the experimental infection has been showed in Figure 5.1 for all three tests. Positive NSP reactors were not detected within 5 days of challenge. With ELISA A, positive reactors were first detected on the 7th day and 50% of challenged pigs were positive after the 9-10th day post challenge. The peak detection rate was on the 12th day post challenge. NSP

antibodies were detected earliest with ELISA B on the sixth day and this test detected 50% of challenged pigs after the 7-8th day post-infection. The peak detection rate for ELISA B was 12 days post challenge. With ELISA C, positive reactors were first detected on the 8th day but this test failed to detect more than 50% of the challenged pigs within 13 days. The peak detection rate was 21.4% on the 13th day after challenge.

The proportion and 95 % confident intervals of NSP antibody reactors from monthly collection of sera post challenge is shown in Figure 5.2 for all three tests. ELISA A and B have comparable sensitivity but they had significantly higher sensitivity than ELISA C at 30, 60 and 90 days post challenge. Additionally, both ELISA A and ELISA B had significantly higher sensitivities at 30 and 60 days post challenge compared with 150 and 180 days post challenge, respectively. This was not unexpected as NSP antibody levels decline over time. However, this was not observed at these times for ELISA C.

5.3.2 Post-infection development of antibodies to NSPs in natural infected pigs

Kinetics of post-infection antibody responses in sequentially collected sera following natural infection is shown in Figure 5.3. With ELISA A, 83.3% of pigs were positive by the second week after infection. Peak sensitivity was 95% four weeks post infection and sensitivity then declined. The sensitivity was 54.3% at week 25 of convalescence, at the

end of sampling. With ELISA B, peak sensitivity was 87.5 % two weeks post infection and sensitivity declined gradually thereafter. The sensitivity was 38.6 % at the end of sampling (week 25). With ELISA C, only 39.9% of pigs were positive at week two after infection. Peak sensitivity for ELISA C was 55.6% reached at the 7th week post infection and sensitivity declined gradually thereafter. The sensitivity was 22.2 % at the end of sampling (week 25).

5.3.3 Detecting NSP antibody in the outbreak herd

The serum NSP antibody reactivity for the three assays in 30 randomly selected sows collected at 60-90 day intervals between 150 and 400 days post exposure to FMDV in the 1997 outbreak are shown in Table 5.1. The ranges of sensitivities with these sera at the different times post exposure for ELISA A, B and C were 33.3 to 60 %, 30 to 60% and 16.7 to 46.7 %, respectively. There was also a substantial difference between sensitivities for ELISA A (60%) and ELISA B and C (33.3% and 26.6%, respectively) in sera at 150-160 days post exposure.

5.3.4 Specificity

The specificity of the assays was evaluated by testing 871 sera collected from naive, prophylactically vaccinated finishers and repeatedly vaccinated sows (Tables 5.2 and

5.3, respectively). The three assays had 100% specificity in naïve swine without vaccination. For sera collected from pigs vaccinated once in 2003, the specificity for ELISA A was 98.3%, for ELISA B 100% and for ELISA C 99.1%. The specificity results for pigs that had received two vaccinations on sera that had been collected one week before slaughtering were different between 1997 and 2003. In 1997, the specificities were 87.8, 93.9 and 80.9 % for ELISA A, B and C, respectively. In 2003, the specificities were 95.8, 99.1 and 98.3 %, respectively. Test results of sera collected from sows that had received multiple FMD vaccinations were also different between 1997-1998 and 2004. In 1997-1998, the specificities were 64.7, 83.8 and 83.8% for ELISA A, B and C, respectively. In 2004, the specificities were 84.3, 100 and 99.2%, respectively. Significance of differences in specificities of the three tests within years and between 1997-1998 and 2003-2004 are shown in Tables 5.2 and 5.3.

5.4 Discussion

Antibodies to FMDV NSP have been shown to be reliable indicators of previous infection in pigs both in challenge tests and outbreaks. However, in comparative evaluations of commercialized NSP kits for use in pigs, other researchers have not included sample numbers of the order suggested by OIE (O.I.E., 2004a) and also assessment of the NSP kits for testing the duration and kinetics of NSP antibody

responses in pigs has not been included in these studies (Brocchi et al., 2006; Chung and Liao, 2003; Lee et al., 2004; Wang et al., 2001). In this study, three different ELISA tests for swine, based on NSP antigens from protein 3B and 3ABC, were compared for sensitivity and specificity on well-characterized stored samples from infected (Figures 5.1, 5.2 and 5.3), naïve (Table 5.2), vaccinated (Table 5.2) pigs, and repeatedly vaccinated sows (Table 5.3), including serum samples sequentially collected following exposure or vaccination. The peak detection percentages of antibodies to NSPs in ELISA C were significantly delayed in sera from challenged infected pigs compared to ELISA A and B. Also ELISA C failed to detect NSP antibody in 50% of challenged pigs within 13 days for challenge. ELISA A and B had better sensitivities for all post-infection times sampled than ELISA C. The results showed that ELISA A and B were capable of detecting antibodies earlier, from 8-14 days post challenge, and had a much better sensitivity than ELISA C. These findings are similar to observations in experimentally challenged pigs reported by others (Lee et al., 2004; Wang et al., 2001). Previous studies have suggested that the antibody response of NSPs can occur within one to two weeks after infection in pigs (Chung et al., 2002; Lee et al., 2004; Rodriguez et al., 1994). In the current study NSP antibody was detected as early as 6, 7, and 8 days post-challenge by ELISAs B, A and C, respectively. This can be used as an indicator for the estimation of actual exposure time in an FMD outbreak. If antibodies of NSPs in

pigs with clinical lesions cannot be detected by these kits, the time course of the outbreak is likely to be within one week.

In addition, the results demonstrate the decline of NSP antibody detection by the kits decreased over time in convalescent pigs in the experimental and natural challenge studies. Similar observations have been reported previously for experimentally challenged cattle and pigs (Lee et al., 2004; Moonen et al., 2004). Even using the best tests, ELISA A and B, the sensitivity had dropped to 30- 60% by the end of the study period (25-26 weeks) from a peak of 96% and 100% respectively at 12 days post challenge. Therefore, negative results for antibodies to NSPs cannot be used to definitively exclude individual pigs from having been infected with FMDV, especially, if sera were collected at longer time intervals after infection. The lower sensitivity found with the ELISA C kit was similar to that reported by Bronsvoort et al. in cattle in Africa (Bronsvoort et al., 2004). However, the results shown in our studies are in contrast to those in a challenge study in cattle (Moonen et al., 2004), which reported a similar sensitivity for ELISA A and C.

Despite variations in the sensitivity of the 3 NSP ELISA kits throughout the challenge studies and their relatively low sensitivity by 150-180 days post-challenge, all kits were

able to detect NSP antibodies in sera of some convalescent sows up to 400 days post infection following the 1997 outbreak. The reason for the persistence of NSP antibodies in convalescent sows is not certain but is most likely linked to repeated infections in herds without vaccination. During the outbreak of FMD in Taiwan in 1997, a high infection rate (more than 90%) was experienced in the naïve herds (Chen, personal observation). The sera from convalescent sows were collected during and after the outbreak while the prevalence in herds was extremely high and the prolonged detection of NSP antibodies may represent multiple challenge and re-infection episodes. There was also a peak in the percentage of reactors using all three NSP ELISA kits at 330-340 days post infection. As well as the high herd prevalence at that time, this was also likely to have been influenced by repeated use of the unpurified FMD vaccines that were available in Taiwan from 1997 to 1998 on these farms. The vaccines in use at that time were able to induce NSP antibodies in a proportion of uninfected pigs as shown in Tables 5.2 and 5.3.

In the evaluation of the NSP ELISA kits in convalescent sows (Table 5.1) the reason for the substantially higher sensitivity for ELISA A than ELISA B or C in pigs at 150-160 days post-exposure was not determined. ELISA A measures antibody to the 3B protein whereas ELISA B and C measure antibody to a 3ABC polyprotein. Also ELISA A

tended to have higher proportion of reactors in the early phase after natural challenge (Figure 5.3). As indicated above these pigs were also probably being exposed to multiple challenge or re-infection episodes. A combination of these factors may have cause the substantial difference in sensitivity at that time but overall the difference in sensitivity between ELISA A and ELISA B was not significant.

In the present study, we have examined a relatively large group of unexposed pigs vaccinated with commercialized vaccines. This is representative of the current situation in Taiwan where pigs are compulsorily vaccinated. Results from sequential serum samples obtained from swine vaccinated against O Taiwan/97 showed that ELISA B and C had somewhat better specificity than ELISA A, which is similar to the findings in pigs, albeit with smaller sample sizes, in the NSP kit evaluation studies by Brocchi and colleagues (Brocchi et al., 2006). In the current NSP surveillance program in Taiwanese pig farms, ELISA A and B have been chosen as the screening and confirmatory tests, respectively. When ELISA A was used there were around 3-5% positive reactors in the sera collected. However, few reactors (0.03%) were detected when ELISA B was used on these reactive sera (Chen, unpublished data). This has facilitated comprehensive trace back studies of all reactors within current manpower availability in Taiwan. All farms that have NSP reactors after screening by ELISA A and confirmatory testing by

ELISA B, undergo a farm visit with intensive investigation and testing.

The specificities of NSP immunoassays for testing cattle have been shown to be influenced by the presence of residual NSPs in commercial FMD vaccines, particularly after multiple immunizations (Bergmann et al., 2000; Mackay et al., 1998). Similar results in pigs have also been reported in Taiwan (Lee et al., 2004). In our study, the three immunoassays began to identify a modest level of FMDV NSP antibody reactors in sera from finishers after two vaccinations in 1997, but not before vaccination. Sows that had been vaccinated with commercially available vaccines up to 10 times in 1997-1998 showed lowered specificities with these assays. This observation is in contrast to test results with sera in this study collected in 2004 and in a previous report (Chung and Liao, 2003) that showed sera collected from sows vaccinated more than 10 times were negative by a 3AB NSP ELISA. The difference between 1997-1998 and subsequent test results can be ascribed to differences in the NSP content of the vaccines used for multiple immunizations. The performance of the FMD NSP assays can be affected by the quality of the vaccines used in different studies. Therefore, results of NSP testing of vaccinated finishers and repeatedly vaccinated sows should be interpreted with caution. In our results sera from vaccinated pigs tested by ELISA B and ELISA C showed enhanced specificity in pigs vaccinated with more purified vaccines.

Use of more purified FMD vaccines is currently required by OIE in countries or regions that use FMD vaccination to control FMD (O.I.E., 2004b).

Overall, it is not reliable to use the present commercialized NSP ELISA kits to identify individual pigs that have been infected by FMD viruses. However, the kits can be applied as a herd test to demonstrate the absence of FMDV circulation by showing that vaccinated pig herds are free from antibody to FMD NSPs. These tests have been applied in Taiwan for pig farm surveillance by testing appropriate numbers of serum samples from the finishers, with numbers dependent upon the estimated prevalence of disease in the country (Chung et al., 2003). This established surveillance method of sampling farms for reactivity to NSP, in conjunction with the tracing back of reactors to detect swine with clinical signs and to conduct follow-up serological testing has been successfully applied in Taiwan to show the lack of FMD virus circulation in the past three years. The positive serum samples detected during the year round surveillance were all shown by further intensive investigation of involved farms to be false positives. Other factors which can cause false positives, such as bacterial contamination and degradation and complement activation in contaminated sera (Sorensen et al., 2005), need to be considered and further investigated. Alternatively, other high specificity confirmatory tests such as the enzyme-linked immunoelectrotransfer blot assay could be

used, as is done with surveillance of cattle for FMD in South America (Bergmann et al., 2000), to check NSP reactors from the surveillance programme in Taiwan.

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Table 5.1. Sensitivity of NSP antibody detection by three ELISA kits on convalescent sows (that had also been vaccinated) from three farms after the 1997 FMD outbreak in Taiwan.

Days post-infection	No. tested	ELISA A			ELISA B			ELISA C		
		No. (+)	Sen* (%)	Herd sen(%)	No. (+)	Sen* (%)	Herd sen(%)	No. (+)	Sen* (%)	Herd sen(%)
150-160	30	18	60	100	10	33.3	100	8	26.6	100
240-250	30	12	40	100	9	30	100	11	31.7	100
330-340	30	18	60	100	18	60	100	14	46.7	100
390-400	30	10	33.3	100	11	36.6	100	6	16.7	100

* Individual animal sensitivity

Table 5.2. Diagnostic specificity of three ELISA kits on sera from naïve pigs or pigs vaccinated with one of the various commercial FMD vaccines in Taiwan

	No. tested	ELISA A		ELISA B		ELISA C	
		No. negative	Specificity (%)	No. negative	Specificity (%)	No. negative	Specificity (%)
Sera before outbreak	130	130	100	130	100	130	100
SPF swine serum	40	40	100	40	100	40	100
Pre-vaccination sera in 1997	80	80	100	80	100	80	100
One-week before marketing with twice vaccination sera in 1997	66	58	87.8 [a]	62	93.9 [b]	55	80.9 [a]
Pre-vaccination sera in 2003	120	120	100	120	100	120	100
Four-week after first vaccination sera in 2003	120	118	98.3	120	100	119	99.1
One-week before marketing with twice vaccination sera in 2003	120	115	95.8 [c]	119	99.1 [c]	118	98.3 [c]

Specificity values with different superscript letters are significantly different as determined by χ^2 test. ($P<0.05$)

Table 5.3. Diagnostic specificity of the three ELISA kits on sera from repeatedly vaccinated sows in 1997-8 or 2004 (10 times vaccination)

	No. tested	ELISA A		ELISA B		ELISA C	
		No. negative	Specificity (%)	No. negative	Specificity (%)	No. negative	Specificity (%)
Repeatedly vaccinated sows in 1997-8	68	44	64.7[a]	57	83.8[b]	57	83.8[b]
Repeatedly vaccinated sows in 2004	127	107	84.3[c]	127	100[d]	126	99.2[d]

Values with different superscript letter are significantly different as determined by χ^2 test. ($P<0.05$)

Figure 5.1. Development of NSP antibodies in naïve pigs after experimental intradermal challenge. Percentage of animals positive per number tested using three NSP ELISA kits. (N=42)

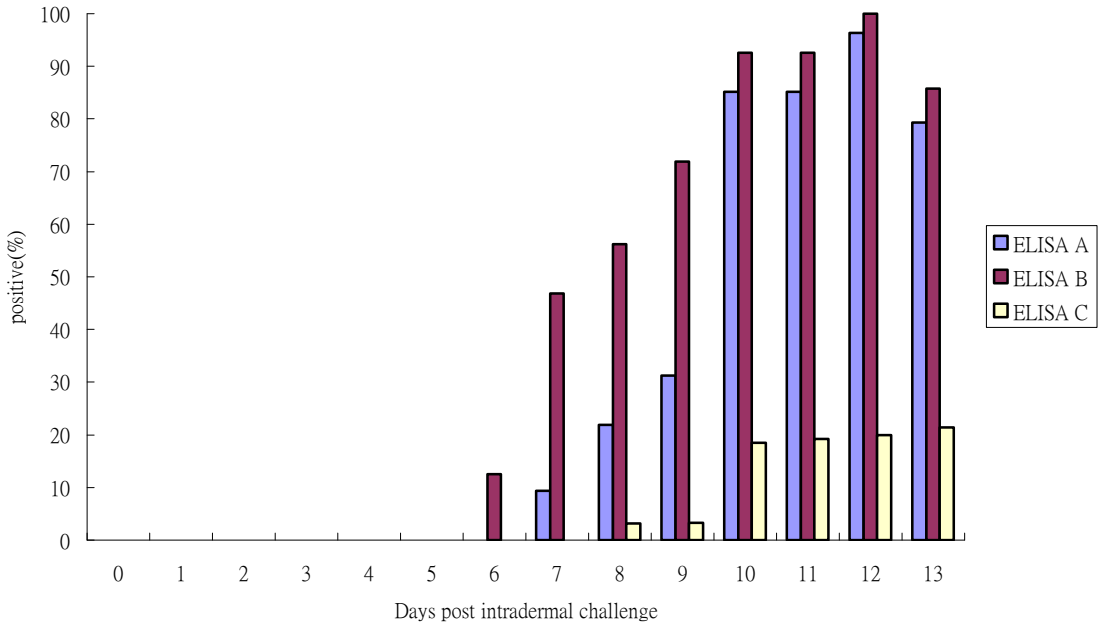


Figure 5.2. Percentage of pigs positive for NSP antibodies at monthly intervals after challenge using three NSP ELISA kits (Number tested = 42). Error bars indicate the 95% confident interval.

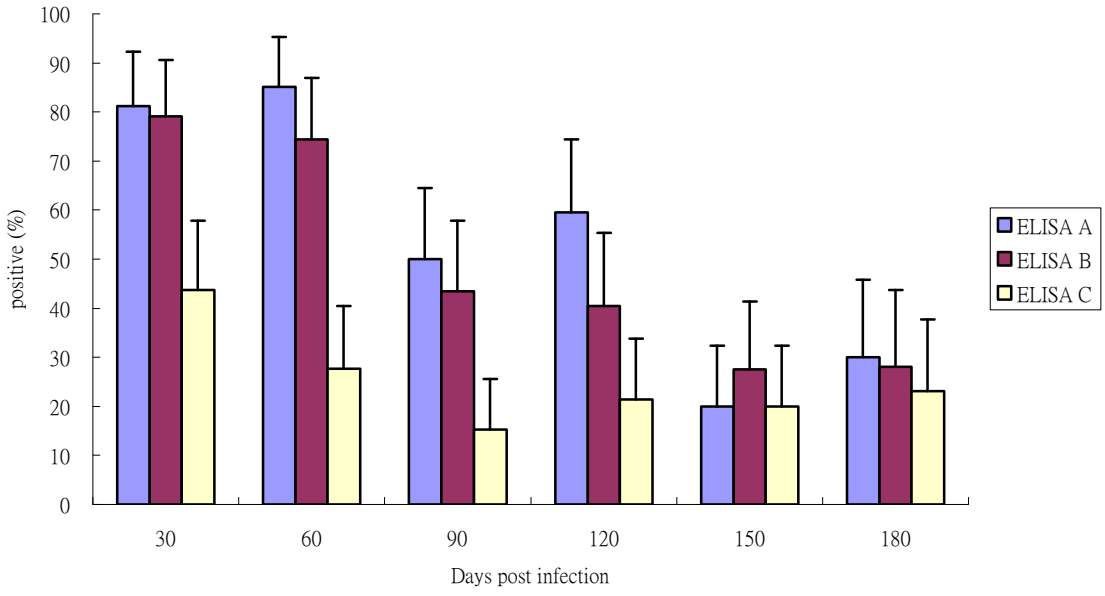
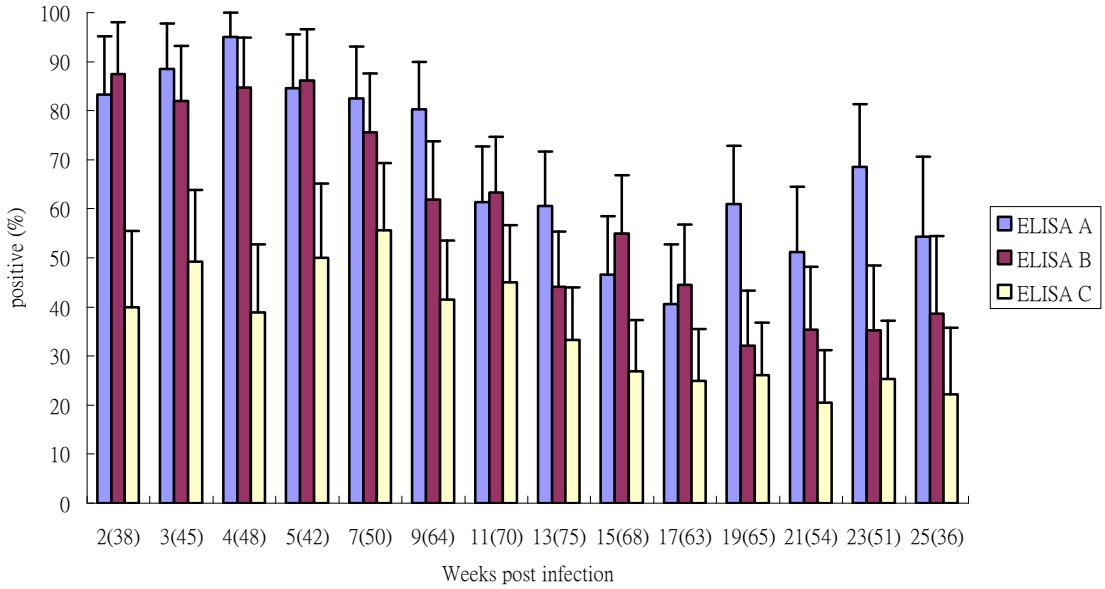


Figure 5.3. Percentage of pigs positive for NSP antibodies at intervals after natural FMD challenge using three NSP ELISA kits. Number tested at each time point is shown in brackets. Error bars indicate the 95% confident intervals.



Chapter 6 - Immune responses of pigs to commercialized emulsion FMD vaccines and live virus challenge

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Abstract

The immune response to structural and non-structural proteins (NSPs) was studied on sequential serum samples in swine from O/Taiwan/97 FMDV challenge studies, outbreaks and after vaccination. The results showed that pigs vaccinated with a commercial vaccine prior to or after infection maintained high neutralizing antibody titers with gradual decline from peak titers over the duration of this study. However, neutralizing antibody titers in non-vaccinated pigs only reached moderate levels 2-4 weeks post infection and remained low thereafter. For the 3B and 3ABC NSP antibody ELISA responses, there were gradually decreasing levels of NSP antibody over time. In multiple vaccinations, all pigs showed significant increases in neutralizing antibodies after booster vaccination. For the 3B NSP antibody ELISA after vaccination, the mean S/P ratios for pigs vaccinated with all three FMD vaccines were all below the 0.23 cut-off value set by the manufacture, but some sera from individual vaccinated pigs gave results above this cut-off after primary or secondary vaccination. However, with the 3ABC NSP antibody ELISA, all sera from vaccinated pigs had negative results for NSP antibody for all time points.

Key words: Foot-and-mouth disease; Diagnosis; Neutralizing antibodies; Vaccines; Non-structural proteins; ELISA (enzyme linked immunosorbent assay)

6.1 Introduction

Foot-and mouth disease (FMD) is an acute, febrile, and contagious vesicular disease infecting cloven-hoofed animals, including cattle, sheep, goats, deer, and pigs. The typical lesions and duration of infectivity varies between species, with pigs and cattle showing more profound clinical signs of infection than other ruminants. The outbreak of FMD in Taiwan in 1997 involved strain specific infection in pigs only and no other animals were affected [1]. Although pigs play an important role in spreading FMD there are few studies investigating the control of FMD in endemic areas by vaccination in this species.

Successful control of FMD outbreaks with emergency vaccination has occurred in Taiwan in 1997 [1], in Korea in 2001 [2] and in The Netherlands in 2001 [3]. This stimulated thinking of alternative methods for FMD control. In Taiwan, a blanket vaccination program was implemented which led to a large reduction in new outbreaks and quickly brought the outbreak under control in 1997. Subsequently, the disease has been largely eradicated from Taiwan by compulsory vaccination campaigns of susceptible animals, which has prevented the spread of foot and mouth disease virus (FMDV) between farms. The last reported case caused by O Taiwan/97 in pigs was in a slaughterhouse in 2001. Since then no further outbreaks have been reported and in May

2003 Taiwan was given FMD free status with vaccination by the OIE [4].

Surveillance studies of convalescent sows in Taiwan after the 1997 type O FMD outbreak showed presence of high neutralizing antibody titers which lasted for more than three years after outbreaks which was in marked contrast to recent studies in pigs [5, 6]. A long duration of immunity following recovery from infection has only been demonstrated with cattle. However, previous reports had also shown that persistence of antibody in pigs, measured by mouse protection tests, was short lived and infected pigs may succumb to re-infection 3-6 months after natural infection [7, 8].

Vaccines in pigs generally promote protective immunity within 21 days following primary vaccination and a secondary injection is required to maintain immunity at protective levels for about 6 months [9, 10]. Thereafter, further re-vaccinations at regular intervals are required to maintain protective immunity. In recent studies the longevity of antibody responses following vaccination with high potency emergency FMD vaccines has demonstrated that peak titers can be maintained for up to 7 months in pigs. This information could potentially be used both in the control of outbreaks and for routine vaccination campaigns by permitting an increased interval for revaccination [11].

As well as antibody to FMDV structural proteins that are detectable in vaccinated and infected pigs the latter also have antibody to FMD non-structural proteins (NSP) [6]. Recently, various ELISA-based kits for detection of antibodies to NSP have become available, including those to the baculovirus expressed 3ABC peptide and the synthesized 3B peptide. These tests have been reported to be sensitive and specific when tested with pig sera [12, 13]. Currently these two tests are commercially available and are used in the Taiwanese FMD surveillance program.

The use of vaccines demonstrated to be free of NSP residues is an important prerequisite for FMD vaccination programs to enable differentiation of infected from vaccinated pigs. This is recommended in the FMD Chapter of the OIE manual[14]. The method proposed to support claims that the vaccine does not induce antibody to NSP is to vaccinate animals at least three times over a period of 3-6 months and then test 30-60 days after the last vaccination for the presence of antibody to NSP.

To date there has been no report on the duration of the immune response to both structural and NSP in pigs after challenge with the pig adapted type O strain of FMDV. To provide such information, we have studied singly and multiple vaccinated pigs using

commercial imported FMD vaccines and pigs naturally infected or experimentally challenged with O Taiwan FMDV in order to understand the differences in the persistence of antibody between previous pig studies and the observations from the Taiwanese FMD outbreaks.

6.2 Materials and methods

6.2.1 Field data

During the 1997 Taiwanese outbreak of FMD (type O/Taiwan/97), 36 pigs were transferred from an affected farm, in which approximately half of the pigs only had been vaccinated (due to limitations in the supply of vaccine), to bio-containment facilities for investigation of FMDV carrier status. The conventionally reared 24-week-old pigs were allocated to two groups of 18 pigs, based on whether they had received vaccination with a commercially available monovalent double-oil-in-water emulsion (DOE) vaccine containing inactivated FMD type O 4174 virus as antigen before the outbreak of FMD on the farm (Group 1) or they had not been previously vaccinated (Group 2). The pigs, which had been naturally infected by FMDV, were housed in a high-containment unit with each group housed in separate rooms. Oropharyngeal (OP) fluid and sera were collected serially from these pigs at 1-week intervals for the first 5 weeks then 2-week intervals up to 23 weeks post infection. At

week 7, half of the pigs (9 pigs) in group 2 were vaccinated with DOE FMD vaccine (subsequently designated as group 3) and the other 9 pigs remained unvaccinated as group 2.

6.2.2 Challenge study

This experiment involved a challenge study of naïve piglets born to unvaccinated and unexposed sows in Taiwan. In this study, 42 non-vaccinated 6-weeks old piglets were tested serologically by serum neutralization tests to type O/Taiwan/97 FMDV and shown to be negative for FMD neutralizing antibody titers at the time of challenge. The piglets were inoculated intradermally at the heel bulb of the right forefoot with approximately 10^5 fifty percent tissue culture infective doses ($TCID_{50}$) of FMDV O Taiwan/97 strain. Following inoculation, serum samples were collected and clinical signs were observed daily for the first 2 weeks post-challenge and then serum was collected monthly for 6 months. All 42 pigs developed FMD lesions, had FMD confirmed by virus isolation or RT-PCR and were included in the immune response to infection study. All of these pigs were housed in the bio-containment facilities.

6.2.3 Vaccination study

One hundred and eighty pigs on a farm that had not been exposed to FMDV and had

been confirmed FMD-free by virological and serological testing were randomly divided into three groups which were vaccinated with one of three commercially available monovalent FMD serotype O Taiwan/97 oil-emulsified vaccines. Vaccines A, B and C were imported from England, Argentina and Russia, respectively. All pigs received the first FMD vaccination at 12 weeks of age and a secondary vaccination at 16 weeks of age. Serum samples were collected from pigs at 12, 16, 18 and 24 weeks of age to monitor FMD neutralizing antibody titers and for detection of FMD NSP antibodies.

To test for evidence of residual NSP in two of the vaccines (Vaccines B and C), 20 pigs were vaccinated with a double dose of vaccine on three occasions (12, 16 and 20 weeks of age) as recommended in the OIE manual [14].

6.2.4 Measurement of serum neutralizing antibody

Serum neutralizing antibody (SN) titers to FMDV type O Taiwan were measured in a micro-neutralization assay according to the method described in the OIE manual [14] . Briefly, sera were diluted in a two-fold dilution series in 96-well microtiter plates. Then 50 μl of virus suspension containing 100 $TCID_{50}$ of FMD viruses (O Taiwan/97) was added to each well and incubated for 1 hour at 37°C. Baby hamster kidney cells (BHK21 cells), $4.0x10^5$ cells/ml, were then added to each well. The wells were

examined for cytopathic effect after 48 hours incubation at 37°C. End-point titers were calculated as the reciprocal of the highest serum dilution to neutralize 100 $TCID_{50}$ of the virus. Results were expressed as the log 10 SN titer.

6.2.5 Measurement of antibody to NSPs

Serum antibodies to FMDV NSPs were measured by two different commercially available ELISAs. The commercial kits used were Cedi FMDV-NS for the NSP 3 ABC peptide (Cedi-Diagnostics B. V., the Netherlands) and FMDV NSP ELISA for the NSP 3B peptide (United Biochemical Inc. (UBI), New York, USA). The former test is a blocking ELISA which measures the competition between test sera and a NSP specific monoclonal antibody for the binding to the 3ABC NSP of FMDV [15]. The latter test is an indirect ELISA which measures the binding of antibodies to 3 B peptide [16]. Both assays were performed according to the manufacturers' instructions. To standardize the results among different ELISA plates, the OD obtained from the Cedi kit was expressed as the percentage inhibition (PI) of the monoclonal antibody binding. The optical density (OD) obtained from the UBI kit was expressed as the signal to positive (S/P) ratio, the OD value of the sample was divided by that of the positive controls on the plate. According to the manufacturers specifications the PI cut-off value for the Cedi ELISA is 50% and the cut-off for the S/P ratio for the UBI ELISA is 0.23.

6.2.6 Statistical analysis

Data were analyzed by the analysis of variance (ANOVA) using general linear model procedures. Differences in means of SN and NSP titers between different vaccine groups were tested by Duncan's new multiple range test. A P-value of less than 0.05 was considered significant.

6.3 Results

6.3.1 Field data

Figure 6.1 shows the neutralizing antibody results for pigs which were exposed to FMDV infection with or without prior FMD vaccination and also the effect on neutralizing antibody titers in pigs vaccinated after infection. A significant difference ($P<0.05$) in neutralizing antibody titers was found between pigs vaccinated or non-vaccinated prior to natural infection and also between pigs vaccinated and non-vaccinated after natural infection. Neutralizing titers in infected pigs vaccinated either before or after the infection were higher than those of non-vaccinated infected pigs. The pigs vaccinated with a commercial vaccine prior to and post infection maintained high neutralizing titers with gradual decline from peak titers over the duration of this study. However, neutralizing antibody titers in non-vaccinated pigs only

reached moderate levels 2-4 weeks post infection and remained low from 5 weeks onward to the end of this study.

6.3.2 Neutralizing antibody titers following experimental infection

Neutralizing antibody responses for pigs over the first 13 days following experimental infection are shown in Figure 6.2. Figure 6.3a shows the profile of neutralizing titers from 30 to 180 days post infection. Antibody was first detected at 3 days post-infection and in most animals titers had peaked by 5 days post-infection with only low mean neutralizing antibody titers (1.11-1.21 log10 SN titer) detected at different time points in these pigs up to 180 days post-infection.

6.3.3 NSP antibody response following experimental infection

Using UBI and Cedi kits for the detection of 3B and 3ABC NSP antibody responses (Figures 6.3b and 6.3c), respectively, there were gradually decreasing levels of NSP antibody as indicated by the mean S/P and PI values over time.

6.3.4 Neutralizing antibody titers following vaccination

Before vaccination, all pigs used were seronegative for neutralizing antibody to FMDV. Figure 6.4a displays the sequential neutralizing antibody titers in pigs after primary

vaccination and booster vaccination 4 weeks later for the three different FMDV vaccines. For all three vaccines geometric mean neutralizing antibody titers peaked by two weeks after booster vaccination and had declined by 12 weeks post-vaccination but were still higher at that time than titers 4 weeks after primary vaccination. Highly significant differences ($P<0.01$) were found between vaccine A and vaccines B and C at two weeks after booster vaccination and 12 weeks post-vaccination.

Figure 6.5a shows the sequential neutralizing antibody titers in pigs vaccinated with double dose of vaccines B and C at 12, 16 and 20 weeks of age and tested for antibodies at 12, 16, 20 and 24 weeks of age. For both vaccines geometric mean neutralizing antibody titers increased four weeks after each vaccination.

6.3.5 NSP antibody responses following vaccination

The mean S/P ratios for NSP antibody in the 3B ELISA for pigs vaccinated with all three FMD vaccines are shown in Figure 6.4b. While the mean S/P ratios were all below the 0.23 cut-off value set by the manufacturer, 10 sera from individual pigs vaccinated with vaccine A and 6 sera vaccinated with vaccine C were above the cut-off after the primary or secondary vaccination. All pigs vaccinated with vaccine B were lower than the cut-off at all time points after vaccination.

The mean PI values for NSP antibody in the 3 ABC ELISA for pigs vaccinated with all three FMD vaccines are shown in Figure 6.4c. All vaccinated pigs had negative results for NSP antibody for all time points and none of the vaccination groups showed any significant change in the mean PI value over time.

In Figures 6.5b and 6.5c the sequential 3B and 3ABC NSP ELISA results respectively in pigs vaccinated with double dose of vaccines B and C at 12, 16 and 20 weeks of age are displayed. The mean S/P ratios were all below the 0.23 cut-off value, but the sera of three pigs vaccinated with vaccine B and two sera from pigs vaccinated with vaccine C were above the cut-off for the 3B ELISA after the primary, secondary or tertiary vaccination. The mean S/P ratio was close to the cut-off value four weeks after the third vaccination in pigs that received vaccine B.

The mean PI values for NSP antibody in pigs with the 3 ABC ELISA for both vaccines did not exceed the 50% inhibition cut-off, but there was an increase in the PI value four weeks after primary vaccination which was maintained at that level after booster vaccination.

6.4 Discussion

The persistence of neutralizing antibody to FMD virus in pigs has been shown previously to be short lived and pigs often only remain immune to re-infection for a few months post-infection [7]. In this study, naive pigs experimentally infected with the pig adapted FMDV O/Taiwan/97 showed similar results to those for pigs infected with other strains in that antibody titers rose to a peak and declined quickly to a stable plateau within 1 month [17]. However, naturally infected pigs that had been vaccinated either prior to or post infection were able to induce very high neutralizing antibody titers of similar magnitude to those found in sows after the outbreaks of FMD in Taiwan in 1997 [6]. Persistent high neutralizing antibody levels were not due to the persistence of infection in pigs. Detailed investigations using probang samples, tested by RT-PCR and viral isolation (data not shown), from pigs from natural outbreaks and experimental challenge studies clearly demonstrated that FMD infection did not persist in these pigs.

In experimental infection studies strong NSP antibody responses were observed by 30 days post infection when tested by both 3B and 3ABC ELISA kits. The decline in the antibody response to 3B or 3 ABC NSP from 30 days to 180 days after experimental challenge showed that the strongest NSP antibody responses in infected pigs occurred in the first two months post infection. Samples taken after 150 days had mean ELISA

results at or below the cut-off value. The NSP antibody response profile from our studies differs from that reported by Elbe et al [18] in two pigs challenged with FMD O/Taiwan 3/97 where 3ABC NSP antibody measured by Ceditest ELISA lasted for more than 119 days after challenge. In one of these pigs the 3ABC ELISA PI values persisted at >80% whereas in the other pig the PI value dropped below the cut-off (50%) by day 49 but then re-bounded after day 56 and was >80% at day 119 post challenge. In contrast only one of 42 pigs in our study showed a PI value >90% and this persisted for more than one year (data not shown). Therefore in pig herd surveillance programs using FMD NSP ELISA, if samples are collected at long periods after outbreaks, when test sensitivity would be expected to have reduced significantly, potentially infected herds may be missed.

Immune responses to NSP of FMD virus have been studied in cattle and sows that have been given multiple repeated vaccinations [19, 20], but this has not been reported for fattening pigs. In the repeat vaccination studies in calves inoculated at 1 month intervals one of six calves were positive for NSP antibodies using one of five vaccines by the 3ABC ELISA but not the 3B ELISA[20]. In our study the results indicated that if pigs received a number of doses of FMD vaccine from which NSPs were not completely removed, positive reactors might be found with the 3B ELISA kit but not the 3ABC

ELISA. This was also the case with two of the vaccines (Vaccines A and C) when pigs were vaccinated according to the routine vaccination regime recommended by vaccine manufacturers in Taiwan. With two vaccines (Vaccines B and C), when repeated double-doses of vaccine were given according to the protocol in the OIE manual significant residual levels of NSP were not present when using the 3ABC ELISA, but were if sera were tested by the 3B ELISA. The third vaccine (Vaccine A) was not included in this part of the study. Although in previous studies vaccine A had performed well in Taiwan [21, 22], it gave significantly lower neutralizing antibody responses than vaccines B and C in the current studies, had failed to pass the recent government vaccine potency tests as previously described [20] and was not available for the multiple dose repeat vaccination study. The lower apparent potency of Vaccine A in pigs showed a similar trend to that with vaccination of calves in another study [20] and it cannot be explained by inappropriate vaccine transportation or storage. All of the vaccines used in this study were sent by one freight forwarding company directly to our Institute and were stored in the same refrigerator during the study period. This difference in efficacy in pigs is more likely related to differences in antigen load and other factors associated with production procedures and the adjuvant used in the vaccine.

The reason for the contrast between NSP antibody reactivity in the 3B and 3ABC

ELISA in vaccinated calves and vaccinated pigs is not explained from these studies. It may relate to species differences in immunogenicity of the 3ABC or to 3B peptides in the vaccinated animals, or the apparent species-related differences in NSP reactive may be due to the different levels of component NSP peptides in the vaccines used in this study.

In conclusion, this study has confirmed that pigs that are infected with type O/Taiwan/97-like FMD viruses and are vaccinated before or after exposure will show persistence of SN antibody at relatively high titers for prolonged periods and this provides an explanation for the long persistence of such antibodies in sows after the 1997 Taiwanese outbreak despite absence of infection with FMDV. The study also identified optimum times for use of NSP ELISA tests for investigation of suspect FMD outbreaks in pig herds infected with pig-adapted FMDV such as O/Tiawan/97.

6.5 Acknowledgements

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Figure 6.1. The profile of neutralizing antibody titers in pigs, either unvaccinated or FMDV vaccinated before or after infection by O/Taiwan/97 FMDV. The error bar indicates the standard error of mean of each sampling time

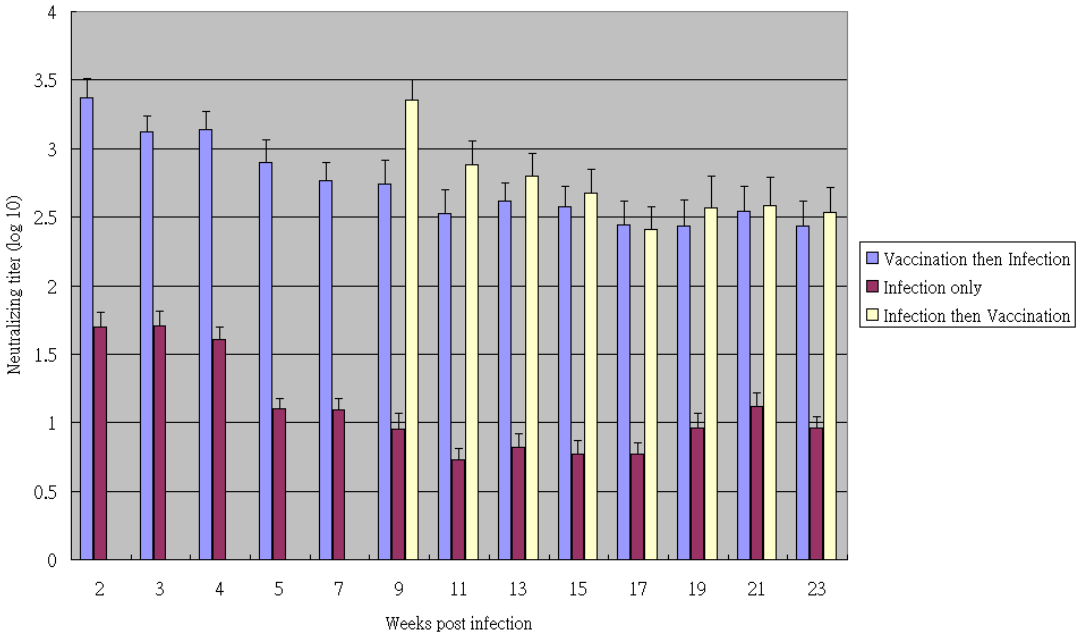
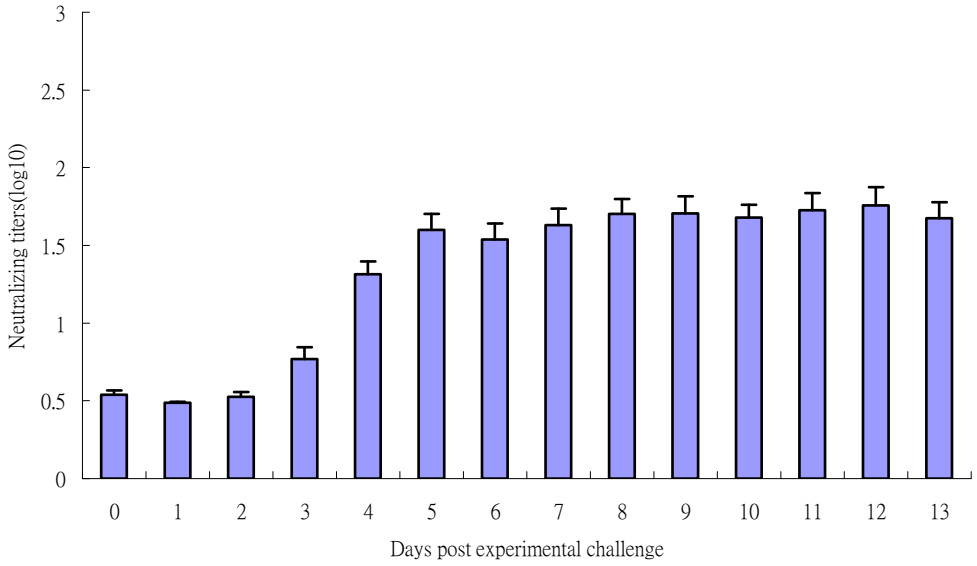


Figure 6.2. The profile of neutralizing antibody titers in pigs within 13 days after experimental infection with FMDV O/Taiwan/97. The error bar indicates the standard error of mean of each sampling time.



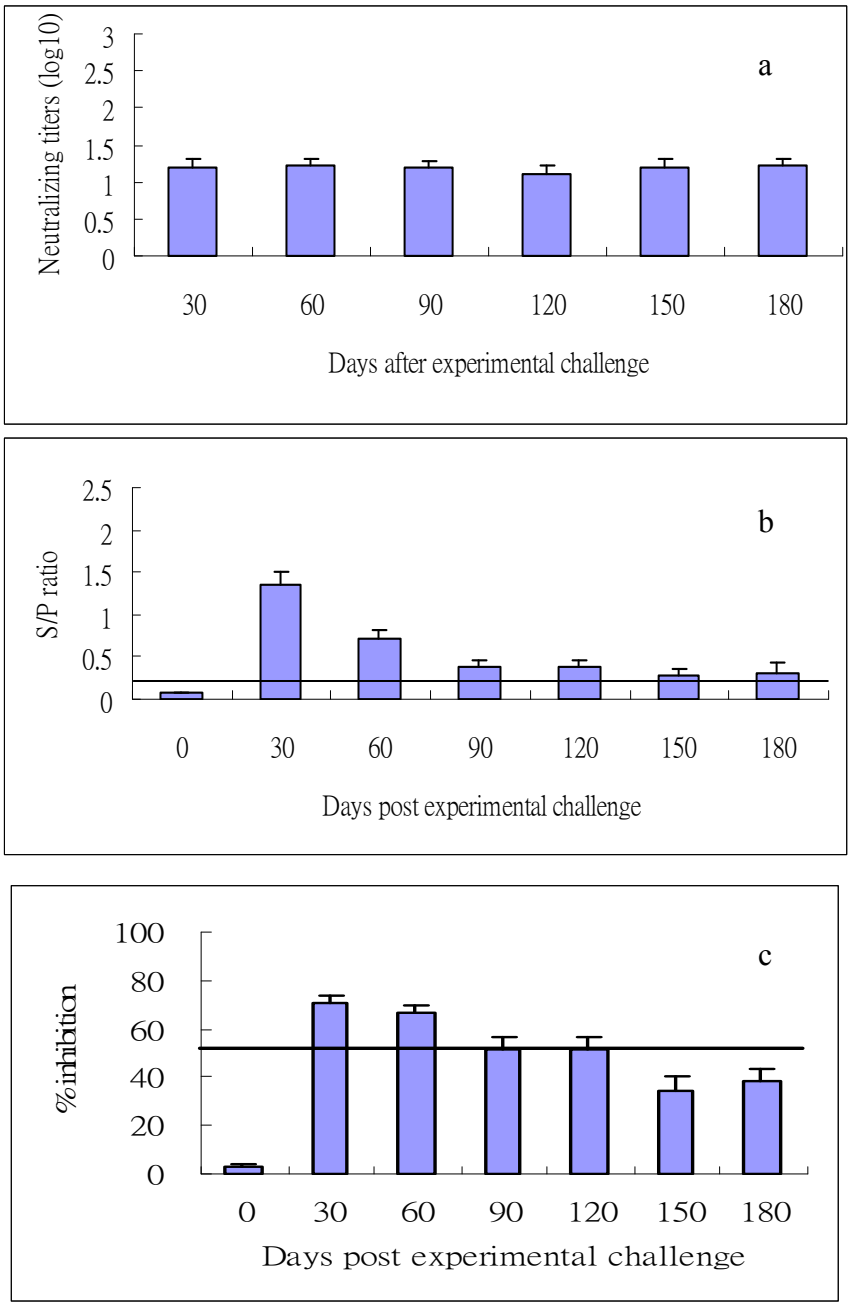


Figure 6.3. The monthly profile of neutralizing antibody titers and NSP antibody responses in pigs after experimental infection with FMDV O/Taiwan/97. The error bar indicates the standard error of mean of each sampling time. (a) The profile of neutralizing antibody titers; (b) 3B NSP antibody ELISA results (S/P ratio); (c) 3ABC NSP antibody ELISA results (% inhibition) . The ELISA test cut-off value is shown as a horizontal line in (b) and (c).

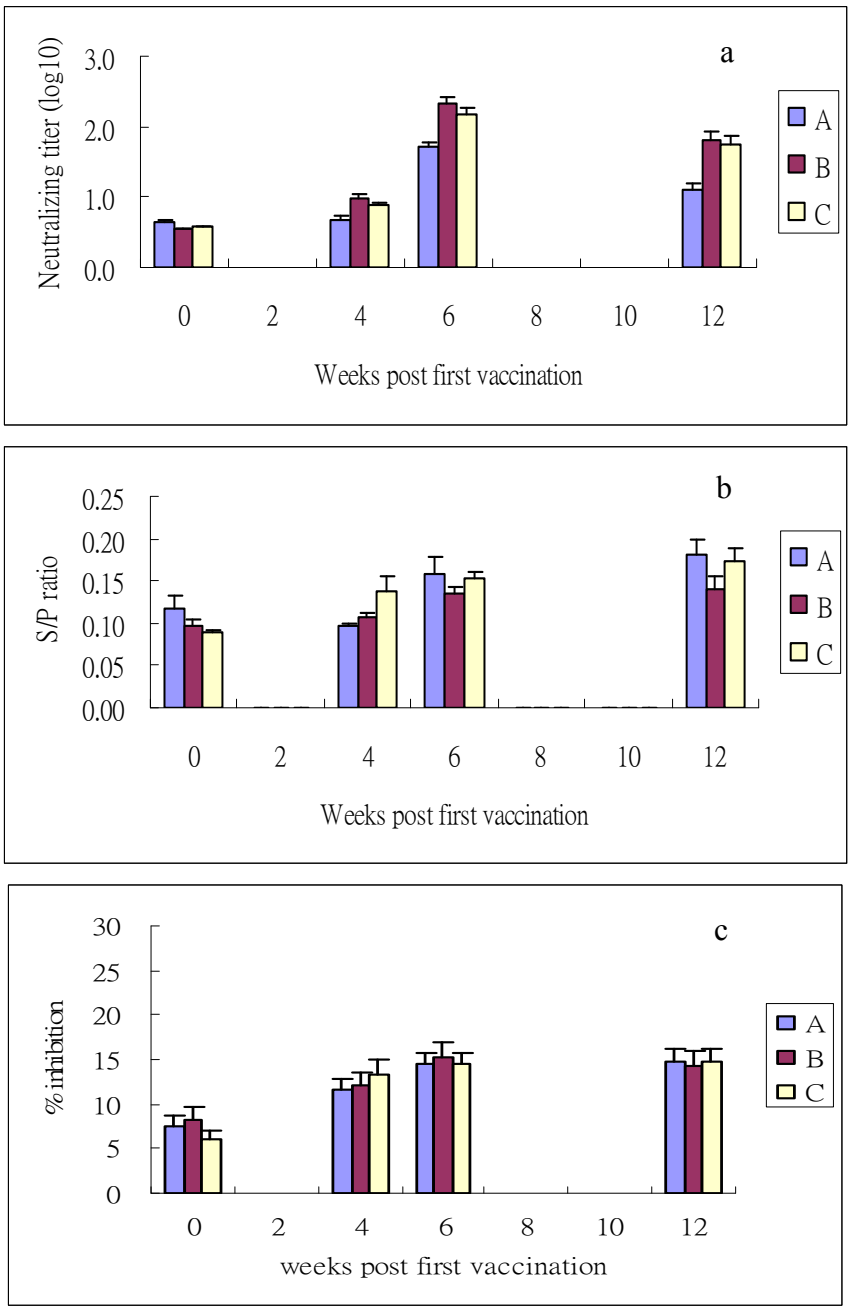


Figure 6.4. The profile of neutralizing antibody titres and NSP antibody ELISA results in pigs after vaccination with three commercial type O FMD vaccines (A, B, C). The error bar indicates the standard error of mean of each sampling time. (a) Neutralizing antibody titers; (b) 3B NSP antibody ELISA results (S/P ratio cut-off = 0.23); (c) 3ABC NSP antibody ELISA results (% inhibition cut-off = 50%) .

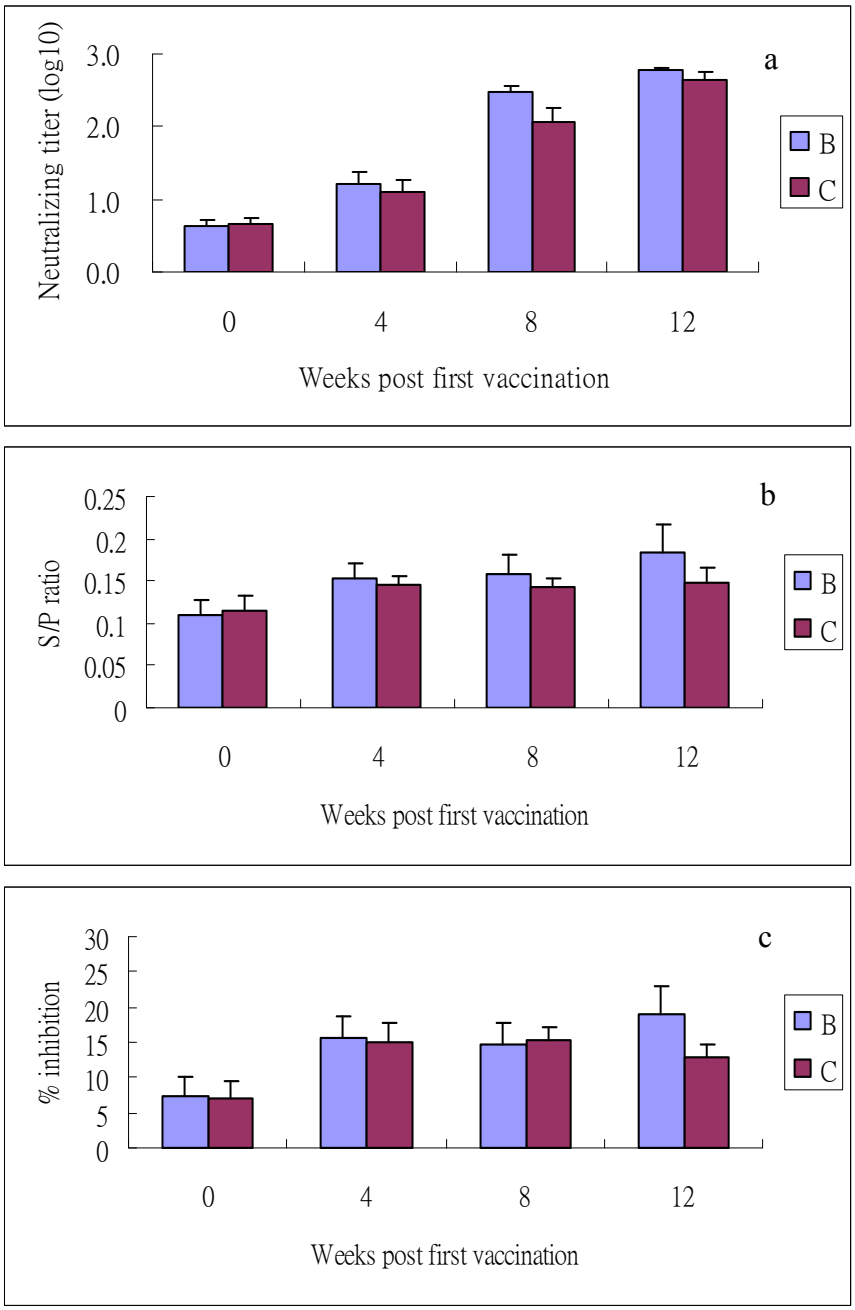


Figure 6.5. The profile of neutralizing antibody titres and NSP antibody ELISA results in pigs after vaccination with double doses of two commercial type O FMD vaccines (B, C). The error bar indicates the standard error of mean of each sampling time. (a) Neutralizing antibody titers; (b) 3B NSP antibody ELISA results (S/P ratio cut-off = 0.23) ; (c) 3ABC NSP antibody ELISA results (% inhibition cut-off = 50%).

Chapter 7 - Immune responses to foot and mouth disease virus in pig farms after the 1997 outbreak in Taiwan

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Abstract

This paper reports on a retrospective study of the antibody responses to structural and non-structural proteins of FMD virus O Taiwan/97 in 6 pig herds in Taiwan in the year after the 1997 Taiwanese FMD outbreak. All herds were vaccinated against FMD vaccination after the outbreak as part of the country wide control program. Three of the herds had confirmed FMD infections (herds N, O and P) and three herds remained non-infected (herds K, L and M). The serum neutralizing antibody titers and the non-structural protein ELISA (NSP) antibody responses in sows and one month old pigs in the infected herds were higher than in the non-infected herds, but over time a number of positive NSP reactors were detected. From the serological studies and the herd monitoring and investigations it was considered that the FMD NSP positive reactors may not have constituted a true reservoir of FMD virus infection especially in herds where susceptible pigs were no longer present post-exposure or post-vaccination. Pigs vaccinated with an unpurified FMD type O vaccines being used at that time also showed false positive responses for NSP antibodies.

Key words: Foot-and-mouth disease; Non-structural protein; Anitbody response; Outbreak

7.1 Introduction

Foot-and-mouth disease (FMD) is caused by infection with FMD virus, a member of the Aphthovirus genus of family Picornaviridae and is one of the most contagious infectious diseases of cloven-hoofed animals. The virus strain, O Taiwan/97, however, has been shown to have a species-specific adaptation to pigs (Dunn and Donaldson, 1997) and only caused overt clinical signs in pigs in the 1997 outbreak of FMD in Taiwan (Shieh, 1997).

Vaccination has been successfully used for controlling FMD in Taiwan (Yang et al., 1999), which has now been recognized as FMD free with vaccination. To achieve this it was necessary to demonstrate freedom from virus circulation in the field and that required effective means of differentiating vaccinated from convalescent animals. This is a major requirement for countries with compulsory FMD vaccination programs (O.I.E., 2004). Recently, new methods such as NSP ELISA kits have become available that provide a reliable procedure to detect infected animals in vaccinated populations.

Several commercialized FMDV NSP ELISA kits have recently been validated and shown to be useful in monitoring NSP antibody responses in cattle (Brocchi et al., 2006) and pigs (Chen et al., 2007a). In general, there is consensus that polypeptide 3ABC used

in the kits is the most appropriate antigen because of its high immunogenicity. In cattle, an additional OIE approved reference test-PANATOSA kit for NSP detection has been developed and used to confirm specificity of the antibody response to NSP in cattle (Bergmann et al., 2000) but this test has so far not been validated for use in pigs.

In studies with pigs in Taiwan (Chen et al., 2007a), both the Ceditest and UBI ELISA kits performed with high sensitivity in detecting infected pigs up to two months post-infection. However, the antibodies to NSP gradually declined over time and the detection rate in pigs known to have previously been infected was approximately 33% with the Ceditest kit at 6 months post infection. Neutralizing antibodies were significantly higher in pigs which had been infected and then vaccinated with FMDV vaccine in comparison with those in pigs after natural infection only.

This paper describes the different profiles in NSP and neutralizing antibody responses in herds vaccinated after infection with Type O Taiwan/97 FMD in comparison to vaccinated herds that were not infected. This data was used to evaluate how long the virus continued to circulate in infected pig herds and also to determine the levels of herd immunity over time in infected and vaccinated herds after the 1997 Taiwanese FMD outbreaks.

7.2 Materials and methods

7.2.1 Collection of pig sera after the emergency FMD vaccination program

A Taiwan-wide emergency vaccination program was undertaken on all pig farms after the 1997 outbreak with vaccination commencing within one month after the outbreak started. Sows were vaccinated every 4 or 6 months and the fattening pigs were vaccinated when they were 8 and 12 weeks old.

To evaluate the level of antibodies to structural and non-structural proteins in pig herds after the emergency FMD vaccination program and the subsequent prophylactic FMD vaccination program in Taiwan three infected herds and three non-infected herds were selected. These herds were all multi-aged, continuous flow herds that were isolated from other pig farms. Herd O was the largest herd of those selected. In Taiwan, at the time of this study, there was little or no investment in replacement sows due to the poor pork prices and uncertainty about the future of the industry after the loss of the pork export market to Japan. Artificial insemination was used routinely in these 6 herds. At 6, 9 and 12 months after the outbreak commenced serum was collected from 10 randomly selected young pigs at each of 1, 2, 3, 4, 5 and 6 months of age and from sows in these herds.

The infected herds (N, O and P) had all experienced severe outbreaks with high mortality rates in nursery piglets in the early stage of the outbreak, losses of some pigs due to secondary bacterial infection and severe FMD lesions in pigs of all age groups. In these three herds erosive lesions of feet and snouts were evident 4-6 weeks after the start of the outbreak. After the outbreak the pigs in these herds were visited and closely monitored at monthly intervals for clinical evidence (recent or healing feet and snout lesions in growers and fattening pigs) and virological evidence (virus culture of probang samples from 10 sows per farms per visit) of FMD but no further clinical or subclinical evidence of FMD was detected in them during the study period or subsequently.

Herds N and O received emergency vaccination and this was continued with a prophylactic vaccination program with sows routinely vaccinated every 4 months and fattening pigs vaccinated at 8 and 12 weeks of age. Pigs in Herd P only received emergency vaccination with no further vaccination by the end of study. The uninfected herds (K, L and M) were similarly vaccinated with sows routinely vaccinated every 4 months and fattening pigs vaccinated at 8 and 12 weeks of age.

At the start vaccine G was used to vaccinate the pigs in all six farms but on farms N and

L after the secondary sampling time vaccine A replaced vaccine G.

7.2.2 Vaccination study

A vaccination study was undertaken to determine the optimum vaccine for continued use in the prophylactic program. Seventy pigs, 8-week-old, on farm L were randomly allocated into one of 7 groups and vaccinated with one of 7 commercially available monovalent FMD serotype O_1 Manisa, O 4174 or O_1 Campos oil-emulsified vaccines that were used in the field for emergency and prophylactic vaccination in Taiwan (Vaccines A-H - see Table 7.1). All pigs received the first FMD vaccination at 8 weeks of age and a secondary vaccination at 12 weeks of age. Serum samples were collected from pigs at 8, 12, 16 and 23 weeks of age for measurement of FMD neutralizing antibody titers and for detection of FMD NSP antibodies.

7.2.3 Measurement of serum neutralizing antibody

Serum neutralizing antibody (SN) titers to FMDV type O Taiwan were measured in a micro-neutralization assay according to the method described in the OIE manual (O.I.E., 2004). Briefly, sera were diluted in a two-fold dilution series in 96-well microtiter plates. Then 50 μl of virus suspension containing 100 $TCID_{50}$ of FMD viruses (O Taiwan/97) was added to each well and incubated for 1 hour at 37°C. Baby hamster kidney cells

(BHK21 cells), $4.0x10^5$ cells/ml, were then added to each well. The wells were examined for cytopathic effect after 48 hours incubation at 37°C. End-point titers were calculated as the reciprocal of the highest serum dilution to neutralize 100 $TCID_{50}$ of the virus. Results were expressed as the $\log_{10}$ SN titer.

7.2.4 Measurement of antibody to NSPs

Serum antibodies to FMDV NSPs were measured by Cedi FMDV-NS for the NSP 3 ABC peptide (Cedi-Diagnostics B. V., the Netherlands). The test is a blocking ELISA which measures the competition between test sera and a NSP specific monoclonal antibody for the binding to the 3ABC NSP of FMDV (Sorensen et al., 2005). The assay was performed according to the manufacturer's instructions. To standardize the results among different ELISA plates, the OD obtained from the Cedi kit was expressed as the percentage inhibition (PI) of the monoclonal antibody binding. According to the manufacturer's specifications the PI cut-off value for the Cedi ELISA is 50%.

7.2.5 Statistical analysis of differences between herds

Data were analyzed by the analysis of variance (ANOVA) using general linear model procedures. Differences in means of SN and NSP titers between different vaccine groups and herds were tested by Duncan's new multiple range test. A P-value of less

than 0.05 was considered significant.

7.3 Results

7.3.1 Monthly clinical observations in the herds

Monthly clinical inspections of pigs from all age groups on these farms were undertaken as part of the herd health monitoring program. Particular attention was paid to feet and snouts to detect evidence of FMD lesions. No clinical evidence of new FMD infection was present on any of these farms after the outbreak.

7.3.2 Neutralizing antibody titers and NSP antibody responses in the infected and non-infected herds

Figures 7.1 and 7.2 show the neutralizing antibody titers (NA) and NSP antibody responses for infected and non-infected herds, respectively. Herds that experienced FMDV infection show high NA titers and NSP responses in pigs, especially in sows that received post- outbreak vaccinations compared with those from non-infected herds. In Herds N, O and P that experienced FMD infection the neutralizing antibody titers in sows, and one and two month old piglets (prior to vaccination) were higher than those in herds K, L and M. For the fattening pigs (4-6 months old), in the infected herds the neutralizing antibody titers at the first sampling (6 months post infection) were higher

than those at the second and third sampling, except for herd N at the third sampling where elevated NA titers was found in 4 to 6 months old pigs. Also there were no significant differences between the first, second or third sampling times for the three months old pigs in herds N and P. The neutralizing antibody titers and NSP antibody responses in herds K, L and M were low except for the third sampling in herd L which showed moderate neutralizing antibody titers in sows, and one, three and four months old fattening pigs.

The NSP antibody responses in sows and one month old piglets (prior to vaccination) in herds N, O and P were higher than those in herds K, L and M except the secondary and third sampling times for one month old pigs in herd O. For the fattening pigs (3-6 months old), there were higher NSP titers in 5 and 6 months old pigs in the first sampling of herd O and in 4 months old pigs in the third sampling of herd N in infected herds and 4 and 5 months in the third sampling of herd L in non-infected herds.

7.3.3 Positive reactors for NSP antibody in herds

Figure 7.3 shows the percentage of pigs positive for NSP antibody in the 6 herds. In Herd N positive reactors were found in sows, in 20% of one month old piglets at 9 months post-outbreak and in 20% of the four months old pigs at 12 months

post-outbreak. In Herd O positive reactors were found mainly in sows and in 10% of four and five months old pigs at 6 and 9 months post outbreak; and in Herd P positive reactors were also found in sows and 10% of one month and four month old pigs at 6 months post-outbreak. The only NSP reactors detected in the uninfected herds were in Herd L which had 30% and 10% of pigs detected positive in the three and four months old pigs respectively at 12 months post-outbreak.

7.3.4 Immune responses to the different vaccines evaluated

Figure 7.4 shows the neutralizing antibody titers, NSP antibody responses and percentage of pigs positive for NSP antibody in pigs vaccinated with the seven different vaccines. Figure 7.4a shows that the mean neutralizing antibody titers increased sharply in all seven groups by 14 days after booster vaccination. However, the mean neutralizing titers induced by vaccines E, F, and G were significantly lower ($p < 0.05$) than those for pigs vaccinated with vaccines A, B, C, and D. Figures 7.4b and 7.4c show that pigs vaccinated with all vaccines, except vaccine A, did not produce a significant NSP antibody response after the initial vaccination course. However, Vaccine A induced a positive NSP antibody response in 50% of pig by 15 weeks post vaccination.

7.4 Discussion

Measurement of NSP antibody and neutralizing antibody levels in pigs in infected and uninfected herds after the 1997 Taiwanese type O FMD outbreak has provided further understanding the epidemiology of this outbreak and will be helpful in developing control programs for any future outbreak caused by pig-adapted type O FMD viruses. We have demonstrated a prolonged duration of persistence of neutralizing and NSP antibodies in herds infected and then given vaccination. Also the effect of using unpurified FMD vaccines which could induce the NSP titers in vaccinated pigs was shown and this would interfere with the ability of NSP testing to differentiate infected from vaccinated pigs in the control program.

In previous studies (Chen et al., 2007b) infected pigs subsequently given FMD vaccination produced persistent high neutralizing antibody titers for more than six months, but NSP antibody responses (with Ceditest ELISA) in convalescent pigs and sows only persisted for the first few months after outbreaks and declined over time so that only a small proportion of positive reactors were detected by 6 months post-infection. Similar results were found from these field investigations, where there were a proportion of sows between 6-12 months after the outbreak in Herds N, O and P and at the 6 month sampling a proportion of older fattening pigs with persistent high

neutralizing antibody titers. There were also a small proportion of sows with low NSP positive reaction 6-12 months after the outbreak despite there being no evidence of active or ongoing FMD infection in these herds from herd inspections of growers and fattening pigs for feet or snout lesions and negative virus culture results on probang samples from randomly selected sows at monthly intervals over this time.

In the infected herds there was also a low percentages of NSP ELISA reactors in growers and fattening pigs. By randomly collecting 60 grower or fattening pigs in each herd at each time point and testing them by this NSP ELISA we would expect to detect 5% sero-prevalence in an infected herd with 95% confidence. In the outbreak of FMD in naïve pig herds in Taiwan, the clinical infection rate was as high as 90% in infected herds (Chen, personal observation) so it was not unreasonable to use a sampling rate to detect 5% sero-prevalence in these herds. The positive NSP reactors in sows in all three infected herds 6 month post-outbreak are most likely to be due to persistent antibody responses in some sows in herds that experienced the massive FMD outbreak in March 1997 and had subsequent FMD vaccinations with FMD vaccines containing residual NSP as part of the control program. By 9 and 12 months post-outbreak, sows in these herds had received emergency FMD vaccination and in Herds N and O additional prophylactic FMD vaccinations with vaccines containing some residual NSP such as

was subsequently demonstrated with vaccine A. This resulted in an increased number of NSP reactors in sows in the latter herds at 9 and 12 months post-outbreak.

The NSP reactors in 1-month old pigs in Herd P at the 6 months post-outbreak sampling and herd N at the 9 month post-outbreak sampling clearly resulted from maternal antibody transfer from their antibody positive sows. Neutralizing antibody and NSP antibody can be detected in piglets up to 2-3 months of age in infected farms when they have acquired passive immunity from sows which had been infected and subsequently vaccinated. This finding is similar to that demonstrated by Chung et al (Chung et al., 2002) that NSP antibodies could be detected in the sera of their progeny when piglets ingested the colostrum derived from previously infected sows.

In herd O the NSP antibody in one of the 5 months old pigs at the first sampling time and in herd P in one of the 4 months old pigs at first sampling time is most likely associated with persistent antibody in piglets infected at the end of the outbreak. A similar low proportion of NSP reactors was demonstrated in infected pigs for 5 to 6 months after natural or experimental challenge (Chen et al., 2007a; Chen et al., 2007b). In the 4 months old pigs at the second sampling in herd O (9 months post outbreak), there was a single positive reactor (1 out of 10) with a 53 % inhibition classed as weak

positive. The mean % inhibition for that group was 6.5(±5 S.E.M.) percentage which was far below the cut off value (50%). As reported above there was no evidence of persistent FMD virus circulation in this farm. Therefore, it is highly likely that the weak positive reactor was a false positive. False positives have been detected in the previous studies with this test in animals after vaccination (Chen et al., 2007a; Lee et al., 2006). In the third sampling, the positive reactors in herds L and N can be ascribed to the use of vaccine A in these herds prior to this sampling. As shown above this vaccine contained significant levels of residual NSP and induced high NSP antibody responses in 50% of experimentally vaccinated pigs.

From this study the best age group to monitor for evidence of FMD virus circulation in vaccinated herds by NSP testing would be fattening pigs provided that vaccines contained no residual NSP. Convalescent sows that had not been culled after FMD infection or young pigs that had received maternal NSP antibody from these sows would not be suitable for this testing. By 12 months post-outbreak and with absence of ongoing disease in all of the study herds, the increasing numbers of positive NSP reactors in sows in Herds N and O and NSP reactors in 3 or 4 month old grower pigs from previously infected herd N and non-infected herd L related to the use of FMD vaccines contaminated with NSP. Such vaccines, like Vaccine A in this study, were able

to produce NSP responses in naïve pigs after repeated revaccination which would give a false positive reaction when NSP tests are used to detect active FMD infection (such as occurred in herd L in this study). Therefore, it is very important to use the purified FMD vaccines in those countries which implement vaccination as part of their FMD control program in order to be able to differentiate vaccinated from infected animals. This is the reason why the OIE has made a clear recommendation that countries using FMD vaccination should use purified FMD vaccines to facilitate use of NSP antibody testing for differentiation of infected from vaccinated herds in post-outbreak surveillance (O.I.E., 2005). Taiwan currently requires the use of purified FMD vaccines as recommended by OIE and FMD vaccines are monitored batch by batch to ensure they meet this requirement. Positive post-vaccination NSP reactors are now rarely found in the pigs that have received multiple vaccinations of the commercial FMD vaccines currently available (Chen et al., 2007b).

At the time of the 1997 outbreak in Taiwan the decision was taken to use control with vaccination and to determine the appropriate vaccine strain antigenic comparison studies were conducted between O Taiwan/97 and other type O FMD vaccine strains by the World FMD Reference Laboratory, Pirbright. Appropriate vaccine strains related to O Taiwan/97 were determined based on the evaluation of r_1 values (Kitching et al., 1989;

O.I.E., 2004; Yang et al., 1999). The r_1 values for O_1 Manisa, O 4174 and O_1 Campos were 1, 1 and 0.7, respectively. However, vaccines E, F and G formulated with O_1 Manisa induced lower neutralizing titers than those vaccines with O_1 Campos and O 4174. Other factors which can cause a difference in vaccine efficacy include antigen mass contained in vaccines, vaccine production procedures and antigen adjuvant interactions (Doel, 2003). The reason for the poorer immunogenicity of vaccines E, F and G may be due to the antigen mass in the vaccine because for one of the vaccines at least (Vaccine G) use of 3x the dose gave a similar neutralizing antibody responses to those of vaccines A to D (Data not shown). Additionally another vaccine, subsequently available in Taiwan, initially performed poorly in field evaluation but gave substantially stronger neutralizing antibody titers when the antigen payload was increased from 6 PD_{50} to 15-21 PD_{50} (A. S. Toloknov, personal communication). Clearly, it is very important to examine the quality of vaccines before use in control programs as recommended by OIE (O.I.E., 2004). Vaccines E, F and G were subsequently not able to be imported to Taiwan for use in the FMD control program because they did not pass Taiwanese Government's vaccine efficacy test.

Poulin and Christianson demonstrated that on-farm control of FMD was achievable in a closed pig herd which controlled the outbreak with vaccination and strict biosecurity

methods. The disease and infection was eradicated after one-year closure of this herd with no spread to other herds (Poulin and Christianson, 2006). In infected herd P, which only had one emergency FMD vaccination after the outbreak plus good biosecurity, the follow-on monitoring clearly demonstrated that virus circulation had been shut down as no further clinical evidence for FMD was present and neutralizing and NSP antibodies were not detected in the fattening pigs in the second and third sampling times. This provides further evidence that control of pig-adapted FMD viruses like O/Taiwan/97 in pigs can be achieved by closure of herd with emergency vaccination if the stamping out policy is not being implemented for FMD control.

7.5 Acknowledgements

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7.6 References

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Table 7.1. Summary details of the seven commercially available FMD vaccines used in the vaccination experiments.

Vaccine designation	Origin of vaccine	Virus strain
A	Argentina	O_1 Campos
B	Argentina	O_1 Campos
C	Argentina	O_1 Campos
D	England	O 4174
E	Germany	O_1 Manisa
F	Turkey	O_1 Manisa
G	Holland	O_1 Manisa

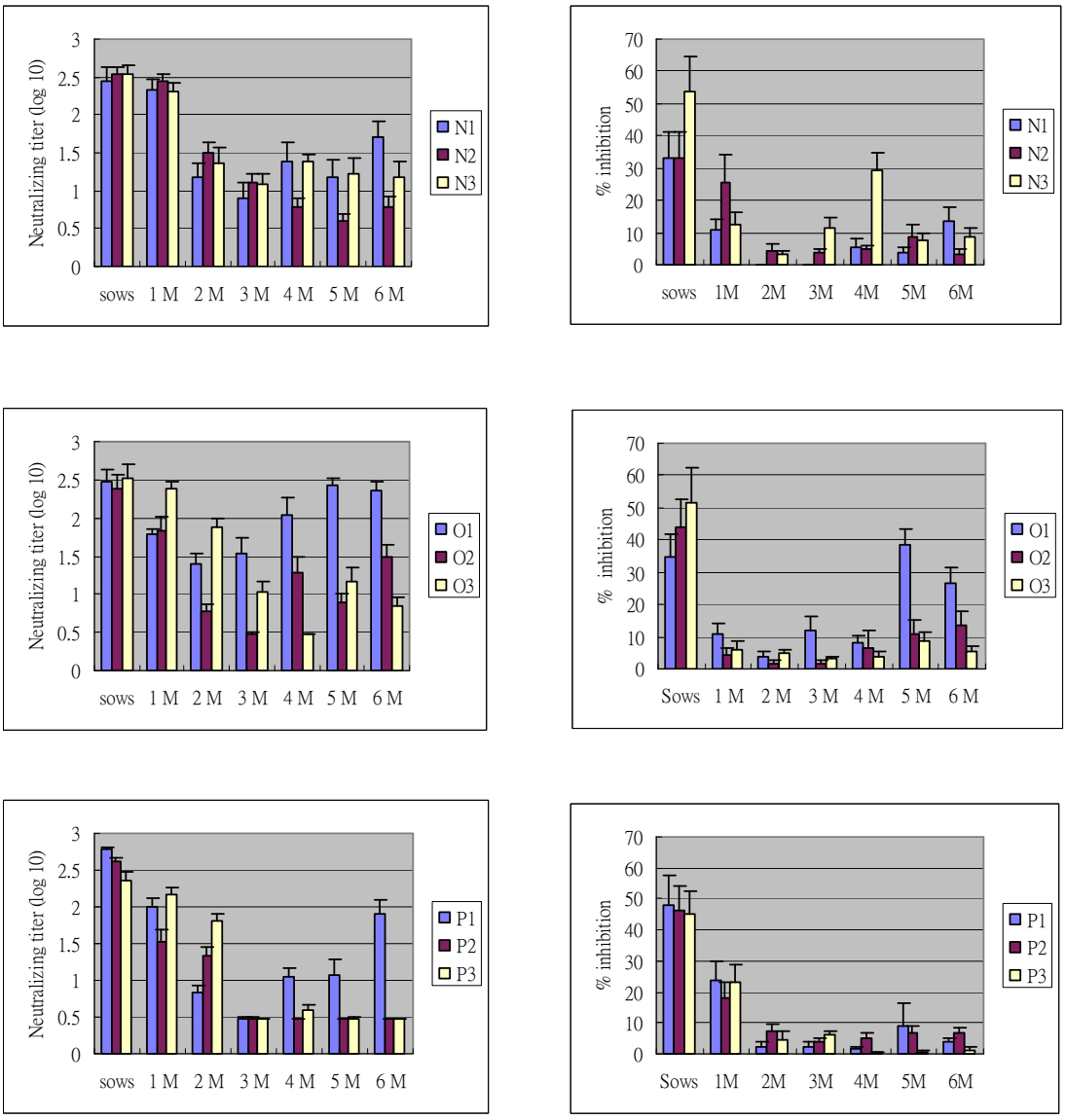

Figure 7.1. The profile of neutralizing antibody titers and NSP antibody responses in various ages of pigs in three herds that had FMD infection during the 1997 Taiwanese FMD outbreak and were subsequently vaccinated. The neutralizing antibody titers and the NSP antibody responses (% inhibition) of herds N, O and P are shown in the left and right columns, respectively. N1, N2, and N3 represent the first, second and third sampling time, respectively, for herd N and so on for herds O and P. The error bar indicates the standard error of the mean for each sampling group and time.

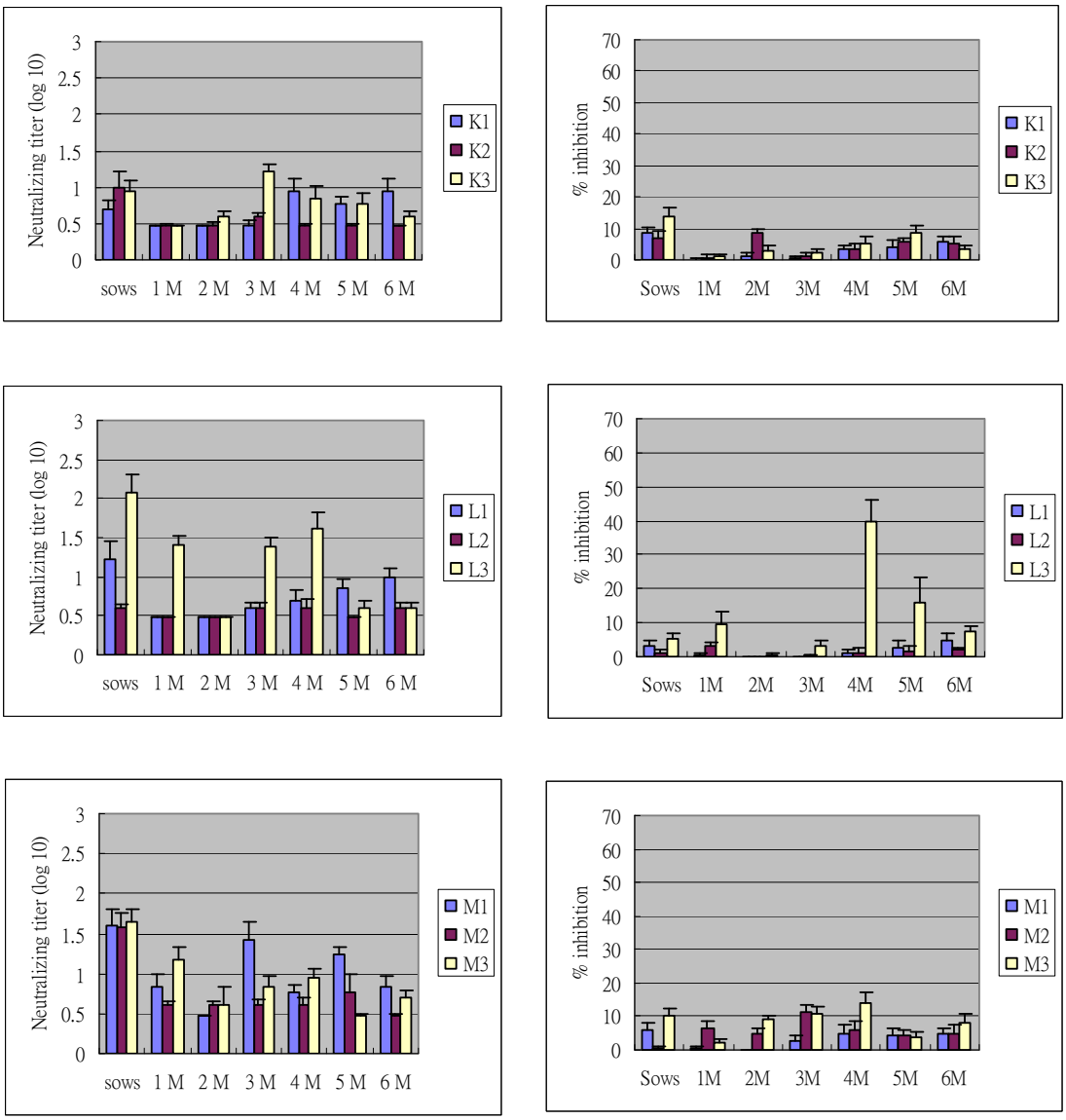

Figure 7.2. The profile of neutralizing antibody titers and NSP antibody responses in various ages of pigs in three herds that were not infected during the 1997 Taiwanese FMD outbreak, but were subsequently vaccinated. The neutralizing antibody titers and the NSP antibody responses (% inhibition) of herds K, L, and M are shown in the left and right columns, respectively. K1, K2, and K3 represent the first, second and third sampling time, respectively, for herd K and so on for herds L and M. The error bar indicates the standard error of the mean for each sampling group and time.

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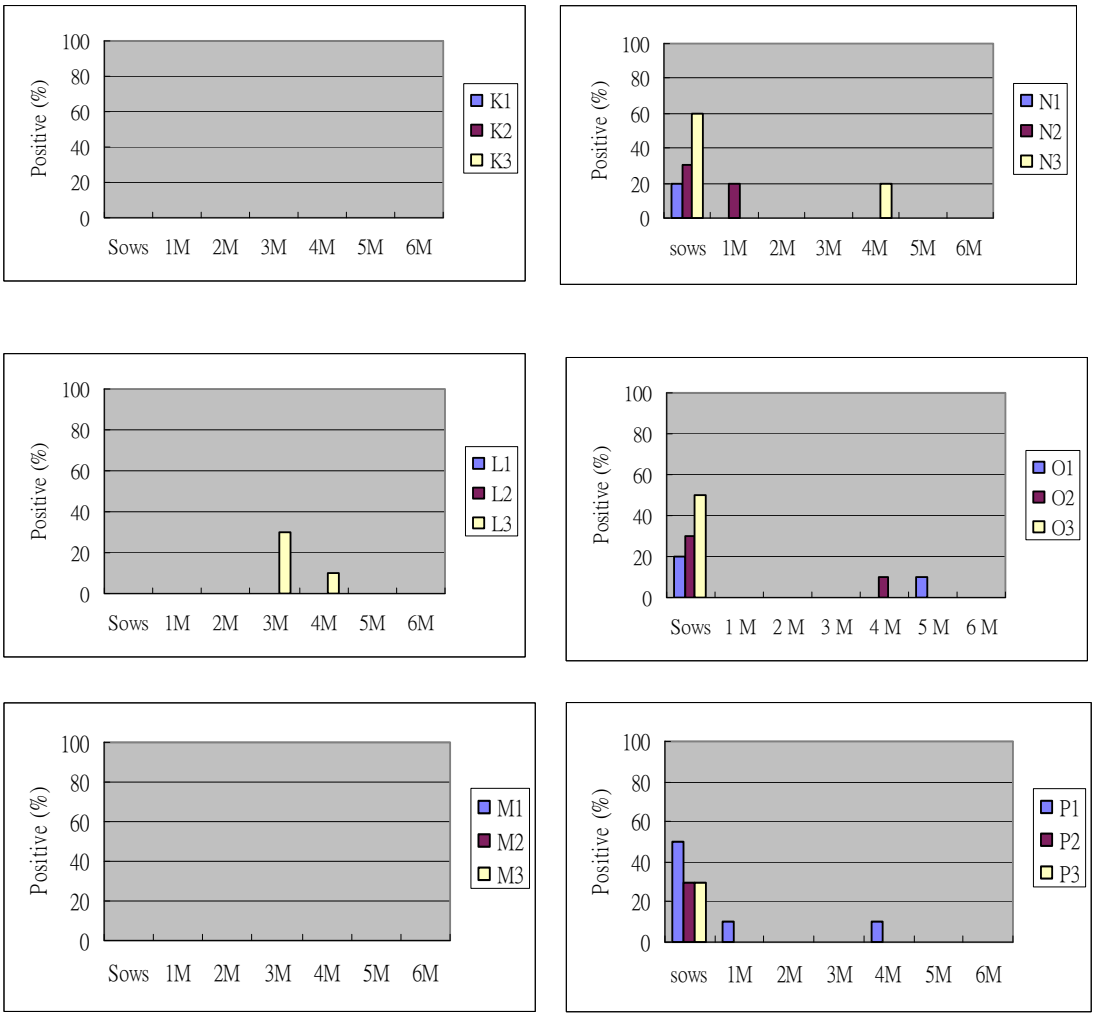
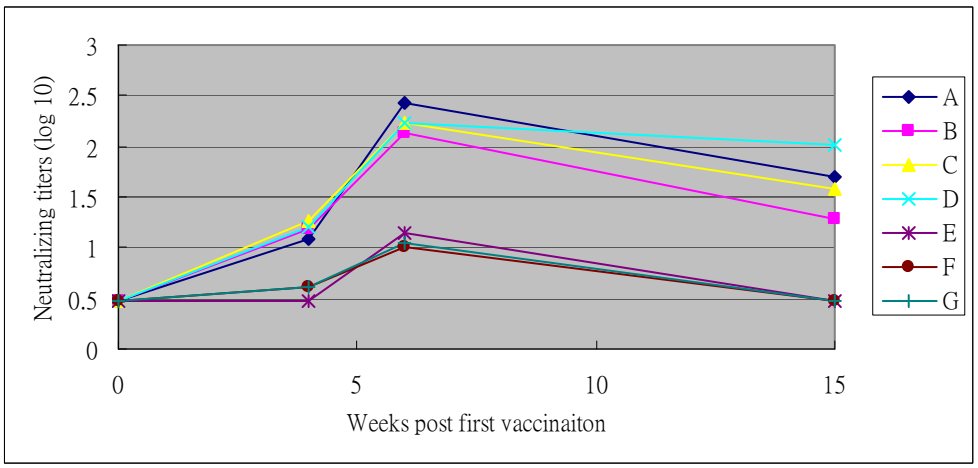
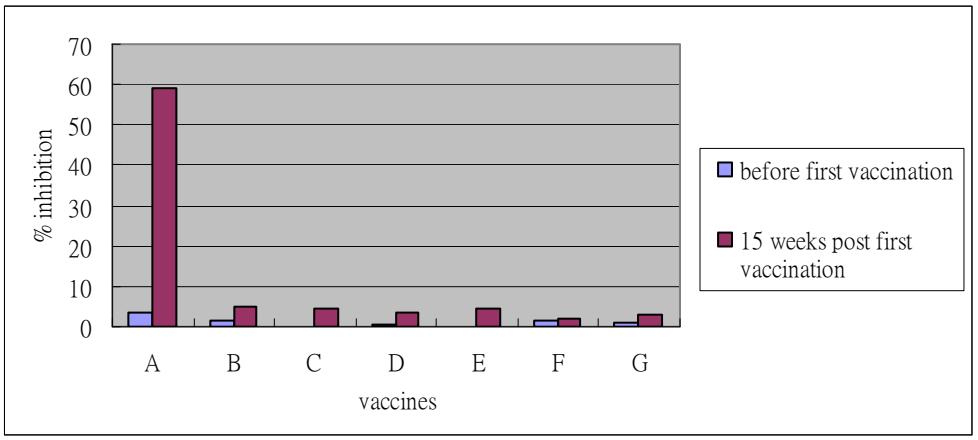


Figure 7.3. Percentage of pigs at different ages positive for NSP antibody in herds after the 1997 Taiwanese FMD outbreak. Herds K, L and M were uninfected and Herds N, O, and P were infected. N1, N2, and N3 represents the first, secondary and third sampling time, respectively, for N herd and so on for other farms.





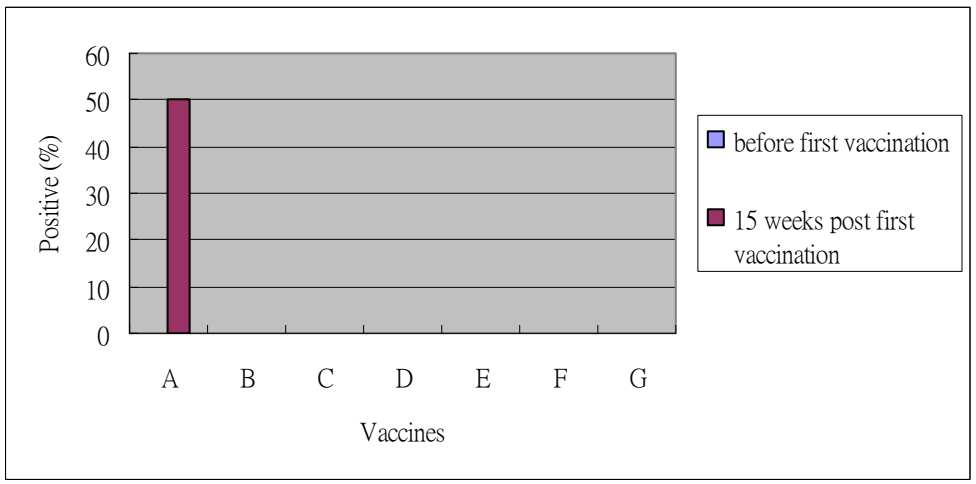


Figure 7.4 The profiles of neutralizing antibody titers, NSP antibody response (NSP 3ABC ELISA - % inhibition) and NSP antibody % positive rate in pigs vaccination with one of 7 FMD vaccines. (a) The profile of neutralizing antibody titers; (b) NSP ELISA (% inhibition); (c) NSP antibody % positive rate

Chapter 8 - Application of non-structural protein ELISA kits in nationwide FMD surveillance in pigs to substantiate freedom from foot-and-mouth disease in Taiwan

8.1 Introduction

After the FMD epidemic in 1997, Taiwan experienced an endemic phase when FMD control was maintained by a national vaccination program. The last case of FMD was reported in 2001 and currently Taiwan is recognized as FMD free with vaccination by the World Organisation for Animal Health (OIE). During this period continuing surveillance of FMD NSP antibody in pigs was conducted to monitor for FMDV circulation using UBI and in-house 3 AB NSP ELISA kits.

Testing for antibody to FMD NSP in infected animals, has been used widely to differentiate vaccinated from infected cattle in the field (Robiolo et al., 2006). Recently, a number of ELISA's with high sensitivity and specificity for detection of antibody to FMD NSP in pigs have also been reported as suitable for large scale FMD eradication programs such as in Taiwan (Chung et al., 2003). In one study there was an evaluation of the analytical sensitivity and specificity of three NSP assays (UBI, Danish Veterinary

Institute for Virus Research (DVIVR) and Checkit) (Lee et al., 2004). The study by Chung et al (2002b), evaluated an in-house kit based on the 3AB antigen obtained from Dr Sorensen that was not commercially available at that time and now is commercialized as the Ceditest. This kit now uses 3ABC as the antigen for detecting NSP antibody.

There is also an OIE reference NSP ELISA test developed by PANATOSA, which has been widely applied to demonstrate freedom from FMD after prophylactic vaccination or post-outbreak vaccination in cattle. This is followed up by a confirmatory test (O.I.E., 2004b). Brocchi et al (2006) compared six ELISA kits in cattle and showed that the Ceditest ELISA kit was comparable to the reference test. As reported in Chapter 5, a similar evaluation of three commercialized NSP ELISA kits was conducted in pigs and this showed that two kits (UBI and Ceditest ELISA kits) had advantages in terms of sensitivity and specificity in testing pigs for NSP antibody (Chen et al., 2007a). In Chapter 6 longitudinal studies showed that the sensitivities of the NSP ELISA kits for pig testing steadily declined over time post-infection. As the prevalence of FMD reached an extremely low level in Taiwan (Chung et al., 2003) the issue of test specificity became very important. The positive predictive value tends to be lower when the prevalence of the disease is very low (Noordhuizen et al., 2001). Therefore, to

combine optimal sensitivity and specificity in NSP antibody testing in the field and to improve the predictive value of a positive test result, two commercially available ELISA kits, UBI and Ceditest, were used in series to test the sera collected during the 2005 FMD NSP sero-surveillance in Taiwan. The UBI test was considered as the screening test as it has slightly higher sensitivity but lower specificity and the Ceditest was used as the confirmatory test as it has a high specificity. The results of this testing are reported in this Chapter. As with any sero-surveillance, reactors will occur that need further investigation. This Chapter also reports on the results of traceback, clinical and serological investigations on farms with NSP ELISA reactors from the 2005 sero-surveillance program in Taiwan.

8.2 Materials and Methods

8.2.1 Sampling method

In Taiwan, fattening pigs for slaughtering are sent by trucks from farms to auction markets for sale. There are 22 auction markets in Taiwan (Figures 8.1 to 8.3). Pigs are sold from 8 am to 12 noon in each auction market daily except for Sunday. Pigs are blood sampled in the auction market after they are placed in the sale pens. As part of national FMD control program, animal technicians employed by Bureau of Plant and Animal inspection and Quarantine collected blood samples from pigs in the auction

markets after they are placed in the sale pens. The pigs are systematically sampled with one serum sample being taken from each 50 pigs from the same farm. Before the blood samples are taken the staff examine the pigs. Any with abnormal hoof lesions or abnormality of gait are sampled, otherwise the pigs are randomly selected.

8.2.2 Samples collected

Sera were collected from all auction markets each month throughout the year as shown in Table 8.1. The numbers collected were proportional to the pig population density throughout Taiwan with most samples coming from the five major pig production prefectures: Pingtung, Kaoshuang, Tainan, Yunlin and Chunghua. A total of 77,674 serum samples were collected from pigs in the auction markets in 2005.

8.2.3 The NSP ELISA kits used in the serosurveillance

Two commercially available ELISAs for detection of antibodies directed against FMD non-structural proteins (NSP ELISAs) were used. The assays were UBI (UBI FMDV NS EIA Swine; United Biomedical Inc., Nauppauge, USA) and Ceditest (CEDI Diagnostics BV, Lelystad, The Netherlands). UBI was designed to detect antibodies to a synthetic peptide of FMDV protein 3B. Ceditest tests for competitive binding of antibody to the 3ABC protein versus a 3ABC specific monoclonal antibody. The assays

were conducted according to the manufacturers' instructions as described in Chapter 2.1.2.1 and 2.1.2.2 respectively. The UBI and the Ceditest ELISA kits were used as the screening test and confirmatory test, respectively.

8.2.4 Follow-up procedure in case of positive test results

Pigs with positive reactions were traced back to the farms of origin where a thorough investigation was conducted using relevant clinical, epidemiological and serological examinations. The official veterinarians in the prefecture checked records to determine if FMD had occurred previously on the farm or if the farm had epidemiological links with known outbreak farms. They visited the farm to examine hoofs and snouts of fattening pigs of all age groups for evidence of current clinical lesions or healing lesions resembling FMD as described in Chapter 2.5. Further blood sampling was also performed, preferably from those batches soon to be sold. For logistic reasons, blood samples were collected from 15 fattening pigs which would be expected to detect at least one positive reactor by NSP ELISA tests, given a herd prevalence of 20 %, with 95% confidence.

8.3 Results

8.3.1 Samples tested with two NSP ELISA kits used

Table 8.2 shows the test results for the 77,674 samples collected in 2005 from which 4155 samples gave positive NSP ELISA reactions with the screening test (UBI). When the 4155 UBI positive samples were further retested with the confirmatory test (Ceditest), 33 were positive. They were evenly distributed throughout the year: 2 in January, 2 in February, 6 in April, 3 in May, 2 in June, 6 in July, 3 in August, 3 in September, 2 in October, 2 in November and 2 in December. No positive sample was found in March. The positive samples were located in various prefectures: 6 in Pingtung, 6 in Taoyuan, 5 in Changhua, 3 in Yulin, 3 in Kaohsiung, 3 in Chiayi, 2 in Taichung, 1 in Tainan, 1 in Nanto, 1 in Hsinchu, 1 in Hualian and 1 in Penghu. No positive reactors were found in the same district within the same prefecture in the same month and there were no epidemiological links detected between these reactor farms. Consequently, there was no indication of clustering of NSP reactor farms found from this surveillance.

8.3.2 Follow-up procedure in case of positive test results

The NSP ELISA testing using both UBI and Ceditest on the 15 follow-up serum samples from all the herds with NSP reactors in 2005 gave negative results in all cases.

Clinical inspection of pigs from all age groups on these farms was undertaken as part of the herd follow-up monitoring and blood sampling program. Particular attention was

paid to feet and snouts to detect evidence of FMD lesions. No clinical evidence of FMD infection was present on any of these farms.

8.4 Discussion

This study provided more information on how to apply the NSP ELISA kits nationally to substantiate FMD free status of a country which has practiced compulsory vaccination in pigs. The system involved clinical surveillance in the auction markets in Taiwan combined with targeted or random serology screen testing and then confirmatory testing. From any herds with reactors, follow-up testing included clinical inspection of the herd and the collection of 15 blood samples for testing by NSP ELISA to determine the disease status. This has been proved to be an effective model to monitor field FMD cases and to substantiate the disease free status when practicing vaccination in pigs. It provided valuable information for government officers in the decision to stop vaccination and proceed toward country freedom from FMD without vaccination.

Results from the challenge studies clearly demonstrated that profound clinical signs could be readily detected one to two days after challenge with O Taiwan/97 FMDV and lesions characteristic of FMD could be observed for 14 days post-challenge. Thereafter

some evidence of resolving lesions could be observed in some pigs for up to 2 months post-challenge. Although experienced personnel could recognize these lesions within two months of challenge, after this time testing with an NSP ELISA was useful for confirming lesions suspected to be FMD lesions and hence confirm the circulation of FMDV in the field. We have demonstrated that NSP antibody titres in a small proportion of pigs could last for more than one year in challenge tests and some pigs after natural outbreaks (Chen et al., 2007b). In this surveillance, we have shown that the NSP ELISA tests could be used to further substantiate the FMD free status of Taiwan even while the national compulsory vaccination program is ongoing.

Collection of blood samples in auction markets was considered to be the most effective active surveillance strategy as there is fast turnover of pigs on farms (at 6-7 month intervals) and the majority of pigs are sold through this channel in Taiwan. The collection of blood samples from pigs at auction markets thoughout the year gave a good representation of most pig farms in the country. Additionally if cases of FMD were occurring some pigs would have evidence of lameness or resolving foot lesions when sent to the auction market. This presented the opportunity for experienced staff to observe large numbers of pigs daily for clinical evidence of FMD at the time blood samples were being collected. If pigs with any suspicious lesions were found, they

would be the target pigs for that farm and would be tested with the NSP ELISA kits (Chen et al., 2007a).

NSP ELISA kits can be applied as a herd test to demonstrate the absence of FMDV circulation by showing that vaccinated pig herds are free from antibody to FMD NSPs. These tests have been applied in surveillance of cattle in Argentina (Robiolo et al., 2006) and in pigs in Taiwan (Chung et al, 2003) by testing appropriate numbers of serum samples from slaughterhouses or auction markets. In order to cover the majority of pig herds in this study, large numbers of sera were collected in the various auction markets from different prefectures which covered nearly 98% of pigs marketed in Taiwan. This large scale surveillance for reactivity to NSP in herds, in conjunction with the follow-up investigations involving clinical examination and serological testing of positive reactor herds after screen testing by UBI and confirmatory testing by Ceditest, provides solid scientific evidence to indicate the lack of FMDV circulation in the field in Taiwan.

In previous studies (Chen et al., 2007a; Chung et al., 2002b; Lee et al., 2004), we have demonstrated that the UBI ELISA has compatible sensitivity but lower specificity than the Ceditest ELISA. The range of specificities for the UBI and Ceditest ELISA's are 93.1-100% and 99.1-100%, respectively, depending on whether samples are derived

from naïve or vaccinated pigs. With the compulsory vaccination program, the large numbers of pigs being tested by NSP ELISA's and the specificity, especially of the UBI test, some level of false positive reactors would be expected. As there has been no FMD outbreaks reported in the past five years, the prevalence would be expected to be extremely low. In this situation the specificity of screening tests must be as high as possible to ensure a high positive predictive value for the test. Therefore screening was conducted using serial testing with initial screening with UBI and then confirmatory testing with the higher specificity Ceditest. In this way the specificity was increased up to 99.94-100%. Even with this high specificity level we could still have expected up to 47 (0.06%) positive reactors from 77,674 tests. In fact we have had 33 positive reactors in the 2005 nationwide surveillance which were all shown by further intensive investigation of involved farms to be false positives. Other factors, which can cause false positives, such as bacterial contamination and degradation and complement activation in contaminated sera (Sorensen et al., 2005), need to be considered and investigated further. Alternatively, other high specificity confirmatory tests such as the enzyme-linked immunoelectrotransfer blot assay could be used to check NSP reactors from the pig surveillance programme in Taiwan, as is done with surveillance of cattle for FMD in South America (Bergmann et al., 2000),.

In a previous report on the comparison of three commercialized NSP ELISA kits (Chen et al., 2007a) (Chapter 5), we have demonstrated that the Ceditest kit had a higher specificity than that of the UBI kit, especially in pigs with prophylactic FMD vaccination. Potentially we could have applied only the Ceditest for detecting positive reactors during the nationwide surveillance while the prevalence was expected to be extremely low. However, in the above studies the UBI test tended to have marginally higher sensitivity in pigs at longer times after challenge so both kits were used in series with UBI positive reactors evaluated with confirmatory testing by Ceditest. This also meant that government resources could be efficiently applied to rapidly conduct full on-farm investigations and testing of all reactors after confirmatory testing. An additional benefit of the decision to continue using both kits was that the price of both test kits dropped considerably. Prices of both kits were discounted by 50% when the companies were competing for tenders for supplying large numbers of kits to Taiwan.

After five years without any outbreaks of FMD and negative results in the NSP ELISA sero-surveillance programme, the Taiwanese Government decided to ban the routine national vaccination program starting on an isolated island, Penghu prefecture, located in the south west of Taiwan. No outbreaks and no positive reactors with NSP ELISA testing have been found in this prefecture since vaccination was stopped in 2006. This

further strengthened confidence for the cessation of FMD vaccination of pigs among pig producers, governmental officers and the Taiwanese swine association and has led to a plan for the cessation of FMD vaccination in the whole country from July 2008.

Table 8.1. The number of sera collected in different months from the various prefectures in Taiwan in 2005. Sera were mainly from five major pig production prefectures: Pingtung, Yunlin, Tainan, Chunghua and Kaohsiung.

	Jan	Feb	Mar	April	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Total
Pingtung P	1,431	941	1,486	1,273	1,280	1,217	1,289	1,393	1,422	1,333	1,623	1,361	16,049
Yunlin P	1,157	787	1,170	1,243	1,000	1,084	1,152	1,349	1,347	1,441	1,351	1,087	14,168
Tainan P	958	712	1,163	919	855	807	930	1,030	970	888	1,056	970	11,258
Changhua P	824	511	856	717	655	738	835	867	754	888	849	840	9,334
Kaohsiung P	648	422	666	628	473	505	567	596	640	588	634	613	6,980
Chiayi P	433	347	548	517	519	400	439	392	398	382	517	406	5,298
Hualien P	185	199	254	253	198	243	257	264	255	227	239	225	2,799
Yilan P	301	99	253	209	238	246	256	299	212	195	147	125	2,580
Taichung P	164	75	223	170	184	151	202	173	158	224	186	140	2,050
Miaoli P	151	104	160	154	165	120	147	146	135	152	126	104	1,664
Nanto P	144	90	121	125	114	105	114	92	137	140	151	127	1,460
Taitung P	72	80	74	81	65	86	82	80	98	40	84	84	926
Hsinchu P	84	38	67	78	78	69	74	61	77	72	79	57	834
Taoyuan P	59	24	66	67	57	59	60	65	54	72	99	73	755
Kinmen P	41	42	42	42	43	42	42	44	42	42	42	42	506
Penghu P	52	25	25	27	26	26	23	26	24	28	25	26	333
Tainan C	17	13	14	18	9	14	17	20	20	18	19	19	198
Taichung C	1	0	12	11	10	5	18	14	11	15	12	11	120
Hsinchu C	1	0	13	5	7	5	10	14	16	15	11	18	115
Taipie P	14	7	9	7	5	8	11	11	12	11	9	9	113
Kaohsiung C	7	1	9	8	7	8	8	9	5	9	4	8	83
Chiayi C	9	7	4	4	3	6	4	4	4	3	2	1	51
Total	6,753	4,524	7,235	6,556	5,991	5,944	6,537	6,949	6,791	6,783	7,265	6,346	77,674

P = Prefecture; C = City

Table 8.2. The number of positive NSP ELISA reactors found by month in different prefectures in Taiwan during the FMD sero-surveillance programme in 2005

	Jan	Feb	Mar	April	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Total
Pingtung P		1		1					1	1	1	1	6
Yunlin P	1						2						3
Tainan P						1							1
Changhua P	1				1		1		1	1			5
Kaohsiung P					1		1	1					3
Chiayi P				1	1				1				3
Hualien P		1											1
Yilan P													0
Taichung P				1								1	2
Miaoli P													0
Nanto P				1									1
Taitung P													0
Hsinchu P											1		1
Taoyuan P				1		1	2	2					6
Kinmen P													0
Penghu P				1									1
Tainan C													0
Taichung C													0
Hsinchu C													0
Taipie P													0
Kaohsiung C													0
Chiayi C													0
Total	2	2	0	6	3	2	6	3	3	2	2	2	33

P = Prefecture; C = City

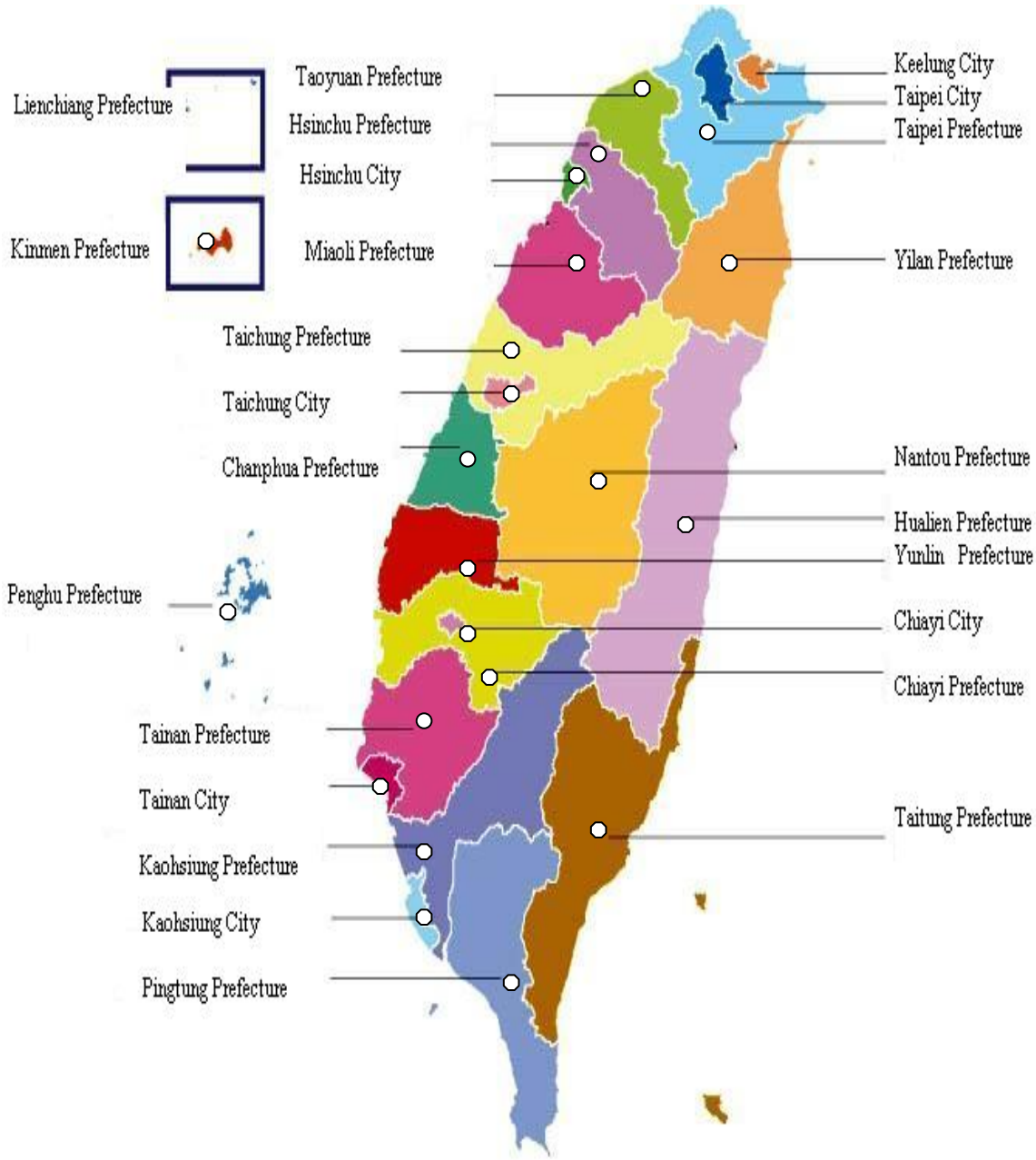


Figure 8.1. Twenty-two auction markets of live pigs were located in different prefectures and cities in Taiwan.

Figure 8.2 An auction market in Miaoli Prefecture where fattening pigs are sold.

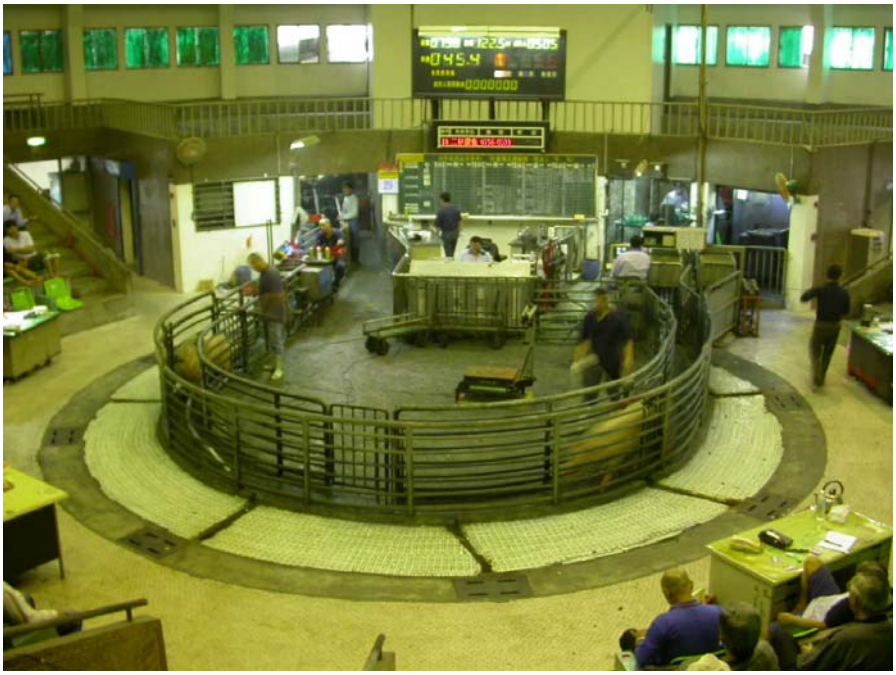


Figure 8.3 Pigs are moved through the exhibition raceway and buyers bid for the pigs with the highest bid acquiring the target pig.

Chapter 9 - General discussion

The major hypotheses of this research, as outlined in Section 1.11 of the Introduction and Literature Review, have all been effectively achieved. The individual components of the research included assessment of the mode of transmission of pig-adapted O/Taiwan/97 FMDV; examining the relationship between post-vaccinal SN antibody titres and protection from disease; evaluation of commercial NSP ELISA tests for monitoring FMDV infection status; examining immune responses to structural proteins and NSP after vaccination with commercial FMD vaccines and live virus challenge; examining immune responses to FMDV in pig farms after the 1997 outbreak in Taiwan; and application of NSP ELISA tests in the nationwide surveillance to substantiate freedom from FMD in Taiwan. The individual components of the study are discussed sequentially below and this demonstrates how the work has provided knowledge to support the policy decisions needed to proceed to OIE freedom from FMD without vaccination in Taiwan and the tools for an effective response to any future incursions of pig-adapted FMDV in Taiwan or elsewhere.

Airborne transmission of FMDV from infected pigs has been considered as a significant risk for spread of infection in some countries (Donaldson et al., 1982). This route of

transmission was suspected to have been involved during the type O FMD outbreak in Taiwan in 1997 (Yang et al, 1999). However, the current studies provide evidence for the lack of airborne transmission of pig adapted type O FMD strains from Taiwan through examining transmission by close direct or indirect contact between challenged pigs and sentinel pigs. This is in line with the findings of Alexandersen et al. (2003a) which demonstrated that pigs challenged with O Taiwan/97 and estimated to be excreting $10^{4.6}$ $TCID_{50}$/24 hrs for 24-48 hours were not able to infect sentinel pigs by the airborne route if there was a physical barrier. The current studies demonstrated that pigs in direct contact with challenged pigs became infected but none of the close-contact pigs became infected. These experiments clearly demonstrated that the pig adapted strain O Taiwan/97 was only efficiently transmitted by direct contact. This indicated that for the control of such pig-adapted type O FMDV strains, conventional methods with restriction of animal movements, culling of pigs from infected premises and maintaining good farm biosecurity should be sufficient to contain these outbreaks. These results are in contrast to air borne transmission studies with other FMD viruses in which pigs infected with Type O Lausanne FMDV could shed $10^{8.6}$ $TCID_{50}$ of FMDV per 24 hrs (Donaldson et al., 1982) and under appropriate weather conditions, aerosolised virus can potentially spread a considerable distance, particularly if the source is a large infected pig herd. In such situations airborne spread has occurred over

a distance of around 250 km (Gloster et al., 1982). The findings in the current studies have supported previous findings that pigs are very resistant to infection by airborne FMDV as compared to cattle and sheep. The most common method of transmission for FMD is direct contact between healthy pigs and those infected with FMD (Alexandersen et al., 2002a; Alexandersen and Donaldson, 2002; Alexandersen et al., 2003a).

Furthermore from the result of the intranasal challenge experiment (Experiment 6, Chapter 3), it would be expected that a very high virus dose would be required to infect pigs by the airborne route with the pig-adapted FMDV. In contrast the pigs were easier to infect by challenge via the intramuscular or subcutaneous routes than by the intranasal route. Further studies are needed to understand the mode of transmission and the minimum infectious dose by various routes for pig adapted FMDV strains like O/Taiwan/97, which clearly differ from other type O FMDV. The reports have confirmed previous research indicating that pigs are remarkably more resistant to airborne FMDV than cattle and sheep (Alexandersen et al., 2002a; Alexandersen and Donaldson, 2002; Alexandersen et al., 2003a; Gloster et al., 2007). The study by Alexandersen et al. (2003a) has shown that the aerosol excretion of O Taiwan/97 (approximately $10^{4.6}$ $TCID_{50}$ /24 hrs) from challenged pigs was similar to that of other

type O viruses. However, the excretion of virus in pigs with O Taiwan/97 is much less than in pigs challenged with C Noville virus strain ($10^{7.6}$-$10^{8.6}$ $TCID_{50}$/24 hrs) (Alexandersen et al., 2003a; Donaldson et al., 1982). If infected pigs only excrete $10^{4.6}$ $TCID_{50}$ of O Taiwan/97 virus by aerosol in 24 hours and a dose of 10^5 $TCID_{50}$ of O Taiwan/97 virus instilled intranasally did not infect pigs in the current experimental studies, it suggests that aerosol transmission with this pig-adapted FMDV is unlikely and this is consistent with field observations in Taiwan.

If aerosol spread was unlikely, there must have been other factors that contributed to the rapid spread of FMD in Taiwan in 1997. Mechanical transmission through vehicles, personnel and fomites is a possible explanation for this. In Taiwan the marketing of pork is through the auction markets in which live fattening pigs (120 kg) are transported by trucks from the major pig production areas in the south-western part of Taiwan to the north every day via major highways. After arrival at the different auction markets throughout Taiwan, pigs are unloaded into and held in the lairage area of the markets. Usually auction markets and abattoirs occupy the same site. Consequently potentially contaminated trucks and personnel leave the auction markets or abattoirs to go to other farms to pick up further loads of fattening pigs. This most probably played an important role in the rapid spread of FMDV in the early stage of the 1997 FMD outbreak in

Taiwan. This has been recognised as a major risk factor and since 1997 a strict system of thoroughly cleaning and disinfecting trucks before they leave the auction markets/abattoirs has been introduced. This pig adapted strain can spread through physical contact with contaminated areas in auction markets and abattoirs and this was shown to be a major source for FDMV transmission in Taiwan (Yang, 2002). Recent studies of methods to prevent transmission of FMDV by people (Amass et al., 2003; Amass et al., 2004) indicated that contaminated personnel did not transmit FMDV to susceptible pigs after hand washing or showering, and changing their protective clothing. Foot and Mouth Disease Virus was not detected in nasal secretions of investigators. Thus, an extended period of avoiding animals does not appear to be necessary to prevent the transmission of FMDV (O/TAW/97) by people to pigs provided organic material is removed through hand washing/showering and wearing of clean clothing. During the outbreak in 1997 the above personal biosecurity procedures were not being followed by the personnel who were involved in the transportation of live pigs. If this was done, the spread of disease might have been be minimised.

Other socio-economic factors may have resulted in the rapid spread of FMD in Taiwan in 1997. For example socio-economic studies in Australia showed that farmers tend to mistrust governments to provide adequate and timely compensation or assistance to

farmers when facing serious or exotic diseases and they saw the authorities as someone who wanted only to confiscate or cull their livestock. Farmers were less concerned about biosecurity risks for the industry than they were about loss of their property or production (Dr Sarah Palmer, personal communication, 2007). Similarly, at the time of the 1997 FMD outbreak in Taiwan it was possible that some farmers may have not reported or ignored pigs with vesicular lesions and sold pigs with mild lesions to other farms or auction markets before the final diagnosis of FMD was confirmed. Some farmers might also have regarded the lesions as those of swine vesicular disease which is seen sporadically in Taiwan (Straw, 2006). A similar situation occurred with classical swine fever (CSF), another OIE list A disease, in pigs in the Netherlands. In that epidemic which lasted 14 months (1997-98) and resulted in more than 10 million pigs being culled, there were a number of instances of intentional movement of apparently healthy pigs from infected areas before movement restrictions could be put in place. There was some delay in confirming the diagnosis of CSF and imposing movement restrictions due to the mild clinical signs (Stegeman et al., 2000). The detection of clinical signs in FMD should not have been a problem for farmers. The clinical signs in pigs infected with FMD are striking, especially when sows are infected with concurrent high mortality rate in sucking piglets (Chang et al 1997). Therefore, education of farmers on the importance of early detection and reporting of suspect exotic diseases to

government officers is an important aspect of exotic disease contingency planning.

Another social factor that was active in Taiwan was the demand for freshly killed meat. The majority of Taiwanese prefer to purchase meat that has been killed on the day of purchase. Consequently this made it harder to close down the auction markets and delayed their closure during the outbreak of FMD in 1997. Once the auction markets were closed the supply system was shut down and the source of fresh meat was interrupted. These factors previously facilitated the spread of diseases in Taiwan (Yang et al, 1999). Therefore, for those countries with similar pig industries, marketing systems and demand for fresh meat, these factors have to be considered when formulating contingency plans for the control of exotic diseases.

Overall it is reasonable to conclude that the rapid spread of FMDV in the 1997 outbreak in Taiwan can be explained by a combination of movement of pigs and indirect spread via contaminated vehicles, personnel and fomites. This was most likely facilitated by the large volume of movements of trucks and people between farms, auction markets and abattoirs with minimal attention to biosecurity and personal disinfection; a delay in recognition or reporting of early cases and the need to keep auction markets and abattoirs functioning to meet the demand for freshly killed pork in the community.

Results from the current study showed that it was highly improbable that aerosol transmission played any role in the rapid spread. This is important for contingency planning for any future incursions of pig-adapted type O FMDV and would point to the need to concentrate on enforcing strict movement control and establishment of effective biosecurity measures and to focus on surveillance activities related to breakdown of these activities rather than consideration of aerosol transmission.

The measures used to control the Taiwanese FMD outbreak in 1997 were initially the slaughter of whole herds in the infected premises. However, with the rapid spread and large numbers of cases present, the decision was taken to use blanket compulsory vaccination of pig herds to control the outbreak when sufficient supplies of vaccines were organized. Type O FMD vaccines were imported from a number of major FMD vaccine manufacturers from around the world. Initially, vaccine efficacy for the imported vaccines was tested by measurement of neutralizing antibody titres in vaccinated pigs. Therefore it was important to establish what neutralizing antibody titre would be sufficient to protect pigs after challenge with O/Taiwan/97 FMDV. To establish the relationship between serum neutralizing titres and protection from FMD in pigs after vaccination, challenge studies were conducted with O/Taiwan/97 FMD in vaccinated pigs. In Taiwan, the field analysis of the previous outbreaks of FMD caused

by pig adapted type O FMD virus has shown that heavily contaminated areas and the introduction of infected pigs from unknown sources were the most likely transmission routes to cause disease outbreaks (Yang, 2002). Therefore, it was important to establish what FMD neutralizing antibody titres in vaccinated pigs would be expected to equate with protection in order to evaluate vaccination programs and the risk of the recurrence of outbreaks after compulsory vaccination. The study showed that absence of FMD SN antibody or low SN antibody titres (≤32) were correlated with the presence of more severe disease and higher lesion scores after challenge. Even though the contact challenge method is likely to be much more severe than a single intradermal challenge there was a good negative correlation between neutralizing titres and lesion scores of infected pigs challenged with a pig adapted type O FMDV strain. Pigs with higher SN titres tend to be protected or had significantly lower lesion scores after challenge with FMDV. These studies have indicated that pigs with neutralizing antibody titres greater than 1:64 are essentially protected from contact challenge. Therefore, for evaluation of vaccine potency and to have confidence in the protection level in a compulsory vaccinated pig population, it is important to ensure that the vaccinated pigs have titres higher than 1:64 to prevent infection and to control outbreaks caused by pig-adapted type O FMD viruses.

In Taiwan evaluation of imported vaccines was performed by measurement of SN titres in naïve pigs three or four weeks post vaccination. The “pass” titre initially used was 1:16 but this was altered to 1:64 after a study examining protection after subcutaneous challenge in vaccinated pigs (Lin et al, 2004). In our unpublished data, there is positive relation between antigen mass contained in imported vaccines (data provided by a vaccine company) and mean group SN titres (Chapter 6) three weeks post vaccination in naïve Taiwanese pigs. Vaccine evaluation by measurement of SN titres is currently not recommended by the OIE (O.I.E., 2004b). In the OIE manual, a challenge test should be applied in the target animals which have been vaccinated three or four weeks prior to challenge. These animals must be vaccinated with different dosages (1/16, 1/4 or full dose) in the protocol used in Europe or a group of 16 animals must be vaccinated with a full dose for the protocol used in South America and the percentage of protection against generalised foot infection is determined. The vaccine used for routine vaccination should contain 3 PD50 (50% protective doses) according to the European protocol or 12 out of 16 animals should be protected in the method used in South America. The OIE manual does allow for assessment of vaccine potency by measuring SN titres if a statistical evaluation has demonstrated a satisfactory correlation between SN titre to that serotype and protection measured by the potency test in cattle. In this project studies were conducted to examine the relationship between protection and SN

titres in pigs with the pig-adapted O Taiwan/97 FMDV (Chapter 4) and the results confirmed there was a good correlation between SN titre ≥ 1:64 and protection after challenge in pigs. This method of FMD vaccine evaluation has some benefits for Taiwan by omitting the need for challenge studies that tie up the high security animal facility with high costs and further unnecessary sacrifice of pigs.

Antibody responses to structural (neutralizing antibody) and non-structural proteins (NSP) were evaluated in vaccinated pig herds after primary and secondary vaccination in herds infected before and after vaccination. A significant difference ($P<0.05$) in SN titres was found between vaccinated or non-vaccinated pigs prior to natural infection and also between vaccinated and non-vaccinated pigs after natural infection. Neutralizing titres in infected pigs vaccinated either before or after the infection were higher than those of non-vaccinated infected pigs. The pigs vaccinated with a commercial vaccine prior to and after infection maintained high neutralizing titres with gradual decline from peak titres over the duration of this study. However, neutralizing antibody titres in infected but non-vaccinated pigs only reached moderate levels 2-4 weeks post infection and remained low from 5 weeks onward to the end of this study at week 12.

The data from the evaluations of NSP antibody tests (Chapter 5); monitoring of immune responses to structural (SN Tests) and non-structural proteins in vaccinated and challenged pigs in pen studies (Chapter 6); and in the field studies after the 1997 outbreak (Chapter 7); and use of NSP antibody tests for the nationwide FMD surveillance in Taiwan (Chapter 8) have provided valuable information for interpreting these tests in serological investigations during control and eradication programs for pig-adapted type O FMD. Some examples of how these tests can be used and interpreted are discussed below.

For socio-economic reasons it is not unusual for farmers in Taiwan to vaccinate their pigs when they suspect some outbreaks of viral diseases, if vaccines are available (eg. hog cholera). Similar tactics are possible when facing a suspect outbreak of FMD where they may vaccinate and not report it to government officers. With surveillance testing in such a situation we could expect to have higher SN titres and a narrow distribution range of SN titres in sera from fattening pigs. The antibody profile of such a herd would be quite different from an uninfected herd with a regular vaccination program in place. In the latter case the SN antibody titres would only be high with a narrow distribution range for one to two months post vaccination and then they would gradually decline and SN titres would be lower and more variable when pigs reach market age 3 to 4 months

after vaccination.

A herd FMD SN antibody profile with high titres and narrow SN titre distribution range in a group of vaccinated pigs has to be interpreted with caution because it might be caused by infection or by a delay in the vaccination of pigs. Some farmers may delay their scheduled vaccination program in pigs, mainly because of the presence of other diseases on their farms. In Taiwan porcine diseases such as pseudorabies, hog cholera, porcine circovirus type 2 related diseases and porcine reproductive and respiratory syndrome (PRRS) are relatively common and can cause heavy losses of grower pigs. Farmers will delay vaccination against FMD if outbreaks of these diseases are present. However, if vaccinated pig farms were suspected of having had an outbreak of FMD, high SN titres in market aged pigs could provide additional evidence to the results from herd NSP ELISA testing and examination of feet for evidence of chronic clinical lesions.

The profile of SN titres in vaccinated pigs in the study has shown that high titres will not persist and will gradually decline (Chapter 6). With some vaccines some vaccinated pigs have already had SN titres drop below 1:64 by 12 weeks post-vaccination and these pigs may then be susceptible to infection. This highlights the need for strict biosecurity

in conjunction with vaccination for prevention and control of FMD on individual farms and this has been emphasized in the modern livestock production system. Serological monitoring of market age pigs from vaccinated herds by SN testing can be incorporated as part of herd management in FMD vaccination programs and can be used to evaluate the risk of an outbreak occurring. These studies suggest that herds should be aiming to have market age pigs with SN titres ≥ 1:64 and choice of vaccines that can achieve and maintain this level of immune response are recommended.

In order to be able to monitor the circulation of virus in vaccinated pig populations, valid diagnostic kits based on the detection of antibody against NSP were required. These tests needed to be evaluated against pig sera derived from challenge studies and natural FMD outbreaks, especially in countries which apply compulsory vaccination for the control of FMD. It was also crucial to determinate if vaccinated pigs would develop any antibody to NSP from residual NSP in vaccines. Three commercially available ELISAs (Cedi, UBI and Checkit), which were available to differentiate infected from vaccinated pigs, were tested and the results (Chapter 5) showed that the Cedi test had the optimal sensitivity and specificity for pig adapted type O FMD testing. The UBI test had high sensitivity but lower specificity, especially when vaccinated but uninfected pig sera were tested. Checkit had a high specificity but low sensitivity. The Cedi test was

used to retrospectively evaluate sera from infected and non-infected pig herds collected sequentially in the year after the 1997 FMD outbreak in Taiwan (Chapter 7). These studies also showed that the early vaccines used stimulated NSP antibody production in herds that were vaccinated but not infected. This resulted in the requirement for purified FMD vaccines to be used when monitoring programs for FMD infection by NSP testing were in place. In these studies, it was also demonstrated that the purified FMD vaccines used later in the control program did not induce NSP antibody (when tested by Cedi and Checkit ELISA kits) after multiple double dosage to pigs.

For effective control of FMD early detection of outbreaks is very important so that quarantine and movement restrictions can be imposed as early as possible. This has been demonstrated in the recent outbreak of FMD in the UK (Anonymous, 2007) compared with the situation in the outbreak in 2001 (Knowles et al., 2001). In investigation of outbreaks of FMD the estimation of the time of onset of the outbreak becomes very critical for contingency planning for FMD control. Time course data for antibody responses to structural or NSP in pigs infected with O Taiwan/97 virus from this study can provide valuable information to be able to indicate time of onset of pig herd infection. NSP antibody was detected as early as 6, 7, and 8 days post-challenge by Cedi, UBI and Checkit, respectively (Chapter 5). If antibodies to NSPs in pigs with

clinical lesions cannot be detected by these kits, the time course of the outbreak is likely to be within one week. In countries not using FMD vaccination SN titres can be used to determinate the time course of infection. SN titres were detected 3 to 4 days after infection and if SN titres are not detected in pigs in an outbreak, the infection time course of the outbreak is likely to be within four days. The level of NSP ELISAs antibody response values can also be used as an indicator for estimating the time of the outbreak especially within the first two months post-outbreak when antibody values will be high. For example when using the Cedi test for pigs which have been infected within two months, the NSP antibody levels expressed as percentage inhibition values in most infected sera would be close to 70-80%. For each of the commercial NSP ELISA kits it was also possible to get some estimation of the time course of infection from the mean levels of antibody responses at times up to 6 months post-infection as indicated in Chapter 5.

Although clinical FMD appeared to be successfully controlled with the vaccination program adopted in Taiwan it was essential for the eradication plan to maintain active surveillance for NSP reactors in the pig population. The UBI and Cedi NSP kits were applied as screening and confirmatory tests, respectively, to pig sera collected in auction markets distributed around Taiwan to monitor for evidence of the circulation of FMDV.

Herds with positive reactors were followed-up by clinical inspection and 15 sera from suspected herds were collected for further testing. Negative results were obtained from all these investigation. With the absence of clinical outbreaks and the lack of evidence of FMDV circulation in the field from the NSP reactor surveillance, the FMD eradication plan in Taiwan has moved to a progressive cessation of vaccination, commencing with banning of vaccination on one isolated island in December 2006. The absence of outbreaks on that island, will pave the way for further cessation of FMD vaccination in Taiwan from July 2008.

The available diagnostic kits and vaccines made it feasible to monitor and control pig adapted FMD in Taiwan. These commercialized NSP kits have previously been validated by OIE for cattle and sheep but not in pigs. In these studies these kits have been evaluated on sera from pigs infected with the pig adapted O Taiwan/97 FMDV (Chapter 5). These NSP kits were also shown to be valid when applied to monitor the circulation of virus in the field (Chapter 7). They clearly demonstrated current disease status throughout the control program in Taiwan, provided data to support Taiwan's status of freedom from FMD with vaccination and have provided solid evidence for government officers in making decisions to sequentially cease FMD vaccination in Taiwan.

If further incursion of a FMD pig adapted strain occurs, there will be a need to rapidly identify and characterise the new virus. In Taiwan there is currently a method available to perform rapid confirmation of a pig adapted FMD strain by PCR (Dr. Pang, personal communication). With such quick identification of a pig-adapted FMD strain a modified contingency plan could be applied in future outbreaks. Because of the lack of evidence of an airborne transmission mode, strict quarantine and control of pig movements on infected premises could be imposed immediately with ring vaccination using vaccines with high antigen mass to prevent further spread by mechanical transmission. The infected premises could be stamped out in a timely manner if single or only limited numbers of herds are involved. Otherwise strictly enforced quarantine and a program of emergency vaccination on the closed premises with strict biosecurity procedures on the infected premises can be considered as an alternative control strategy if widespread outbreaks have already started. It was clearly demonstrated in Chapter 7 that at the time of the 1997 outbreak one of the infected farms had controlled FMD using this procedure without spread to neighbouring farms or recurrent outbreaks on that farm. A similar approach of control and eradication of FMD with vaccination has been studied in closed farms elsewhere in Asia (Poulin and Christianson 2006). The systematic surveillance of market age pigs through the auction markets has been shown to be an effective

monitoring tool during the current control program and would be used in any future FMD incursions. These studies have helped to validate the approach used in Taiwan for FMD surveillance during the control and eradication program. This includes the process of inspection for gross lesions and testing for NSP antibody with the UBI test and confirmatory testing with the Ceditest of pig sera from the auction markets with follow-up on-farm investigations of positive reactor herds involving clinical examination and further NSP antibody testing. This has supported and given confidence to veterinary authorities in their decisions on the management of FMD control and the final eradication of FMD and will be the basis for the control of any further incursions of pig-adapted FMDV in Taiwan.

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Appendix I-Testing procedures for UBI, Idexx and Cedi NSP ELISA kits

1. UBI Test Procedure

The test procedures followed the manufacturer's test manual as described below:

(1) Separate dilution microplates are provided for specimen dilution.

(i) Use two wells (A1and B1) for FMDV non-reactive control.

(ii) Use two wells (C1and D1) for FMDV reactive control (RC).

(iii) Use one or two wells for each specimen (Note: The reliability of the test is increased if replicate samples are tested.)

(iv) Place the dilution microplate over the microwell locator label (optional).

(v) Dilute the controls and specimens as follows:

- Add 200uL of specimen diluent into each control or specimen well of the dilution microplate;
- Dispense 10uL of control or specimen into the assigned wells of the dilution microplate. Mix thoroughly by pipetting up and down 3 times. Use a separate pipette tip for each diluted control or specimen.

(2) Cut open the foil pouch and remove the reaction microplate. If necessary, remove any excess antigen-coated strips from the frame and return them to the foil pouch.

Seal the extra strips securely inside the foil pouch making sure to include the desiccant provided. Return to storage at 2°C to 8°C immediately. It may be necessary to replace these strips with blank strips (not provided in the kit), depending on the washing system used.

(3) Transfer 100uL of the diluted controls and specimens from each well of the dilution microplate to its corresponding well in the reaction microplate. Dispose of the dilution microplate and unused liquid as biohazardous waste.

(4) Cover the reaction microplate with the enclosed plate cover or equivalent and incubate for 60±5 minutes at 37±2°C.

(5) Prepare working conjugate solution, as described in the preparation of reagents section prior to washing the microplates.

(6) Uncover the reaction microplate and wash with diluted wash buffer.

(i) Automatic Microplate Washer-Use six washes with at least 300uL/well/wash. It is recommended that the plate be reoriented in the washer following the first three wash cycles, by turning 180 degrees to assure uniform treatment.

(ii) Manual Microplate Washer-Wash six times, using at least 300uL/well/wash. Fill the entire plate, then aspirate in the same order.

(iii) Hand-held Multichannel (8 or 12) Pipettor-Wash six times using at least 300uL/well/wash.

(iv) Discard liquid as biohazardous waste.

(7) After washing is complete, excess liquid may be removed from the reaction microplate by inverting and tapping on absorbent paper until no further moisture appears on the paper. Dispose of the absorbent paper as biohazardous waste.

(8) Add 100uL of the working conjugate solution (1:101) to all wells of the reaction microplate.

(9) Cover and incubate for 30±2 minutes at 37±2°C.

(10) Uncover and repeat the wash procedure as in steps 6 and 7.

(11) Add 100uL of TMB substrate solution to each well of the reaction microplate.

(12) Cover and incubate in the dark for 15±1minute at 37±2°C.

(13) Uncover and add 100uL of stop solution to each well of the reaction microplate. Mix by gently tapping the side of the reaction microplate.

(14) Read the absorbance of each well at 450nm. Blank on air. If a dual filter instrument is used, the reference wavelength should be within the range of 620 to 690 nm.

(i) Absorbance should be read within 15 minutes of the addition of the stop solution to the reaction microplate.

(15) Review absorbance values for the controls. Refer to CALCULATION OF RESULTS for control validation parameters and calculation procedures.

Calculation of the cutoff value: cutoff value= (0.23) × (RC)

2. CEDI Test procedure

The Cedi test is manufactured by Cedi Diagnostics B. V., Lelystad, The Netherlands. The test procedures adopted followed the manufacturer's test manual as described below:

■ Day 1:

(1) Incubation with test serum

(i) Dispense 80 µl ELISA buffer to all wells.

(ii) Dispense 20 µl of negative control to wells A1 and B1.

(iii) Dispense 20 µl of weak positive control to wells C1 and D1.

(iv) Dispense 20 µl of positive control to wells E1 and F1.

(v) Dispense 20 µl of test samples to the remaining wells.

(vi) Seal the test plate(s) using the enclosed plate sealers.

(vii) Shake the test plate(s) gently.

(viii) Incubate overnight (16–18 hrs) at room temperature (20–25°C).

■ Day 2:

(2) Incubation with conjugate and chromogen/substrate solution

(i) Empty the test plate(s) after the incubation period and wash the plate(s) six times with washing fluid. Tap the plate(s) firmly after the last washing.

(ii) Dispense 100 µl of the working dilution of the conjugate (mouse anti-3ABC Mb) to all wells.

(iii) Seal the test plate(s) using the enclosed plate sealers.

(iv) Incubate for 1 hour at room temperature (20-25°C).

(v) Empty the test plate(s) after the incubation period and wash the plate(s) six times with washing fluid. Tap the plate(s) firmly after the last washing.

(vi) Dispense 100 µl of chromogen (Horseradish peroxidase-conjugated anti-mouse IgG antibody) / substrate (3,3',5,5-tetramethylbenzidine;TMB) solution to all wells.

(vii) Incubate for 20 minutes at room temperature (20-25°C).

(viii) Add 100 µl of the stop solution to all wells.

(ix) Mix the content of the wells of the test plate(s) prior to measuring.

NB Start the addition of stop solution 20 minutes after the first well was filled with chromogen/substrate solution. Add the stop solution in the same order and at the same pace as the chromogen/substrate solution was dispensed.

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

(i) Measure the optical density (OD) of the wells at 450 nm preferable within 15 minutes after colour development has been stopped.

(ii) Calculate the mean OD450 value of wells A1 and B1 (negative control = OD max).

(iii) The percentage inhibition (PI) of the controls and the test sera are calculated according to the formula below.

NB The results of all samples are expressed as Percentage Inhibition (PI) relative to the mean OD450 of the negative control (ODmax).

$$PI = 100 - \left(\frac{\text{OD450 test sample}}{\text{OD450 max}} \times 100\right)$$

3. Checkit test procedures

The test procedures used followed the manufacturer's manual as described below:

(1) Predilute each sample and control 1:100 in a tube using CHEKIT-FMD-3ABC Sample Diluent. For example, add 5 ul of sample or control to 495 ul CHEKIT-FMD-3ABC Sample Diluent.

(2) Dispense 100 ul of prediluted samples and controls into the appropriate wells of the microtitre plate. Final dilution::1:100.

(3) Cover the microtitre plate with a lid and incubate for 60 minutes°C ±10minutesat

37±2°C in a humid chamber.

(4) Wash each well with approximately 300ul CHEKIT wash solution three times. Aspirate liquid contents of all wells after each wash. Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.

(5) Dispense 100 ul of the CHEKIT-FMD-3ABC-Anti-swine-lgG-PO conjugate into each well.

(6) Cover and incubate the microtitre plate for 60 minutes±10minutes at 37±2°C in a humid chamber.

(7) Repeat step (4).

(8) Dispense 100 ul CHEKIT-TMB Substrate into each well.

(9) Incubate the substrate at room temperature for 15 minutes.

(10) Stop the colour reaction by adding 100 ul CHEKIT-Stop Solution TMB per well. The stop solution should be dispensed in the same order and at the same speed as the substrate.

(11) Read the results using a photometer at a wavelength of 450nm.

(i) To validate the assay, the OD of the positive control should not exceed 2.0 and the OD of the negative control should not exceed 0.5. The difference between the positive and the negative control must be > 0.4. Make sure to

read the plates within 2 hours after the addition of the stop solution.

(ii) Corrected ELISA result = OD_{sample}-OD_{neg}/OD_{pos}--OD_{neg}×100

Interpretation: < 20 →negative; 20 to 30→suspect; >30→positive