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Foot-and-Mouth Disease risk management project

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Abstract

Australia is currently free from foot-and-mouth disease (FMD) and an outbreak would seriously threaten out meat and livestock industries. This project was conducted to better prepare Australia, should an outbreak occur, by examining FMD viruses circulating on our region, determining the efficacy of vaccines in the Australian Vaccine Bank (AVB) against FMD viruses that might enter Australia, and developing Australia's laboratory capability to diagnose FMD. The project facilitated an improved understanding of the FMD viruses in South East Asia (SEA), establishing networks to ensure continued monitoring of the evolution of FMD virus strains. Vaccine trials confirmed that the AVB contains suitable vaccines and results support the continued inclusion of vaccination in Australia's FMD response plan. High-potency vaccines were effective in cattle and sheep, but less so in pigs. New techniques simplified sample collection from animals being tested for FMD. The project also focused on improving laboratory tests and procedures to ensure that Australian laboratory staff could confidently diagnose FMD. These outcomes have enhanced Australia's preparedness for an outbreak. This was the second phase of a program that commenced in 2010, with Phase I concluded in 2015 (project P.PSH.0558).

Executive summary

Foot-and-mouth disease (FMD) is an infectious viral disease affecting cloven-hoofed livestock, including cattle, sheep and pigs. While the disease does not usually cause death of adult animals, the economic impact of an FMD outbreak would be enormous (due primarily to trade restrictions), with estimates reaching in excess of $50 billion over a 10 year period in the event of a wide-spread outbreak in Australia.

The Matthew's Report[1] highlighted areas of improvement in Australia's preparedness for an outbreak and the Foot-and-Mouth Disease Risk Management Project (FMD RMP) addressed some of these. The project was funded by the government and livestock industries between 2010 and 2016. It was conducted to increase understanding of the FMD viruses circulating in South East Asia (SEA), improve Australia's laboratory capability to diagnose FMD, and to determine if the vaccines in Australia's Vaccine Bank (AVB) would be effective against FMD viruses that might enter Australia. Much of the project was conducted off-shore, in collaboration with laboratories in SEA and elsewhere. The project involved evaluating efficacy of FMD vaccines in cattle, sheep and pigs, and improving laboratory tests and procedures to ensure that Australian laboratory staff could confidently confirm diagnosis of FMD if it were to occur. It was conducted in two phases, with Phase-I (project P.PSH.0558) concluded in 2015.

Antigenic and genetic comparisons between the vaccines in the Australian Vaccine Bank and field viruses from South East Asia

Foot-and-mouth disease virus changes rapidly, which may result in vaccines failing to protect against clinical infection. The FMD RMP collaborated with laboratories in SEA to conduct antigenic vaccine matching tests and genetic comparisons to estimate the protective capacity of the vaccines in the AVB. The study focused on serotypes O and A, as viruses from these serotypes are presently circulating in SEA. Four of the vaccine strains in the AVB belong to these serotypes, two for serotype O and two for serotype A.

The laboratory data for serotype O revealed that one of the AVB vaccine strains, O3039, is likely to provide better protection than the other, O1 Manisa, against isolates recently circulating in SEA.

Analysis of serotype A viruses showed greater genetic variability of these viruses, and the emergence of new clusters of viruses. Either of the serotype A vaccine strains, A22/IRQ or A/MAY/97, should protect against viruses from the region, but more evaluation is needed to see which one would be most suitable. Combining A/MAY/97 and A22/IRQ strains in the same vaccine preparation might be beneficial.

Testing the efficacy of vaccines in the Australian Vaccine Bank

The AVB contains several vaccine strains that are kept as inactivated, purified and concentrated, deep-frozen antigen preparations. In the event of an outbreak of FMD, the Australian Animal Health Laboratory (AAHL) would determine the serotype responsible and *in vitro* assays to determine which vaccine strain would be most relevant to control the outbreak virus. The vaccine manufacturer would formulate the vaccine using the chosen antigen and ship it to Australia.

In vitro vaccine matching assays are not always accurate in predicting whether or not a vaccine will protect against a field strain, so live animal challenges remain the only definitive way to determine this.

[1] Matthews K, 2011. A review of Australia's preparedness for the threat of FMD. http://www.agriculture.gov.au/SiteCollectionDocuments/animal-plant/pests-diseases/animal-pests-diseases/footandmouth.pdf

The FMD RMP tested a number of the AVB vaccine strains in cattle, sheep and pigs against recent FMDV isolates from SEA. The vaccines were tested for the ability to protect against clinical disease with challenge at different times post-vaccination.

The animal trials aimed to:

- Determine if high-potency vaccines provide clinical protection against challenge within a week after vaccination and 21 days post vaccination (dpv)
- Determine if vaccination will decrease virus excretion
- Determine if vaccination has an impact on the development of persistent infection in cattle and sheep
- Determine if vaccination, together with strict biosecurity, could prevent airborne transmission of FMDV to other pigs
- Determine if oral and nasal swabs could be used to detect virus/viral RNA in vaccinated and challenged cattle, sheep and pigs for diagnostic purposes.

Efficacy of vaccines in cattle

In the majority of the trials performed in cattle, the AVB vaccines provided complete protection at 21 dpv, and partial protection at 7 dpv.

Efficacy of vaccines in sheep

The FMD vaccines appeared to be effective at minimising clinical disease and reducing virus excretion in sheep, even with only a few days between vaccination and challenge.

Efficacy of vaccines in pigs

Vaccination in pigs did not provide the same level of clinical protection as observed in cattle and sheep. Protection varied significantly based on the challenge strain used.

Summary

The results indicated that even when vaccination does not clinically protect all animals, it could reduce the quantity of virus excreted once infected, and this would help to slow down virus spread in an outbreak while other control measures take effect.

Vaccination and persistent infection in cattle and sheep

Foot-and-mouth disease virus may persist in the pharynx and associated lymph nodes of ruminants for a variable time beyond 28 days, termed persistent infection (so-called ‘carrier state’). The occurrence of persistent FMDV infection is the reason why infected animals are destroyed in an outbreak response. Pigs do not become persistently infected.

In most of the cattle and sheep trials performed by the FMD RMP, vaccination did not prevent persistent infection in a proportion of the animals. It was not possible to measure if vaccinated cattle and sheep maintained virus for shorter periods of time compared to unvaccinated animals (as experiments did not run beyond 35 days post challenge). It was also not possible to determine if persistently infected vaccinated cattle and sheep transmitted disease. These experiments are difficult to perform as they need large numbers of animals or long periods in containment that are prohibitively expensive.

Swabs as diagnostic tools during and after outbreaks

Most FMD lesions rupture within 2–3 days post infection and it is then not possible to confirm the presence of virus from the rapidly healing lesions. Antibodies can only be detected from

around 7–10 days after infection which means it may be difficult to find suitable samples to confirm infection between 3 and 7 days after exposure.

The FMD RMP found that nasal and oral swabs are useful to detect virus/viral RNA in infected as well as vaccinated and infected cattle, sheep and pigs between 1 and 10 days after infection. Swabs were able to detect virus/viral RNA in sub-clinically infected animals, in the absence of obvious clinical signs. Bulk sampling methods, such as rope chews are an easy and effective way to collect oral fluid samples from pigs (for surveillance purposes), followed by laboratory assays to detect FMDV/viral RNA

Diagnostic test validation

In addition to having efficacious vaccines, Australia needs access to fit-for-purpose laboratory diagnostic services to ensure the ability to rapidly detect and respond to potential cases of FMD. Through the FMD RMP, various samples (not containing infectious virus) were imported to AAHL and used locally to validate diagnostic tests. Tests requiring the use of infectious virus were conducted overseas.

Demonstration of the absence of antibodies to non-structural proteins (NSP) of FMD virus will be an important aspect of proving freedom from infection to our trading partners after an outbreak has been controlled. The NSP assay is a valuable tool to identify infected animals and will be a key component in post-outbreak surveillance. The AAHL in-house assay was compared with two commercial kits, using a panel of well-characterised sera obtained from various animal trials carried out in the FMD RMP. Preliminary data suggest that the performance of the AAHL assay is comparable to that of the two commercial kits.

The FMD RMP team tested and compared the reagents used for routine diagnostic testing at AAHL and made recommendations to improve the tests.

Genetic information using nucleotide sequencing of the outbreak virus would be an important tool during an outbreak of FMDV. A conventional sequencing method for obtaining high quality partial genome sequences was established, which will be useful to confirm the serotype and lineage of virus during an outbreak and its possible region of origin. Using next generation sequencing (NGS) methods, full-length FMDV genome sequence data were obtained directly from clinical samples, including those containing very low concentrations of viral RNA, without having to grow the virus in cell culture.

Capacity building in Australia and SEA

By closely working with the national and reference laboratories for FMD in SEA, Australian scientists have had opportunities to work with live FMDV to enhance their expertise. In addition, staff in SEA have been trained by the FMD RMP team, helping them become more confident diagnostics and research. The FMD RMP team is regarded as a trusted advisor in many of the SEA laboratories. Improved diagnostics could lead to improved control measures in SEA, thereby decreasing the risk to Australia.

Conclusions and recommendations

The direct benefits resulting from the project are:

- Confidence that the AVB contains suitable vaccine strains and an understanding of their utility in different species
- An appreciation of the behaviour of different FMDV isolates/serotypes in different livestock species
- Determining the best samples to collect at different times post infection to aid in detection

- The establishment of fit-for-purpose, validated diagnostic assays and a store of samples of known origin to serve as controls in these assays
- An improved understanding of the FMD situation in SEA, with established networks to ensure continued monitoring of the genetic and antigenic (immunological) evolution of FMDV strains, important for ensuring the relevance of AVB vaccines
- Improved capability in laboratories in SEA to characterise field isolates and so address the risk at source
- More staff in Australia with direct experience identifying lesions in FMDV infected animals, processing infected samples, and performing molecular, cell culture and serological assays to detect and characterise FMDV.

These all contribute to Australia's preparedness for an outbreak of FMD and in doing so, contributes to protecting the livestock industries. However, due to the constantly changing epidemiological situation of FMD, constant vigilance is needed and it is essential that monitoring in SEA and globally continues.

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1 Background

Foot-and-mouth disease (FMD) is one of the most infectious viral diseases affecting cloven-hoofed livestock, including cattle, sheep and pigs. While the disease does not cause death of adult animals, the economic impact of an FMD outbreak, due to international trade restrictions, would be enormous. For example, the cost of an outbreak of FMD to Australia, including lost trade in animals and their products, is estimated at around $50 billion over a 10 year period[2].

Although FMD is endemic in many parts of the world, disease-free countries and zones exist and are maintained through costly disease control measures. As a result, the countries/zones free from FMD will not accept live animals or animal products from high-risk areas. There has been no recorded outbreak of FMD in Australia since 1872 and this disease-free status, together with the absence of various other infectious diseases, provides Australia access to lucrative animal product export markets.

There are seven different serotypes[3] of FMD virus (FMDV - O, A, C, Asia1, SAT1, SAT2 and SAT3), each with a distinct geographical distribution, grouped as pools of circulating serotypes (Fig. 1.1). For instance, serotypes O, A, SAT1, 2 and 3 are prevalent in certain parts of Africa, while FMD viruses belonging to serotypes O, A and Asia1 are predominantly found in livestock populations of South East Asia (SEA), along with other parts of Asia and the Middle East.

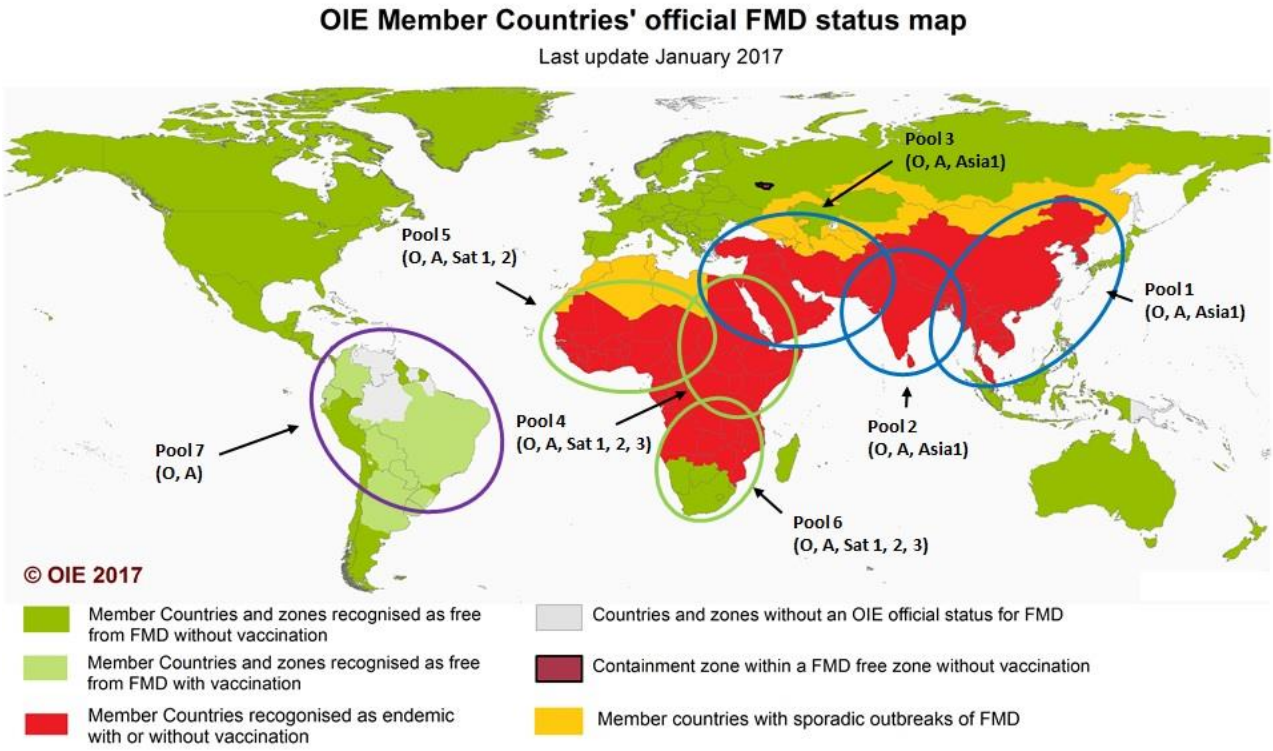

Fig. 1.1: Global distribution of FMDV serotypes indicating the seven virus pools

[2] Buetre et al, 2013, Potential socio-economic impacts of an outbreak of foot-and-mouth disease in Australia, ABARES research report, Canberra, September. CC BY 3.0.
[3] Serotypes are groups of viruses that cross-react with each other, but not to those belonging to a different serotype.

During 2010–2016, FMD outbreaks continued to affect countries in the established endemic regions of the world. Progress was made in FMD control in some countries, with the Philippines, Kazakhstan and Russia obtaining freedom from disease without vaccination. There have also not been any outbreaks in South America since August 2011, where vaccination is used to help control FMD. Serotype C was last detected in 2004 and efforts are underway to restrict work with live serotype C viruses and encourage the cessation of vaccination to this serotype, mainly still occurring in South America.

However, recently a number of viral lineages of serotypes O and A have emerged from their established endemic pools and crossed geographical boundaries to cause significant outbreaks in distant locations (Fig. 1.2). No single factor underpins these changes, but the movement of FMDV since 2010 may be influenced by the migration of people from North Africa and the Middle East, as well as new trading patterns and demand for meat and meat products in South East Asian countries.

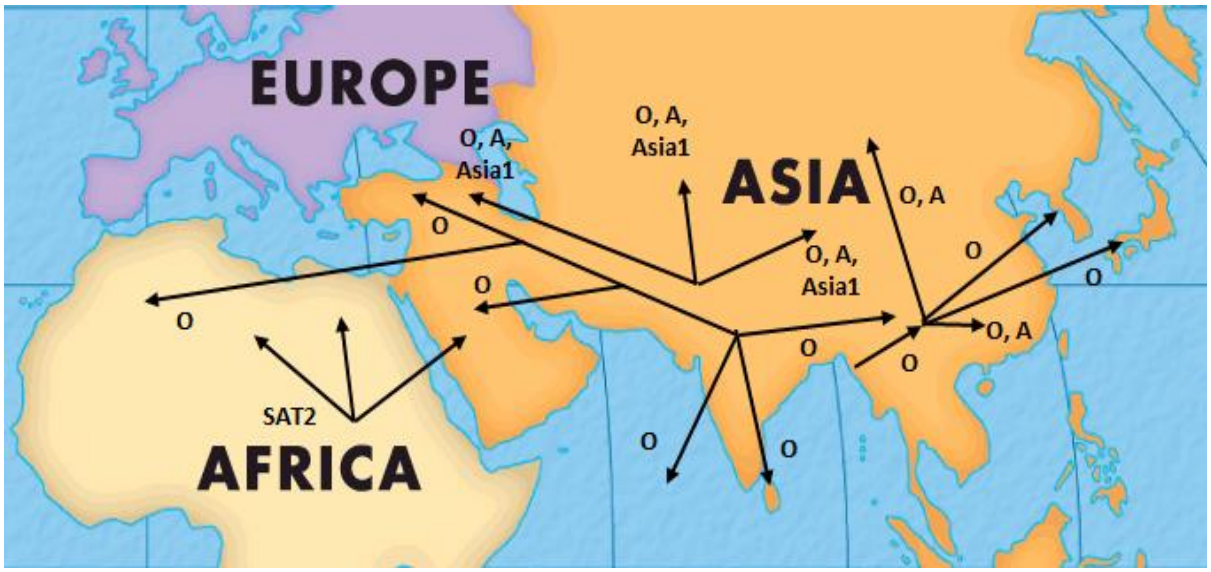

Fig. 1.2: FMDV movements within Africa and Asia (2010–2016).
Arrows represent the movement of different virus lineages and genotypes from their endemic pools in Africa, South Asia and South East Asia into new geographical areas[4].

During 2010, the O/SEA/Mya-98 lineage of virus spread through South East Asia (SEA) and into South Korea and Japan, which had previously been free from FMD. There was also the emergence of new A/SEA-97 variants in SEA, leading to vaccine failures in Thailand and other South East Asian countries in 2012. Since 2013, the O/ME-SA/Ind2001 lineage spread from the Indian sub-continent into the Middle East and North Africa and further south to Mauritius in the Indian Ocean, as well as east to Sri Lanka and then Laos, Vietnam and Myanmar. This was followed by the emergence of the A/ASIA/G-VII lineage into Iran, Armenia and Turkey, from South Asia in 2015. There is concern that these lineages could become established and outcompete indigenous serotype O and A lineages. Variants of serotype Asia1/Sindh-08 lineage emerged from Pakistan and Afghanistan and spread to the Middle East.

According to internationally agreed standards through the World Organisation for Animal Health (OIE), countries/zones can receive official disease status as follows[5].

[4] Adapted from the OIE-FAO FMD Reference Laboratory Report 2015

- FMD free country or zone where vaccination is not practised
- FMD free country or zone where vaccination is practised
- FMD free compartment
- FMD infected country or zone

The OIE Terrestrial Animal Health Code[6] recommends animal health measures for safe international trade in terrestrial animals and their products. Countries that are free from FMD without vaccination can trade animals and their products without FMD-related restrictions. Exports of live animals from countries/zones that use prophylactic vaccination is not allowed to countries/zones where vaccination is not used, and meat from vaccinated animals needs to be treated (matured, deboned and major lymph nodes removed) before being accepted by most markets. It is therefore of great trade benefit to retain FMD-free status without the use of vaccination.

If an outbreak occurs in a country/zone previously free where vaccination is not usually applied, it is accepted that all infected animals should be destroyed, with the option to use vaccination to assist with disease control. Emergency vaccination can be beneficial to slow the spread of disease by reducing the likelihood and number of animals becoming infected, thereby assisting other control measures, such as movement control and removal of infected animals, to take effect. Australia has a national FMD vaccination policy (see below).

Policy Statement

Given the developments in vaccine technology, changing international attitudes and the recent experiences of countries experiencing FMD outbreaks around the world, Australia no longer views vaccination as a measure of last resort.

Australia will consider the potential role of vaccination as part of the response strategy from the day an incursion of FMD is detected.

The role of vaccination in an FMD response will depend upon the unique nature of each outbreak, and will vary depending on a wide range of factors. Therefore Australia will maintain a flexible policy that allows decision-makers to determine the role of vaccination appropriate for each specific outbreak scenario.

Australia will prepare as though vaccination will be used in the event of an FMD incursion, to allow adequate preparatory measures to be put in place.

Adapted from http://www.agriculture.gov.au/pests-diseases-weeds/animal/fmd/review-foot-and-mouth-disease/national_foot-and-mouth_disease_vaccination_policy

However, to regain the OIE agreed disease-free status after an outbreak has been brought under control, various waiting periods apply (see below).

[5] http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_fmd.htm
[6] http://www.oie.int/international-standard-setting/terrestrial-code/access-online/

Article 8.8.7

Recovery of free status

When a FMD case occurs in a FMD free country or zone where vaccination is not practised, one of the following waiting periods is required to regain this free status:

- three months after the disposal of the last animal killed where a stamping-out policy, without emergency vaccination, and surveillance are applied in accordance with Articles 8.8.40. to 8.8.42.; or

- three months after the disposal of the last animal killed or the slaughter of all vaccinated animals, whichever occurred last, where a stamping-out policy, emergency vaccination and surveillance in accordance with Articles 8.8.40. to 8.8.42. are applied; or

- six months after the disposal of the last animal killed or the last vaccination whichever occurred last, where a stamping-out policy, emergency vaccination not followed by the slaughtering of all vaccinated animals, and surveillance in accordance with Articles 8.8.40. to 8.8.42. are applied. However, this requires a serological survey based on the detection of antibodies to nonstructural proteins of FMDV to demonstrate no evidence of infection in the remaining vaccinated population.

The country or zone will regain the status of FMD free country or zone where vaccination is not practised only after the submitted evidence, based on the provisions of Article 1.6.6., has been accepted by the OIE.

The time periods in points 1a) to 1c) are not affected if official emergency vaccination of zoological collections has been carried out following the relevant provisions of Article 8.8.2.

Where a stamping-out policy is not practised, the above waiting periods do not apply, and Article 8.8.2 applies.

http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_fmd.htm

Based on the far-reaching economic consequences of an FMD outbreak, government and livestock industries are investing in risk reduction measures to help prevent the introduction of this disease into Australia, and to reduce the impacts if it is introduced. One such initiative is establishment of the Australian Vaccine Bank (AVB), which will be activated in the event of an FMD outbreak in Australia. For logistical reasons, the AVB holds a limited number of vaccine strains. It is therefore important to know that these vaccine strains would be effective against virus strains of greatest geographical risk to Australia. Due to cost limitations and a prohibition on the use of infectious FMDV in Australia, there had been little investigation into testing the efficacy of available AVB vaccines against FMDV challenge until the commencement of this project.

To address these challenges, the Foot-and-Mouth Disease Risk Management Project (FMD RMP) was established. As a jointly funded industry and federal government initiative, the FMD RMP focuses on:

- the protection of livestock against clinical FMD using the AVB vaccines

- pathogenesis of SEA virus serotypes in Australian livestock

- validation of diagnostic assays
- molecular epidemiology of FMD in SEA, and
- capacity building in the SEA region. Given the volume of trade and travel between Australia and South East Asian countries, this region is perceived as the biggest risk for illegal introduction of FMD into Australia, and therefore a large part of the study focused on this region.

All vaccine efficacy studies and laboratory assays described in this report were performed offshore, since no infectious FMDV is allowed in Australia.

2 Project objectives

There were five inter-related components of the project (Fig. 2.1). The project had a number of objectives and required a multitude of approaches and scientific techniques in the areas of virology, immunology, pathology, molecular biology, epidemiology, and bioinformatics.

The objectives were:

a. Gain comprehensive knowledge about FMDV strains that pose a geographic high risk to Australia and their comparable likely behaviour in Australian livestock species

b. Devise control strategies tailored to Australian circumstances and store appropriate bulk serum derived reagents for future Australian use

c. Improve laboratory diagnostic capability for FMDV to rapidly isolate or detect FMDV and confirm a primary diagnosis by providing Australian Animal Health Laboratory (AAHL) staff the opportunity to work with live virus off shore and so gain experience with FMDV culture and recognition of cytopathogenic effects

d. Validate diagnostic tests (including DIVA) for use in local animal species and breeds, and to conduct genetic ‘fingerprinting’ (sequencing) on the virus(es) isolated in support of molecular epidemiology and vaccine selection

e. Enhance the epidemiological and virological understanding and thus help model virus spread

f. FMD vaccination response policies included in AUSVETPLAN and associated national standard operating procedures that are soundly technically based

g. Produce experimental data on the efficacy (in terms of protection against challenge with circulating high-risk virus isolates) of the vaccine strains and the vaccine potency in Australia’s FMD vaccine bank, to maximise the benefits arising from investment in the vaccine bank and to inform FMD response planning

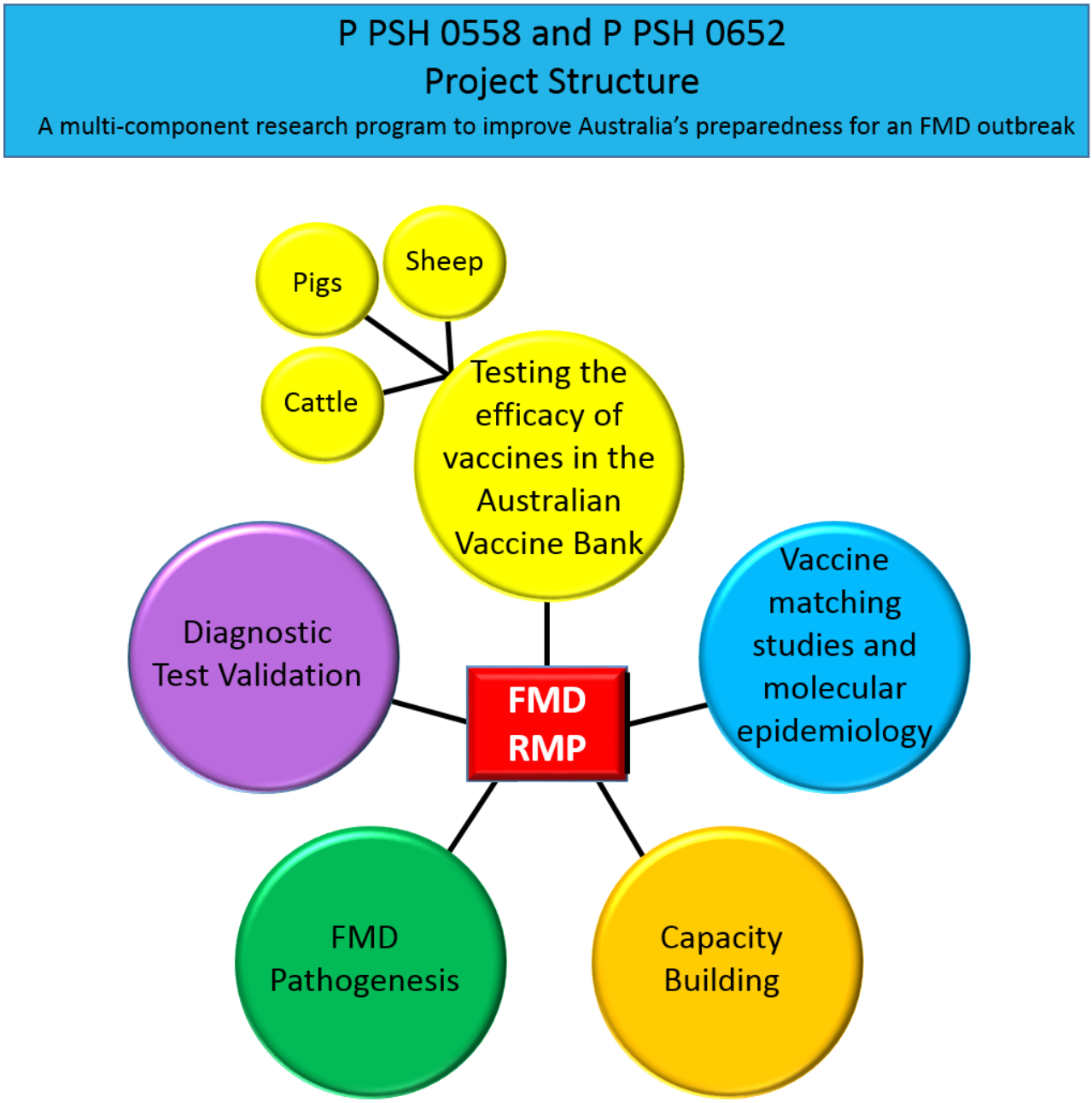

Fig. 2.1: Components of the FMD RMP

3 Vaccine matching studies and molecular epidemiology of FMD viruses from SEA

Foot-and-mouth disease virus is a small RNA virus that exists as seven serotypes. Within each serotype, a large number of variants exist that results in different levels of cross-reactivity between isolates belonging to the same serotype. This variation has implications for vaccines and diagnostic tests. If the variation is significant, the cross-protection within a serotype is poor, for example, a serotype O vaccine may not protect animals against clinical disease from all serotype O isolates, necessitating more than one vaccine strain per serotype.

For these reasons, it is important to continually study the circulating viruses in endemic areas such as SEA, which is the most likely source of potential future entry of disease into Australia, to determine whether or not new variants have emerged, or if new introductions from elsewhere have occurred.

3.1 Antigenic matching of vaccine strains to viruses circulating in SEA

3.1.1 Introduction

There are two ways to determine whether or not a vaccine will protect animals against clinical signs as a result of FMDV infection.

1. *In vivo* studies, where animals are vaccinated and challenged with a live virus, and
2. *In vitro* studies, performed in the laboratory using serological assays.

While *in vivo* studies are considered the principal means of measuring protection, they are expensive, time consuming and involve ethical questions regarding animal use. It is therefore not possible to rapidly generate *in vivo* results when an outbreak is diagnosed, unless prior vaccine efficacy data are available. In contrast, *in vitro* studies assist in screening a large number of viruses, can be performed in a matter of hours and would be used to decide on the most appropriate vaccine strain in the event of an outbreak in Australia. In addition, the *in vitro* data can be used to select viruses with a poor match to the vaccine strains for subsequent testing *in vivo*.

3.1.2 Methods

3.1.2.1 Virus strains

Four vaccine strains from the AVB, A/MAY/97, A22/IRQ, O1 Manisa and O3039 were used as reference strains. Serotype A (n=42) and serotype O (n=40) cell culture adapted field viruses, isolated during 2011–2015 from Thailand, Laos and Vietnam, were used for antigen matching at Regional Reference Laboratory (RRL). Serotype A (n=18) and serotype O (n=42) viruses, isolated during 2008–2015 from Vietnam, were used for antigen matching studies at the Regional Animal Health Office 6 (RAHO6), Vietnam. The viruses were isolated from tongue epithelium of animals with suspected FMD lesions using primary lamb kidney or BHK-21 cells at RRL, and BHK-21 cells at RAHO6. The list of viruses used in this study, including their origin and passage levels, are provided in Appendices 1–4.

3.1.2.2 Test reagents

The vaccine antigens, A/MAY/97, A22/IRQ, O1 Manisa and O3039, were provided by the AAHL and used in the *in vitro* assays with their homologous rabbit, guinea pig and bovine vaccinated sera.

3.1.2.3 Antigen matching protocol

The field viruses, and the inactivated and purified reference vaccine viruses, were titrated using a double antibody sandwich ELISA in a homologous system (Hamblin *et al.,* 1984[7]). The antigen dilution selected for use in the antigen matching assay was 2-fold higher (more concentrated) than that which gave an OD value of 1.0–1.5. The antigen matching protocol was similar to that described by Samuels *et al*. (1990)[8], which used reference bovine sera raised against the vaccine strains. The antibody titres were calculated based on 50% inhibition of the OD compared to the virus control. The 'r' value, the relationship between the field and the vaccine strain, was derived as the antilog of the negative value of the log differential of homologous and heterologous titre, and was interpreted as per Samuels *et al*. (1990)[8].

Heterologous log titre =	x
Homologous log titre =	y
log differential (*d)* =	x-y
r_1 value =	$10^{-(x-y)}$

If $r_1 > 0.39$ – Homologous
If $r_1 = 0.19$-0.39 – Intermediate
If $r_1 < 0.19$ – Heterologous

3.1.3 Results

Serotype O and A viruses isolated from countries including Cambodia, Lao PDR, Thailand and Vietnam were selected and tested against the relevant vaccine strains in the AVB (O1 Manisa & O3039 and A/MAY/97 & A22/IRQ)[9].

For serotype O, nearly half of the viruses tested were homologous or intermediate to O1 Manisa; the others were heterologous to the vaccine strain or did not show any binding, which indicates a very significant difference between the vaccine and the outbreak viruses. O3039 demonstrated a much better match with 87% of the isolates related to the vaccine strain, indicating that the vaccine is likely to protect (Table 3.1).

[7] Hamblin et al (1984). A rapid enzyme-linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues. Veterinary Microbiology, 9, 435-443.
[8] Samuels et al (1990). Antigenic analysis of serotype O foot-and-mouth disease virus isolates from the Middle East, 1981 to 1988. Vaccine, 8, 390-396.
[9] Outbreaks due to Asia-1 have not been reported in the SEA region since 2006.

Table 3.1: Summary of antigenic matching results between the vaccine strains in the AVB and viruses circulating in SEA since 2010

	Serotype O		Serotype A	
Vaccine strain	*O1 Manisa*	*O3039*	*A22/IRQ*	*A/MAY/97*
No of isolates tested	82	84	60	60
Homologous	28%	67%	28%	50%
Intermediate	15%	20%	25%	18%
Heterologous	22%	1%	42%	32%
Poor binding*	35%	12%	5%	0%

*Very significant difference with the vaccine strain

There was no obvious increase or decrease in relatedness of field strains to the vaccine strains over time (Fig. 3.1; Appendix 1).

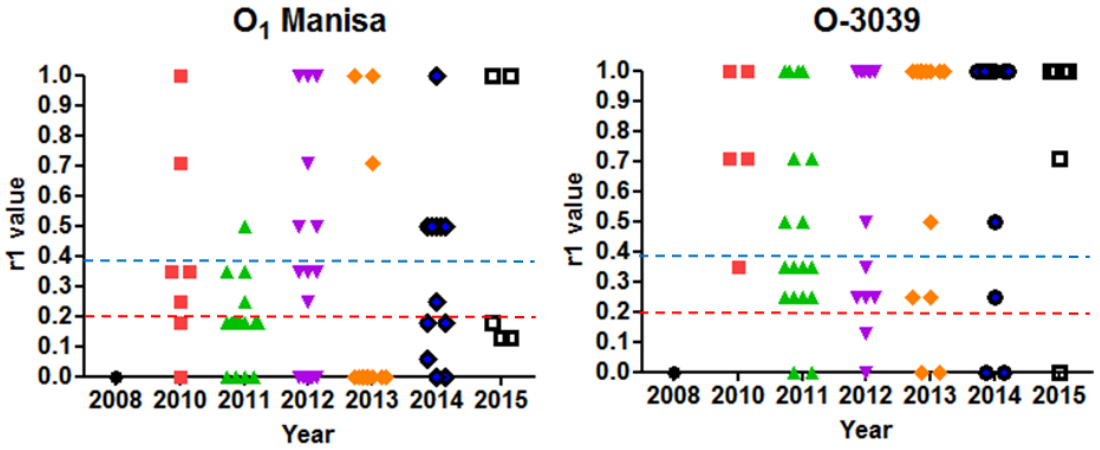

Fig. 3.1: Antigenic values, using LP-ELISA, of isolates from SEA (2008 to 2015) against the AVB serotype O vaccine strains.
The respective r1 cut-off values are indicated by dashed lines.

For serotype A, 53% of the isolates were homologous or intermediate to A22/IRQ, and 68% to A/MAY/97 (Table 3.2). The results indicated that the number of field isolates with heterologous or intermediate r1 values to the serotype A vaccine strains increased between 2012 and 2014. That is, the trend was for the field viruses to become less closely matched over time.

The temporal trend of the homology values for the field to the reference vaccine strains differed for each of the serotype A vaccines in the AVB (Fig. 3.2). The more recent 2014 and 2015 SEA field isolates matched less well (heterologous or intermediate r1 values) with A22/IRQ compared to A/MAY/97 (intermediate or homologous r1 values).

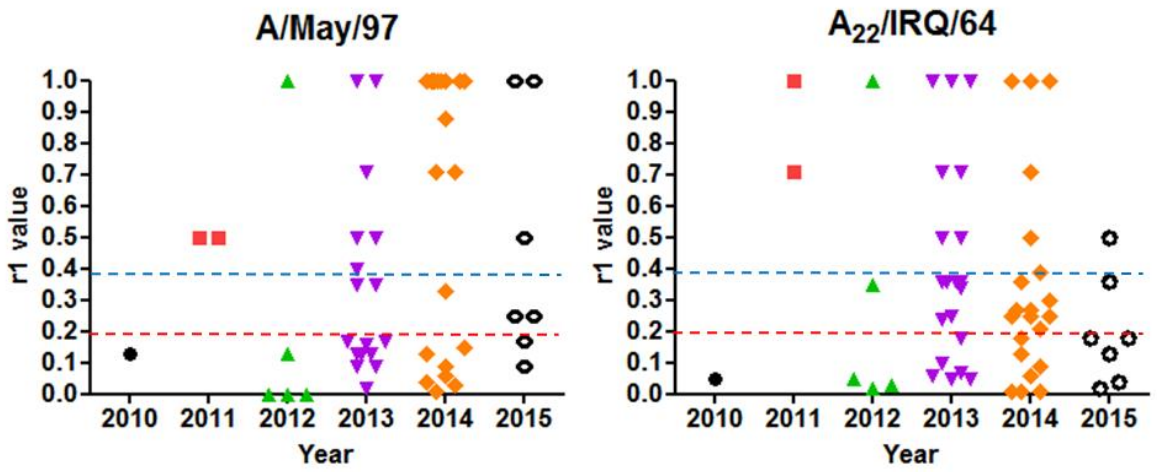

Fig. 3.2: Antigenic values, using LP-ELISA, of isolates from SEA (2010 to 2015) against the AVB serotype A vaccine strains.
The respective r1 cut-off values are indicated by dashed lines.

3.2 Genetic comparisons of viruses from SEA

3.2.1 Introduction

The relationships between viruses and vaccine strains, using the information from their genetic material (RNA), provide valuable information regarding relationships and evolution of viruses. If an outbreak were to occur in Australia, genetic sequencing would be used to

- determine the relationship between the outbreak virus and vaccine strains[10], and
- trace the possible region of origin of the outbreak.

3.2.2 Methods

3.2.2.1 Phylogenetic analysis

The complete capsid protein VP1 sequences of the FMD genome from selected isolates, generated by RRL and RAHO6, were used to determine the phylogenetic relationship of FMD viruses in the region in comparison with the reference sequences obtained from GenBank. The phylogenetic trees were drawn using the Neighbour-Joining method in MEGA version 6.2[11].

3.2.3 Results

3.2.3.1 Phylogenetic relationships of serotype O

The majority of the SEA serotype O isolates clustered with the main variants of the O/SEA/Mya-98 lineage (A and B) (Fig. 3.3). All of the isolates from Thailand were grouped under the variants of O/SEA genotype/topotype or one of the two sub-lineages of O/SEA/Mya-98 lineage, A or B.

[10] The nucleotide sequences of vaccine strains are not available, but those of closely related viruses will be used as proxy.
[11] Tamura et al (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

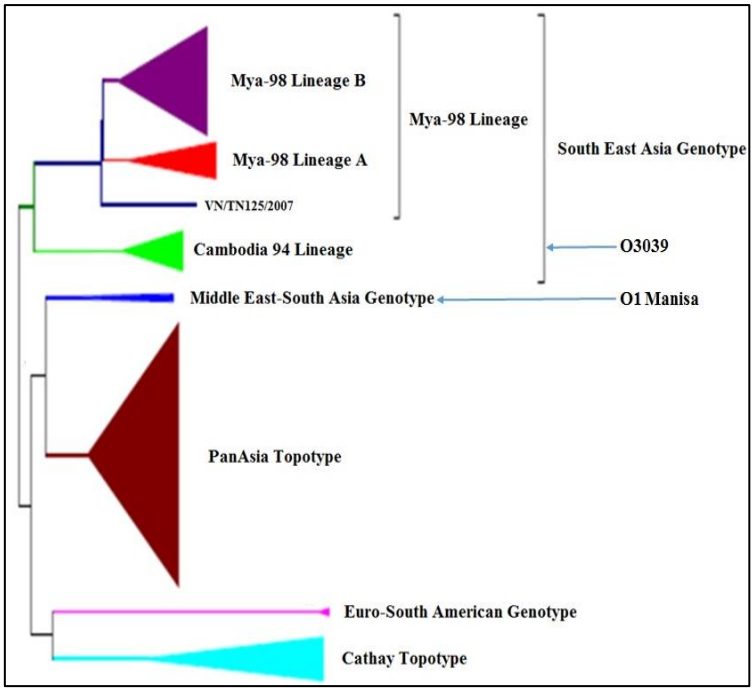

Fig. 3.3: Phylogenetic tree based on the complete sequences of the VP1 coding region from representative serotype O isolates from SEA.
Brown and purple shading depict groupings for which sequences of the field isolates were available. The position of vaccine isolates O3039 and O1 Manisa is indicated by arrows. Sequences kindly provided by RRL and RAHO6. African genotypes are not represented.

In addition, O/PanAsia1 continues to circulate in SEA and viruses from this lineage have been detected in Cambodia, Lao PDR, Thailand and Vietnam (Fig. 3.3).

In 2015, isolates from Lao were grouped under O/ME-SA/Ind 2001d sub-lineage, the first time this lineage has been observed in SEA.

3.2.3.2 Phylogenetic relationships of serotype A

Three main variants of the A/SEA-97 lineage were identified, encompassing the majority of field isolates tested (Fig. 3.4). This genetic drift has been evident since 2004, and three clusters have emerged (2004–2008, 2010–2012 and 2014–2015). Two subgroups were apparent in the 2014–2015 cluster and their significance should be further investigated by sequencing additional recent field isolates (Appendix 1). This new trend needs to be carefully followed to determine if both clusters will become established and what impact that could have on the choice of vaccines to be included in the AVB.

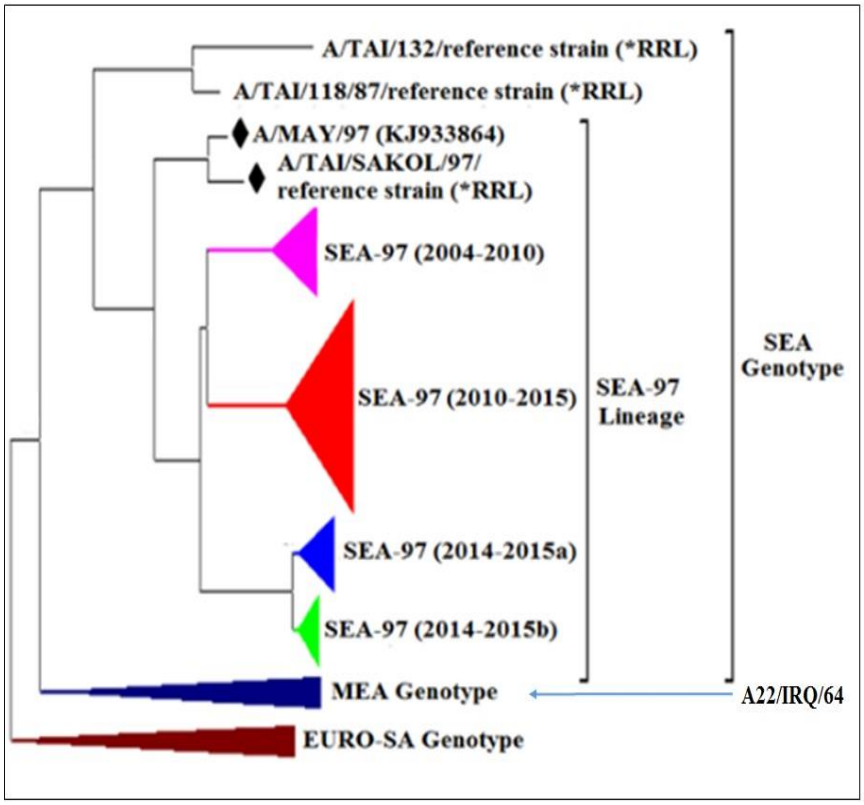

Fig. 3.4: Phylogenetic tree based on the complete sequences of the VP1 coding region from representative serotype A isolates from SEA.

Coloured shading depicts groupings for which sequences of the field isolates were available. The position of reference vaccine isolates is shown with a diamond (◆) for A/MAY/97 and A/TAI/SAKOL/97 and with an arrow for A22/IRQ/64. Sequences kindly provided by RRL and RAHO6.

3.3 Conclusions

The data for serotype O revealed that the isolates in SEA are generally intermediate or heterologous to the O1 Manisa vaccine, and this vaccine strain may not provide adequate protection against the viruses currently circulating in SEA. However, the data for O3039 suggest that it is likely to provide better protection against these viruses and should be the vaccine strain of choice (see Section 4).

Phylogenetic analysis revealed O/Mya/98 as the major variant in circulation, along with minor outbreaks of PanAsia1. Of concern is the recent occurrence of a new lineage, O/Ind/2001d, previously confined to the Indian subcontinent. The route of introduction has not been determined with certainty.

The serotype A viruses from SEA are more variable, and new variants are continuously emerging. This is evident from the antigen matching studies that show a slow drift, in a direction either less or more related to the vaccine strains in an unpredictable manner. However, both A strains currently in the AVB would seem to be useful for protecting against viruses from the region.

Phylogenetic analysis showed the emergence of new clusters of serotype A over time, with new variants arising in 2014–2015. These changes could be due to inadequate vaccination that drives viral evolution, and warrants further investigation.

By closely working with the national and reference laboratories for FMD in SEA, Australian scientists can obtain first-hand information on the emerging FMD virus isolates and their relationships with the strains in the AVB. The information gained in these studies will assist Australia in making informed decisions on the composition of the vaccine bank and improve preparedness for FMD management and disease control, as well as modelling spread with and without vaccination.

4 Testing the efficacy of vaccines in the Australian Vaccine Bank

The AVB contains several vaccine strains that are kept as deep-frozen, purified and concentrated, inactivated virus antigen preparations. FMD vaccines can contain different amounts of virus antigen and are classified by protective ability of the vaccine, or 50% Protective Dose (PD_{50})[12] value. There AVB vaccines are high-potency vaccines, containing a dose of >6 PD_{50}.

The FMD RMP focused on three serotypes of FMD that are prevalent in SEA, Asia and the Middle East, namely O, A and Asia1. For serotype O, the studies involved AVB vaccine strains O1 Manisa and O3039, for serotype A, vaccine strains A22/IRQ and A/MAY/97, and for Asia1, Asia1/Shamir vaccine strain. Each vaccine strain belongs to a specific genetic cluster (Table 4.1).

Table 4.1: Summary of the vaccine strains tested in the FMP RMP indicating their origin, year of isolation and the genetic cluster.

Serotype	Strain	Genotype/Topotype	Origin	Year
O	O1 Manisa O3039	O/Middle East-South Asia O/South East Asia	Turkey Thailand?	1969 1989
A	A22/IRQ A/MAY/97	A/ASIA/G-IV O/South East Asia	Iraq Malaysia	1964 1997
Asia1	Asia1/Shamir	Asia1/G-II	Israel	1989

All of the challenge trials had to be performed offshore and several collaborations were established over the six years. The details of each trial are summarised in Table 4.2.

[12] The PD_{50} is the dose that protects 50% of challenged animals.

Table 4.2: Summary all the vaccine efficacy testing work

# of exp	Species	Vaccine tested	Challenge Virus	Outcomes	Animal work complete	Lab work complete	Location
Cattle trial 1	Cattle	O1 Manisa O/SKR/ 2010/vac	O/SKR/8/2010	O1 Manisa vs O/SKR/8/2010 - heterologous challenge PD50 = 3.47, O/SKR/2010/vac vs O/SKR/8/2010 - homologous challenge PD50 = 7.94	Sep-13	Feb-14	Pirbright Institute, UK
Cattle trial 2	Cattle	O1 Manisa	O/SKR/2010	50% 7 dpv, 0% 4 dpv	Sep-14	Aug-15	SENASA, Argentina
		Test whether vaccination prevents the carrier state		Viral RNA detected in 100% of cattle	Sep-14	Aug-15	
Cattle trial 3	Cattle	A/MAY/97 A22/IRQ	A/VIT/2012	Both vaccines 40% 7 dpv, A May 96 100% and A Iraq22 60% at 21 dpv	Sep-14	Dec-14	CVI, Netherlands
		Test whether vaccination prevents the carrier state		Viral RNA detected in 100% of cattle	Sep-14	Dec-14	
Cattle trial 4	Cattle	O1 Manisa O1Man + O3039	O/ALG/3/2014	O3039 and bivalent O1 Man/O3039 - 100% 21 dpv; O3039 - 60% 7 dpv; O1 Man/O3039 80% 7 dpv	Oct-15	Aug-16	CVI, Netherlands

# of exp	Species	Vaccine/Objective	Challenge Virus	Outcomes	Animal work complete	Lab work complete	Location
Cattle trial 4	Cattle	Test whether vaccination prevents the carrier state		Probang samples tested positive at 11 dpc; RNA/virus detected intermittently until 33 dpc in some vaccinated animals; RNA/virus consistently detected in one of three control animals	Oct-15	Aug-16	CVI, Netherlands
Cattle trial 5	Cattle	A22/IRQ A/ May/97	A/IRN/22/2015	A22/IRQ 29% and A/MAY/97 71% at 21 dpv	Dec-16	Feb-16	CVI, Netherlands
Sheep trials 1 & 2	Sheep	Compare different routes of challenge in sheep CB, INP, AI and DC	O/SKR/2010	CB and INP resulted in the best progress to disease, AI and DC were also successful	Jul - Dec 13	Jun-14	PIADC, USA
		Test lower doses of O1 Manisa vaccine at 7 dpv (1ml, 0.5ml and 0.25ml) using CB challenge	O/SKR/2010	No protection	Jul - Dec 13	Jun-14	
		Test higher doses of O1 Manisa vaccine at 7 (2ml) and 14 dpv (1ml and 2 ml) using CB challenge	O/SKR/2010	2ml vaccine 7 dpv = 43% protection 1ml vaccine 14 dpv = 57% protection 2ml vaccine 14 dpv = 57% protection	Jul - Dec 13	Jun-14	
Sheep trial 3	Sheep	O1 Manisa	O/SKR/2010	100% 4 dpv	Aug-Sep 13	Apr-14	NCFAD, Canada

# of exp	Species	Vaccine/Objective	Challenge Virus	Outcomes	Animal work complete	Lab work complete	Location
Sheep trial 4	Sheep	A22/IRQ DC challenge	A/VIT/15/2012	83% 4 dpv	Jun-14	Dec-14	NCFAD, Canada
Sheep trial 5	Sheep	Adapt Asia1 Sind-08 lineage strain Asia1 PAK/19/2014 to cattle		Virus adapted in 1 pass	Mar-15	Mar-15	NCFAD, Canada
		Challenge dose trial in sheep with using INP route of challenge	Asia1 PAK/19/2014	Challenge dose of $10^{4.5}$ was selected for optimal results	Mar-15	Mar-15	
		Pathogenicity study	Asia1 PAK/19/2014	Experiment and laboratory work completed, IHC on hold	Mar-15	Aug-15	
		Asia1 Shamir	Asia1 PAK/19/2014	Protection: 100% 21 and 7 days, 80% 4 days	Jul-15	Aug-15	
		Test whether vaccination prevents the carrier state		20-40% sheep in each grp became carriers except the 7 day grp which had no carriers	Jul-15		

# of exp	Species	Vaccine/Objective	Challenge Virus	Outcomes	Animal work complete	Lab work complete	Location
Pig trial 1	Pigs	Adapt O/VIT/2010 to pigs	O/VIT/2010	Virus adapted in 3 passes	Jan - Feb 12	Jan-15	NAVETCO &, RAHO6, Vietnam
		O1 Manisa	O/VIT/2010	Protection - 60% in 4 dpv CB, INP, AI and DC 80% in 7 dpv; no transmission to contact pigs	Feb - May 12		
Pig trial 2	Pigs	Adapt A/VIT/2005 to pigs	A/VIT/08/2005	Virus adapted in 3 passes	Aug-12	Apr-14	NAVETCO &, RAHO6, Vietnam
		A/MAY/97	A/VIT/08/2005	Protection - 100% in 4 dpv, 75% in 7 dpv; no transmission to contact pigs	Aug 12 - Mar 13		
Pig trial 3	Pigs	O1 Manisa O/SKR/2010/vac	O/SKR/2010	Both vaccines 0% 5dpv	Jun-13	Feb-14	Pirbright Institute, UK
Pig trial 3	Pigs	O1 Manisa O/SKR/2010/vac	O/SKR/2010	O1 Manisa 20% 21 dpv, O/SKR2010 60% 21 dpv	Jul-13	Feb-14	Pirbright Institute, UK
Pathogenesis study	Pigs	Pathogenicity study	O/VIT/2010	Experiment and laboratory analysis completed; IHC on hold	Nov/Dec 13	Jul-15	NAVETCO &, RAHO6, Vietnam
Pig trial 4	Pigs	A22/IRQ A/MAY/97	A/TAI/2013	Both vaccines 20% at 21 dpv 0% at 7 dpv	Nov-15	Apr-16	NCFAD, Canada

# of exp	Species	Vaccine/Objective	Challenge Virus	Outcomes	Animal work complete	Lab work complete	Location
Pig trial 4	Pigs	A22/IRQ/A/MAY/97	A/TAI/2013	Protection - 80% at 21 dpv 0% at 7 dpv	Jul-16	Sep-16	
Pig trial 5	Pigs	Test the efficacy of intradermal vaccination using A22/IRQ		To be completed in 2017	Apr-17	Sep-17	NCFAD, Canada

dpv - days post vaccination; dpc - days post challenge; PD50 - 50% protective dose; TCID50 - tissue culture infectious doses; RT-qPCR - reverse transcriptase quantitative polymerase chain reaction; NSP - non-structural proteins; AEC - Animal Ethics Committee; IHC - immuno-histochemistry; CB – coronary band; INP – intranasal pharyngeal instillation; AI – aerosol infection; DC – direct contact
SENASA - National Food Safety and Quality Service; PIADC - Plum Island Animal Diseases Centre; CVI - Central Veterinary Institute; NCFAD - National Centre for Foreign Animal Diseases; NAVETCO - National Veterinary Company; RAHO6 - Regional Animal Health Office 6

4.1 Vaccine efficacy trials in cattle

4.1.1 Introduction

Cattle are the most economically important livestock species in Australia. They are highly susceptible to FMDV infection, partly due to their large respiratory volume. Infected cattle develop painful blisters and erosions in the mouth, which stop them from eating, and on the coronary band and feet that can lead to lameness. In dairy cattle, there can be a severe drop in milk production. Although most animals recover within two weeks, some never recover their previous or potential levels of production and condition. Some animals may become persistently infected with FMDV (the so-called 'carriers'), and there is a perceived risk that these persistently infected animals may infect susceptible animals, although this has never been demonstrated in experimental studies.

Vaccination in cattle has been shown to be an effective way of controlling FMD in endemic regions, and for preventing FMD in regions at risk of infection from neighbouring FMD-affected areas. In some endemic regions, vaccinating cattle only (and not other FMD susceptible species such as sheep, goats and pigs) is sufficient to prevent outbreaks. Modelling has shown that, for most outbreak scenarios in regions historically free of FMD without the use of vaccination, emergency vaccination of only cattle, in combination with other control measures, could assist in containing an outbreak.

Five vaccine efficacy trials have been completed in cattle since 2012 as part of this project: three with serotype O vaccines and two with serotype A vaccines (Table 4.2). All vaccines tested were high-potency vaccines similar to those which would be used during an outbreak in Australia (>6PD_{50}). All cattle were challenged by injecting FMDV into the dermal layers of the tongue (IDL).

4.1.2 Comparison of the potency of O1 Manisa and O/SKR/2010 high payload vaccines in cattle using O/SKR/2010 as challenge virus

In 1998, a variant strain of FMDV serotype O, South East Asian topotype (O/SEA/Mya-98), emerged from Myanmar and spread across SEA causing major outbreaks in Thailand, Vietnam, Lao PDR and Cambodia. Due to reports of vaccine failure using the O1 Manisa vaccine, Merial developed a new vaccine strain, using the outbreak virus O/SKR/2010.

Antigen matching studies performed at the World Reference Laboratory (WRL), Pirbright Institute, showed apparently poor relationships between the O1 Manisa vaccine and outbreak viruses (r1<0.3).

4.1.2.1 Methods

The initial trial was a potency test as described by the OIE and European Pharmacopeia.

Virus: The challenge virus, O/SKR/2010, was provided by Merial, UK.

Vaccine: Monovalent O1 Manisa and O/SKR/2010/vac double oil adjuvant vaccines, both at an antigen payload of >6 $PD_{50,}$ were prepared by Merial, UK.

Experimental Design:

Fifteen Holstein-Friesian cross-bred cattle were randomly allocated to each of the 2 vaccines (monovalent double-oil adjuvant O1 Manisa and O/SKR/2010) and further divided into groups of 5 animals each (Table 4.3).

Table 4.3: Experimental design

Group No.	No of Animals	Vaccine	Vaccine Dose	Challenge
1	5	O1 Manisa	Full (2 ml)	O/SKR/2010
2	5	O1 Manisa	¼ (0.5 ml)	O/SKR/2010
3	5	O1 Manisa	1/16 (0.125 ml)	O/SKR/2010
4	5	O/SKR/2010	Full (2 ml)	O/SKR/2010
5	5	O/SKR/2010	¼ (0.5 ml)	O/SKR/2010
6	5	O/SKR/2010	1/16 (0.125 ml)	O/SKR/2010
7	3	Unvaccinated controls	-	O/SKR/2010

The animals were challenged with 10,000 cattle infectious dose (CID_{50}) virus and were observed daily for appearance of clinical signs of FMD up to 8 days post challenge (dpc) and again at termination at 14 dpc.

Clotted blood for serum (for detection of viral RNA and antiviral antibodies) was collected on 0, 7, 13, 14, 18 days post vaccination (dpv) and 0-8, 10 and 14 dpc. Saliva samples (for detection of viral RNA) were collected on 0–8, 11 and 14 dpc. Oro-pharyngeal samples for detection of live virus and viral RNA were collected on 0, 2, 4, 6, 8, 11 and 14 dpc.

4.1.2.2 Results

None of the cattle that received a full dose of O/SKR/2010/vac showed any clinical lesions up to 8 dpc, while two of those vaccinated with ¼ dose and three of those with 1/16 dose demonstrated disseminated disease (Fig. 4.1). This resulted in a PD_{50} = 7.94.

In the heterologous challenge cattle were vaccinated with O1 Manisa vaccine and challenged with O/SKR/2010. As with the homologous challenge, all five cattle that received a full dose were protected, however, three of those that received the ¼ and all five that received the 1/16 dose had clinical signs indicating FMD infection resulting in a PD_{50} of 3.47.

Although the O1 Manisa vaccine had a lower protective potential compared to the homologous vaccine, it was still considered sufficiently potent to protect animals from infection. In addition, for both vaccines, all the cattle that received the full dose of vaccine were fully protected against clinical disease (see Table 4.8; Appendix 2). Merial found in subsequent work that the new vaccine strain, O/SKR/2010/vac, did not cross-react with other viruses from the same lineage and ceased the development process.

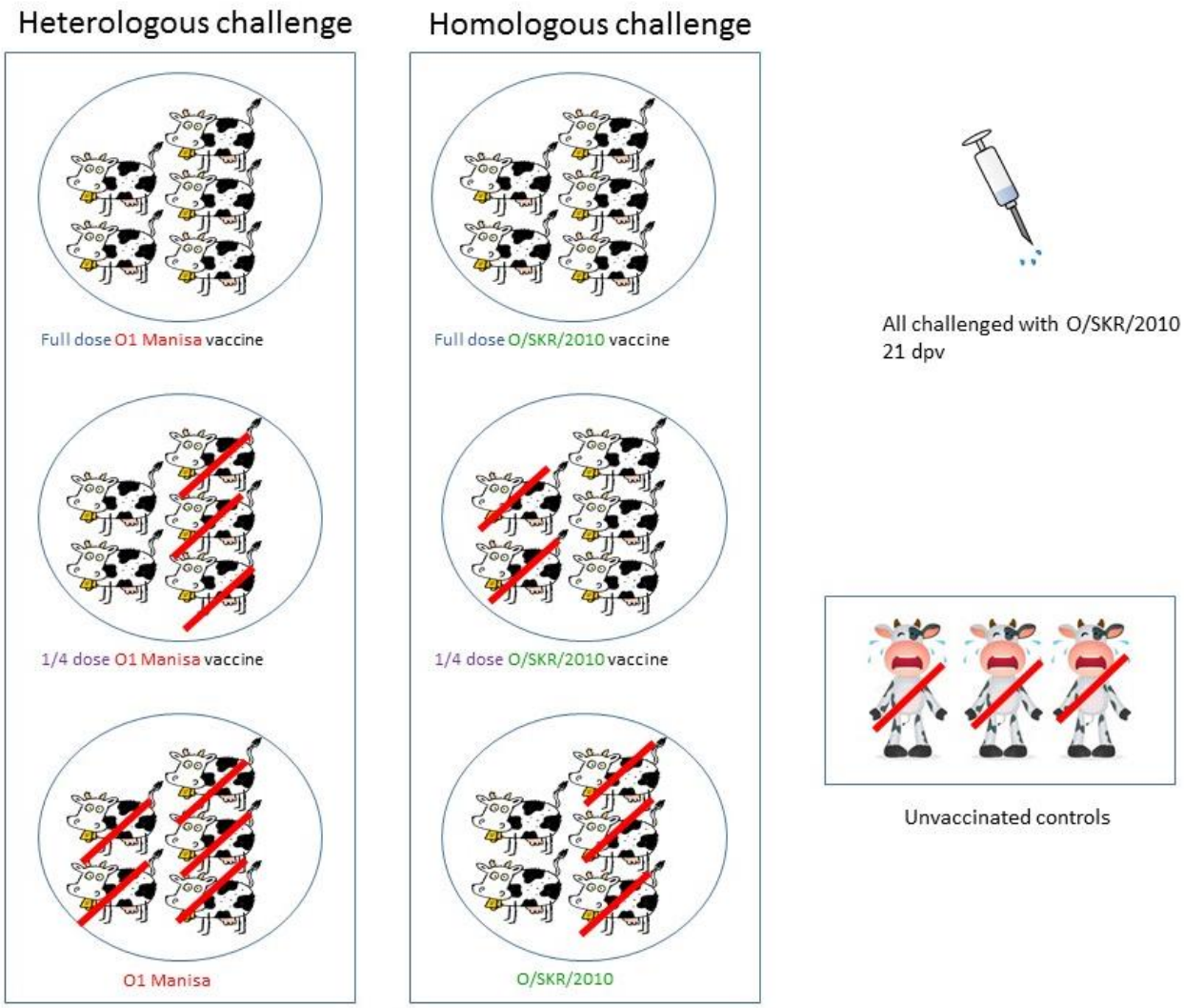

Fig. 4.1: Schematic representation of the potency trials performed to test O1 Manisa and O/SKR/2010/vac vaccines against challenge with O/SKR/2010 in cattle.
Red lines indicate the cattle with clinical disease, dpv – days post vaccination.

4.1.3 Vaccine efficacy trial in cattle with FMDV O1 Manisa monovalent vaccine using FMDV O/SKR/2010 as challenge virus

A next trial was performed as a follow-up of the previous trial where the cattle were challenged at 21 dpv, and the commercially available O1 Manisa vaccine provided clinical protection to all animals. The question remained whether or not the vaccine would protect at earlier time points post vaccination as cold be expected during an outbreak. In a subsequent trial, cattle were challenged at 4 and 7 dpv.

4.1.3.1 Methods

Virus: The challenge virus was O/SKR/2010, provided by Merial, UK.

Vaccine: Monovalent O1 Manisa double oil adjuvant vaccine at an antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design:

Twenty-two 12–18 month old male Hereford cattle were used in three groups (Table 4.4). All cattle were challenge by IDL inoculation with 10^4 MID_{50} in 1 ml, administered in approximately four locations in the tongue.

Table 4.4: Experimental design

Group	No. of animals	Vaccine	Challenge
V7	10	O1 Manisa	O/SKR/2010, 7 dpv
V4	10	O1 Manisa	O/SKR/2010, 4 dpv
UV	2	None	O/SKR/2010

All of the cattle were monitored for the development of clinical signs such as lameness, salivation, nasal discharge and the development of vesicles. Clotted blood for RT-qPCR (to detect viral RNA) and serology (virus neutralisation test (VNT), non-structural protein (NSP) ELISA) and nasal and saliva swabs (for RT-qPCR) were collected at -7 dpc (V7 cattle only), -4 dpc (V7 and V4 cattle only) and at 0, 1, 3, 5, 7, 10, 14, 21 and 28 dpc (all cattle), at which point the experiment was terminated. Oro-pharyngeal fluid was collected at -0, 7, 10, 14, 21 and 28 dpc.

4.1.3.2 Results

None of the cattle were protected at 4 dpv, while 50% of cattle were protected at 7 dpv, indicating that partial early protection is possible with the O1 Manisa vaccine (see Table 4.8). Vaccination did not prevent the development of persistent infection in the early protection groups (4 and 7 dpv), but reduced the amount of virus that was excreted from the nasal secretions. The results showed that high-potency O1 Manisa vaccine can be used in case of an outbreak but only with partial protection in the first week or two after vaccination (Appendix 3).

4.1.4 Vaccine efficacy trial in cattle with monovalent O3039 and combination O3039/O1 Manisa vaccines using O/ALG/3/2014 as challenge virus

During 2013, a new sub-lineage of serotype O, O/ME-SA/Ind-2001d lineage, previously restricted to South Asia, caused widespread outbreaks in the Middle East and Northern Africa. The predictions from the WRL showed a poor antigenic match with O1 Manisa vaccine strain (r1<0.3). Independent of the FMD RMP, the Pirbright Institute performed a potency test using a high potency O1 Manisa vaccine (>6 PD_{50}) against this newly emerged variant (O/ALG/2014) and, although the vaccine had an acceptable final protective value (PD_{50}=3.47), two of the five cattle that received the full dose of vaccine were not protected against clinical disease at 21 dpv. This trial indicated that the O1 Manisa vaccine could be used in an outbreak and should provide some protection in an emergency situation, but that a more efficacious vaccine would be preferable. For this reason, Merial is suggesting a combination serotype O vaccine containing both O1 Manisa and O3039, which costs double.

A vaccine efficacy trial with O3039 vaccine alone and another with O3039 in combination with O1 Manisa were performed in cattle, with animals challenged at 7 and 21 dpv with a virus from the O/ME-SA/Ind-2001d lineage.

4.1.4.1 Methods

Virus: The challenge virus, O/ALG/3/2014, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent O-3039 or a combination with O3039 and O1 Manisa double oil adjuvant vaccines at antigen payloads of >6 PD_{50} were prepared from the AVB by Merial, UK.

Experimental Design:

Twenty three Holstein-Friesian heifers were divided into five groups (Table 4.5). Vaccinated animals received 2 ml vaccine intramuscularly. Animals were challenged by IDL inoculation with 10^5 plaque forming units (pfu) (equivalent to 10^4 CID_{50}) with cattle-derived FMDV O/ALG/3/2014.

Table 4.5: Experimental design

Vaccine	Group	Day of challenge
O-3039 + O1 Manisa	1 (5 cattle); O/Combo-21	21 dpv
	3 (5 cattle); O/Combo-7	7 dpv
O-3039	2 (5 cattle); O3039-21	21 dpv
	4 (5 cattle); O3039-7	7 dpv
Unvaccinated controls	5 (3 cattle); UVC	0 dpv

The animals were observed daily post vaccination and post challenge. Clinical signs for FMD were monitored between 1 and 7 dpc. Temperatures were monitored using rectal probes on all days.

Clotted blood was collected on -21, -18, -14, -10, -7, -4, 0, 1–7, 11, 14, 18, 21, 25, 28 and 32 dpc (30 ml each) for RT-qPCR (to detect viral RNA) and serology (VNT, NSP ELISA). At 0, 1–7, 11, 14, 18, 21, 25, 28 and 32 dpc, saliva and nasal swabs were collected for RT-qPCR. Oro-pharyngeal fluid samples were collected on 0, 11, 14, 18, 21, 25, 28 and 32 dpc.

4.1.4.2 Results

All animals in the 21 dpv group were protected, irrespective of the vaccine used. Partial protection (60–80%) was observed in cattle that were vaccinated and challenged at 7 dpv (Fig. 4.2; see Table 4.8). Although there was evidence that some cattle became persistently infected (intermittent detection of virus/viral RNA in the probang samples), there was less development of persistent infection post challenge when vaccinated with either vaccine (Appendix 4).

There was no statistically significant difference in the level of clinical protection between the three vaccines (O1 Manisa monovalent - done by the Pirbright Institute; O3039 monovalent and O1 Manisa and O3039 bivalent vaccines – done by the FMD RMP) using the same challenge virus. A comparison of the virus excretion results between the different experiments is needed to determine if any of these vaccines resulted in decreased shedding of virus before a recommendation can be finalised.

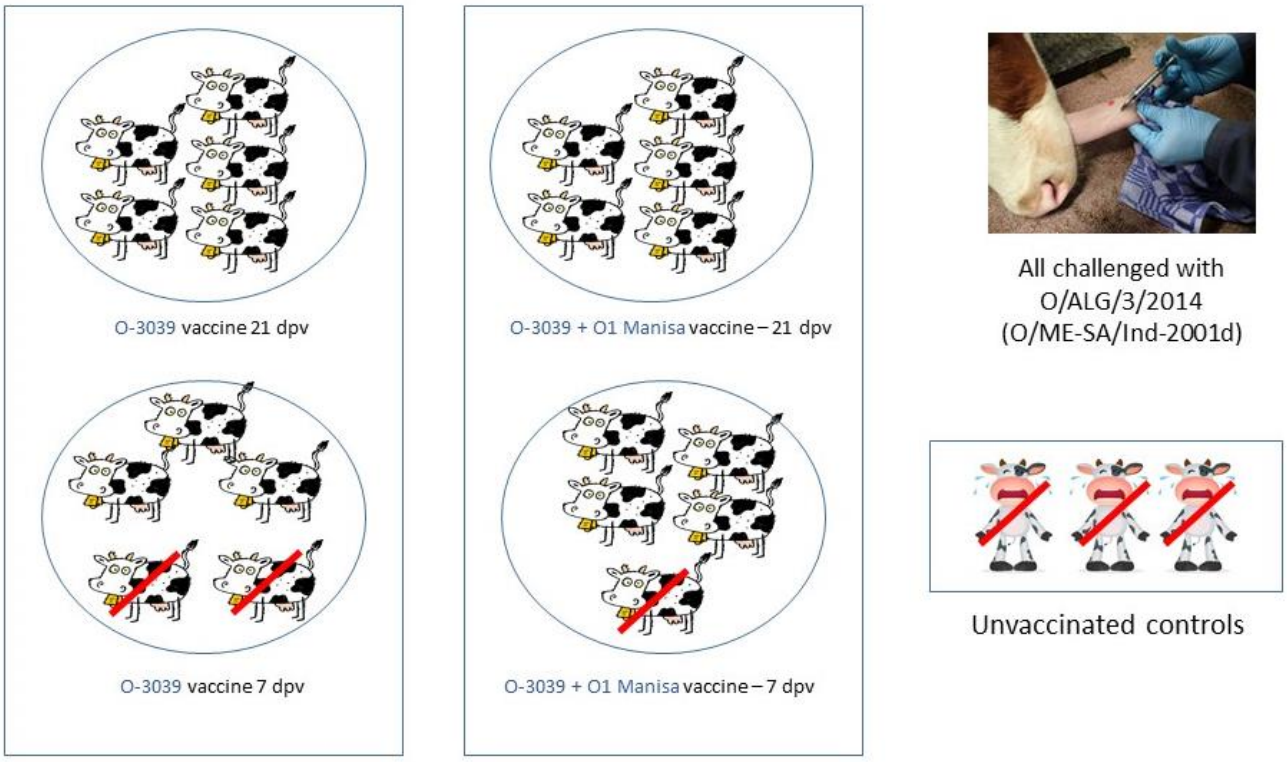

Fig. 4.2: Schematic representation of the potency trials performed to test O3039 and O1 Manisa vaccines against challenge with O/ALG/2014 in cattle.
Red lines indicate the cattle with clinical disease; dpv – days post vaccination.

4.1.5 Vaccine efficacy trial in cattle with monovalent A22/IRQ and A/MAY/97 vaccines using A/VIT/15/2012 as challenge virus

During 2011–2012, a new variant of the A/SEA-97 lineage emerged in Thailand leading to vaccine failures. This prompted the vaccine manufacturer in Thailand to develop a new vaccine strain for local use. Australia does not have access to this new Thai vaccine strain[13] and it was essential to determine whether or not the serotype A vaccine strains in the AVB, A/MAY/97 and A22/IRQ, would protect against this newly emerged virus. Work done by the FMD RMP team showed poor to moderate antigenic match in vitro (r1<0.19 for A/MAY/97 and 0.19–0.39 for A22/IRQ).

4.1.5.1 Methods

Virus: The challenge virus A/VIT/15/2012 was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent A22/IRQ and A/MAY/97 double oil adjuvant vaccines at antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design:

Twenty three Holstein-Friesian heifers were divided into 5 groups (Table 4.6). Vaccinated animals received 2 ml vaccine intramuscularly. Animals were challenged by IDL inoculation with 10^5 pfu (equivalent to 10^4 CID_{50}) with FMDV A/VIT/15/2012. Vaccination was staggered across the different groups such that all the animals were challenged on the same day.

Table 4.6: Experimental design

Vaccine	Group	Day of challenge
A22/IRQ	1 (5 cattle)	21 dpv
	3 (5 cattle)	7 dpv
A/MAY/97	2 (5 cattle)	21 dpv
	4 (5 cattle)	7 dpv
Unvaccinated controls	5 (3 cattle)	0 dpv

The animals were observed daily post vaccination and post challenge. Clinical signs for FMD were monitored between 1–7 dpc. Temperatures were monitored using rectal probes on all days.

Clotted blood was collected on -21, -18, -14, -10, -7, -4, 0, 1, 3, 5, 7, 10, 14, 21, 28 and 35 dpc (30 ml each) for serology; 150 ml clotted blood was collected on 0 dpc from cattle vaccinated 21 days earlier as standard for r-value determination. At 0–7, 10, 14, 21, 28, and 35 dpc mouth swabs were collected using Salivitte tubes and saliva was extracted using 0.5 ml of phosphate buffered saline. Nasal swabs were collected on the same days using sterile cotton swabs into 2 ml of phosphate buffered saline. The probang samples were collected on -7, 0, 7, 10, 14, 21, 24, 28, 31 and 35 dpc.

[13] Only the commercially available strains in the AVB are licenced for use in Australia.

4.1.5.2 Results

Both vaccine strains provided full protection at 21 dpv, with partial early protection by 7 dpv (80% protected when vaccinated with A22/IRQ and 60% protected with A/MAY/97 – which is not a statistically significant difference) (Fig. 4.3; see Table 4.9). Although there was evidence that some cattle became persistently infected with intermittent detection of viral RNA in probang samples, the levels of viral RNA were at a very low level (Appendix 5). Therefore, in the event of an incursion with this variant of virus, either A/MAY/97 or A22/IRQ could be used, as either would reduce the clinical signs and virus shedding in oral and nasal secretions.

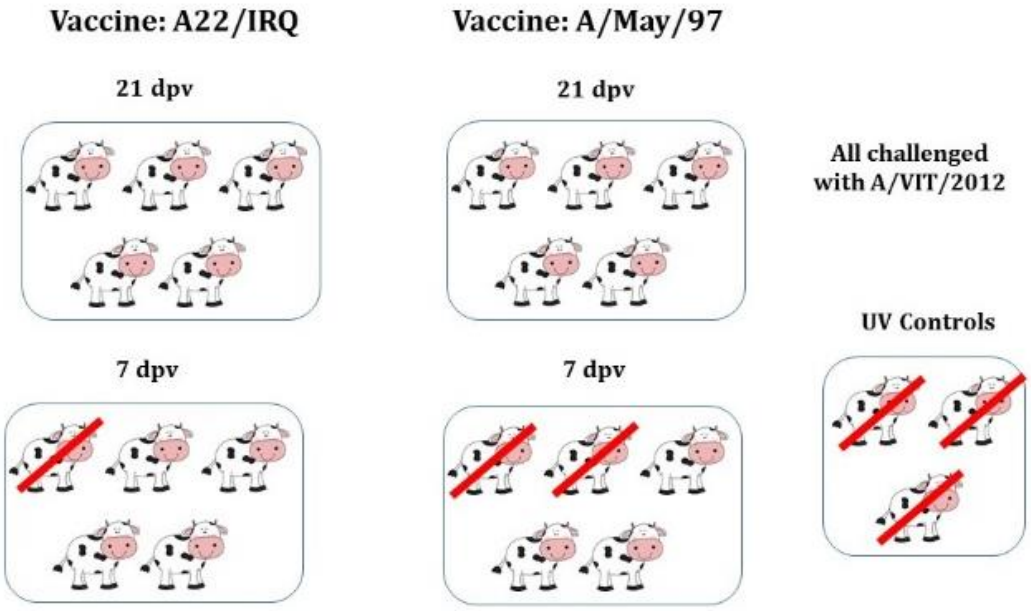

Fig. 4.3: Schematic representation of the potency trials performed to test A22/IRQ and A/MAY/97 vaccines against challenge with A/VIT/2012 in cattle.
Red lines indicate the cattle with clinical disease; dpv – days post vaccination; UV - unvaccinated.

4.1.6 Vaccine efficacy trial in cattle with monovalent A22/IRQ and A/MAY/97 vaccines using A/ASIA/G-VII as challenge virus

4.1.6.1 Introduction

Recently, FMD outbreaks have occurred in large parts of West Eurasia, caused by a new lineage of serotype A, A/ASIA/G-VII, previously restricted to South Asia (Indian subcontinent). There were reports from several countries that the established FMD vaccines used in this region did not seem to provide sufficient protection against clinical disease, while the cross-protective capacity of vaccines, as predicted by serological tests at the WRL, was poor ($r1<0.3$).

The WRL performed a potency test using the standard potency, commercially available vaccine in Saudi Arabia that contains seven different strains from various serotypes. For serotype A it contains A/IRN/05 ($r1 = 0$) and A/Saudi/95 ($r1 \sim 0.2$). The potency study found that only 56% of the cattle were protected against challenge with a 2015 isolate from Iran (A/ASIA/G-VII lineage).

Since this was of concern to all vaccine banks, the Pirbright Institute, CVI Lelystad and AAHL collaborated to identify whether or not the AVB vaccine strains, A22/IRQ and A/MAY/97, would protect against virulent challenge with strain A/ASIA/G-VII.

4.1.6.2 Methods

Virus: The challenge virus, A/IRN/22/2015 that belongs to the A/ASIA/G-VII lineage, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent A22/IRQ and A/MAY/97 double oil adjuvant vaccines at antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design:

Vaccinated animals received 2 ml vaccine intramuscularly. Two groups of 7 cattle each were vaccinated with the monovalent vaccines respectively and challenged by IDL inoculation with 10^5 pfu (equivalent to 10^4 CID_{50}) with FMDV A/IRN/22/2015 at 21 dpv (Table 4.7). Three cattle were left unvaccinated and also challenged.

Table 4.7: Experimental design

Vaccine	Group	Day of challenge
A22/IRQ	1 (7 cattle)	21 dpv
A/MAY/97	2 (7 cattle)	21 dpv
Unvaccinated controls	3 (3 cattle)	0 dpv

The animals were observed daily post vaccination and post challenge. Clinical signs for FMD were monitored between 1–8 dpc. Temperatures were monitored using rectal probes on all days.

Clotted blood was collected on -21, -18, -14, -10, -7, -4, 0, 1-8 dpc (30 ml each) for serology; 250 ml clotted blood was collected on 0 and 8 dpc from cattle vaccinated 21 days earlier as standard for r-value determination. Nasal swabs were collected on 0-8 dpc using sterile cotton swabs into 2 ml of phosphate buffered saline.

4.1.6.3 Results

The cattle vaccinated with A/MAY/97 were better protected than those vaccinated with A22/IRQ (72% and 29% protection, respectively). Virus excretion was less in the A/MAY/97 group than the A22/IRQ and unvaccinated groups. Final laboratory results are awaited[14].

This apparent deficiency in protection at 21 dpv leaves some cause for concern, but A/MAY/97 still protected 5 of the 7 cattle. There are no commercial vaccine strains currently available to fully protect against this lineage and A/MAY/97 seems to be the most promising vaccine strain. Merial is developing a new strain and the FMD RMP team will provide feedback to the AVB when results are available.

4.1.7 Detection of virus/viral RNA in nasal and oral swabs

In all the cattle trials, oral swabs were regularly collected (e.g. 0–10, 14, 18, 21, 24, 28, 31 and 35 dpc) to see if virus and /or viral RNA could be detected. Viral RNA was detected

[14] As part of a collaboration between the Pirbright Institute and CVI, the laboratory assays for this trial will be performed by staff at CVI. In addition, one more challenge experiment will be performed in the first half of 2017 to test other vaccine strains available to international vaccine banks, as well as a new vaccine strain under development at Merial.

between 1 and 7 dpc in all the cattle, possibly due to development of lesions in the tongue after IDL inoculation.

Nasal swabs, collected at the same time points, showed that virus/viral genome could be detected between 1–10 dpc from both vaccinated and unvaccinated cattle. Clinical material such as epithelium and/or vesicular fluid could be collected from mouth samples only up to 3–4 days post infection. Swabs therefore provide a much wider window for detecting infected cattle and can be an alternative/additional sampling method for surveillance purposes.

4.1.8 Conclusions

The cattle trials aimed to:

- Determine if high-potency vaccines provided clinical protection against challenge within a week after vaccination and 21 dpv,
- Determine if a newly developed serotype O vaccine would provide better protection than the commercially available vaccines,
- Determine if vaccines would decrease virus excretion,
- Determine if vaccines have an impact on persistent infection, and
- Determine if oral and nasal swabs could be used to detect virus/viral RNA in vaccinated and challenged cattle.

The continual emergence of new viral variants and the spread of variants beyond their previous distribution ranges remain a concern when estimating the potential of vaccines to assist with disease control. In the majority of the trials performed in cattle in this project, the high-potency vaccines provided clinical protection at 21 dpv, and partial protection at 7 dpv (Tables 4.8 and 4.9). The A/ASIA/G-VII lineage is of most concern at present, as the best protection we've seen from our available vaccines was about 70%.

In all the trials performed, vaccination decreased the amount of virus excreted into the environment, which could be useful to assist with control of spread during an outbreak.

In most cases the vaccinated and challenged cattle became persistently infected (virus or viral RNA detected in probang scrapings 28 dpc). The unnatural route of challenge used in these studies (injection of virus into the tongue), necessary to ensure that all challenged animals received the same infectious dose, could have resulted in the persistence of virus in the oropharyngeal area. Since it was not possible to keep the animals for longer than 30–35 dpc due to costs, the impact of vaccination on persistent infection over a longer period of time could not be determined (i.e. it was not possible to determine if vaccinated cattle maintained virus for shorter periods of time compared to unvaccinated cattle). It was also not possible to determine if persistently infected vaccinated cattle could transmit the disease. These experiments are difficult to perform as they need large numbers of animals or long periods in containment that are prohibitively expensive. Earlier studies have shown that the percentage of animals that remain persistently infected after an outbreak of FMD is up to 50% in unvaccinated herds, decreasing considerably with vaccination. The risk of transmission from these animals is very low[15].

It was found that nasal and oral swabs are useful to detect virus/viral RNA in infected as well as vaccinated and infected cattle. These swabs were positive in some animals as early as one day post infection, and in some remained positive for up to 10 days. In addition, swabs

[15] Tenzin et al., 2008; Risk Analysis 28(2): 303-309

were often positive for virus/RNA in the absence of obvious clinical signs. Swabs would therefore be very useful during an outbreak to detect infection and for surveillance.

Table 4.8: Summary of the vaccine efficacy trials in cattle using vaccines against serotype O. Table indicates the proportion protected against clinical disease when challenged with two different serotype O isolates at various time points post vaccination.

Vaccine	Virus	4 dpv	7 dpv	21 dpv	PD_{50}
O1 Manisa	O/SKR/2010	-	-	100%	3.47
O/SKR/2010/vac	O/SKR/2010	-	-	100%	7.94
O1 Manisa	O/SKR/2010	0%	50%	-	-
O3039	O/ALG/2014	-	60%	100%	-
O3039 + O1 Manisa	O/ALG/2014	-	80%	100%	-

dpv – days post vaccination; PD_{50} – 50% protective dose.

Table 4.9: Summary of the vaccine efficacy trials in cattle using vaccines against serotype A. Table indicates the proportion protected against clinical disease when challenged with two different serotype A isolates at various time points post vaccination.

Vaccine	Virus	7 dpv	21 dpv
A/MAY/97	A/VIT/2012	60%	100%
A22/IRQ	A/VIT/2012	80%	100%
A/MAY/97	A/IRN/2015	-	72%
A22/IRQ	A/IRN/2015	-	29%

dpv – days post vaccination

4.2 Vaccine efficacy testing in sheep

4.2.1 Introduction

Sheep represent a large component of the world's FMD-susceptible livestock and, in some recent outbreaks, including the UK in 2001, sheep were important in spreading the infection. The clinical signs of FMD in sheep can be severe, but are commonly mild or inapparent, facilitating spread of the virus if cases go undetected. Furthermore, sheep, like other ruminants, can become persistently infected with FMDV. Vaccination using high potency vaccines has been shown to be effective in protecting FMD-susceptible livestock challenged as early as 4 dpv, reducing virus excretion, lowering the likelihood of transmission and potentially minimising the duration and intensity of an outbreak.

4.2.2 Defining the most effective route of infection for sheep

Although sheep are epidemiologically important in maintenance and spread of FMD, vaccines are usually tested in cattle following a standardised methodology. However, for sheep such standard methods have not been described and, before testing of vaccines could commence, it was necessary to first determine which route of challenge would result in reproducible clinical disease. This is essential for vaccine testing as the method of challenge should be as natural as possible, but result in every challenged animal receiving the same infectious dose and unvaccinated control animals developing clinical disease.

Direct contact between infected and susceptible animals is the most natural route of infection, but it is not possible to quantify the infectious dose nor ensure that each animal will even receive a standardised (infectious) dose.

4.2.2.1 Methods

The challenge virus, O/SKR/2010, belongs to the South East Asia (SEA) topotype, Myanmar 1998 (Mya-98) lineage. The virus was passed once in Holstein cattle before being used to inoculate sheep. Four different challenge methods were tested: direct contact (DC) with infected sheep, inoculation into the coronary band (CB), intranasal pharyngeal instillation (INP) and aerosol infection (AI) using 10^6 CID_{50} (Table 4.10). Each group consisted of four sheep.

Table 4.10: Experimental design

Route	No of sheep
Direct contact (DC)	4
Coronary Band (CB)*	4
Intranasal Pharyngeal Instillation (INP)	4
Aerosol Infectin (AI)	4

*also served as challenge for the DC group

4.2.2.2 Results

All routes led to infection, with CB and INP providing the most reproducible results. However, DC and AI also gave acceptable results (Appendix 6). The disadvantage of the DC method is the higher number of animals required for challenge experiments, while the AI route is very time consuming and not suitable for large experimental studies.

4.2.3 Dose response in sheep

4.2.3.1 Introduction

The recommended dose of most commercial oil adjuvanted FMD vaccines for small ruminants is half the cattle dose (i.e. 1 ml). However, potency tests for FMD vaccines are usually carried out in cattle, using 2 ml of vaccine and homologous virus challenge. There is little scientific evidence to support the use of half a cattle dose, except the size difference between the species. It is also not known how well vaccines will protect against clinical disease if the challenge is heterologous.

Using smaller doses means that more animals can be vaccinated with the vaccine available in the AVB, which could be advantageous during a more widespread outbreak. The FMD RMP team decided to determine what dose of vaccine would provide early clinical protection in sheep, using O1 Manisa vaccine against the O/SKR/2010 virus that caused severe outbreaks in South Korea.

4.2.3.2 Methods

Virus: The challenge virus, O/SKR/2010, was obtained from the PIADC, USA.

Vaccine: A high potency monovalent O1 Manisa double oil adjuvant vaccine (>6 PD_{50} in 2 ml, bovine dose) was prepared from the AVB by Merial Company Limited, United Kingdom.

Experimental Design:

Six groups of 7 animals each received different doses of vaccine (Table 4.11). In Phase 1, animals received 1 ml, 0.5 ml and 0.25 ml and were challenged with 400 µl FMDV O/SKR/2010-PI-BovP1 (10^6 $BTID_{50}$) coronary band intradermal inoculation 7 dpv. Four controls were not vaccinated and challenged. For Phase 2, 7 sheep per group were vaccinated with 2 ml of vaccine and challenged at 7 dpv, while two groups were challenged at 14 dpv after being vaccinated with 1 ml or 2 ml respectively.

Table 4.11: Experimental design

Phase (1)	Group	Vaccine dose (2)	Day of challenge (3)	No of animals
1	1	0.25 ml (1/4X)	7 dpv	7
1	2	0.5 ml (1/2X)	7 dpv	7
1	3	1 ml (1X)	7 dpv	7
2	4	2 ml (2X)	7 dpv	7
2	5	1 ml (1X)	14 dpv	7
2	6	2 ml (2X)	14 dpv	7
1 and 2	7	Naïve	-	8

All the sheep were observed and sampled for 6 or 35 days, for phases 1 and 2, respectively. In phase 1, sera were was collected on -7, 0-6 dpc, nasal swabs were collected on 0-6 dpc. In phase 2, sera were collected on -14, -7, 0-10, 14, 21, 28, 35 dpc, nasal swabs on 0-10, 14, 17, 21, 24, 28, 31, 35 and probang samples were collected on 14, 17, 21, 24, 28, 31, 35 dpc.

4.2.3.3 Results

Doses of 1 ml or less (0.5 ml and 0.25 ml) provided no protection at 7 dpv when challenged via the CB, but 1 ml protected 57% of sheep at 14 dpv. When vaccinated with 2 ml vaccine, 43% of sheep were protected at 7 dpv, and 57% at 14 dpv (Table 4.12).

Table 4.12: Summary of dose trial in sheep indicating the different doses, the day of challenge post vaccination and the proportion protected.

Dose (ml)	Day of challenge (post vaccination)	Protected
1	7	0
0.5	7	14%
0.25	7	0
1	14	57%
2	14	57%
2	7	43%
UV	0	0

Virus and viral RNA could be detected in nasal swab samples from most sheep between 1 and 6 dpc. Significantly lower virus excretion was observed in sheep vaccinated with higher doses or challenged at 14 dpv, compared to animals vaccinated with lower doses and the unvaccinated controls. Intermittent virus shedding in nasal secretions was detected between 1–35 dpc in some sheep in all vaccine groups and all the unvaccinated controls, but persistent infection only occurred in 2 out of 21 sheep in the vaccinated and challenged groups compared to 3 out of 4 unvaccinated and challenged sheep (Appendix 6).

At 14 dpv only moderate protection was seen with both 1 ml and 2 ml vaccine doses. The challenge virus used in this study (O/SKR/2010) is virulent in sheep (also in pigs; see section 4.3.3) and these results are attributed to the challenge route used, and high challenge dose. In another study with the same vaccine and challenge virus, 1 ml vaccine in sheep was sufficient to provide 100% protection as early as 4 dpv, when animals were challenged by direct contact with infected and diseased sheep (see below).

4.2.4 Vaccine efficacy trial in sheep with monovalent O1 Manisa vaccine using O/SKR/2010 as challenge virus

Following the dose trial which indicated only moderate protection when challenged via the CB, a vaccine efficacy study was performed in sheep using the DC challenge method and with just 4 days between vaccination with 1 ml O1 Manisa and exposure to O/SKR/2010, reflecting what could happen in an outbreak scenario.

4.2.4.1 Methods

Virus: The challenge virus was O/SKR/2010, provided by Merial, UK.

Vaccine: Monovalent O1 Manisa double oil adjuvant vaccine, at an antigen payload of >6 $PD_{50,}$ was prepared by Merial, UK.

Experimental Design:

In six replicate rooms, two donor sheep (infected by CB inoculation) were used to challenge two contact sheep (Fig. 4.4). The 12 donor sheep were each inoculated intradermally into the CB with $10^{6.5}$ $TCID_{50}$ of virus in a volume of 0.5 ml and kept with the vaccinated sheep for the duration of the trial.

All of the sheep were monitored for the development of clinical signs such as pyrexia, lameness and development of vesicles, daily to 14 dpc.

Clotted blood for serum (for detection of viral RNA and antiviral antibodies) was collected at -4 dpc, daily between 0 and 14 dpc and then weekly to 35 dpc. Nasal swab and saliva samples (for detection of viral RNA) were collected at the same time points. Oro-pharyngeal samples for detection of live virus and viral RNA) were collected on -1, 7, 10, 14, 21, 28 and 35 dpc.

4.2.4.2 Results

Ten of the twelve unvaccinated and infected donor sheep showed multiple lesions in the feet, mouth and tongue, and all were shedding virus from 1 to 14 days following infection.

The vaccinated in-contact sheep were all negative for anti-FMDV antibodies at the time of challenge. However, all were protected, with no lesion developing and no detection of virus or viral RNA in nasal or oral secretions throughout the experiment. All of these sheep seroconverted to FMDV structural proteins by 6 dpc, and there was no detection of antibodies to NSPs, indicating that the virus did not replicate in these vaccinated sheep (Appendix 7). Of the unvaccinated in-contact sheep, three were infected, as evidenced from seroconversion to NSP, but only two developed clinical disease.

The findings of this study indicated high-potency O1 Manisa vaccine was effective at protecting a proportion of sheep challenged with O/SKR/2010 by DC, just 4 dpv.

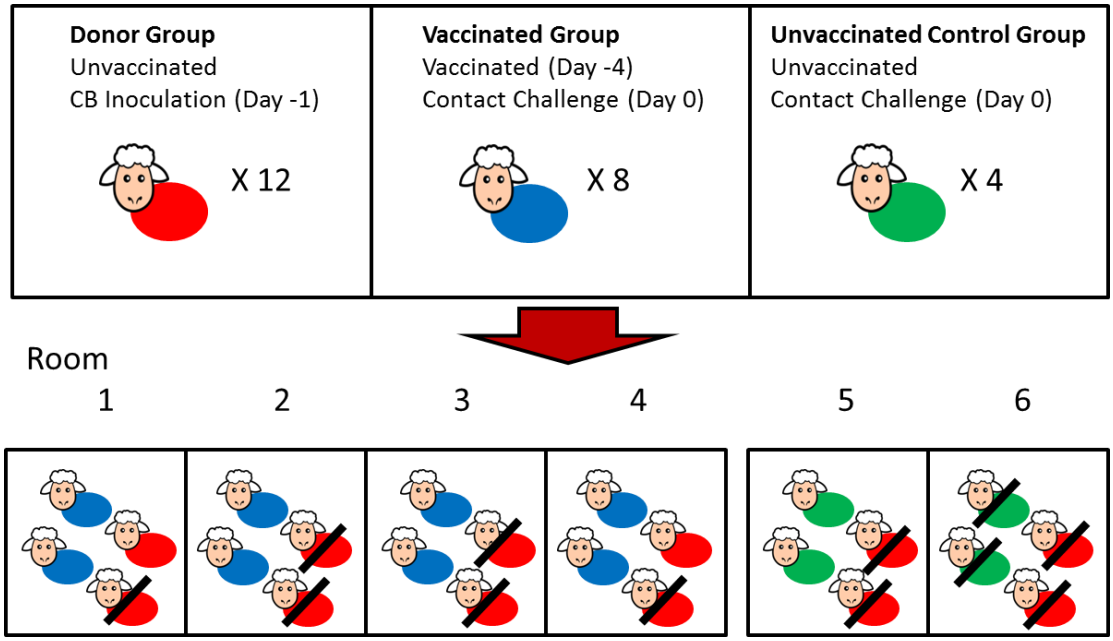

Fig. 4.4: Schematic representation of the potency trials performed to test O1 Manisa vaccine against challenge with O/SKR/2010 in sheep.
Black lines indicate the sheep with clinical disease.

4.2.5 Vaccine efficacy trial in sheep with monovalent A22/IRQ vaccine using A/VIT/15/2012 as challenge virus

Due to the emergence of new sub-lineages of serotype A viruses, it was necessary to test the efficacy of the A22/IRQ vaccine against a virus isolated in Vietnam in 2012 (A/VIT/15/2012).

4.2.5.1 Methods

Virus: The challenge virus, A/VIT/15/2012, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent A22/IRQ and A/MAY/97 double oil adjuvant vaccines at antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design:

The study design was similar to that used in the serotype O experiment, with DC used to provide a natural challenge method, 4 dpv. The study comprised six replicate rooms with three donor sheep, infected via the CB, and one vaccinated contact and one unvaccinated contact sheep per room (Fig. 4.5). Sheep belonging to donor group were challenged by coronary band injection using 100 µl (10^6 CID50/ml) of cattle passed A/VIT/15/2012 virus.

All of the sheep were monitored for the development of clinical signs such as pyrexia, lameness and development of vesicles, daily to 14 dpc.

Clotted blood for serum (for detection of viral RNA and antiviral antibodies) was collected at -4 dpc, daily between 0 and 14 dpc and then weekly to 35 dpc. Nasal swab and saliva samples (for detection of viral RNA) were collected at the same time points. Oro-pharyngeal

samples for detection of live virus and viral RNA) were collected on -1, 7, 10, 14, 21, 28 and 35 dpc.

4.2.5.2 Results

All CB inoculated donor animals developed generalised FMD with multiple lesions on the feet, mouth and tongue as early as 2 dpc. Nasal and oral swab samples from most donor sheep were positive for FMDV RNA from as early as 1 dpc, and in some animals up to 9 dpc, with viral loads generally decreasing from 4–6 dpc.

At the time of challenge (4 dpv), none of the vaccinated sheep had detectable neutralising antibodies to the vaccine, A22/IRQ. Despite this, 83% (5/6) of the vaccinated sheep were clinically protected, and only three excreted virus. All seroconverted to FMDV structural proteins by 9 dpc and to NSP by 14 dpc, the latter indicating that the sheep had become infected in the absence of clinical signs. Two animals became persistently infected. In contrast, five of the six unvaccinated contact sheep developed clinical FMD, and infectious virus and viral RNA were detected in oral and nasal swab samples from these 5 sheep between 5 and 10 dpc. Two of the unvaccinated contact sheep seroconverted to FMDV structural proteins by 9 dpc and a further three by 14 dpc (Appendix 8). Comparison of the effect of vaccination on viral excretion (detection of viral RNA in nasal and oral swab samples) showed no significant difference between the vaccinated and unvaccinated contact sheep.

These findings indicate that the A/VIT/15/2012 virus is pathogenic in sheep and, while vaccination with A22/IRQ did not provide sterile immunity after 4 days, it was able to reduce the occurrence of clinical disease, and it is possible that at later time points, vaccination may decrease virus excretion further.

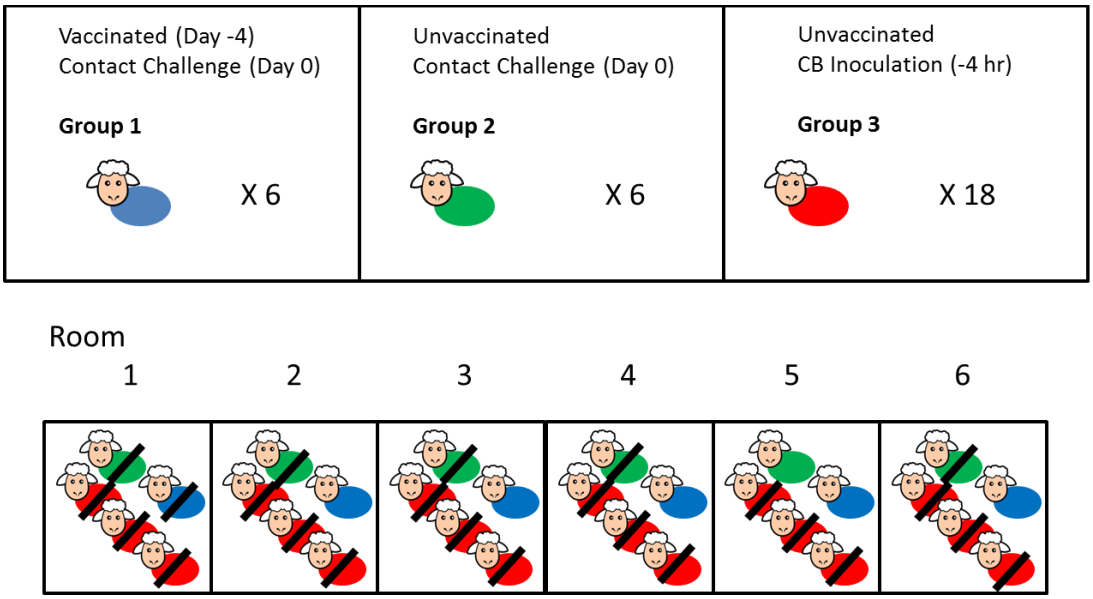

Fig. 4.5: Schematic representation of the potency trials performed to test A22/IRQ vaccine against challenge with A/VIT/2012 in sheep.
Black lines indicate the sheep with clinical disease; CB – coronary band; hr – hour.

4.2.6 Vaccine efficacy trial in sheep with monovalent Asia1/Shamir vaccine using Asia1/PAK/19/2014 as challenge virus

The AVB also contains a vaccine strain against Asia1. In addition to serotypes O and A, Asia1 is prevalent in countries considered high risk to Australia and has recently been responsible for a number of outbreaks in India, Bangladesh, Pakistan and Turkey. While a number of genetic lineages of Asia1 viruses exist, only one vaccine strain (Shamir) is available in the AVB. *In vitro* vaccine matching performed at the WRL identified a number of contemporary circulating viruses with poor or no matching to Asia1/Shamir. One of these strains, Asia1/PAK/19/2014, was selected for this study. The INP method was used as this provides a direct challenge, reduces animal numbers (as no donors are required) and is more close to a natural method of infection than CB inoculation.

4.2.6.1 Methods

Virus: The challenge virus, Asia1/PAK/19/2014, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent Asia1/Shamir double oil adjuvant vaccine at an antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design:

Twenty three sheep were divided into 5 groups (Table 4.13). Sheep were vaccinated with Asia1/Shamir 4, 7 or 21 days prior to INP challenge with $10^{4.5}$ CID_{50} Asia1/PAK/19/2014.

Table 4.13: Experimental design

Group	Vaccine	No. of Animals	Challenge
V21	Asia 1 Shamir	5	Yes - 21 dpv
V7	Asia 1 Shamir	5	Yes -7 dpv
V4	Asia 1 Shamir	5	Yes - 4 dpv
UV	Unvaccinated controls	5	Yes
VO	Vaccinated only controls	3	No

All of the sheep were monitored for the development of clinical signs such as pyrexia, lameness and development of vesicles, daily to 14 dpc.

Clotted blood for serum (for detection of viral RNA and antiviral antibodies) was collected at -4 dpc, daily between 0 and 14 dpc and then weekly to 35 dpc. Nasal swab and saliva samples (for detection of viral RNA) were collected at the same time points. Oro-pharyngeal samples for detection of live virus and viral RNA) were collected on -1, 7, 10, 14, 21, 28 and 35 dpc.

4.2.6.2 Results

All V21 sheep were protected from clinical disease and there was sterile protection (no anti-FMDV NSP antibodies detected) in 4/5 (80%) sheep. All V7 were protected from clinical disease and there was sterile protection in 2/5 (40%) sheep. In the sheep vaccinated 4 days prior to challenge, 4/5 were clinically protected but all developed antibodies to NSP, indicating infection and virus replication. There was no FMDV excretion detected in the V21 sheep and FMDV was detected in only the oral swab samples from one V7 sheep between 3

and 6 dpc. FMDV was detected in oral swabs from 3/5 and nasal swabs from 2/5 V4 sheep between 1 and 7 dpc (Table 4.14; Appendix 9).

In contrast, all unvaccinated sheep developed clinical FMD, were excreting FMDV in oral and nasal secretions between 1 and 7 dpc, and seroconverted to NSP. Two animals were euthanized before the end of the study for ethical reasons.

Table 4.14: Summary of the outcomes when sheep were vaccinated with Asia1/Shamir and challenged 21, 7 and 4 dpv.

Group	Clinical protection	Virus excretion	NSP response
V4	80%	60%	100%
V7	100%	20%	60%
V21	100%	None	20%
UV	0%	100%	100%

The number of persistently infected sheep was very low with virus only isolated from probang samples from one V21 sheep and one unvaccinated sheep beyond 28 dpc.

Vaccination with Asia1/Shamir was effective at protecting sheep from clinical and sub-clinical FMD following challenge with Asia1/PAK/19/2014, and at reducing virus excretion. However, the time between vaccination and challenge is important, with better protection observed after 1 week compared to 4 dpv.

4.2.7 Conclusion

The broad aims of testing vaccines in sheep were to:

- Determine if vaccination would clinically protect sheep against circulating viruses,
- Determine if vaccination decreases virus excretion,
- Determine if vaccination inhibits the development of persistently infected sheep,
- Determine if oral and nasal swabs could be used to detect virus/viral RNA for diagnosis of FMD, and
- Identify a suitable vaccine dose and challenge method in sheep.

Regardless of serotype or strain, high-potency FMD vaccines appear to be effective at minimising clinical disease in sheep, even with only a few days between vaccination and challenge (Table 4.15). While the reduction or prevention of virus excretion is less consistent, it is clear the ability of vaccination to limit excretion improves greatly with additional time for the development of an immune response to the vaccine. However, vaccination appears to have no effect on the occurrence of persistent infection in sheep.

Table 4.15: Summary of the vaccine efficacy trials in sheep using vaccines against serotype O, A and Asia1.
Table indicates the proportion protected against clinical disease when challenged with different isolates at various time points post vaccination.

Vaccine	Virus	4 dpv	7 dpv	21 dpv
O1 Manisa	O/SKR/2010	100%	-	-
A22/IRQ	A/VIT/2012	83%	-	-
Asia1/Shamir	Asia1/PAK/19/2014	80%	100%	100%

dpv – days post vaccination

The vaccine efficacy studies performed in sheep showed significant variation in severity of disease with different FMDV strains and when using different challenge methods. Overall, the INP method was effective and reproducible without being as severe and 'unnatural' as CB inoculation. Our findings also indicated a 1 ml dose of vaccine would be suitable for sheep when challenged by natural exposure to other infected sheep in contrast with the more severe CB infection.

Nasal and oral swabs can be used to detect virus / viral RNA in clinically and sub-clinically infected sheep for periods up to 14 days post infection and where the challenge is high, up to 35 days post infection. However, the number of positive samples decreased over time, indicating that as the window between infection and sampling increases, more animals would need to be tested to detect virus.

4.3 Vaccine efficacy testing in pigs

4.3.1 Introduction

Pigs are called the **amplifier** hosts of FMDV, because when they become infected they produce large quantities of virus. This virus is shed into the environment, including through virus-containing aerosols breathed out by the pigs.

Intensive pig farms have high densities of pigs, which, if infected with FMDV, could produce 'plumes' or 'clouds' of virus. Virus-laden aerosols pose a risk for FMD transmission to ruminants, particularly cattle, which are susceptible to infection by inhalation. Historically, it has been suggested that plumes of aerosols have dissipated over large distances causing distant outbreaks[16]. However, it is unlikely that the specific conditions required for this to happen will regularly occur in Australia.

Pigs are more resistant to aerosol infection than cattle, and FMD spread between pigs generally occurs by direct pig-to-pig contact.

Because of the risk infected piggeries pose to other FMD-susceptible species of livestock, it is essential that control options are available to either prevent infection of pigs, or to control spread of infection between pigs and from pigs to other animals such as cattle and sheep. In addition to movement controls and other quarantine measures, vaccination of pigs is an option that could assist with disease control.

4.3.2 Vaccine efficacy trial in pigs with monovalent O1 Manisa vaccine using O/VIT/2010 as challenge virus

Due to the outbreaks caused by serotype O viruses of the O SEA/Mya-98 lineage in SEA and South Korea, two trials focused on the efficacy of the O1 Manisa vaccine against challenge with viruses that belonged to this lineage but fell into different sub-lineages as a result of genetic differences. The first trial used a virus that was circulating in 2010 in Vietnam (O/VIT/2010).

[16]Gloster J, Sellers RF, Donaldson A. 1982. Long distance transport of foot-and-mouth disease virus over the sea. Vet Rec. 110:47-5

4.3.2.1 Methods

Virus: The challenge virus, O/VIT/2010, was obtained from the RAHO6, Ho Chi Minh City, Vietnam.

Vaccine: Monovalent O1 Manisa double oil adjuvant vaccine, at an antigen payload of >6 $PD_{50,}$ was prepared by Merial, UK.

Experimental Design:

Two groups of five pigs each were vaccinated and challenged by heel bulb inoculation with 10^5 $TCID_{50}$ pig derived virus by inoculation in two sites in the left-hind foot pad (0.2 ml/site); group 1a at 7 dpv, and group 2a at 4 dpv. Group 3a was not vaccinated, but challenged in a similar manner. Five unvaccinated pigs were kept in the same room, but not in direct contact with the challenged pigs to determine if vaccination sufficiently reduced virus excretion to prevent aerosol transmission in the same room (Table 4.16; Fig. 4.6).

Table 4.16: Experimental design

Group	No of animals	Vaccine	Challenge	Day of challenge	Route
1a	5	O1 Manisa	O/VIT/2010	7 dpv	Heel bulb
2a	5	O1 Manisa	O/VIT/2010	4 dpv	Heel bulb
3a	5	Unvaccinated and control	O/VIT/2010	0 dpc	Heel bulb
1b	5	Unvaccinated	O/VIT/2010	0 dpc	Indirect contact by housing with Group 1a
2b	5	Unvaccinated	O/VIT/2010	0 dpc	Indirect contact by housing with Group 2a
3b	5	Unvaccinated	O/VIT/2010	0 dpc	Indirect contact by housing with Group 3a

The groups were observed daily for the development of generalised disease and appearance of secondary lesions of FMD. Clinical material including nasal secretions, saliva and blood were collected daily for virus isolation. Rectal temperatures were recorded daily.

4.3.2.2 Results

The O1 Manisa vaccine clinically protected 60% of the pigs challenged with the O/VIT/2010 virus 4 dpv and 80% at 7 dpv. Vaccination also prevented virus transmission to pigs that were kept in the same room, but without direct contact (Fig. 4.6; Appendix 10). The overall virus excretion patterns from nasal and oral swabs between the vaccinated and unvaccinated pigs post-challenge was significantly different (P=0.0121), but there was no significant difference between the two vaccinated pig groups, 1a and 2a (P>0.05).

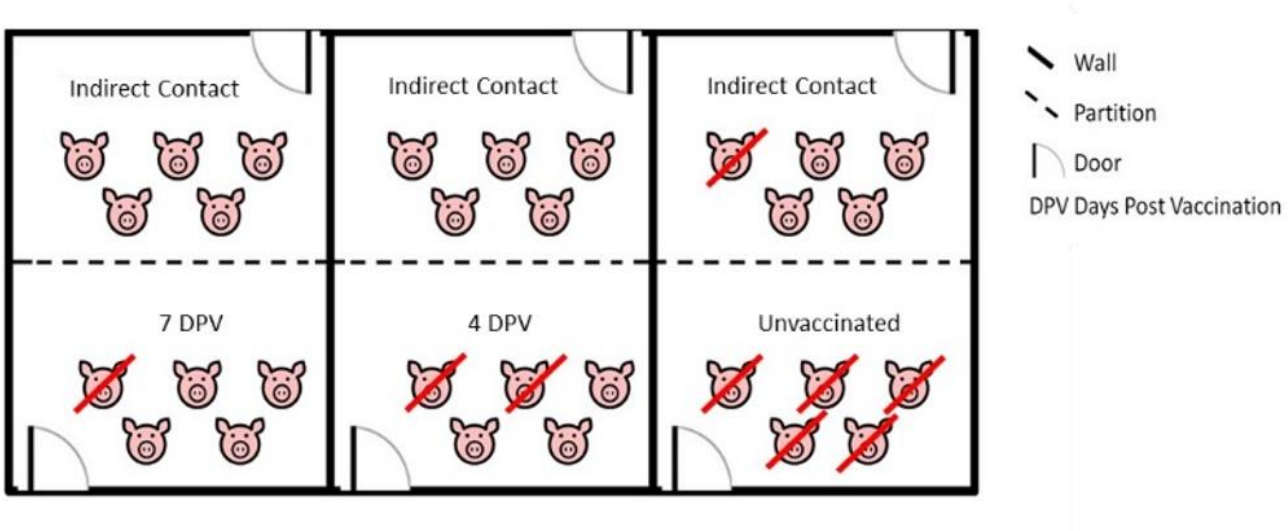

Fig. 4.6: Schematic representation of the vaccine efficacy trial performed to test O1 Manisa vaccine against challenge with O/VIT/2010.
Unvaccinated pigs were kept in the same rooms as vaccinated and challenged pigs. Red lines indicate the pigs with clinical disease.

4.3.3 Vaccine efficacy trial in pigs with monovalent O1 Manisa and O/SKR/2010/vac vaccines using O/SKR/2010 as challenge virus

The second serotype O trial in pigs used the virus that caused extensive outbreaks in 2010 in South Korea. Reports on the apparent failure of the O1 Manisa vaccine against the South Korean virus (O/SKR/2010) prompted Merial to develop a vaccine strain from the outbreak virus (O/SKR/2010/vac), which would be considered a vaccine homologous to the outbreak virus. Both the O1 Manisa and O/SKR/2010/vac were tested against the O/SKR/2010 outbreak virus.

4.3.3.1 Methods

Virus: The challenge virus, O/SKR/2010, was obtained from the Merial, UK.

Vaccine: Monovalent O1 Manisa double oil adjuvant vaccine, at an antigen payload of >6 $PD_{50,}$ was prepared by Merial, UK.

Experimental Design:

The experiment was performed in two phases where two groups of 5 pigs each (1 and 2) were vaccinated with O1 Manisa and O/SKR/2010/vac respectively and challenged at 5 dpv. Two unvaccinated controls were included (group 3a). The second phase followed the same design, but the pigs were challenged at 21 dpv (groups 4, 5 and 3b) (Table 4.17). Pigs were challenged with at least 10^4 $TCID_{50}$/ml of the cattle adapted challenge virus by inoculation in three sites in both of the hind feet pad, 0.1 ml in total.

Table 4.17: Experimental design

Group	Vaccine	No of pigs	Day of challenge	Challenge virus
1	O1 Manisa	5	5 dpv	O/SKR/2010
2	O/SKR/2010/vac	5	5 dpv	O/SKR/2010
3a	Unvaccinated control	2	5 dpv	O/SKR/2010
4	O1 Manisa	5	21 dpv	O/SKR/2010
5	O/SKR/2010/vac	5	21 dpv	O/SKR/2010
3b	Unvaccinated control	2	21 dpv	O/SKR/2010

The groups were observed daily for the development of generalised disease and appearance of secondary lesions of FMD. Clinical material including nasal secretions, saliva and blood were collected daily for virus isolation. Rectal temperatures were recorded daily.

4.3.3.2 Results

When pigs were vaccinated with monovalent O1 Manisa or O/SKR/2010/vac and challenged with O/SKR/2010 5 dpv, none were protected and all had to be euthanized at 3 dpc due to the severity of the disease. At 21 dpv, O1 Manisa provided 20% protection and O/SKR/2010/vac 60% protection (Table 4.18). Although the new vaccine provided improved protection, it was still not considered adequate for a high-potency vaccine (Appendix 11). The virus excretion patterns could not be studied in this trial due to humane intervention and the short duration of the study. Merial subsequently found that the new vaccine strain did not cross-react with other viruses from the same lineage and so did not continue with the production of this vaccine. Although both O/VIT/2010 and O/SKR/2010 belong to the same serotype O lineage (O SEA/Mya 98 lineage), the latter virus was more pathogenic in pigs.

Table 4.18: Summary of the vaccine efficacy trials in pigs using vaccines against serotype O
Indicating the proportion protected against clinical disease when challenged with two different serotype O isolates at various time points post vaccination.

Vaccine	Virus	4/5 dpv	7 dpv	21 dpv
O1 Manisa	O/VIT/2010	60%	80%	-
O1 Manisa	O/SKR/2010	0%	-	20%
O/SKR/2010/vac	O/SKR/2010	0%	-	60%

4.3.4 Vaccine efficacy trial in pigs with FMDV A/MAY/97 monovalent vaccine using FMDV A/VIT/08/2005 as challenge virus

A trial was performed using a virus that was first isolated in 2005[17] in Vietnam, with pigs vaccinated with A/MAY/97 challenged 4 and 7 dpv.

[17]Efforts to adapt a more recent virus to pigs were unsuccessful and we therefore chose an older isolate that belonged to the same lineage as the current viruses.

4.3.4.1 Methods

Virus: The challenge virus, A/VIT/08/2005, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent A/MAY/97 double oil adjuvant vaccine at antigen payload of >6 PD_{50} was prepared from the AVB by Merial, UK.

Experimental Design: Two groups of eight pigs each were vaccinated and challenged by heel bulb inoculation with 10^5 PID_{50} (50% pig infective dose) of the pig-derived virus by inoculation in two sites in the left-hind foot pad (0.2 ml/site); group 1a at 7 dpv, and group 2a at 4 dpv. Group 3a was not vaccinated, but challenged in a similar manner. Five unvaccinated pigs were kept in the same room, but not in direct contact with the challenged pigs to determine if vaccination sufficiently reduced virus excretion to prevent aerosol transmission in the same room (Table 4.19; Fig. 4.7).

Table 4.19: Experimental design

Group	No of animals	Vaccine	Day of challenge*	Route
1a	8	A Malaysia 97	7 dpv	Heel bulb
2a	8	A Malaysia 97	4 dpv	Heel bulb
3a	8	Unvaccinated and control	0 dpc	Heel bulb
1b	5	Unvaccinated	0 dpc	Indirect contact by housing with Group 1a
2b	5	Unvaccinated	0 dpc	Indirect contact by housing with Group 2a
3b	5	Unvaccinated	0 dpc	Indirect contact by housing with Group 3a

*The challenge virus was A/VIT/08/2005

The animals were observed and sampled daily for 14 days, and rectal temperatures recorded. Nasal secretions, saliva and faeces were collected daily. Clotted blood for serum was collected on −7, −4, 0, 5, 7, 10 and 14 dpc. Whole blood was collected in EDTA tubes on 0, 1–7, 9, 10 and 14 dpc.

4.3.4.2 Results

All the pigs were protected against clinical disease at 4 dpv, and 75% at 7 dpv (Fig. 4.7). Disease did not spread to unvaccinated pigs that were kept in the same room, but with no direct contact (Appendix 12). This apparent anomaly between the 4 and 7 dpv groups could be due to the small numbers of pigs used in the trial (n=8), and is not statistically significant. The overall virus excretion pattern was significantly different between the vaccinated and unvaccinated groups post-challenge (P=0.002).

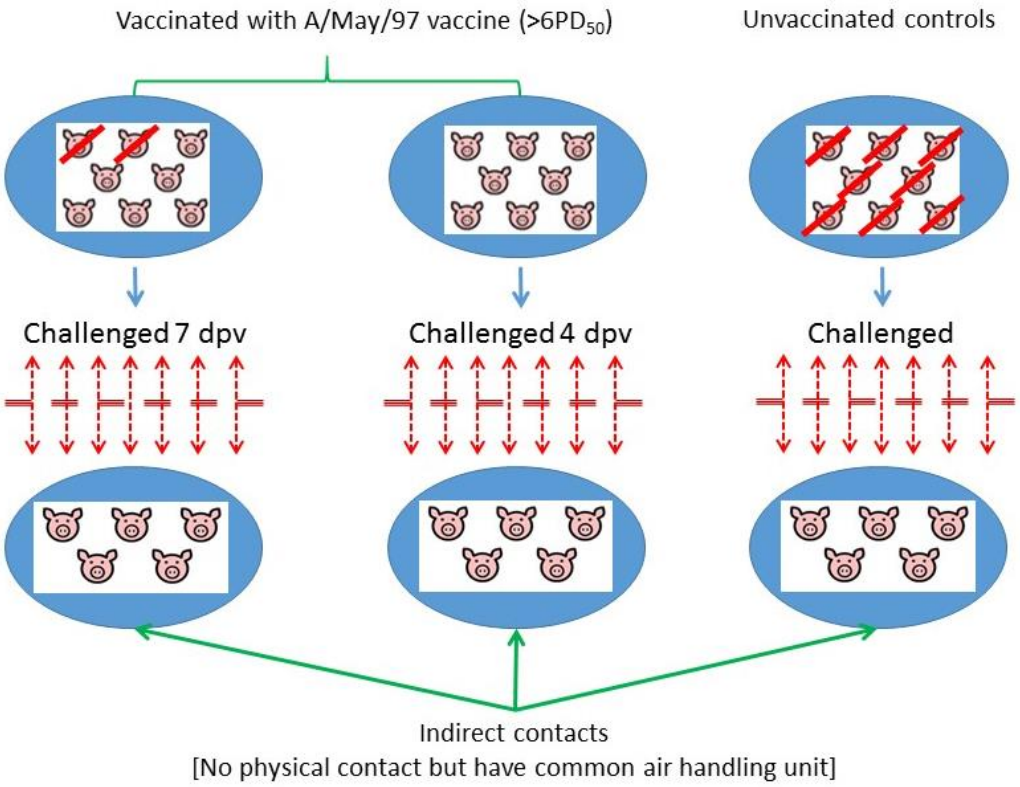

Fig. 4.7: Schematic representation of the vaccine efficacy trial performed to test A/MAY/97 against challenge with A/VIT/2005.
Unvaccinated pigs were kept in the same rooms as vaccinated and challenged pigs. Red lines indicate pigs with clinical disease; dpv – days post vaccination.

4.3.5 Vaccine efficacy trial in pigs with FMDV A22/IRQ, A/MAY/97 and A22/IRQ/A/MAY/97 combination vaccines using FMDV A/TAI/15/2013 as challenge virus

After the completion of the above-mentioned trial, a new variant of serotype A was detected in SEA that showed very poor match to the A vaccine strains included in the AVB, A/MAY/97 (r1 = 0.05) and A22/IRQ (r1 = 0.10). The efficacy of the two vaccines, as monovalent vaccines and as a bivalent combination, was tested using a virus isolated in 2013 in Thailand.

4.3.5.1 Methods

Virus: The challenge virus, A/TAI/15/2013, was obtained from the WRL, Pirbright Institute, UK.

Vaccine: Monovalent A/MAY/97 and A22/IRQ double oil adjuvant vaccines (at antigen payloads > 6 PD_{50}), and a combination A22/A May double oil adjuvant vaccine (with an antigen payload of > 6 PD_{50} of each strain) were prepared from the AVB by Merial, UK.

Experimental Design: Each vaccine was administered to two groups of five animals each, with one group being challenged at 7 dpv and the other at 21 dpv. Each trial had two unvaccinated control animals (Table 4.20). All pigs received 2 ml of vaccine and were

challenged by the heel bulb route using 0.2 ml of virus inoculum (equivalent to 10,000 $TCID_{50}$) divided equally between two sites.

Table 4.20: Experimental design

Group	Vaccine	Animals	Day of vaccination
V21May	A Malaysia 97	5 pigs	-21 dpc*
V7May		5 pigs	-7 dpc
VOMay		2 pigs	-21 dpc, not challenged
V21A22	A22 IRQ 64	5 pigs	-21 dpc
V7A22		5 pigs	-7 dpc
VOA22		2 pigs	-21 dpc, not challenged
V21Comb	A22/A May	5 pigs	-21 dpc*
V7Comb		5 pigs	-7 dpc
VOComb		5 pigs	-21 dpc, not challenged
UV	Unvaccinated controls	10 pigs	Not vaccinated

The animals were monitored for development of clinical signs consistent with infection by FMDV such as pyrexia, lameness and development of vesicles. Clinical samples were collected from the pigs daily for 14 days including vesicular fluid, oral and nasal swabs, blood for serology and PCR.

4.3.5.2 Results

None of the pigs vaccinated with monovalent vaccines and challenged 7 dpv were protected and only 20% were protected at 21 dpv. None of the pigs vaccinated with the bivalent combination and challenged at 7 dpv were protected, while 80% were protected at 21 dpv (Table 4.21; Appendix 13). It is therefore clear that the combination vaccine provided better protection, but since this vaccine contained double the amount of antigen compared to the monovalent vaccines, it is not clear whether this was due to the increased amount of antigen or the provision of broader protection from using two strains together.

4.3.6 Conclusion

The broad aims in testing vaccines in pigs were to:

- Determine if vaccination would clinically protect pigs against viruses circulating in SEA,
- Determine if vaccination decreased virus excretion and by how much,
- Determine if vaccination, together with strict biosecurity, could prevent airborne transmission of FMDV to other pigs, and
- Determine if oral and nasal swabs could be used to detect virus/viral RNA for diagnosis of FMD.

Vaccination in pigs did not provide the same level of clinical protection compared to cattle and sheep. The high potency vaccines did not provide early protection (4–7 dpv) but, in some cases, protected a number of pigs once the immune response had time to fully develop (21 dpv) (Table 4.21). However, the vaccines may need more antigen or a different route of administration to be effective in pigs.

Although the results indicated that vaccination did not protect pigs from clinical disease, it could assist to reduce the volume of virus they produce once infected, and therefore help slow down spread while other control measures take effect.

While experiments involve small numbers of animals compared to intensive farms, this work has demonstrated the value of strict biosecurity and personal decontamination to prevent disease transmission to other cohorts that don't have direct contact. By decreasing the amount of virus in the air using vaccines, it may be possible to slow down large-scale spread of FMD in piggeries and allow time for other control measures to take effect.

Oral and nasal swabs have proven to be valuable tools in detection of virus/viral RNA and, in all the experiments, FMDV genome was detected between days 1 and 10 consistently, and intermittently thereafter until at least 14 days post infection.

The results also indicated that a novel vaccination approach is needed to prevent FMD in pigs.

Table 4.21: Summary of the vaccine efficacy trials in pigs using vaccines against serotype A.

Vaccine	Virus	4 dpv	7 dpv	21 dpv
A/MAY/97	A/VIT/08/2005	100%	75%	-
A/MAY/97	A/THAI/2013	-	0%	20%
A22/IRQ	A/THAI/2013	-	0%	20%
A/MAY/97 + A22/IRQ	A/THAI/2013	-	0%	80%

Table indicates the proportion protected against clinical disease when challenged with two different serotype A isolates at various time points post vaccination.

5 Disease pathogenesis

The clinical presentation of FMD differs between species, with varying duration of viremia, virus shedding, severity of lesions and persistence of infection. Foot-and-mouth disease viruses also vary, with some more adapted to a specific species: for example, more prone to cause disease in pigs.

Pigs are considered more refractory to FMDV infection compared to ruminants, requiring a higher minimum infectious dose to establish infection (Fig. 5.1a,b). Infection can happen by various routes such as inhalation, ingestion, artificial insemination and through abrasions in the skin. In some endemic settings it is possible that outbreaks are maintained through scavenging in free-range pigs and feeding of swill. Clinical signs range from small lesions on the tongue, snout and on the bulb of the hoof, to large lesions, detached claws and death in young piglets (due to cardiomyopathy). Pigs recover within 2 weeks of infection and do not become persistently infected.

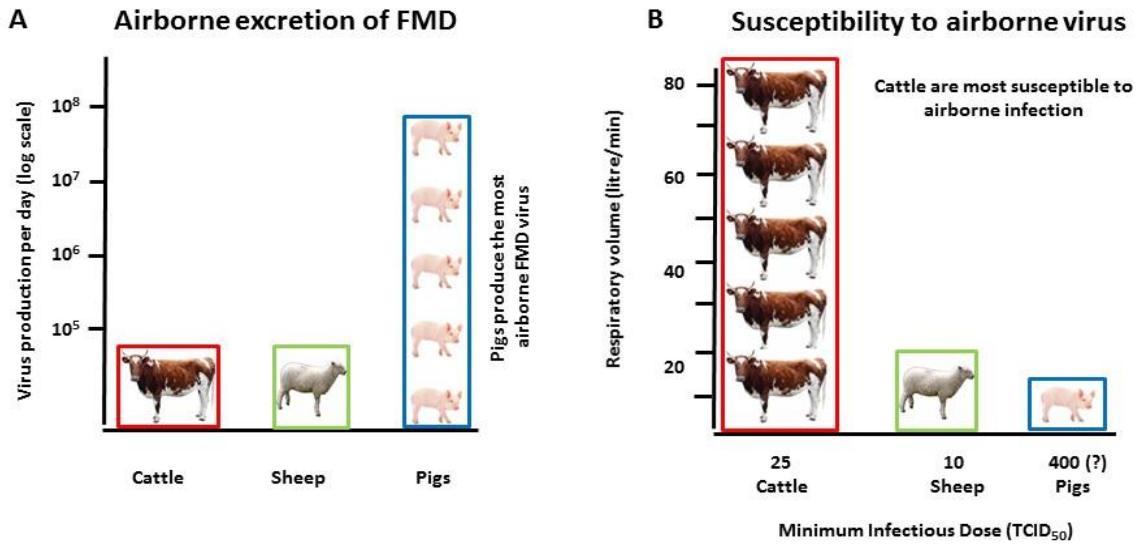

Fig. 5.1: Graphical representation of A) the relative amount of FMDV excreted by cattle, sheep and pigs per day, and B) the relative susceptibility of cattle, sheep and pigs to FMDV infection, and the minimum infectious dose for each by inhalation.

Sheep are considered less susceptible to FMDV than cattle (Fig. 5.1b), but once infected naturally they can show mild to moderate signs of the disease. The disease may circulate unnoticed in flocks, with only occasional lameness and small, inapparent lesions. In some endemic areas with large numbers of sheep, they seem to play an important role in the epidemiology of FMD, but in southern Africa, for example, where there are smaller numbers of sheep, vaccinating only cattle is sufficient to prevent outbreaks. During the outbreaks of FMD in the UK in 2001, sheep played a major role in transmission of the disease to other susceptible livestock. Sheep also become persistently infected with FMDV post infection, but remain so for shorter periods than cattle.

Given the variation in pathogenesis between pigs and sheep, the pathogenesis of some emerging FMDV variants was studied.

5.1 Pathogenesis in pigs

5.1.1 Introduction

A serotype O isolate from Vietnam (O/VIT/2010), belonging to the O/SEA/Mya-98 lineage of viruses, was used to study the pathogenesis in pigs. A model of direct contact infection (a natural route of infection) was established and the development of clinical disease in naïve pigs after different periods of contact compared with pigs infected by heel pad inoculation.

5.1.2 Methods

Groups of unvaccinated pigs were exposed to two infected donor pigs, and two in-contact pigs were euthanised and studied at various time points following exposure (Table 5.1).

Table 5.1: Experimental design

Group	No of pigs	Route of challenge
Donor	8	Heel pad
Contact	2	Direct contact for 2 hrs
Contact	2	Direct contact for 4 hrs
Contact	2	Direct contact for 6 hrs
Contact	2	Direct contact for 8 hrs
Contact	2	Direct contact for 10 hrs
Contact	2	Direct contact for 12 hrs
Contact	2	Direct contact for 24 hrs
Contact	2	Direct contact for 48 hrs

Various tissue samples were collected from both the infected and in-contact pigs for analysis by RT-qPCR (to determine how much viral RNA was present in tissues), to search for the predilection sites (primary and secondary sites of viral replication) and follow the progress of infection in the different organs where transient replication can occur. In addition to monitoring the development of clinical signs, the primary site of virus replication post-exposure and the spread of virus across the different tissues was studied.

5.1.3 Results

Generalised clinical signs of FMD were observed by 3 dpc in 6 out of the 12 donors, and 9 out of 12 donors were showing virus replication at different sites. The three donor pigs that did not show any clinical signs of infection or virus replication could have received a low challenge dose due to variation during the heel bulb inoculation procedure. The coronary band (close to the site of inoculation) appeared to be the primary site of replication. The major secondary sites of replication were the tonsillar epithelium, cervical lymph nodes, coronary band epithelium of the other feet and epithelium on the snout (Fig. 5.2a). Prior to this experiment, it was expected that pigs should develop clinical signs by 2 dpc and any later signs were presumed to result from infection by in-contact infected pigs and not as a result of the heel bulb inoculation. However, this experiment indicated that clinical disease, due to heel bulb inoculation, could take up to 5 days to develop.

The contact pigs, which received a natural challenge from the pigs previously infected via the heel bulb, were kept for various time points after a two hour exposure to infected pigs, before being euthanised and sampled. None of the pigs sampled at early time points had clinical signs, except one pig that was euthanised 48 hours post exposure. With direct contact, the primary site of virus replication was the tonsillar epithelium and the retropharyngeal lymph nodes, gradually spreading to spleen and then localising to other sites of replication such as the lymph nodes around the pharynx and larynx before infecting the upper respiratory tract (pharynx, larynx and trachea), and gradually spreading to the lower respiratory tract (bronchi and lungs) by 48 hours (Fig. 5.2b). With aerosol challenge models, clinical signs appeared earlier (2–3 dpc) than in the heel bulb challenge model (3–5 dpc) (Appendix 14), possibly as a result of natural (contact with infected pigs) versus unnatural (injection into the heel bulb) challenge. These results also indicated that the route of infection will impact on the time to detection of disease.

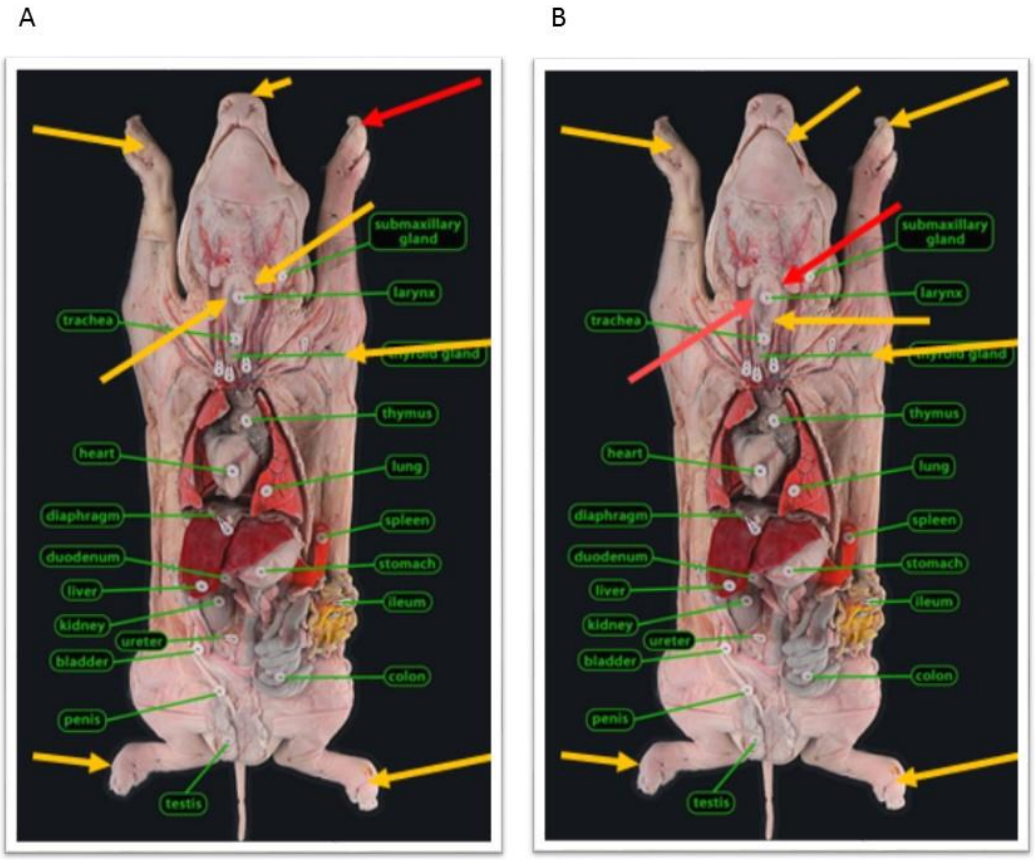

Fig. 5.2: Replication sites in pigs infected with FMDV O/VIT/2010.
The primary site of virus replication is indicated by red arrows and the secondary sites of replication by gold arrows. A) Pigs infected via the coronary band, B) pigs infected via direct contact.

5.2 Pathogenesis in sheep

5.2.1 Introduction

Little information is available on the pathogenesis of Asia1 in sheep. The early pathogenesis of an Asia1 virus isolated from Pakistan (Asia1/PAK/19/2014), belonging to the Asia1/Sind-08 lineage, in sheep was investigated to examine virus replication and subsequent movement between tissues.

5.2.2 Methods

Sheep were infected by INP challenge with $10^{4.5}$ CID_{50} Asia1/PAK/19/2014 and two sheep were euthanized at 3, 6, 12, 24, 48, 72 and 96 hours post challenge (hpc). As with the pigs, tissues from different organs and locations were collected during necropsy for analysis by RT-qPCR, in order to follow the progress of infection in the different organs (Appendix 9). Blood, nasal swab and oral swab samples (collected daily or at the time of euthanasia) were also tested for FMDV and FMDV RNA.

5.2.3 Results

Lesions were observed in only two sheep: one culled 72 hpc and one at 96 hpc, suggesting with this strain, 3–4 days were required for the establishment of infection at secondary sites following systemic spread. No virus or viral RNA was detected in any blood or swab samples between 0 and 24 hpc. All animals were viraemic between 24 and 96 hpc and FMDV was detected in most nasal and oral swab samples between 48 and 96 hpc.

No FMDV RNA was detected in any tissues 0–12 hpc. Of the two sheep culled 24 hpc, one was positive only in the nasopharyngeal tonsil, however all tissues from the 48 h sheep were negative. At 72 and 96 hpc, FMDV RNA was detected in various lymph nodes, the soft palate, dental pad and palatine tonsil, as well as lesion sites on the feet. Depending on the virus isolate, it therefore took at least 72 hours before virus could be detected in more tissues of infected sheep, and this correlated with the appearance of lesions.

5.3 Conclusions

In pigs, following direct contact challenge, primary virus replication occurred in the tonsillar epithelium and retropharyngeal lymph nodes as early as 12 hours post-contact. Generalisation of disease occurred around 48 hours post infection with viral RNA dissemination to other organs and tissues especially spleen, lymph nodes around the pharynx and larynx, the upper respiratory tract (pharynx, larynx and trachea), followed by gradual spread to the lower respiratory tract (bronchi and lungs). These tissues are therefore important samples to collect for FMD diagnosis during necropsy.

In sheep, following INP infection with Asia1/PAK/19/2014, the levels of viral RNA in the local nasopharyngeal tissues were below the level of detection by RT-qPCR in the first 1–2 days after infection. Following generalised spread after 48 h, virus appeared most predominant at secondary replication sites on the feet and in the oral cavity, and in the lymph nodes that drain these sites. These results suggest low level replication of the virus in the primary infection sites at early time points, contradictory to what has been reported in cattle, and this may be associated with the milder clinical disease commonly seen in these animals.

By comparing the results between the different species and challenge models (Table 5.2) it was concluded that the incubation period (time between infection and development of clinical signs) is affected by the anatomical constraints of the primary infection site and the likely difference in replication rates in the two species. Inoculation of the feet epithelium in pigs resulted in a slower progression to systemic spread than infection of the nasopharyngeal tissues, probably because the former is not the natural route of infection. The nasopharyngeal tissues most likely facilitate significant viral replication, concordant with the high viral excretion reported in pigs by exhalation. Conversely, in sheep a lower replication rate in the nasopharyngeal tissues may account for the longer lag time we observed before the appearance of clinical disease, compared to pigs. In all cases, viral loads appeared highest in the predilection sites for FMD and the lymph nodes that drain these areas, indicating other tissues do not commonly serve as reservoirs for the virus. This is useful for determining tissues to target during necropsy in case the disease is suspected during post-mortem examination of carcases.

Study of the early pathogenesis of FMDV provides important insights into the different infection routes and infection dynamics in different species, and is useful in improving our understanding of key events associated with virus spread and opportunities for detection.

Table 5.2: The major differences in pathogenesis between pigs and sheep.

	Pigs		Sheep
Challenge route	Heel-Bulb	Contact	Intranasal-pharyngeal
Primary site	Coronary band on inoculated limb	Tonsillar epithelium, retropharyngeal lymph nodes	Naso-pharyngeal tonsils
Secondary site	Tonsillar epithelium, cervical lymph nodes, snout	Spleen, lymph nodes around pharynx and larynx	Soft palate, dental pad, palatine tonsils, feet
Appearance of clinical signs	3–5 days	2–3 days	3–4 days

6 Diagnostic test validation

In addition to having efficacious vaccines, Australia needs access to excellent laboratory diagnostic services to ensure the ability to rapidly detect and respond to potential cases of FMD.

The laboratory assays need to be rapid and accurate. The laboratory will play an essential role during a response to an outbreak by:

- Confirming the outbreak and determining the serotype of FMDV present,
- Characterising the outbreak virus (including vaccine matching and genetic sequencing),
- Monitoring the response to vaccines if used, and
- Surveillance during the outbreak, and post-outbreak to provide proof of freedom from disease.

Laboratory preparedness includes ongoing validation of assays under strict quality assurance guidance (accreditation). However, because infectious FMDV and its genetic material (RNA) cannot be imported into Australia, it is challenging for the Diagnosis, Surveillance and Response (DSR) team at the AAHL to ensure all the routine assays remain relevant to the viruses circulating in the SEA region (see section 3).

The FMD RMP team obtained import permits from the Department of Agriculture and Water Resources (DAWR) to transport samples (which were generated during the project and are free from live virus) to AAHL to use for test validation. In addition, it assisted AAHL to stockpile reagents for a large-scale emergency response[18].

6.1 Comparison of AAHL 'in-house' FMD NSP cELISA and two commercial ELISA kits – preliminary report

Serological tests (ELISAs) that detect antibodies to FMDV non-structural proteins (NSP) enable the identification of animals that have been infected by any of the seven serotypes, irrespective of whether or not they have been vaccinated. When animals are infected with FMDV, they develop antibodies to viral structural proteins and NSPs. Uninfected, infected

[18] A high number of serum samples collected during the animal trials have been imported as well as bulk sera that can be used to establish panels for proficiency testing. The FMD RMP also imported cDNA and PCR products for sequencing.

and vaccinated animals develop antibodies to structural proteins of the virus. Because antibodies against NSP are generally only produced following virus replication, the presence of antibody to NSP can indicate FMDV infection (Fig. 6.1)

In highly purified vaccines, such as those in the AVB, there are negligible amounts of NSP and animals would therefore require repeat vaccination before anti-NSP antibodies become detectable.

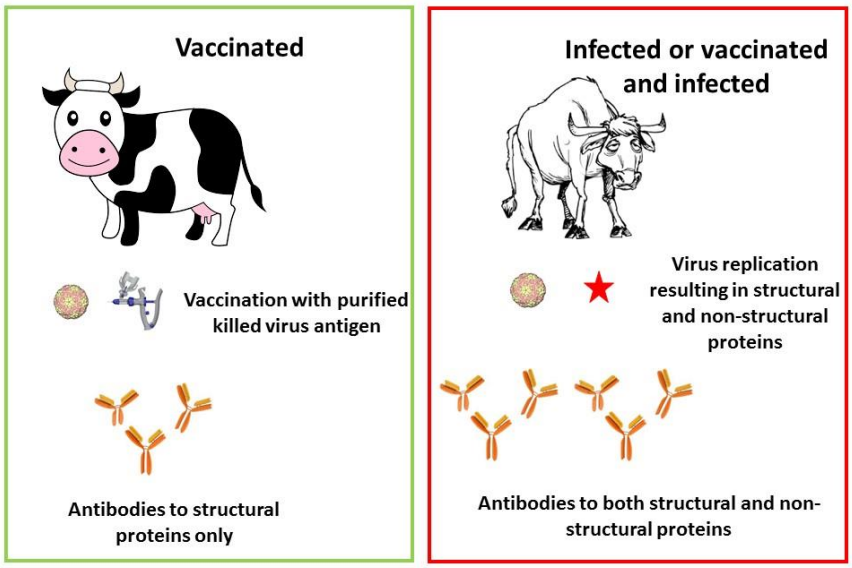

Fig. 6.1: Representation of DIVA testing.
Vaccinated animals develop antibodies to the structural proteins, while infected, and animals vaccinated and infected, develop antibodies to both structural proteins and NSP. The presence of antibodies to NSP indicates virus infection and their absence can be used to prove freedom from infection during post-outbreak surveillance when vaccination is used.

Following eradication of an outbreak, Australia would have to provide evidence to the OIE and its trading partners that all infected animals had been removed and that no FMDV was circulating. If vaccines were used to assist with disease control, and vaccinated animals were retained in the population, the NSP assays would be used for serological proof that no infected animals were present.

The laboratory needs access to at least two NSP tests: one as a screening test and the other to retest all positive sera (confirmatory test). Only those animals that remain positive on the second test would need to be resampled, in addition to other actions required by the OIE, during the proof of freedom stage of the outbreak.

An in-house developed NSP competitive ELISA[19] (cELISA) is the accredited test for routine diagnostic use at AAHL. Two commercial cELISA kits, with no components derived from live virus, are also available for detection of anti-NSP antibodies. These are the

- PrioCHECK FMDV NS, Prionics Lelystad, The Netherlands and
- ID Screen FMD NSP Competition, IDVet, France.

[19] Australian Veterinary Journal 92 (2014) 192-199.

These test kits were imported to compare their performance with the AAHL in-house test, to inform a decision on which tests to use for screening and confirmation of FMDV infection.

To investigate the comparative performance of the AAHL and commercial assays, a selection of positive and negative sheep and cattle sera[20], which were generated during the FMP RMP, were tested and results compared.

6.1.1 Methods

Sera: Sheep (n=352) and cattle (n=324) sera collected from the different vaccine efficacy studies were used to compare the three different NSP assays (Table 6.1).

Table 6.1: Sheep and cattle sera used for comparison of different NSP assays

Species	Naïve / Vaccinated	Challenged / Vaccinated-infected
Sheep	200	152
Cattle	174	150

Assays: The following NSP assays were compared

1. AAHL anti-NSP c-ELISA Assay
2. PrioCHECK FMDV NS, Prionics Assay
3. ID Screen FMD NSP Competition, IDVet Assay

6.1.2 Results

All three assays demonstrated very good specificity (no false positive results) when testing sera from naïve or vaccinated animals, with only one naïve sheep sample incorrectly classified as positive by the Prionics assay. In contrast, considerably more variation was observed when testing sera from infected or vaccinated-infected sheep and cattle. The estimated sensitivity of the AAHL assay (94.7%) was higher than the Prionics (93.0%) and IDVet (74.6%) kits for infected sheep, whereas the estimated sensitivity (78.6%) was lower than the Prionics (96.4%) and higher than the IDVet (58.9%) kit for vaccinated-infected sheep. The Prionics cELISA demonstrated 100% sensitivity for infected and vaccinated-infected cattle, whereas the estimated sensitivity of the AAHL assay was 84.2% and 90.7%, respectively, and the IDVet kit only 68.4% and 57.9%, respectively (Table 6.2).

[20] Most of the pig sera were destroyed by the irradiation on arrival in Australia as required by the import permit, and comparisons were not possible.

Table 6.2: Preliminary estimates of sensitivity and specificity of the three NSP tests, the AAHL assay, Prionics and IDVet kits.
Results are shown separately for cattle and sheep sera. Sera were obtained from naïve and vaccinated animals as well as from animals that were infected, and infected/vaccinated.

NSP antibody assays	Interpretation		Sensitivity				Specificity
	Neg	Pos	% Infected sheep	% Vaccinated-infected sheep	% Infected cattle	% Vaccinated - infected cattle	% Naïve & vaccinated cattle and sheep
AAHL assay	<35 PI	>35 PI			84.2	90.7	100
AAHL assay	<45 PI	>45 PI	94.7	78.6			
Prionics kit	<50 PI	>50 PI	93	96.4	100	100	99.9
IDVet short format kit[21]	<50 PI	>50 PI	74.6	58.9	68.4	57.9	100

PI – percentage inhibition

The analytical sensitivity of the AAHL assay was lower than the two commercial kits, and additional sera will have to be tested to establish the relative performance characteristics of all three assays with more confidence, and determine whether or not the Prionics and IDVet (long procedure) protocols could be used as confirmatory NSP ELISA tests in the Australian context.

It is also possible that gamma irradiation of imported serum samples (required under the permit conditions) had a detrimental effect on the quality of antibody present in the sera, and particular samples or specific assays may have been affected to different extents.

6.1.3 Conclusion

Preliminary data suggested that the specificity of the AAHL assay was comparable to that of the two commercial kits, while the sensitivity was similar to the Prionics kit for infected sheep, with more pronounced differences for vaccinated-infected sheep and cattle, as well as infected cattle (Appendix 15).

The AAHL assay would be suitable as a screening assay, which is also more cost-effective. However, testing with the IDVet long assay (overnight incubation) is still required as part of the current project before reaching conclusions on which assay to recommend as a confirmatory test.

6.2 Evaluation of reagents used at AAHL for FMDV serotyping

If FMD has been confirmed, it will be essential to determine which of the seven serotypes of the virus is causing the outbreak. This information would guide the decision on which vaccine strain to order and determine the set of serological reagents that would be used to detect antibody during vaccination and surveillance activities.

AAHL uses a double antibody sandwich ELISA (Antigen ELISA; Ag-ELISA) for serotyping FMDV isolates[22], based on purchased reagents that are not in a kit format. Seven different sets of reagents are needed, one for each serotype of FMDV. The Ag-ELISA at AAHL can

[21] The kit can also be used in an overnight format (long assay) and preliminary results show that this improved the results.
[22] Roeder PL, Le Blanc Smith PM. Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. Res Vet Sci. 1987;43:225-32.

identify all seven serotypes of FMDV, as well as other vesicular diseases that may cause blisters similar to FMD (swine vesicular disease and vesicular stomatitis (serotypes Indiana and New Jersey)).

An Ag-ELISA kit is available from the Pirbright Institute that is used in most laboratories in SEA. Recently, there have been reports of poor kit performance with many false negative results, especially for serotype O. AAHL also reported false negative results when participating in a proficiency test using its own reagents that are based on those included in the kit.

The FMD RMP team therefore evaluated the AAHL serotype O and A reagents at the RRL in Thailand, testing epithelium samples that had originated from diseased animals in the field. The O1 Manisa reagents did not react satisfactorily with the serotype O isolates from SEA, while O3039 performed well. There were no false negative results with the serotype A reagents.

6.2.1 Conclusion

It was recommended that the Ag-ELISA for serotype O at AAHL needed to be adjusted to also include the O3039 reagents which has been implemented.

6.3 Comparing susceptibility of different cell cultures for FMDV isolation

Virus isolation on cell culture is used for further characterisation of FMDV and for antigen matching studies.

Virus isolation protocols must have maximum sensitivity. Some primary cells, such as bovine thyroid (BTY) are highly susceptible to FMDV, but they are difficult and costly to prepare and lose FMDV susceptibility after multiple passages. For this reason they need to be prepared frequently, which is time consuming, and since AAHL is not allowed to work with live virus, the susceptibility of each new batch cannot be determined.

Immortalised, continuous cell lines, such as baby hamster kidney cells (BHK21), are used as substitutes for FMDV isolation in most of the laboratories where BTY cells are difficult to obtain and culture. However, BHK cells also lose FMDV susceptibility after multiple passages and are not suitable for primary virus isolation for the SAT serotypes. Other cell lines that have been shown to be sensitive to FMDV have been imported to AAHL, including the goat tongue epithelium (ZZ-TR) and porcine kidney cells (LFBK$\alpha_v\beta_6$) that have been modified to express the bovine cell receptors of FMDV (integrins α_v and β_6).

The sensitivity of BTY, BHK21 and LFBK$\alpha_v\beta_6$ cells to FMDV infection were compared at RAHO6, Vietnam, using clinical material from animals infected with viruses from serotypes O and A. Ten serotype O and ten serotype A viruses containing epithelial tissue suspensions were tested using the AAHL Standard Operating Procedures. After virus isolation, the results were confirmed using the serotyping Ag-ELISA.

When comparing results for the same sample, 40–60% cytopathic effect was recorded in BHK-21 flasks after two days of observation, whereas almost 100% cytopathic effect was observed in LFBK$\alpha_v\beta_6$ flasks.

Serotype O viruses could be isolated easily using all three cell culture systems but, for serotype A, more success was observed using BTY and LFBK$\alpha_v\beta_6$. Seven serotype O

isolates and only two serotype A isolates could be isolated using BHK-21 cells, whereas nine serotype O isolates and eight serotype A isolates could be isolated using LFBK$\alpha_v\beta_6$ cells.

Antigen ELISA results showed that the BTY and LFBK$\alpha_v\beta_6$ cells were more successful in FMDV isolation than BHK21 cells.

6.3.1 Conclusion

A recommendation was made to the diagnostic laboratory at AAHL on how to improve the sensitivity of their assays and which cell line would be most suitable to replace the BHK and potentially the BTY cells.

6.4 Progress on sequencing methodologies for genetic comparisons

The genetic information of the outbreak virus would be important to:

- confirm the serotype,
- provide an indication of the broad region of origin of the virus,
- assist in the decision regarding which vaccine to order,
- investigate if the virus is mutating during an ongoing outbreak, as this may have an impact on the sensitivity of diagnostic tests, and
- help trace the movement of virus between properties and potentially guide field services in identifying affected farms.

The FMDV genome consists of a single stranded RNA that codes for 4 major regions, the Leader protease (L^{pro}), the P1 (4 structural proteins - VP1, VP2, VP3 and VP4), P2 (3 non-structural proteins - 2A, 2B, 2C) and P3 (5 non-structural proteins - 3A, 3B, 3C and 3D) (Fig. 6.2).

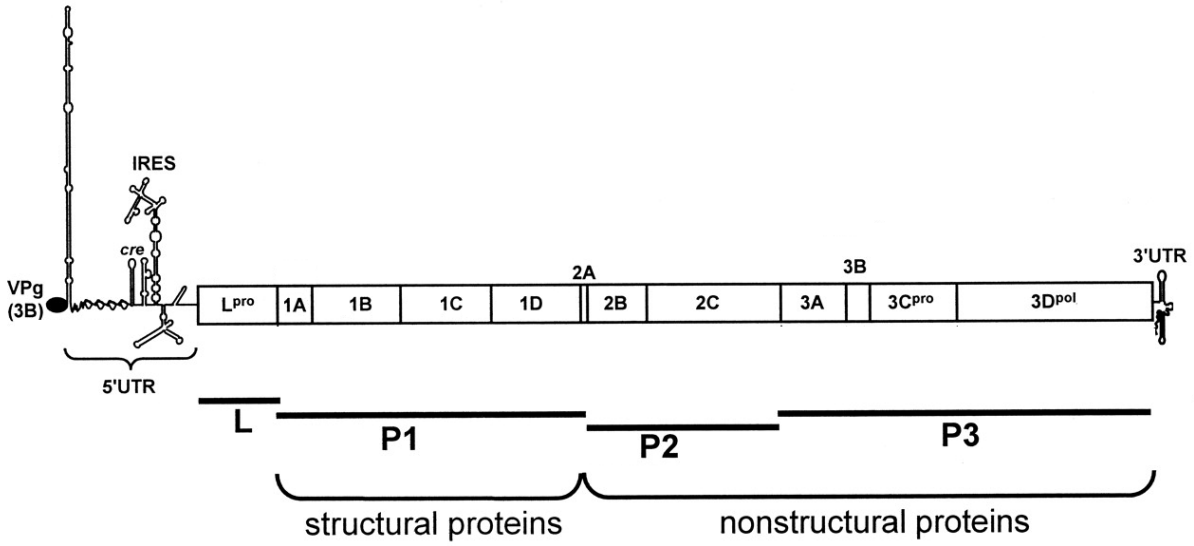

Fig. 6.2: Graphical representation of the FMDV genome.
The P1 region encodes the structural proteins (1A = VP4, 1B = VP2, 1C = VP3 and 1D = VP1) while the P2 and P3 regions encode the non-structural proteins.

The P1 sequence determines the viral serotype, genotype and lineage. In the past, only the 1D genomic region encoding the major antigenic protein, VP1, which enables determination of the serotype and comparison with other virus isolates, was routinely sequenced by laboratories including AAHL. With the developments of sequencing technologies, and publication of comprehensive primer sets targeting this region, it is now possible, and preferable, to sequence the entire P1 region, enabling more detailed information and better comparisons to be made.

6.4.1 Conventional sequencing

AAHL's sequencing capabilities were previously confined to sequencing the 1D region and were challenging to improve due to the restrictions on importing genetic material. The FMD RMP team obtained import permits that allowed the importation of complementary DNA (cDNA) of the virus, which is not infectious, and validation of methods for sequencing the P1 region is ongoing for serotypes O, A and Asia1.

This methodology is not suitable for providing rapid full-genome sequences and another technology needed to be established to achieve this (see below).

6.4.2 Next generation sequencing (NGS)

A single FMDV isolate exists as a heterogeneous mixture of viruses with subtle sequence differences in a given sample (quasispecies). The conventional methods of sequencing cannot identify these sequence variations and, to overcome this limitation, a new technique referred to as Next Generation Sequencing (NGS) is utilised. This technique provides 'deep' sequencing, enabling detection of all the sequence differences within an isolate, even if that sequence occurs at a very low frequency.

The conventional sequencing methods are not very sensitive and usually require that a virus first be grown to a high titre in cell culture, which could result in genome mutations and selection of sub-populations. NGS can overcome this problem, providing sequence data directly from clinical material. In addition, full-genome sequences can be obtained very rapidly.

A collaborative initiative with Duke-National University of Singapore was established to develop and validate a new method of NGS for FMDV that uses a target-enrichment strategy enabling direct sequencing of FMDV in clinical material. This method avoids the potential bias caused by inadvertent selection of viral variants that are better adapted to replication *in vitro*. The technique is based on using hybridisation probes to enrich the sample for FMDV-specific cDNA prior to sequencing. When sequencing a virus using NGS that was first grown in cell culture, the method resulted in a 65-fold enrichment of viral genomic material, compared to the unenriched library, and resulted in a marked improvement in the depth and coverage of the FMDV genome sequence obtained. For the unenriched library only 1.5% of mapped reads were of viral origin (Fig. 6.3a), whereas for the enriched library 97.8% of the total reads were FMDV-specific (Fig. 6.3b). The method also worked well when sequencing directly from swabs, where virus is present at very low levels (Appendix 16).

6.4.3 Conclusion

As a result of this work, proof of concept for obtaining the sequence of the entire P1 region have been demonstrated, from cDNA samples made from RNA obtained from oral and nasal swabs, using conventional sequencing methods. This method would be used to confirm the serotype of virus during an outbreak and its possible region of origin.

In addition, using NGS methods full-length FMDV genome sequence data directly from clinical samples were obtained, including those containing very low concentrations of viral RNA, without isolation and propagation of virus in cell culture. Data generated with this methodology could be used to trace the movement of viruses during an outbreak, and will be further developed in the next phase of the project. It could also be used in research projects to facilitate in-depth study of the viral quasispecies diversity during FMDV infection, with or without vaccination, thereby revealing the location and frequency of single nucleotide polymorphisms within specific viral populations.

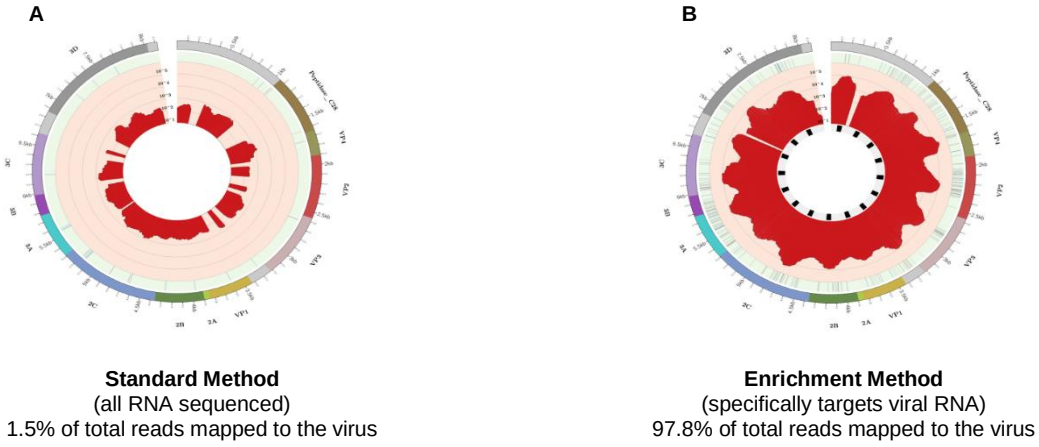

Fig. 6.3: Sequence coverage maps of unenriched (A) and enriched (B) library samples generated during optimisation of the enrichment protocol.
Each map depicts the viral genome in 5' to 3' orientation starting at 12 o'clock and moving in a clockwise direction. The depth of coverage at each position in the genome is indicated by red shading and is shown on a $\log_{10}$ scale.

6.5 Novel ways of FMD surveillance

In high-density farming practices it is important to constantly monitor for infectious diseases, especially for diseases such as FMD that have the potential to spread rapidly between holdings. Pigs are known to amplify FMDV by excreting large amounts of virus and it is therefore important to detect the virus quickly and accurately to minimize the spread of disease. Rope chews are an easy and effective way to collect oral fluid samples from pigs, which can then be tested in the laboratory to detect virus/viral RNA.

The rope sampling was done during two of the pig trials in Vietnam (Section 4.3.2 and 4.3.4), where groups of pigs were infected with a virus from serotype O or serotype A, with or without vaccination, and unvaccinated pigs were kept in aerosol contact. Each rope sample was compared to saliva samples collected from individual animals to determine how effective this method is to detect FMDV.

The sensitivity of the rope sampling varied between 0.67 and 0.92 and the statistical agreement between this method and individual sampling ranged from substantial to moderate for the two different serotypes (Fig. 6.4).

The ease of collecting oral fluids using ropes, together with the high sensitivity of subsequent FMDV detection through PCR, indicates that this could be a useful method to monitor pig populations for FMDV infection. With further validation of the sensitivity of detection of FMD viral RNA, this could be applied as a cost effective, non-invasive diagnostic tool (Appendix 17).

Viral RNA in rope oral fluid samples

	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc	9 dpc	10 dpc	11 dpc	12 dpc	13 dpc	14 dpc
UV	N	P	P	P	P	P	P	P	P	P	P	P	P	ND	P
UVC	N	N	N	N	N	N	N	P	P	N	N	N	P	ND	P
V4	ND	ND	P	N	P	P	P	P	P	N	N	N	ND	P	N
UVC4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
V7	N	P	P	N	P	P	N	N	N	P	N	P	P	N	N
UVC7	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Viral RNA in saliva swab samples

	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc	9 dpc	10 dpc	11 dpc	12 dpc	13 dpc	14 dpc
UV	N	P	P	P	P	P	P	P	P	P	P	P	P	P	P
UVC	N	N	N	N	P	P	P	P	P	N	N	P	P	P	P
V4	N	N	P	P	P	P	P	P	P	N	P	P	P	P	P
UVC4	N	N	N	N	P	N	N	N	N	N	P	P	N	P	P
V7	N	P	P	P	P	P	N	P	P	P	N	P	N	P	N
UVC7	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Fig. 6.4: Comparison of daily results when sampling pigs using ropes and individual saliva swabs.
A group of animals was deemed positive for saliva swabs if at least one of the animals in the group was positive on a given day. UV – unvaccinated group; UVC – unvaccinated contact group; V4 – group vaccinated 4 days before challenge; UVC4 – unvaccinated contact group for V4; V7 – group vaccinated 7 days before challenge; UVC7 unvaccinated contact group for V7; dpc – days post challenge; N – negative; P – positive; ND – not done.

6.5.1 Conclusion

The FMD RMP has contributed to building confidence in the diagnostic assays available at AAHL. The number of well characterised samples has been essential in assisting with test validation and ensuring reagents are available for an outbreak and for proficiency testing.

Imported samples, although they don't contain live virus, are valuable for AAHL staff to maintain their training in working with FMD-related samples and hence help with capacity development.

Novel technologies, especially for sequencing whole virus genomes, have been established and the data management to allow genome comparisons for tracing of virus movement will be developed during the next phase of the project.

7 Other activities to support capacity building

7.1 Global FMD Research Alliance (GFRA)

The Global FMD Research Alliance (GFRA)[23] was initiated in 2003 by Dr Martyn Jeggo, the Director of AAHL at the time. It is an international consortium where the partners have recognised capabilities and expertise in a broad range of advanced technologies, and research capabilities in fields that are of potential strategic, tactical or commercial interest to those working in the area of FMD. Through collaboration, the GFRA is the global leader in scientific and technical knowledge for economically viable, socially and environmentally responsible detection, management and eradication of FMD.

Wilna Vosloo has served as the Chief Executive Officer of the GFRA since 2013. As part of the GFRA mission, a scientific meeting is held every two years to bring together scientists from all over the world to discuss progress with research and identify/prioritise future research needs. In 2015 the GFRA meeting was held in Hanoi, Vietnam, with the aim to involve more scientists from SEA in research and also bring the needs in SEA to the attention of the GFRA partners. Having the meeting in our region also made it more accessible to Australian stakeholders. During the meeting, a workshop was held to discuss the policy issues surrounding disease control options. Of particular interest to Australia was the scenario that dealt with levels of appropriate post-vaccination monitoring for use in an FMD free zone without vaccination, after use of emergency vaccination. The workshop outputs are available as Appendix 18.

The next GFRA scientific meeting will be held in Seoul, South Korea, from 25–27 October 2017. The themes will focus on vaccination in pigs.

In addition to the biennial meetings, the GFRA also performs a regular gap analysis to identify research areas that need attention, sponsored by the European Union FMD Commission. The latest gap analysis was published in Transboundary and Emerging Diseases (see publications below).

7.2 Training of staff at laboratories in SEA

The FMD RMP team provided laboratory training to staff at RRL (Thailand), RAHO6 (Vietnam) and, more recently, the National Animal Health Laboratory (NAHL), Lao PDR, during numerous visits. Between 2010–2013 staff at NAVETCO, Vietnam, received training in animal handling, ethics protocols as well as procedures to work in their newly built animal facility. The FMD RMP team wrote all of their standard operating procedures. Selected staff at NAVETCO and RAHO6, Vietnam, received laboratory training in FMD Ag ELISA, serology and molecular diagnosis of FMD during each visit made to the laboratories. The FMD RMP staff conducted a workshop at the National Institute for Animal Health (NIAH), Pakchong, Thailand, on 5th August 2016.

Whilst working in these laboratories, the FMD RMP team also gained experience in working with live animal models under varying circumstances.

[23] https://www.ars.usda.gov/GFRA/

7.3 Support of SEA scientists to attend meetings

During 2013, the FMP RMP sponsored two people from Vietnam, one from NAVETCO (Dr Nguyen Thi Thu Hong) and one from RAHO6 (Dr Long Thanh Ngo), to attend the GFRA scientific meeting in South Africa.

During 2016, the FMP RMP sponsored Dr Dung Do Huu, from the Department of Animal Health, Vietnam, to attend the EUFMD Open Session in Portugal. He was invited and presented a talk on selection of FMD vaccines in Vietnam.

7.4 Support for AAHL people to work with FMD

The following people were provided either with financial support to work overseas with FMD, or were trained at AAHL:

Gemma Harvey and Frank Wong – conventional sequencing of viral cDNA at AAHL (July 2015 – December 2016)

Leanne McNabb and Ross Lunt – evaluation of NSP ELISAs at AAHL (October 2015 – November 2016)

Jianning Wang – training in Next Generation Sequencing at Duke-NUS, Singapore (May–June 2016)

Michelle Giles and Andrew Davis – animal trial in pigs, NAVETCO, Vietnam (2012–2013)

Dr Mark O'Dea, DPI Western Australia – training on FMD in pigs, NAVETCO, Vietnam (July–August 2012)

7.5 Import of material for test validation

A large number of samples have been imported into AAHL under permit issued by the Department of Agriculture and Water Resources.

- Post vaccination and challenge sera from animal trials in Vietnam, United Kingdom, Argentina, The Netherlands and Canada.
- cDNA from pig trials in Vietnam and sheep trials in Canada.
- Immune rabbit and guinea pig sera for ELISA standards.

8 Discussion

Over the last six years, the FMD RMP has undertaken extensive research and related activities with the overarching goal of increasing Australia's preparedness and response capabilities for an outbreak of FMD. This has been a complex task, with the bulk of the work necessarily being performed off shore.

The key outputs of this project are:

- An improved understanding on the pathogenesis of various FMDV isolates in different species

- An understanding of the efficacy of the vaccine strains in the AVB in three important livestock species
- An improved understanding of the regional epidemiology of FMD
- An enhancement of existing, and development of new, diagnostic and surveillance tools and approaches
- Valuable capacity building, both at AAHL and in regional laboratories within SEA.

The intrinsic features of FMDV, such as high mutation rate and broad host range, influence the complexity of the disease and the subsequent measures required for effective control. The spectrum of disease severity in different species depends on viral factors such as virulence, pathogenicity and transmissibility, and host factors including susceptibility, immune and health status, and route and extent of exposure. In addition, factors such as herd/flock size, stocking density and contact rate between animals, play a role in disease transmission and the rate in which infection can spread between susceptible animals.

The FMD RMP, using different FMDV serotypes in three livestock species of importance to Australia (cattle, sheep and pigs), has enhanced our knowledge of how each species may be affected by contemporary, regional FMDV isolates. In particular, the work has highlighted **how diverse the presentation of the disease can be**, the best approaches for detection, and the efficacy of vaccination in the different species. For example, two distinct isolates belonging to the same lineage (O/SEA/Mya-98) and with high genetic similarity, behaved differently in pigs, with a clear difference in virulence. O/VIT/2010 caused mild lesions and vaccination provided partial protection, while the other isolate, O/SKR/2010, caused severe lesions in pigs and vaccination was not effective. In contrast, O/SKR/2010 was only mildly virulent in cattle and sheep, and vaccination provided complete to partial protection. Similarly, marked differences in virulence in pigs were observed with serotype A isolates. The 2005 Vietnam isolate was less virulent than the 2013 isolate from Thailand, although they both belong to the same A/Asia/SEA-97 lineage. These findings have important implications for recognising disease on farms, as it may not be easily detected.

As it is impossible to predict the virus that may cause an outbreak in Australia, understanding the range of the characteristics of FMDV is of great value. Particularly important is an appreciation of the potential for inapparent, subclinical infections in some species. Unrecognised FMDV-infected animals could represent a potential source for FMD dissemination. In addition to providing crucial data to better guide detection and control efforts, our findings **reiterate the importance of on-farm surveillance and reporting, adhering to biosecurity protocols in and around farms, and being vigilant against practices such as illegal importation of animals or animal products and swill feeding**.

An important consideration in controlling FMD is the level of protection afforded by vaccines. While in many cases, vaccination prevented clinical disease in our experimental animals, it was less common to see a complete absence of infection, and subsequently of virus excretion. It is well-reported that FMD vaccines can protect animals exposed to FMD against clinical disease, but not necessarily against infection. However, vaccination can reduce the amount of virus shed by animals exposed to FMDV, and this may help reduce the rate of spread of the disease and should be accompanied by other measures (e.g. movement controls and biosecurity) to assist with eradication of disease.

An important distinction must be made between the usual outcomes of a natural infection, and those of an experimental infection. In a vaccination/challenge study, the virus dose and route of infection are tailored to ensure reproducible clinical disease in the non-vaccinated

control animals, and are usually much greater than the level of challenge expected in the field. In addition, the routes of experimental infection focus on primary sites of viral replication, presenting an opportunity for the virus to replicate (albeit transiently in vaccinated animals) and generate NSP that leads to the development of antibodies. By definition, all of these animals will be considered infected. Consequently, in experimental settings the parameter of 'absence of clinical disease' serves as a good indicator that a vaccine is efficacious, and a higher level of protection would be expected in the field. In a number of our studies utilising direct-contact challenge (natural exposure), or with 21 days between vaccination and challenge, protection from infection and reduced virus in secretions was observed, despite the high challenge dose and use of heterologous challenge viruses.

Most of the challenge methods adopted in the FMD RMP are standardised for each of the species (IDL in cattle, heel-bulb challenge in pigs), and work was done in the project to determine the most reproducible challenge method for sheep. The FMD RMP showed that the clinical protection offered by vaccination can be significantly influenced by the challenge models used. For example, the results with the near-natural challenge model in sheep trials (INP route of infection) show not only clinical protection, but also sterile immunity, demonstrated by the absence of antibodies to NSP in vaccinated and challenged animals. In contrast, the results with the CB challenge route showed partial clinical protection and all of the challenged sheep developed antibodies to NSP.

Overall, it could be concluded that **vaccination with high-potency vaccines was effective in cattle and sheep, but less so in pigs,** and a model of emergency vaccination (with only 4 or 7 days between vaccination and challenge) was able to provide partial protection in some cases. **Good to moderate protection was observed in cattle**, with no cases of sterile immunity recorded. However, reduced virus excretion in many vaccinates was observed and overall the results in cattle should be considered favourable. In every trial, cattle were challenged with IDL inoculation, which is a method of significantly greater intensity than natural aerosol exposure.

The most comprehensive vaccination protection was seen in sheep, even at early time points. Traditionally sheep are described as being less clinically affected than cattle or pigs, however, in our studies, significant clinical disease was observed in many animals, varying with serotype/virus isolate and challenge route. Sheep have been instrumental in FMD outbreaks in countries with high sheep populations and where undetected sub-clinical infections have facilitated spread. Consequently, sheep could potentially have a key role in spreading disease in Australia.

The varied challenge methods used in the sheep studies highlighted the importance of selecting the right method to obtain the most informative results. We found INP instillation was a highly suitable challenge method in sheep, resulting in reproducible disease, but with a challenge severity that permitted the observation of progressive vaccine effects, such as differences due to the time between vaccination and challenge. These effects may be less clear if sheep are challenged with extremely high doses of virus and/or by a less natural route. The INP method has recently been shown to also be effective in cattle[24] and may be an approach worth investigating in the next phase of the project to examine protection with a more natural challenge. Our previous cattle studies were performed following the methods of the European Pharmacopoeia (that is, with IDL inoculation) as it is standard protocol adopted around the world and allows better comparison between studies.

The poorest vaccine protection was seen in the pigs. Overall pigs took longer to develop a neutralising antibody response, and partial protection and reduced virus excretion were

[24] Pacheco JM, Stenfeldt C, Rodriguez LL, Arzt J. 2016. Infection Dynamics of Foot-and-Mouth Disease Virus in Cattle Following Intranasopharyngeal Inoculation or Contact Exposure. J Comp Pathol. 155(4):314-325.

only observed when the challenge strain caused mild disease or in animals challenged 21 dpv. Similar results have been observed by others, and have suggested that pigs take at least 21–28 days to develop sufficient neutralising antibodies post vaccination[25]. These results signal the need for either pig-specific vaccines or alternative vaccination methods, and a better understanding of the immune response to vaccination. The availability of vaccines that induce a rapid protective response and are effective at reducing virus excretion in pigs could have ramifications on the vaccination policies for these animals, with 1) countries free of disease including pigs in emergency vaccination campaigns and 2) improvements in the global efforts to control the disease in endemic countries with large pig populations.

An additional objective of the vaccine trials in cattle and sheep was to assess any effects of vaccination on the development of persistent infection (the so-called 'carrier status'). The occurrence of persistent FMDV infection is the reason why infected animals are destroyed in an outbreak response. The definition of persistent infection stems from an historic, arbitrarily assigned 28 day cut-off for virus detection. This definition is less convincing if based on the detection of viral RNA, as residual nucleic acid may be detected in the absence of infectious virus. It has never been shown experimentally that persistently infected livestock can transmit infection, and their importance in disease spread and management decisions is a matter of debate. Despite this, the occurrence of persistence has had a dramatic influence on trade restrictions and control policies globally, and in Australia, current policy dictates that all infected animals will be culled, negating concerns about the effect of vaccination on prevalence of persistence.

Overall, there was no observable decrease in the prevalence of persistence in vaccinated animals in our studies, however, the effects of challenge dose/route could not be determined. Most of the vaccinated animals were infected by direct challenge (not a natural route of infection). Importantly, regardless of vaccination status, all infected animals were detected using the NSP assays. Therefore, irrespective of the low risk of persistently infected animals spreading infection, we can be confident the assays available will detect these animals when vaccination is used.

The *in vivo* vaccine trials performed in this project have provided invaluable information on the protective capacity of the vaccines in the AVB, including the protection at early (4–7 days) and intermediate (14–21 days) times post vaccination. However, in order to evaluate more FMDV isolates, *in vitro* screening alternatives must be utilised. *In vitro* vaccine matching has been performed in Vietnam and Thailand, testing contemporary viruses of different serotypes against the vaccines in the AVB. This work has provided important information about the trends in antigenic evolution of viruses in SEA. For example, while there was no clear trend in antigenic drift for serotype O, for serotype A, it was found that new viral clusters are constantly evolving. This has implications for the predicted efficacy of the two serotype A vaccines, A22/IRQ and A/MAY/97. The results of these assays are expressed as r-values, representing the antigenic similarity between the vaccine and field isolates. In all *in vivo* studies, challenge viruses were selected based on their low match with the vaccine strain. **An important conclusion from these studies was that in most cases, regardless of low r-values (poor matching), good quality, high-potency vaccines do have the capacity to protect against clinical disease.** Therefore, while a good match *in vitro* may indicate protection *in vivo*, a poor match does not necessarily correlate with no protection. An alternative *in vitro* method for improved ability to predict protection, thereby better guiding decision makers in the event of an outbreak, is desirable. However, currently available techniques are still of value and continued monitoring of circulating strains using these techniques is essential to maintain awareness of the antigenic changes in these continuously evolving viruses.

[25] http://www.fao.org/ag/againfo/commissions/docs/research_group/borovet/app31.pdf

An additional output from the vaccine trials has been information on suitable samples for diagnosis, and windows for detection of infection in different species. Probang samples (scrapings taken from the oro-pharyngeal area using a probang cup) may be useful for detection of virus or viral RNA for a number of months following initial infection, however with increasing time, the number of animals a returning positive test decreases and more frequent and comprehensive sampling would be required to detect cases. Moreover, this method is unreliable and attempting it on poorly restrained animals can be difficult and dangerous, both to the animal and the sample collector. Collection of lesion material can facilitate successful isolation of live virus, but the ideal time to collect is limited. A burst and healing lesion will likely be devoid of infectious virus, and lesions may only be 'fresh' for one or two days. The FMD RMP work has indicated nasal and/or oral swab samples as alternative samples to assist in diagnosis. In all species tested, these samples were positive in most infected animals over the first 10 days following infection, in some cases up to 14 days, and in rare cases for even longer. This was also true for sub-clinically infected animals. Nasal/oral swabs can be positive for FMDV prior to the onset of clinical signs, serving as a method for early detection. Nasal and oral swab samples could prove useful for screening dangerous contact premises or confirming suspect cases where there are no visible lesions. **Considering this, nasal and oral swabs represent a good addition to lesion and serum samples for diagnosis and we recommend they are included in sample collection guidelines.**

Following an outbreak, proof of freedom from FMD virus will be key to achieving a rapid return to trade. Assays (ELISAs) to detect antibodies against FMDV NSPs are the current gold standard for detecting infected animals (including those that have recovered). The FMD RMP has worked to determine the most appropriate ELISAs from those commercially available, as well as the AAHL in-house ELISA. The vaccine and pathogenesis studies in cattle, sheep and pigs have provided serum samples representing all possible scenarios (uninfected, vaccinated and vaccinated and infected), which have been tested to validate and compare the ELISAs. This work has enabled a two-tier strategy to be determined, using one assay for screening large numbers of sera, as would be expected during a large-scale post outbreak surveillance campaign, and a second to confirm any positive results. This approach is in line with strategies accepted by the OIE, and ensures that field based follow-up actions will be limited to only true laboratory positive results. With the experimental samples, we found that all clinically diseased animals seroconverted to NSP and no anti-NSP antibodies were detected in vaccinated non-challenged animals. Therefore, the NSP ELISA is a valuable tool to identify clinically and sub-clinically infected animals and will be a key component in post-outbreak surveillance.

The currently available diagnostic tests (such as RT-qPCR, ELISA) play an important role in detection and monitoring infection and transmission. However, with new approaches and technologies, complementary assays that will increase the amount of information available are important. We have investigated the use of rope sampling as a method of oral fluid collection to obtain diagnostic material from pigs. **Ropes appear to be a cost-effective, non-invasive, sampling tool to detect FMD** in a pig pen, which can assist with disease surveillance.

Sequencing technologies provide additional information regarding the outbreak virus that can be valuable for decisions regarding control options. Partial sequences obtained by conventional methods facilitate the rapid and accurate identification of the serotype and subtype, which could guide the choice of vaccine strain, and can provide information on the probable origin of the outbreak. The development of next generation full-genome sequencing methodologies allows identification of virus isolates directly from clinical samples with low levels of virus present. Use of this method in outbreak analysis can help identify the path of disease transmission within a population. Changes in the genome of FMDV accumulate over time, and by identifying these changes in samples from different

premises, cases can be put in chronological order and spread traced backwards to potentially identify premises where infection was missed. This sequencing method has been successfully developed in collaboration with Duke-NUS, Singapore, and we are in the process of establishing the technique at AAHL, providing a crucial tool in tracing virus during an outbreak. This essential tool and data analysis will be further developed in the next phase of the project.

An additional component of the FMD RMP has been **capacity building**. Materials collected during animal experiments (such as serum or cDNA) have been sent to AAHL, and in collaboration with the DSR group, serve as valuable reagents/samples for assay development and validation. This is paramount to ensuring we have the right tools and experienced staff to detect, characterise and monitor FMD in the event of an outbreak. Through the various animal experiments performed around the world, a number of AAHL staff have also had significant first-hand experience at observing the disease in the different susceptible species, which enhances our understanding of the disease and ensures relevant expertise in Australia.

Building capacity in our region has also been a priority of the project. By working collaboratively in laboratories in Thailand, Vietnam, Lao PDR and Myanmar, our FMD RMP staff have added to the skill levels of the personnel in the participating laboratories. These personnel have received training in virus isolation protocols, serological methods to detect anti-FMDV antibodies, molecular methods for FMDV detection, animal challenge models and working within BSL3 requirements. We have also provided assistance to RRL, Pakchong to perform sequence analysis using the latest tools, and draw conclusions from the phylogenetic tree outputs. In return, the FMD RMP team has gained experience in working with high-throughput technologies and has been enriched by their interaction with diverse cultural groups.

The coordination and successful execution of the work in the FMD RMP would not have been possible without extensive collaboration with laboratories and institute around the world as well as the funding provided by the visionary livestock industries and local partners. **Through this work, Australia has attained significant international recognition in the field.** We are actively involved in the international arena with members of the FMD RMP team serving on international bodies such as the GFRA, SEACFMD and OIE. We have built on existing international relationships and developed new ones, of note in SEA and China. This has facilitated an exchange of knowledge and expertise and a strong collaborative effort to tackle FMD from a regional and global perspective.

In conclusion, the direct benefits resulting from the activities of this project included:

- Confidence that the AVB contains suitable vaccine strains and an understanding of their utility in different species
- An appreciation of the behaviour of different FMDV isolates/serotypes in different livestock species, shedding light on transmission risks and the best samples to collect at different times post infection to aid in detection
- The establishment of fit-for-purpose, validated diagnostic assays and a store of samples of known origin to serve as controls in these assays
- An improved understanding of the FMD situation in SEA, with established networks to ensure continued monitoring of the evolution of FMDV strains and the relevance of AVB vaccines
- Improved capability in laboratories to characterise field isolates and so address the risk at source

- More staff in Australia with direct experience identifying lesions in FMDV infected animals, processing infected samples, and performing molecular, cell culture and serological assays to detect and characterise FMDV.

8.1 The extent to which each project objective was met

1. *Gain comprehensive knowledge about FMD virus strains that pose a geographic high risk to Australia and their comparable likely behaviour in Australian livestock species.*

The project focused on SEA as an area of high risk to Australia due its close proximity to Australia, to the endemic nature of the disease in most countries, and the amount of trade and people movement between SEA and Australia.

We have established close collaborations with the FAO-OIE Regional Reference Laboratory for FMD in SEA in Pakchong, Thailand, and the Regional Animal Health Office VI in Ho Chi Minh City, Vietnam (also the designated national laboratory for FMD), to study the antigenic and genetic characteristics of the FMDV isolates in SEA. Antigen matching studies focusing on recent isolates available in these two laboratories were undertaken to identify the vaccine strains in the AVB that would best match these field viruses. Sequence data obtained from these laboratories were used to study the genetic relationship of these isolates and improve our understanding of the virus evolution over time in SEA (Section 3).

Information gathered during the vaccine efficacy trials revealed how the various FMDV isolates presented clinically in cattle, sheep and pigs, and differences in the levels of virus shedding (Section 4). The pathogenesis studies in pigs and sheep more specifically increased our understanding of the early sites of infection, and provided guidance on which samples should be collected for diagnosis during a necropsy (Section 5).

2. *Devise control strategies tailored to Australian circumstances and store appropriate bulk serum derived reagents for future Australian use.*

Controlling FMD requires early detection, containment of infection, and use of appropriate tools to control the spread and ultimately eradicate the disease as quickly as possible. To achieve this objective, the project assessed different sampling methods including oral and nasal swabs in pigs, sheep and cattle, and rope sampling methods in pigs. Our studies showed the usefulness of these samples in detection of virus/viral RNA even in the absence of overt clinical signs of FMD (Sections 4 and 6.5). The effectiveness of biocontainment to prevent virus transmission in pigs was also demonstrated.

The vaccine efficacy trials showed that vaccination could be used in combination with other measures to control FMD, especially in sheep and cattle, but that improvements in vaccines or their administration are needed to provide better protection in pigs. Emergency vaccination as early as 7 days prior to viral challenge can be used as a strategy to reduce clinical signs of the disease and virus shedding, thereby reducing the load of FMDV in the environment (Section 4).

As a result of the vaccine efficacy studies, well-characterised sets of sera and genetic material are now available for test validation and development. In addition, bulk sera were collected that could be used as controls and in proficiency panels to improve the testing capabilities in Australia (Section 7.5).

3. *Improve laboratory diagnostic capability for FMD virus to rapidly isolate or detect FMD virus and confirm a primary diagnosis by providing AAHL staff the opportunity to work with live virus off shore and so gain experience with FMD culture and recognition of cytopathogenic effects.*

A number of the routine diagnostic assays were tested overseas, on behalf of the AAHL Diagnosis Surveillance and Response group, and recommendations on test improvements were made. The sequencing capability has also been improved which will assist with diagnosis and tracing of virus movement during an outbreak (Section 6.4.1 and 6.4.2).

Staff working in the diagnostic groups have been trained in disease detection, sample collection and handling, and sequencing methods, and a number were involved in the animal trials (Section 7.4).

4. *Validate diagnostic tests (including DIVA) for use in local animal species and breeds, and to conduct genetic 'fingerprinting' (sequencing) on the virus(es) isolated in support of molecular epidemiology and vaccine selection.*

Comparisons between the AAHL in-house NSP assay and commercial kits are in progress (slightly delayed due to the time it took to bring back all the sera from various overseas locations). We know the AAHL assay has good sensitivity and specificity, and will be suitable as a screening test. We still need to determine which of the commercial kits will be preferable as a confirmatory test (Section 6.1).

The panels of sera will also be used for development and validation of the existing cELISAs for FMDV structural proteins in the future.

Limited genome sequencing was carried out in SEA. Historical and contemporary virus sequences were used to identify the different lineages of viruses circulating the SEA, indicating the levels of genetic change happening in the region (Section 3).

The sequencing capabilities at AAHL were improved using conventional methods, as well as developing methods to sequence directly from samples with low concentrations of virus using next generation sequencing (Section 6). One staff member from AAHL's molecular detection group has been trained in the method (Section 7.4) and further developments are planned under the next phase of the project.

5. *Enhance the epidemiological and virological understanding and thus help model virus spread.*

By working closely with laboratories in SEA, large amounts of data on vaccine matching and FMDV sequences were generated. In addition, data generated during the vaccine efficacy trials on the virus excretion patterns in different species after virulent FMDV challenge, with and without vaccination, can be used to help model virus spread. We are working closely with DAWR (Graeme Garner) to identify the information required in the models from the raw data generated (one such example is provided in Appendix 19).

6. *FMD vaccination response policies included in AUSVETPLAN and associated national standard operating procedures that are soundly technically based.*

The FMD RMP is not directly involved in AUSVETPLAN, but the information generated during the FMD RMP has been shared with the Vaccine Expert Advisory Group (VEAG) and was considered when deciding which antigens to include when renewing the AVB (2015). In addition, the lessons learned were considered when developing the document on 'Movement controls for vaccinated animals during an FMD outbreak'. We envisage that the FMD RMP will be consulted more regularly in the future when control measures involving vaccination are discussed. The FMD RMP team has contributed to the revision of the 'Field Guide for Australian Veterinarians' aka 'Blue Book' by providing inputs on different samples to be collected from different species to successfully detect an FMD case.

7. *Produce experimental data on the efficacy (in terms of protection against challenge with circulating high-risk virus isolates) of the vaccine strains and the vaccine potency in Australia's FMD vaccine bank, to maximise the benefits arising from investment in the vaccine bank and to inform FMD response planning.*

Multiple trials were performed to test vaccine efficacy in cattle (Section 4.1), sheep (Section 4.2) and pigs (Section 4.3). A summary is also available in Table 4.2.

9 Conclusions/recommendations

9.1 Insights and recommendations

Vaccine Matching

Constant monitoring of the emerging isolates in the SEA and ME-SA regions helps Australia's outbreak preparedness.

Future R&D: The collaborations established in the region should be maintained and expanded into the future. Continue with *in vitro* vaccine matching studies and sequencing to build a better understanding of the epidemiology in SEA by continuing to work with the existing collaborators and also by forging new collaborations in the region.

Testing the efficacy of vaccines in the Australian Vaccine Bank

Vaccines in the AVB were effective in cattle and sheep, but less so in pigs. In most cases, good quality, high-potency vaccines can protect animals from clinical disease when exposed to FMD virus and also help reduce shedding of virus thereby limiting the spread.

Overall, these results support the composition of the AVB.

Future R&D: Continue with the *in vivo* testing of vaccines when novel variants of FMDV arise where *in vitro* analysis indicate a possible vaccine failure.

Pathogenesis studies

Pathogenesis studies have identified which tissues to sample in suspected cases during necropsy, including for early detection of subclinical cases.

Vaccination and persistent infection in cattle and sheep

Since current policy in Australia dictates that all infected animals will be culled, the significance of the effect of vaccination on prevalence of persistence is minor. Importantly, regardless of vaccination status, all infected animals were identified by the detection of antibodies to FMDV non-structural proteins (NSP). Therefore, irrespective of the low risk of persistently infected cattle and sheep spreading infection, we can be confident the NSP assays available will detect these animals even when vaccination is used.

This will ensure that post outbreak surveillance is effective in detecting previously infected animals regardless of vaccination status and will assist with a rapid return to trade after an outbreak.

Future R&D: Continue with the validation of NSP assays and determining the best protocol to follow to increase specificity of the testing. Continue with the development of a faster in-

house screening assay at AAHL. Ensure the test protocol is adopted within a quality system via the LEADDR network.

Vaccination of pigs

Vaccination in pigs was least successful indicating need to improve response to vaccines in pigs.

Future R&D: This will be a focus in the next phase of the project with investigations into the use of trans-dermal vaccination.

Swabs as diagnostic tools during and after outbreaks

Nasal/oral swabs could prove useful for screening dangerous contact premises or confirming suspect cases where there are no visible FMD lesions (inapparent or subclinical infection) in livestock on these properties.

Future R&D: Ensure nasal and oral swabs are included in sample collection guidelines. Continue with the validation of test protocols to ensure the best sensitivity for surveillance and outbreak control. Ensure that virus in samples collected from farms will be inactivated and that no live virus will be shipped to state laboratories that don't have the suitable biosafety levels.

Diagnostic test validation

The FMD RMP activities have contributed to improved diagnostic capabilities that will have a positive impact for diagnosis of the first case, and for surveillance in the event of an FMD outbreak.

Australia is well prepared to provide an excellent laboratory service.

Future R&D: Continue to monitor virus evolution and ensure the FMD diagnostic assays remain sensitive in the face of ongoing change. Continue with the application of NGS for FMDV and transferring the technology to AAHL. Continue with the development of improved NSP testing and ensuring it is deployed to all state labs via the LEADDR network. Monitor new diagnostic developments and validate for use in Australia if needed.

Capacity building in Australia and SEA

By closely working with the national and reference laboratories for FMD in SEA, Australian scientists have been exposed to opportunities to work with infectious FMDV and ensure continued expertise that could be applied in Australia. These training opportunities have been reciprocal. The FMD RMP team is regarded as a trusted advisor in many of the SEA laboratories. Improved diagnostics could lead to improved control measures in SEA, thereby decreasing the risk to Australia.

Future R&D: Via the collaborations in SEA and elsewhere, there will be continuing opportunities for capacity building.

10 Key messages

10.1 Australia's laboratories are better prepared to deal with an FMD outbreak as a result of this project

Through test validation and enhancement of current diagnostic assays, mostly due to the ability to generate material or work with live virus abroad, there is more confidence that Australia can rapidly and accurately diagnose the disease.

10.2 Australia's laboratories should continue to maintain links with laboratories in SEA to monitor the viral change over time

Knowledge about the viruses circulating and emerging in the SEA region is important to ensure the vaccines in the AVB will protect Australian livestock and that diagnostic assays will be sensitive. It adds to Australia's preparedness to know of any major changes in the epidemiology of the disease that may increase the risk of an outbreak.

10.3 Vaccination could be an effective tool to assist in controlling an FMD outbreak by providing time for other control measures to take effect

The role of vaccination as an important additional tool to control an outbreak by decreasing the amount of virus being excreted has been demonstrated. It could therefore assist in controlling and eradicating the disease and once again ensure Australia can return to trade in live animals and animal products faster. The inclusion of vaccination as an option during an outbreak in policy is well supported by the results.

10.4 Vaccination in cattle and sheep are highly effective, but less so in pigs

Vaccination provided clinical protection to cattle and sheep when immunity has developed fully. Even at earlier time points, in the absence of detectable antibodies, vaccination provides partial protection. More importantly, it decreases the amount of virus excreted into the environment and can so assist in preventing further spread of an outbreak.

10.5 New approaches are needed to improve vaccine efficacy in pigs

Vaccination was not very effective in pigs, indicating that novel methods are needed to ensure vaccine protection for pigs. The results indicated that different approaches are needed to provide protection in pigs, if it is critical to control FMD in pigs or prevent them from becoming infected, and act as source of infection to other susceptible species, such as cattle. In a future project, alternative methods of vaccination will be investigated to ensure Australia is prepared to more effectively control FMD in pigs.

10.6 Biosecurity is of utmost importance and avoidance of risk practises will protect Australia against outbreaks of FMD

The viruses used in these studies were all different for their ability to infect cattle, sheep and pigs. Whereas some viruses were virulent and caused severe clinical disease, others caused sub-clinical or low grade FMD. In these cases it will not be easy to detect disease, pointing to the need for farmers and producers to observe their animals very carefully on a regular basis, but more importantly, to avoid high risk practises that could lead to the introduction of disease.

10.7 Alternative and additional sampling methods must be considered for surveillance and screening

In addition to routine sampling procedures where clinical material and blood are collected, oral swabs and nasal swabs should be added to the list of samples for surveillance and screening of in contact premises. Our studies have shown that these samples are valuable to detect virus with sub-clinically infected animals whether they are vaccinated or not and for a time period after clinical lesions have healed. Bulk sampling methods, such as the use of rope chews for sampling pigs can be used for screening diseases including FMD.

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Appendix 9: FMDV Asia1 pathogenesis and vaccine efficacy studies in sheep

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Appendix 11: Report on vaccine efficacy trial with O1 Manisa and O Mya-98 monovalent vaccine against O-SKR (South Korean isolate of the O-Myanmar lineage) in pigs, the Pirbright Institute June-July 2013

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Appendix 15: Preliminary comparison of the AAHL in-house 3NSP competitive ELISA and two alternative commercial ELISA kits for the detection of antibodies to non-structural proteins in sheep and cattle sera - A collaboration between the FMD Risk Management Project and the Diagnostic Emergency Response Laboratory, CSIRO AAHL, Australia

Appendix 16: An improved approach to whole genome sequencing of FMDV in clinical samples - A collaboration between the FMD Risk Management Project, CSIRO Australia and Duke-NUS Medical School, Singapore

Appendix 17: Collection of oral fluids using cotton ropes as a sampling method to detect foot-and-mouth disease virus infection in pigs

Appendix 18: Policy Meets Science

Appendix 19: Sample excel sheet with information for FMD modelling

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13 List of abbreviations/acronyms

A/MAY/97 – A Malaysia 97; a serotype A vaccine strain
A22/IRQ – A22 Iraq/24/64; a serotype A vaccine strain
AAHL – Australian Animal Health Laboratory
Ag-ELISA – Antigen detection ELISA
AI – Aerosol infection
Asia1 Shamir – Asia1 Shamir/Israel/89; A serotype Asia1 vaccine strain
AVB – Australian Vaccine Bank
BHK – Baby Hamster Kidney
BSL – Bio Safety Level
BTY – Bovine Thyroid
CB – Coronary band
cDNA – Complementary Deoxyribonucleic Acid
c-ELISA – Competitive ELISA
CID_{50} – 50% Cattle Infectious Dose
CSIRO – Commonwealth Scientific and Industrial Research Organisation
DC – Direct contact
DIVA – Differentiating Infected from Vaccinated Animals
dpc – day post challenge
dpv – day post vaccination
DSR – Disease Surveillance and Response
Duke-NUS – Duke National University of Singapore
ELISA – Enzyme-Linked Immunosorbent Assay
FMD – Foot-and-Mouth Disease
FMD RMP – FMD Risk Management Project
FMDV – Foot-and-Mouth Disease Virus
GFRA – Global Foot-and-Mouth Disease Research Alliance
G-IV – Genotype IV
G-VII – Genotype VII
HB – Heel bulb
IDL – Intra-dermo lingual
Ind-2001d – India-2001d; A sub-lineage of serotype O virus from ME-SA
INP – Intra-nasopharyngeal
LFBK$\alpha_v\beta_6$ – A recombinant primary porcine kidney cell line that has been modified to express the bovine cell receptors of FMD (integrins α_v and β_6)
LP-ELISA – Liquid Phase blocking ELISA for antibody detection
Merial, UK – M/s Merial Company Limited, United Kingdom
ME-SA – Middle East-South Asia
Mya-98 – Myanmar 1998; a lineage of serotype O virus from SEA
NGS – Next Generation Sequencing
NSP – Non-structural Proteins
O1 Manisa – O1 Manisa/Turkey/69; a serotype O vaccine strain
O3039 – a serotype O vaccine strain
OD value – Optical density value from ELISA assay
OIE – World Organisation for Animal Health (formerly Office International des Epizooties)
PD_{50} – 50% Protective Dose
pfu – plaque forming units
PID_{50} – 50% Pig Infectious Dose 50
RAHO6 – Regional Animal Health Office No: 6 in Ho Chi Minh City, Vietnam
RNA – Ribonucleic Acid
RRL – Regional Reference Laboratory in Pakchong, Thailand
RT-qPCR – Reverse transcription quantitative polymerase chain reaction

SAT – South African Territories; 1, 2 and 3 represent three different FMD serotypes from that region
SEA – South East Asia
SEACFMD – South East Asia China Foot-and-Mouth Disease Campaign
Sindh-08 – Sindh 2008; a lineage of serotype Asia1 virus from Pakistan-Sindh Region
SP – Structural Proteins
$TCID_{50}$ – 50% Tissue Culture Infectious Dose
VNT – Virus neutralisation test
WRL – World Reference Laboratory for FMD, The Pirbright Institute, United Kingdom
ZZ-TR – Primary goat tongue epithelium cell line

Appendix 7: Early protection in sheep against intratypic heterologous challenge with serotype O foot-and-mouth disease virus using high-potency, emergency vaccine

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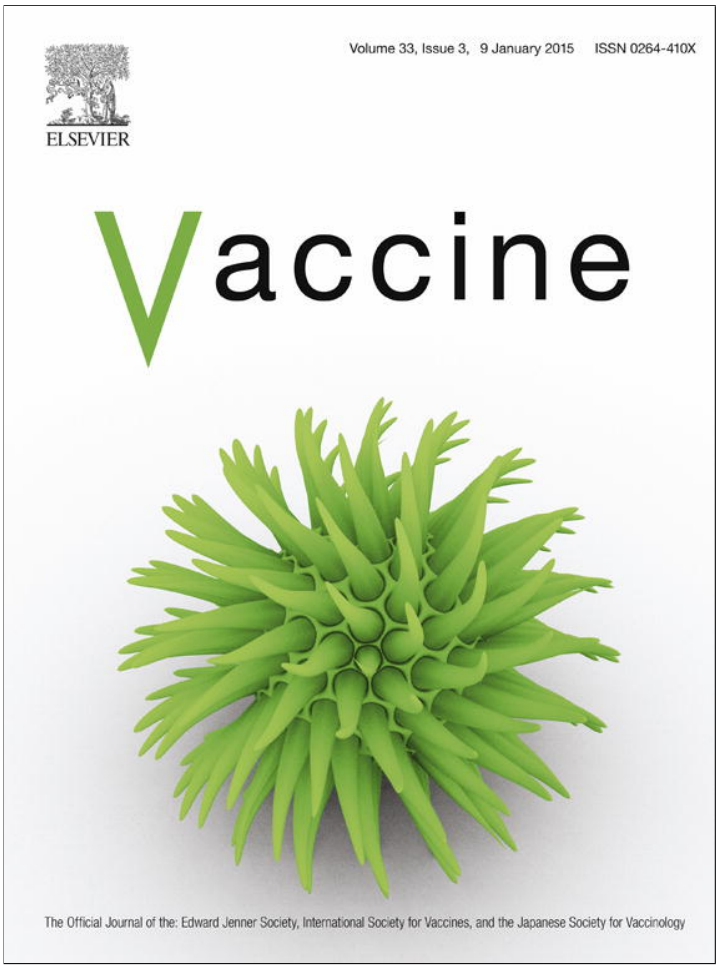
Appendix 7: Early protection in sheep against intratypic heterologous challenge with serotype O foot-and-mouth disease virus using high-potency, emergency vaccine



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Early protection in sheep against intratypic heterologous challenge with serotype O foot-and-mouth disease virus using high-potency, emergency vaccine

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ABSTRACT

In 2009–2011, spread of a serotype O foot-and-mouth disease virus (FMDV) belonging to the South East Asia topotype led to the culling of over 3.5 million cattle and pigs in Japan and Korea. The O1 Manisa vaccine (belonging to the Middle East-South Asian topotype) was used at high potency in Korea to limit the expansion of the outbreak. However, no data are available on the spread of this virus or the efficacy of the O1 Manisa vaccine against this virus in sheep. In this study, the early protection afforded with a high potency (>6 PD_{50}) FMD O1 Manisa vaccine against challenge with the O/SKR/2010 virus was tested in sheep. Sheep ($n=8$) were vaccinated 4 days prior to continuous direct-contact challenge with donor sheep. Donor sheep were infected with FMDV O/SKR/2010 by coronary band inoculation 24 h prior to contact with the vaccinated animals, or unvaccinated controls ($n=4$). Three of the four control sheep became infected, two clinically. All eight O1 Manisa vaccinated sheep were protected from clinical disease. None had detectable antibodies to FMDV non-structural proteins (3ABC), no virus was isolated from nasal swabs, saliva or oro-pharyngeal fluid and none became carriers. Using this model of challenge, sheep were protected against infection as early as 4 days post vaccination.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly infectious disease of cloven-hoofed animals caused by FMD virus (FMDV), a small, positive-sense RNA virus in the Genus *Aphthovirus*, Family *Picornaviridae*. There are seven serotypes of FMDV (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3) and infection or vaccination with one serotype does not confer protection against the other serotypes [1,2]. Within each serotype are distinct genetic lineages known as topotypes [3]. The genetic variation observed within each serotype can also result in antigenic variation, impairing the ability of vaccines to protect against heterologous strains of the same serotype [4,5]. Serotype O FMDV is the most widespread serotype throughout the world and eight topotypes have been designated [6].

Following the 2001 outbreak in the United Kingdom, deliberation on the necessity of mass culling and the non-vaccination policy led to a re-evaluation of FMD control policies. In both Europe and Australia, emergency vaccination will now be considered in an FMDV outbreak (AUSVETPLAN; EU Council Directive 2003/85/EC) and similar changes are occurring in North America. Understanding the outcome of intratypic (viruses of the same serotype), heterologous challenge for the different vaccine strains is critical when determining which strains should be stored in national antigen banks in case of emergency or used during routine prophylactic vaccination where the latter may still be a part of FMD control. *In vivo* studies remain the most accurate way to determine the effectiveness of FMD vaccines against heterologous challenge (reviewed by [7]).

Sheep represent a large component of the world's FMD-susceptible livestock and some recent outbreaks, including that in the UK in 2001, have involved movement of sheep as an important factor for spreading the infection [8–10]. The clinical signs of FMD in sheep are frequently mild or inapparent, which facilitates the spread of infection due to undetected cases. Furthermore, sheep, like other ruminants, can become long-term sub-clinical carriers of FMDV [11,12] although epidemiologically not important. Vaccination using high potency (>6 protective dose $(PD)_{50}$) vaccines has been shown to be effective in protecting animals challenged as

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early as 4 days post-vaccination (dpv) [13–16], reducing the titre and duration of FMDV excretion, limiting the possibility of transmission and potentially minimising the duration and intensity of an outbreak.

In Australia, FMD is of major concern to the livestock industries, with the potential to cause losses in excess of AUD$50 billion over 10 years [17], as a result of many years of lost revenue due to restrictions placed on the export of Australian products. Foot-and-mouth disease is endemic in many countries in South East Asia (SEA), which through the volume of people and potentially illegal products entering, is considered the most likely source of an FMDV incursion to Australia. In 2009–2011, spread of serotype A and O viruses from SEA led to outbreaks of FMD in six countries in the eastern Asia region [18,19]. Spread of serotype O virus belonging to the SEA topotype led to the culling of over 3.5 million cattle and pigs in Japan and Korea [19]. While the *in vitro* antigenic relationship between the O1 Manisa vaccine (belonging to the ME-SA topotype) and field strains was just acceptable (r value ~0.3), use of this vaccine at high potency in South Korea eventually assisted in controlling the outbreaks [20]. However, no data are available on the spread of this virus or the efficacy of the O1 Manisa vaccine against this virus in sheep.

The current study was undertaken to examine the ability of single vaccination with high potency vaccine (>6 PD_{50}) to afford protection in sheep and prevent the development of persistent infection following heterologous challenge. To mimic an emergency vaccination regime in the field, sheep were vaccinated with O1 Manisa monovalent vaccine and 4 days later challenged by direct contact with sheep infected with the serotype O virus strain that caused outbreaks in South Korea during 2010.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the recommendations in the Australian and Canadian codes of practice for the care and use of animals and was endorsed by both the Australian Animal Health Laboratory's Animal Ethics Committee (AEC 1637) and the National Centre for Foreign Animal Disease (NCFAD) Animal Care Committee (C-13-005).

2.2. Animals

Twenty-four Rideau Arcott/Ile de France male sheep aged between 6 and 12 months (~40 kg) were used. All animals were housed in the BSL3 animal facility at the NCFAD, Winnipeg, Canada. The sheep were divided into three groups: unvaccinated, coronary band (CB) inoculated donor sheep (n = 12); vaccinated contact-challenged (VC) sheep (n = 8); and unvaccinated contact-challenged (UC) sheep (n = 4).

2.3. Vaccination

The VC sheep were vaccinated with one full sheep dose (1 ml) of high potency (>6 PD_{50}) FMDV O1 Manisa double-oil emulsion vaccine (Merial Animal Health, Pirbright, UK), administered intramuscularly in the neck region above the left shoulder. Vaccination was given 4 days prior to challenge.

2.4. Challenge

The challenge virus was O/SKR/4/2010 of the SEA topotype (Mya98 lineage), originally isolated from cattle [21], that had been passaged twice in primary bovine thyroid cells (at the FMD World Reference Laboratory, Pirbright Institute, UK) and twice in primary lamb kidney cells (at NCFAD). The P1 capsid coding region of the virus used for inoculation was sequenced and it was confirmed that no known adaptation to cell culture (such as changes to positively charged amino acids mediating binding to heparan sulphate-like moieties) had occurred. The 12 donor sheep were each inoculated intradermally into the CB with 6.5 $\log_{10}$ $TCID_{50}$ of virus in a volume of 0.5 ml. The VC and UC sheep were challenged by direct contact with the donor sheep 24 h later (designated as 0 days post-contact challenge (dpc)). The sheep were arranged into groups of four, housed in separate rooms, with two VC sheep (rooms 1–4) or two UC sheep (rooms 5 and 6) placed in continuous direct contact with two directly inoculated donor sheep.

2.5. Monitoring and sample collection

The sheep were monitored for 35 days after challenge and rectal temperatures and clinical scores were recorded daily to 14 dpc. Sheep showing elevated temperatures (>40.0 °C) were considered as having pyrexia. The tongue, gums and feet were examined for lesions with each site where lesions were observed, not including the inoculation site, given a score of '1' (maximum score of 5).

Blood, for RT-qPCR and serology, was collected at −4 dpc, daily between −1 and 14 dpc and then weekly to 35 dpc. Nasal secretions, saliva and rectal swabs were collected at the same time points. Swabs were placed in tubes containing 1 ml of phosphate buffered saline for RT-qPCR. Oro-pharyngeal fluid (OPF) was collected with a small probang sampling cup and mixed with 2 ml cell culture media. Collection was at −4, 0, 7, 10 14, 21, 28 and 35 dpc. All samples were stored at −70 °C until processed.

2.6. Virus isolation

Oro-pharyngeal fluid samples were examined for the presence of live virus by inoculation on to foetal bovine kidney (LFBK) cells [22] grown in 24-well cell culture trays, according to standard procedures. Cells were examined for cytopathic effect (CPE) after 24, 48 and 72 h and if no CPE was observed, a blind passage followed. Supernatants were tested using an in-house FMDV antigen enzyme-linked immunosorbent assay (ELISA) [23].

2.7. Detection of FMDV RNA by RT-qPCR

The amount of viral RNA in whole blood, OPF and nasal, oral and faecal swab samples was quantified by a TaqMan RT-qPCR assay [24]. Viral RNA was extracted from 50 μl of sample with the MagMAX™-96 Viral RNA Isolation Kit (Life Technologies) using the MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies). One-step RT-qPCR was performed using the AgPath ID One-Step RT-PCR reagents (Life Technologies) on the Applied Biosystems 7500 Real-Time PCR Instrument. All samples were tested in duplicate and samples with poor Ct value correlation in the duplicate reactions were repeated. Samples with a Ct >36 were considered negative.

2.8. Determination of neutralising antibody titre

Heat inactivated (56 °C, 30 min) serum samples were used for neutralisation assays on LFBK cells. Sera with titres >1.5 $\log_{10}$ (1:32) were considered positive (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals).

2.9. Detection of antibodies to non-structural and structural proteins by ELISA

Sera were tested for the presence of antibodies against viral non-structural proteins (NSP) by an in-house competitive ELISA

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Table 1
Pyrexia and lesion scores in donor and vaccinated and unvaccinated contact sheep.

Group	Sheep No.	Pyrexia	Days post-inoculation												
			0	1	2	3	4	5	6	7	8	9	10	11	15
Donor	9	Y[a]	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	Y	0	0	0	0	0	1	2	2	1[c]	1	1	1	0
	11	Y	0	0	1	2	3	3	3	3	3	2	2	1	0
	12	N[b]	0	0	1	1	1	1	1	1	1	1	0	0	0
	13	Y	0	0	1	1	2	3	4	4	4	2	2	2	0
	14	N	0	0	1	2	2	2	2	2	3	2	2	1	0
	15	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
	16	Y	0	0	0	0	2	3	3	3	3	3	3	2	0
	21	Y	0	0	0	0	1	1	1	1	1	1	1	0	0
	22	Y	0	0	0	3	3	4	3	3	1	1	1	0	0
	23	Y	0	0	1	2	2	3	1	1	1	1	1	1	0
	24	Y	0	0	0	1	1	1	1	0	0	0	0	0	0

Group	Sheep No.	Pyrexia	Days post-challenge											
			−1	0	1	2	3	4	5	6	7	8	9	10	14
UC	17	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	18	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	19	Y	0	0	0	0	0	0	0	1	3	4	4	5	4
	20	Y	0	0	0	0	0	0	0	0	0	0	1	1	0
VC	1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	Y	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	N	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	Y	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] yes.
[b] no.
[c] A reduction in score indicates lesions have healed.
Shaded area = generalised lesions.

(3ABC-ELISA) [23] and for the presence of antibodies against FMDV type O structural proteins by O-serotype specific in house solid phase competition ELISA (SPCE) using reagents homologous to O1 Manisa [25].

3. Results

3.1. Donor sheep

Ten of the 12 donor sheep developed generalised clinical disease from 2 days post-inoculation (dpi; 1 dpc), albeit of varying severity (Table 1). Lesions were observed on the CB, heel pad and interdigital spaces of the feet, the tongue and the gums, however lameness was only observed in four of the donors. Pyrexia occurred in 10 of the 12 sheep (Table 1) at 1 dpi, although no correlation between pyrexia and disease severity was observed.

Virus isolation and RT-qPCR indicated viraemia in 11 donor sheep between 1 and 4 dpi (0 and 3 dpc; Fig. 1). In sheep 9, viraemia was only detected at 8 dpi (7 dpc), in the complete absence of clinical signs. Interestingly, sheep 14 demonstrated an extended period of viraemia with FMDV RNA detected between 1 and 7 dpi, although virus was isolated only at 2 and 3 dpi (Fig. 1).

To determine if virus was being excreted, nasal, oral and faecal swab samples were tested for the presence of FMDV RNA by RT-qPCR. Viral genome was detected in the nasal and/or oral swab samples from 11 of the 12 donor sheep throughout the acute phase (1–6 dpi/0–5 dpc), with some animals being positive, particularly in the oral swabs, intermittently up to 29 dpi (28 dpc; Table 2). The highest levels of virus excretion in nasal and oral swabs were seen between 2 and 4 dpi (1 and 3 dpc). Faecal swabs from all of the sheep in the study were negative at all time points (data not shown).

To identify animals that became carriers, OPF samples were investigated for the presence of both infectious FMDV and FMDV RNA genomes by cell culture and RT-qPCR, respectively. Virus was isolated from 7 of 12 and viral RNA detected in 10 of 12 donor sheep at 8 dpi (7 dpc). Six of the sheep in this group established persistent infection with virus isolated at 29 and/or 36 dpi (28 or 35 dpc), although viral RNA was detected in one additional sheep (sheep 14) at these time points (Table 3).

All donor sheep became positive for neutralising antibodies against FMDV O/SKR/4/2010 and O1 Manisa, by 5–6 dpi (4–5 dpc), with the exception of sheep 9 that was not positive until day 12 dpi (data not shown). The antibody titres were 0.5–1 $\log_{10}$ higher against the challenge virus when compared to the vaccine strain (Fig. 2A). All donor sheep, except sheep 9, were positive in the FMDV SPCE from 3 dpi (Fig. 3A).

To further substantiate that virus replication had occurred in the donor sheep, sera were assayed for anti-NSP antibodies. All donor sheep seroconverted to FMDV NSP from 6 dpi, with the exception of sheep 9 that did not produce antibodies to NSP at any time point tested (Fig. 3B). A drop in anti-NSP antibody was seen in sheep 14 between 9 and 15 dpi. The reason for this apparent drop in detectable antibody level is not clear.

3.2. Vaccinated sheep

All of the VC sheep were protected against clinical disease following direct contact FMDV challenge (Table 1). Pyrexia was observed in 3 of the 8 sheep at different times between 1 and 5 dpc. Viraemia was not detected in any of the animals (data not shown). In nasal swab samples, two sheep were positive by RT-qPCR: sheep 4 at 6 dpc and sheep 7 at 3 dpc (Table 2). No FMDV RNA was detected

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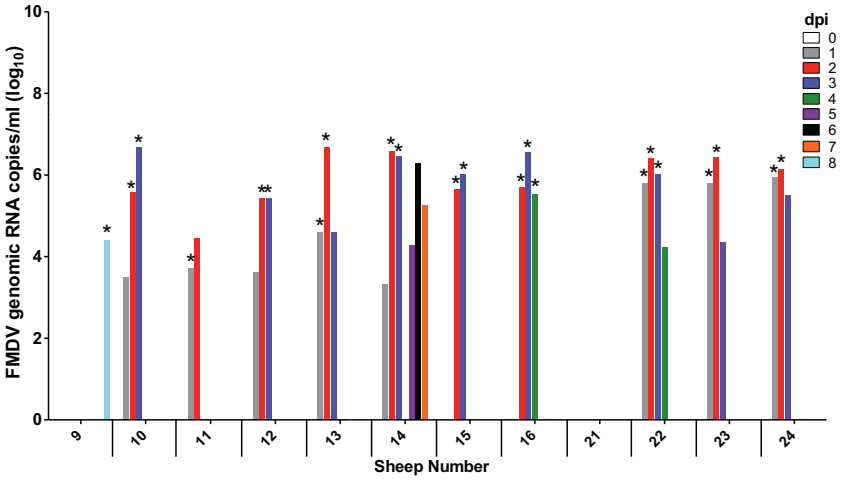

Fig. 1. Viraemia in donor sheep determined by RT-qPCR (FMDV RNA copies per ml whole blood) and virus isolation on LFBK cells (VI positive samples are indicated with *).

in the oral swabs or OPF and no virus was isolated from OPF at any time point (Tables 2 and 3).

Neutralising antibodies as a result of vaccination were below the level of detection at the time of challenge (0 dpc; 4 dpv). All of the VC animals seroconverted to both O/SKR/4/2010 and O1 Manisa 5–10 dpc (9–14 dpv) (Fig. 2B). There was no difference on average in the titres to the vaccine strain and the challenge virus. Overall, the neutralising titres at 21 dpc were lower than those observed in the donor and UC sheep.

The SPCE results indicated that 5 of the 8 sheep had anti-FMDV structural protein antibodies by 3 dpc, however, sheep 2, 4 and 8 did not seroconvert until 6 dpc (Fig. 3 C). In sheep 8, the antibodies started declining at 10 dpc, but increased again at 28 dpc, while the antibody levels in sheep 1 were below the positivity cut-off from 14 dpc. A biphasic response was observed in 7 of the sheep, but the possible reason for this is unclear. Antibodies to FMDV NSP were not detected in any of the VC sheep throughout the study (Fig. 3D).

3.3. Unvaccinated contact sheep

Clinical disease was observed in two of the four UC sheep from the same room (sheep 19 and 20) from 6 to 14 dpc (Table 1), including foot and oral lesions in both animals and lameness in sheep 19. However, viraemia was only detected in sheep 19, by virus isolation but not RT-qPCR, at 4 dpc (data not shown). Viral RNA was detected in the oral and nasal swabs of sheep 19 from 3 to 6 dpc, and in the oral swabs from sheep 20 at 1, 3, 14 and 28 dpc (Table 2). A nasal swab from sheep 17 was positive on one occasion at 14 dpc

Table 2
Detection of FMDV RNA in nasal and oral swabs from donor and vaccinated and unvaccinated contact sheep.

Room No.	Sheep	No.	Days post-challenge											
			−4	0	1	2	3	4	5	6	10	14	21	28
1	VC	1	−[a]/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
		2	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Donor	9	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
		10	−/−	−/−	4.58[b]/−	4.06/6.43	3.70/4.32	−/−	−/−	−/−	−/4.76	−/−	−/−	−/−
2	VC	3	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
		4	−/−	−/−	−/−	−/−	−/−	−/−	−/−	4.41/−	−/−	−/−	−/−	−/−
	Donor	11	−/−	5.42/4.86	−/6.47	5.04/5.76	−/4.41	3.68/4.50	−/−	−/−	−/−	−/5.03	−/−	−/4.28
		12	−/−	−/3.48	6.84/5.76	6.10/4.55	7.12/−	−/5.29	−/−	−/−	−/−	−/−	−/−	−/−
3	VC	5	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
		6	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Donor	13	−/−	−/4.46	6.68/−	5.84/5.68	−/5.38	−/−	−/−	−/−	6.11/−	4.65/−	−/−	−/−
		14	−/−	−/4.87	−/4.55	7.29/−	−/7.86	−/−	−/−	−/−	−/−	−/−	−/−	−/−
4	VC	7	−/−	−/−	−/−	−/−	4.11/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
		8	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Donor	15	−/−	4.97/−	−/−	6.43/−	5.56/4.95	−/6.21	−/−	−/−	−/−	−/−	−/−	−/4.47
		16	−/−	−/−	5.28/−	5.88/−	3.99/−	−/−	4.65	−/−	−/−	−/4.11	−/−	−/−
5	UC	17	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	4.08/−	−/−	−/−
		18	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Donor	21	−/−	−/−	−/3.60	−/−	−/−	−/−	−/−	−/−	−/4.99	−/−	−/−	−/−
		22	−/−	−/4.62	5.68/6.01	5.92/6.70	5.89/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	UC	19	−/−	−/−	−/−	−/−	6.81/−	5.90/−	4.60/6.14	−/4.59	−/−	−/−	−/−	−/−
		20	−/−	−/−	−/3.92	−/−	−/5.71	−/−	−/−	−/−	−/−	−/6.05	−/−	−/4.08
	Donor	23	−/−	−/5.99	−/6.59	−/−	−/−	−/−	−/−	−/−	−/−	−/4.36	−/−	−/−
		24	−/−	−/3.64	6.25/6.01	4.05/5.13	−/4.53	−/4.79	−/−	−/−	−/−	−/−	−/−	−/−

[a] Negative by RT-qPCR.
[b] RNA copies/swab (log_{10}).
−/− = nasal swab/oral swab.

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Table 3
Virus detection in oro-pharyngeal fluid from donor and vaccinated and unvaccinated contact sheep.

Group	Animal	Virus detection in OPF									
		−4 dpc		7 dpc		14 dpc		28 dpc		35 dpc	
		RT-qPCR	VI[a]	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI	RT-qPCR	VI
Donor	9										
	10			4.5[b]	+[c]		+				+
	11			5.0	+	5.6	+	5.6	+	5.0	+
	12					6.4	+	5.3	+		
	13			5.8							
	14			5.2	+			5.0		4.6	
	15			5.5	+			3.3	+	5.2	+
	16			6.6	+	4.2				5.3	+
	21			5.3							
	22			5.2	+						
	23			6.1	+	5.7	+	5.1	+	4.9	+
	24										
UC	17										
	18										
	19			7.5	+	5.0	+	4.8	+	4.1	
	20					7.4	+	5.5		6.5	+
VC	1										
	2										
	3										
	4										
	5										
	6										
	7										
	8										

[a] virus isolation on LFBK cells.
[b] RNA copies/ml (log_{10}).
[c] positive by VI.

(Table 2). No virus was detected in OPF samples from sheep 17 and 18 at any time point, whereas, sheep 19 was positive from 7 dpc and sheep 20 positive from 14 dpc. In both of these animals virus was isolated up to 28 or 35 dpc (Table 3).

Neutralising antibodies to O/SKR/4/2010 (Fig. 2C) and O1 Manisa (Fig. 2D) were detected in sheep 19 from 10 dpc and in sheep 17 and 20 at 21 dpc. As with the donor sheep, neutralising titres were slightly higher to the challenge virus than to O1 Manisa. The higher titres seen in sheep 19 at 10 dpc suggest this animal was infected a few days before sheep 20 and 17, supported by the delay in clinical signs and FMDV RNA detection for sheep 20. No neutralising antibodies were detected in the serum from sheep 18 at any time point, suggesting this animal did not become infected.

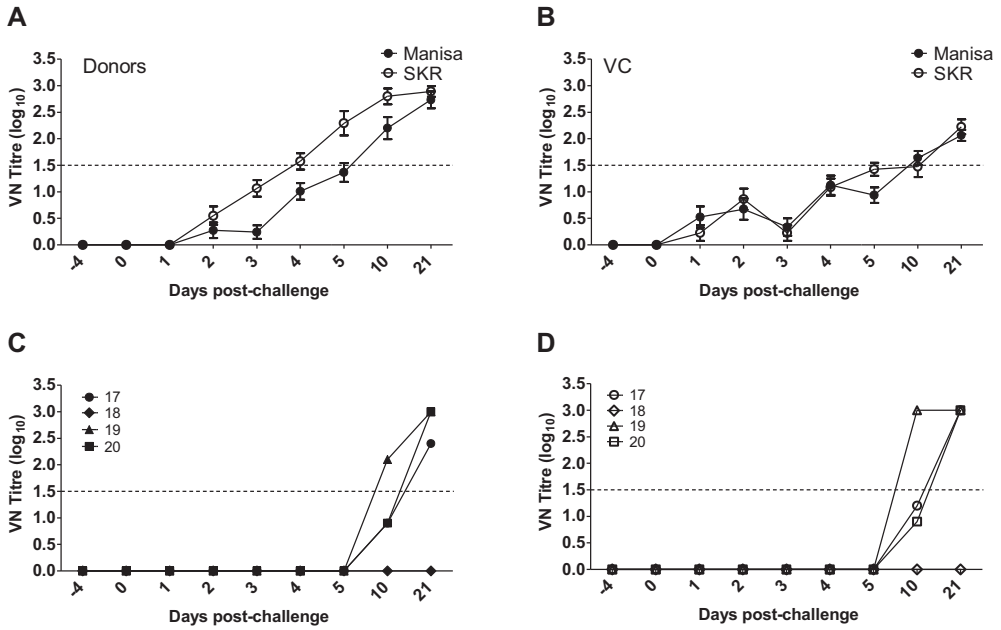

Fig. 2. Mean neutralising antibody response in donor (A) or VC (B) sheep against the vaccine strain O1 Manisa (open circles) or the challenge strains O SKR 4/2010 (closed circles). Neutralising antibody response in the individual UC control sheep (sheep 17–20) against the vaccine strain O1 Manisa (C) and the challenge strains O SKR 4/2010 (D). Titres <1.5 log_{10} were considered negative; titres >3 are reported as 3.

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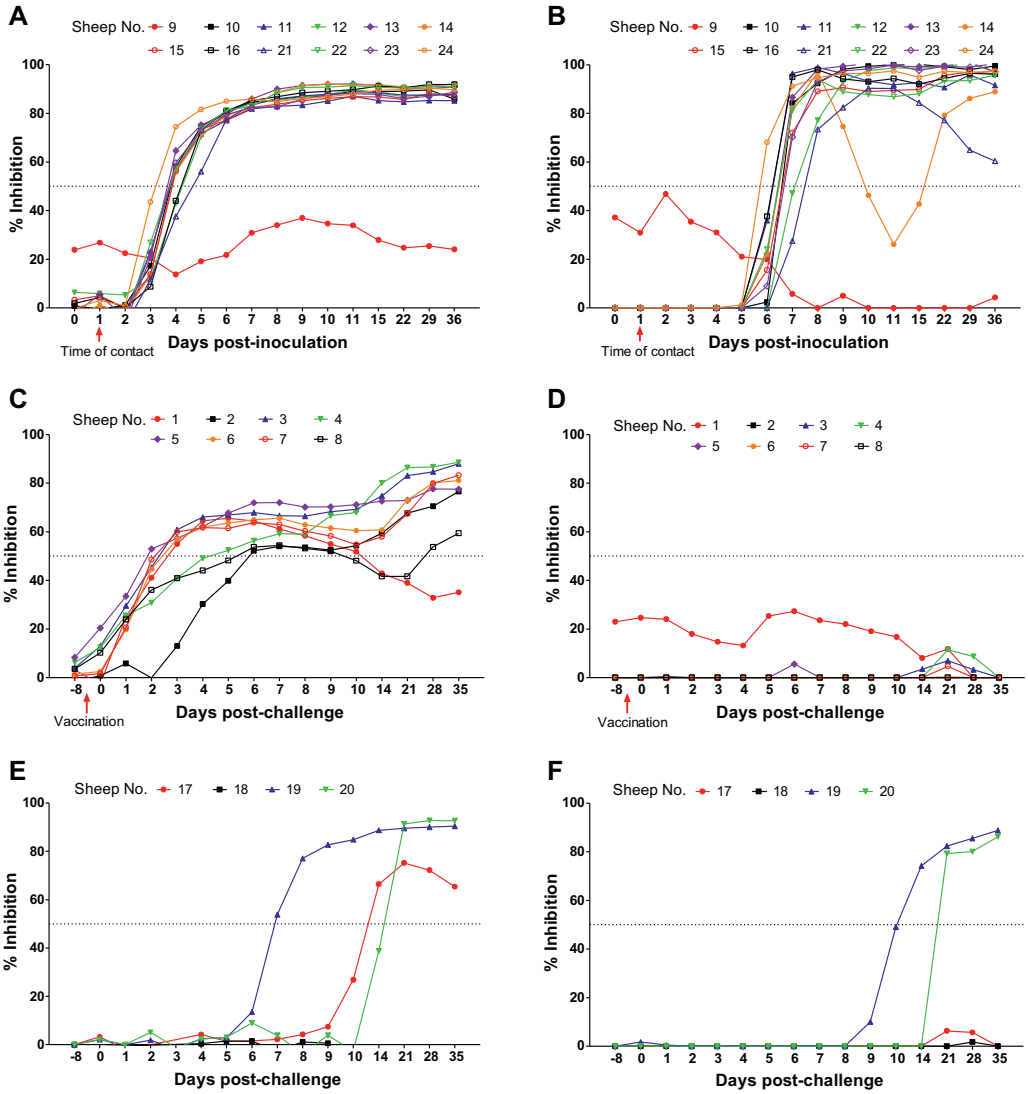

Fig. 3. Antibody to FMDV type O structural proteins (SPCE) and non-structural proteins (3ABC ELISA) in donor sheep (A – SPCE; B – 3ABC); VC sheep (C – SPCE; D – 3ABC) and UC sheep (E – SPCE; F – 3ABC). % Inhibition >50 is considered positive.

In the SPCE, sheep 19 was positive from 7 dpc and sheep 20 from 21 dpc; sampling was weekly from 14 dpc. Sheep 17 was positive from 14 dpc, however by 28 dpc these antibodies appeared to be waning (Fig. 3E). Sheep 18 was negative on all days. Antibodies to FMDV NSPs were detected in sheep 19 from 10 dpc and in sheep 20 from 21 dpc, while no NSP antibody was detected in sheep 17 or 18 (Fig. 3F).

4. Discussion

In the management of FMD, vaccines are important but represent just one of the components required for successful control. Vaccination can often protect against clinical disease but can be less effective at preventing infection, virus excretion or the establishment of persistent infection [26–29]. *In vivo* vaccine experiments expand our understanding of the virus-host relationship but variables such as challenge route and animal numbers limit the significance of any single study and the direct relevance to a specific field situation. In this study, a direct intra-species contact-challenge route was chosen to reflect what is likely to occur in the field. The data indicate, albeit based on a limited number of animals, that the vaccine provided efficient protection in this challenge model with none of the contact-challenged vaccinated sheep developing clinical disease. In addition, there was no evidence of virus replication, with no detection of viraemia or antibodies to NSP. In contrast, three of the four unvaccinated contact sheep became infected, two clinically. Taken together, these results suggest emergency vaccination with high potency O1 Manisa vaccine may play a role in preventing the spread of an intratypic heterologous virus like O/SKR/4/2010 in a sheep population. However, additional research to further elucidate the protective capability of O1 Manisa against O/SKR/2010 in sheep, for example using different routes of infection, is required and is in progress.

The efficacy of the vaccine was measured by assessing the clinical disease, virus replication and virus excretion in the vaccinated animals, compared to unvaccinated control animals. Of the 12 donor sheep, 11 were successfully infected and 10 developed signs of generalised disease. Analysis of swabs and probang samples provided evidence that the donor sheep in each room were shedding virus and provided challenge to the contact animals. While it appeared CB infection of the donor sheep 9 was unsuccessful, this animal did develop FMDV neutralising antibodies from 11 dpc indicating a sub-clinical infection, presumably resulting from exposure to the other donor sheep in this room and

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thereby serving as another contact control. This provided further evidence of challenge to the vaccinated sheep. Sheep are known to excrete relatively low levels of FMDV which can complicate efficacious transmission in an experimental setting. Previous reports describe infection of 33–100% of unvaccinated controls by direct-contact, although often only subclinical [16,30–33]. As 3 of 4 contact control animals became infected, 2 of which developed generalised disease, it can be inferred that the vaccinated animals in this study were sufficiently challenged. However, as no strong anamnestic response was evident and overall VNT titres in the vaccinated sheep were lower than in the unvaccinated sheep, it is difficult to confirm this challenge in the absence of a vaccination only control. Neutralising antibodies were detected sooner in the VC than in the UC sheep (5 dpc/9 dpv compared to 10 dpc), which may suggest a contribution of the vaccine to the immune response after challenge. Viral RNA was detected in nasal swabs from two of the VC sheep, and while this may have resulted from environmental exposure rather than excretion, for sheep 4 at least, the detection was at a time when no virus was detected from the corresponding donors.

A significant difference was observed between the neutralising titres in post-infection sera against the vaccine strain and the challenge strain which disappeared on day 21 (Fig. 2A). However, in sera considered post-vaccination sera (as no boost due to infection was observed) (Fig. 2B), it appears that the *r*-value for the sheep is approximately 1.0, based on the lack of difference between the serum titres against the challenge and the vaccine strains. This finding is of interest since it is not known whether or not there is a systematic difference between cattle and sheep when determining antigenic relationships.

Of the 15 unvaccinated sheep that were shown to be infected, 8 (53%) became carriers. No evidence of carrier status was observed in the VC sheep. Persistence in the absence of disease in vaccinated animals has been reported and can be a deterrent against choosing to vaccinate [34]. However, it seems from this study that efficient vaccination can prevent infection which implies that control over the vaccine strain used and the efficiency of the vaccination campaigns are important aspects in preventing the establishment of carriers. In addition, even if carriers are present at low frequency, a well vaccinated cohort would ensure the disease cannot persist and spread.

There were no correlations between VNT titre or detection of viraemia and either clinical disease or establishment of persistent infection. There were also no differences in the levels of RNA detected in secretions between the donor and UC animals; however, the viral load was, on average, lower in the two nasal swabs from the VC sheep. Despite evidence of sub-clinical infection, no anti-NSP antibody was detected in the sera of sheep 9 or 17. A similar observation has been reported previously and is related to very low levels of viral replication [35,36]. Antibodies to NSP also have shorter half-life in comparison to antibodies to structural proteins [37] and, as sampling was only weekly after day 14, a mild, transient antibody response may have escaped detection.

Pyrexia is reported as a clinical sign of FMD and an association between fever and infection in sheep has been observed in some studies [28,30,32,34]. However, we found no correlation between the occurrence of elevated rectal temperatures and clinical disease or infection, consistent with a number of other reports [14,16,33]. These findings suggest that this parameter cannot be considered a direct correlate of FMD in sheep, though variation in strain, infection method and dose likely play a role in the presentation of this and other clinical outcomes. Despite the development of lesions, lameness was not observed in all diseased sheep highlighting the difficulty in identifying infected sheep in a farm setting.

Sheep comprise a large proportion of FMDV susceptible animals in the world. They form the backbone of Australian livestock industries and any decision on vaccination to control FMD in Australia would consider sheep. Although Orsel et al. [33] showed that O/NET/2001, known to infect sheep, had a reproductive ratio (R0) that only just exceeded the rate necessary for disease spread, and that vaccination would most probably decrease R0 to below 1, the lack of clear clinical signs together with sub-clinical infection as was observed in this study is of concern as it may result in delayed detection of infection as was the case in the UK in 2001. It is therefore probable that FMD infection, if predominately in sheep, will only be reported once a large number of sheep flocks are observed with illness or lameness or when other species become infected. Consequently, preparedness and assessment of the efficacy of vaccines in vaccine banks against contemporary strains is of high priority and provides confidence that this mitigation step in the event of an outbreak will be successful.

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Appendix 10: Efficacy of a high potency o1manisa monovalent vaccine against heterologous challenge with a FMDV O Mya-98 lineage virus in pigs 4 and 7 days post vaccination

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Efficacy of a high potency O_1 Manisa monovalent vaccine against heterologous challenge with a FMDV O Mya98 lineage virus in pigs 4 and 7 days post vaccination

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ABSTRACT

Early protection with a high potency (>6PD_{50}) foot-and-mouth disease (FMD) O_1 Manisa (Middle-East South Asia lineage) vaccine against challenge with O/VIT/2010 (O Mya98 lineage) was tested in pigs. Only two pigs that were vaccinated seven days prior to challenge had any demonstrable antibodies as a result of vaccination at the time of challenge. However, 80% and 60% of pigs that were vaccinated seven and four days prior to coronary band challenge were protected. Vaccination significantly reduced the amount of virus excreted in nasal swabs, saliva and faeces compared to unvaccinated and infected controls. Virus and viral RNA could be detected in some pigs until termination of the experiment 14 days after challenge. Antibodies to the non-structural proteins (NSP) were detected in only one pig that was challenged four days post vaccination (dpv) and transiently in two pigs that were challenged seven dpv at only one time point. For each vaccine and control group, a group of unvaccinated pigs were kept in the same room but with no direct contact with the infected pigs to determine whether vaccination prevented transmission. Despite the presence of live virus and viral RNA in these indirect contact pigs, the groups in contact with the vaccinated and infected pigs did not develop clinical signs nor did they sero-convert. Contact pigs in the same room as unvaccinated challenged controls did show signs of disease and virus infection that resulted in sero-conversion to the NSP. A breach of the wall that separated the two groups at nine days post challenge might have contributed to this finding. This study showed that high potency vaccine can provide protection to pigs soon after vaccination and that aerosol transmission within rooms is a rare event.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease that affects species belonging to the *Artiodactylae*. The disease is characterised by an acute, systemic vesicular disease with lesions developing on areas of friction. Despite low mortality rates in adult animals, FMD severely decreases livestock production and results in devastating trade restrictions.

The causative agent, FMD virus (FMDV), belongs to the *Aphthovirus* genus of the *Picornaviridae* family. Seven serotypes of FMDV (A, O, C, Asia-1, SAT 1, SAT 2 and SAT 3) have been identified on the basis of the ability of viruses to induce cross-protection in animals. This cross-protection is serotype-restricted, and it is not always complete when vaccines contain different subtypes and variants of the same serotype [1].

Highly potent vaccines for emergency use have previously been shown to protect cattle against airborne challenge as early as two

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[2] and four [3] days post immunisation and pigs within four days of vaccination [4,5]. Immunisation of pigs and cattle prior to challenge can dramatically reduce the titre and duration of FMDV excretion [4,6,7]. In these studies the challenge was with a homologous virus. Protection against heterologous challenge has been shown for serotypes A in cattle [8] but similar studies with serotype O did not yield satisfactory results [9,10].

FMD is endemic in many parts of the world and occurs in most countries in South East Asia (SEA) where regular outbreaks of FMDV serotypes O, A, and Asia-1 are reported [11–13]. FMDV serotype O viruses belonging to serotype O/SEA topotype (Mya-98 and Cam-94 strains), O/ME-SA topotype (PanAsia lineage and the derivative Pan-Asia-2 sub-lineage) and O/Cathay topotype, serotype A (ASIA topotype; SEA-97 strain) and serotype Asia-1 have been identified [14–16].

Pigs are seen as the biggest risk of disease dissemination during an outbreak [7]. While it is possible to control the movement of people, animals and their products, it is not possible to control aerosols that have been implicated in previous outbreaks [17–19]. It is therefore important to decrease virus loads and quantify the effect of vaccination on levels of virus excreted during a heterologous challenge. In this paper we report the early protection afforded by the O_1 Manisa high potency vaccine in pigs challenged with a virus isolated in Vietnam in 2010.

2. Materials and methods

2.1. Cell lines, viruses and vaccine

Baby hamster kidney (BHK)-21 cells were used for any virus related work. The challenge virus (O/VIT/2010) belongs to the FMDV O Mya98 lineage [20] and was isolated in 2010 from pig feet tissue and was passaged five times to a titre of $10^{7.05}$ $TCID_{50}$/ml.

A high potency (>6PD_{50}) monovalent O_1 Manisa double oil adjuvant vaccine was prepared by M/s. Merial Company Limited, United Kingdom for this study.

2.2. Preparation of pig challenge virus

The work was performed according to the Australian Animal Ethics Code (AEC1465 and 1497) in the animal facility of NAVETCO, Vietnam. Three month old sero-negative cross-bred Landrace pigs were obtained from a commercial piggery in Vietnam.

Tissue culture adapted virus at $10^{6.5}$ $TCID_{50}$/ml was inoculated either into the foot-pad [21,22] of the left-fore limb at multiple sites (2 ml; 0.1 ml/site in each digit) or intravenously (1 ml) into the ear vein and intramuscularly (1 ml) on the mid neck region in two pigs. Epithelium was collected from lesions other than the inoculation sites and a 10% suspension prepared by homogenizing tissue in PBS (pH 7.4). Two additional pigs were inoculated with 1 ml of the suspension into the foot-pad of left-fore limb at multiple sites and the process repeated in two more pigs. A 10% suspension was prepared from vesicular lesions, titrated and stored at −80 °C.

2.3. Pig immunisation, challenge and sample collection

Three groups of 10 pigs each were divided into sub-groups consisting of five pigs (Fig. 1). Groups O-V7 ($n = 5$) and O-V4 ($n = 5$) were vaccinated intramuscularly in the neck with 2 ml vaccine (21G needle) and challenged seven and four days post vaccination (dpv) respectively. Group O-UV ($n = 5$) was not vaccinated but constituted unvaccinated challenged controls. Groups O-UVC7, O-UVC4 and O-UVC ($n = 5$ each) were used as unvaccinated indirect contacts and housed in the same room as O-V7, O-V4 and O-UV respectively but with a physical partition consisting of a waist-high steel wall between the groups (Fig. 1). Animals shared the same air handling facility but were not in direct physical contact and were provided with separate feed and water troughs.

Animals in O-V7, O-V4 and O-UV were challenged with $10^{5.0}$ $TCID_{50}$ pig derived virus by inoculation in two sites in the left-hind foot pad (0.2 ml/site). Rectal temperatures were recorded daily and animals were observed for 14 days for clinical disease. Nasal secretions, saliva and faeces were sampled daily using cotton swabs for virus isolation (0.5 ml of Eagle's Basal Medium with 10% FCS and field antibiotics) and viral genome detection (0.5 ml of lysis buffer with carrier RNA; Stratec Biomedical, Germany). Swabs were submersed in buffer immediately after collection and stored at −80 °C. Clotted blood for serum was collected on −7, −4, 0, 5, 7, 10 and 14 days post-challenge (dpc) and blood in EDTA buffer on 0–7, 9, 10 and 14 dpc.

Observations and sample collection were performed in O-UVC7, O-UVC4 and O-UVC groups as described above. Clotted blood for serum was collected on days 0, 5, 7, 10 and 14 dpc and unclotted blood on 0, 3, 5, 7, 9, 10 and 14 dpc.

2.4. Virus isolation and antigen ELISA

Virus isolation from the salivary and nasal swabs was performed in 96-well plates by adding 100 μl of $\log_{10}$ dilutions to each well in duplicate and observing for cytopathic effect (CPE) at 24 and 48 h post-infection using standard procedures. Positive samples were confirmed by ELISA [23].

2.5. Real-time quantitative RT-PCR for detection of viral RNA

Total RNA from the salivary, nasal and faecal swab samples and blood collected in EDTA was extracted using the InviMag Virus RNA Mini kit/KF96 (Stratec Molecular, Germany) on an automated nucleic acid extraction system (KingFisher* Flex Magnetic Particle Processor, ThermoFisher Scientific, USA) following the manufacturer's protocol. The RNA was used for quantitative reverse transcription PCR (RT-qPCR) using the Ambion AgPath-ID MasterMix (Life Technologies, USA) as per standard protocols [24].

In vitro transcribed RNA was prepared to determine a standard curve for each RT-qPCR run using Megascript T7 kit (Ambion, USA) from a plasmid pBluescript KS+, cloned using a 550 base region from the 5′UTR region of the FMDV genome [25].

2.6. Serology for virus antibodies to the structural and non-structural proteins (NSP) of FMDV

The solid phase competition ELISA (SPCE) was performed to detect antibodies to the structural proteins on sera samples at 1:5 dilutions in duplicate following standard procedures [26] using rabbit (O_1 Manisa) and guinea pig (O_1 BFS) antisera and O_1 Manisa inactivated antigen. Final OD values were expressed as the percentage inhibition relative to the mean OD of the OD max control wells i.e. $100 - (100 \times (\text{OD test serum mean/OD OD Max control mean}))$. Samples that showed <50% inhibition of the OD max control were scored negative and those ≥50% were considered positive.

A competitive ELISA (c-ELISA) developed at AAHL to detect antibodies to the NSP was performed in duplicate on the sera samples at 1:5 dilutions as described [27] using baculovirus expressed recombinant 3ABC protein and chicken antibodies raised against the protein. The final OD values were expressed as for the SPCE and cut-off values were the same.

2.7. Statistical analysis

Quantitative data were assessed for normality using commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). Quantitative

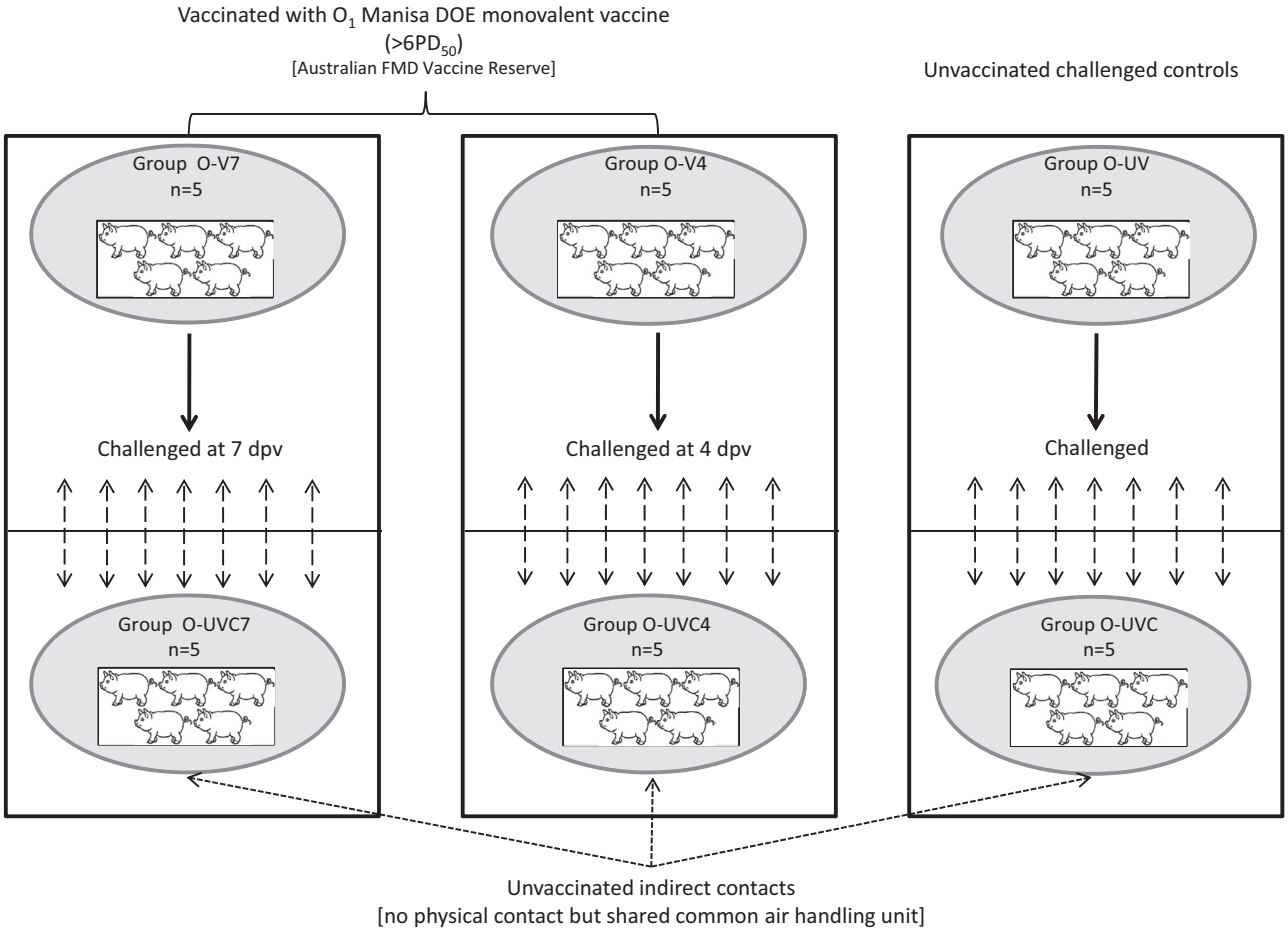

Fig. 1. Experimental lay-out of challenge and indirect contact groups to study the efficacy of high potency O_1 Manisa vaccine against O/VIT/2010 (Mya-98 strain).

data were transformed using the natural logarithm prior to statistical analysis and descriptively presented as the median and range. Categorical variables were compared between groups using chi-square or Fisher exact tests. Quantitative variables were compared among experimental groups at each day using Kruskal–Wallis tests followed by multiple pairwise Mann–Whitney *U* tests with Bonferroni correction of *P* values. Correlation between quantitative variables was estimated using Spearman's rho. Linear mixed models were used to estimate the effect of treatment group on viral titres and rectal temperatures. All models included a random effect term for pig and fixed effect terms for treatment group, experimental day, and barrier status (intact versus breakage). A combined model of multiple specimens also included a fixed effect for sample type. Bonferroni correction was used to adjust *P* values for multiple post-hoc comparisons. Statistical analyses were performed in commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and results were interpreted at the 5% level of significance.

3. Results

3.1. Preparation of pig challenge virus

Initial inoculation of the cell culture adapted virus via the footpads did not result in clinical disease. Intravenous infection of two other pigs resulted in lesions in one pig three days post infection (dpi). At the third passage, both animals had disseminated disease 2–3 dpi and material collected from these pigs was used in the vaccine study.

3.2. Vaccine efficacy study

All animals in O-UV showed generalised disease, defined as lesions at locations other than the inoculation site, within 48–72 h post-challenge (Table 1). One pig (no. 5) died 2 dpc and necropsy showed infarction in the epicardium. The other four animals (nos. 1–4) showed lesions on all sites between 2 and 4 dpc. On 9 dpc the pigs breached the steel wall and a small hole was created where pigs from O-UV and O-UVC could have direct contact. None of the contact animals in O-UVC showed disease until 13 dpc, when one pig (no. 9) showed lesions on the feet and tongue and was removed. At 14 dpc, when the trial was terminated, necropsy examination of pig no. 10 showed heart lesions but no other lesions were noticed in this or the three remaining pigs (Table 1).

In O-V7, one pig (no. 21) showed secondary lesions on all three feet other than the site of inoculation at 2 dpc. One animal (no. 22) showed lines of infarction on the heart musculature upon necropsy at 14 dpc, while O-UVC7 remained clinically normal.

One pig (no. 12) in O-V4 had secondary lesions on the non-inoculated feet and lower lip at 3–4 dpc. Pig no. 15 developed a lesion on the tongue at 4 dpc that resolved quickly. None of the contact animals in O-UVC4 showed any clinical signs (Table 1). The cumulative incidence of clinical signs over the 14 day study period is presented in Supplementary Table 1.

3.3. Pyrexia and viraemia in pigs as determine by RT-qPCR

Pyrexia was intermittent in most cases (Table 1). Three of the pigs in O-UV had detectable RNA in the blood between 3 and 7 dpc;

Table 1
Summary of the clinical outcome and presence of genomic material and live virus after challenge with O/VIT/05/2010.

Days post challenge		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Groups	ID															
O-UV	**1**		N^{GV} S^{V}	♦ N^{G} S^{GV}	■ N^{GV} S^{GV}	N^{GV} S^{GV}	N^{G} S^{GV}	N^{GV} S^{G}	N^{G}	N^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G}			ψ
	2		♦ N^{G} S^{G}	■ N^{GV} S^{GV}	N^{G} S^{GV}	N^{GV} S^{GV}	N^{G} S^{GV}	N^{GV} S^{G}	N^{GV}	N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G}	S^{G}		N^{G}ψ
	3		S^{V}	N^{GV} S^{GV}	■ N^{G} S^{GV}	N^{G} S^{G}	N^{G} S^{G}	N^{GV} S^{GV}	N^{V} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}				ψ
	4		S^{V}	N^{GV} S^{GV}	N^{GV} S^{GV}	N^{G} S^{GV}	■ N^{GV} S^{G}	N^{GV} S^{GV}	N^{GV} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}	S^{G}	N^{G} S^{V}		N^{G} S^{G}ψ
	5		Hψ													
O-UVC	**6**			S^{V}		S^{GV}	S^{G}	N^{V} S^{V}	S^{V}				S^{V}	S^{GV}		N^{G} S^{G}ψ
	7			S^{V}		S^{V}	N^{V}	S^{GV}	S^{G}	S^{GV}	S^{V}			S^{G}		S^{G} ψ
	8				S^{V}	N^{V}	S^{GV}	S^{V}		S^{GV}						S^{G} ψ
	9			N^{V} S^{V}					S^{G}				S^{G}	S^{G}	■ S^{GV}ψ	
	10			S^{V}			S^{V}	S^{G}	N^{V}							H S^{G} ψ
O-V4	**11**		N^{V}	N^{GV} S^{V}	N^{GV} S^{G}	N^{GV} S^{G}	N^{G} S^{G}	N^{G}	N^{G} S^{G}	N^{G} S^{GV}	N^{G}	N^{GV} S^{G}		N^{G} S^{G}	N^{G}	N^{G}ψ
	12			♦ N^{G} S^{G}	■ N^{GV} S^{GV}	N^{G} S^{G}	N^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{GV}		N^{G}			N^{G}	ψ
	13		S^{V}	N^{GV} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}		N^{G} S^{G}	N^{G} S^{G}	N^{G}	N^{G} S^{G}	N^{G}		N^{G}	ψ
	14			N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G}	N^{G} S^{G}		N^{GV} S^{G}		N^{G}	N^{G} S^{G}	N^{G}	N^{G} S^{G}	N^{G}ψ
	15			♦■ N^{G} S^{G}	N^{G} S^{G}	M S^{G}	N^{G} S^{G}	N^{G}	S^{G}	N^{G} S^{G}		S^{G}	N^{G}	N^{V}		N^{G} S^{G}ψ
O-UVC4	**16**		N^{V}	S^{V}	N^{V} S^{V}	N^{GV} S^{V}	N^{G} S^{V}	N^{V}	N^{G}	S^{V}	N^{V} S^{V}	N^{V}	S^{V}	N^{G}	N^{V}	S^{GV}ψ
	17			S^{V}			S^{V}		N^{GV}	N^{GV} S^{V}		N^{G} S^{G}	S^{G}			N^{G}ψ
	18		S^{V}				N^{G}	N^{V}	N^{G}	N^{G} S^{V}		N^{V}	S^{G}	N^{V}	N^{G} S^{G}	ψ
	19			N^{V}	N^{V}	S^{G}	N^{G}	S^{V}	N^{V}	N^{G}	N^{V}	N^{V} S^{V}	S^{G}		N^{G} S^{G}	N^{G}ψ
	20		N^{GV}		N^{G}	S^{V}	S^{V}	N^{GV}	N^{G}	N^{G}		N^{V}				ψ
O-V7	**21**		♦N^{GV} S^{GV}	■ N^{G} S^{G}	N^{G} S^{GV}	N^{GV} S^{G}	N^{GV} S^{G}	N^{G} S^{V}			N^{G}	N^{V} S^{V}	S^{V}	S^{V}	N^{G} S^{V}	N^{V}ψ
	22		♦N^{GV} S^{GV}	N^{GV} S^{GV}	N^{G} S^{G}	N^{G}	N^{V}		N^{G} S^{GV}	N^{G}		N^{G}	S^{V}			Hψ
	23		N^{G} S^{G}	N^{G} S^{G}	N^{G} S^{G}	N^{G}	N^{GV}		N^{G} S^{GV}	N^{GV}	N^{GV} S^{G}	S^{V}	N^{G}		N^{G} S^{G}	S^{V}ψ
	24		♦ ■ N^{G} S^{G}	N^{G} S^{GV}	N^{G}	S^{V}	S^{GV}	N^{G} S^{V}	S^{V}	N^{G} S^{G}			S^{G}		N^{G} S^{V}	N^{G}ψ
	25		N^{G} S^{G}	N^{G} S^{G}	N^{G}	N^{G}	N^{G}	N^{GV}		N^{G}	N^{G}	S^{V}	S^{V}	S^{V}	N^{G}	ψ
O-UVC7	**26**		N^{V}	S^{V}												ψ
	27															ψ
	28			S^{V}												ψ
	29		S^{V}		N^{V} S^{V}											ψ
	30															ψ

♦ Lesion at site of inoculation; ■ Lesion at any other site including feet, mouth, tongue and snout, indicating disseminated disease; H—lesion in heart muscle; ψ Animal died/Euthanised; shaded boxes—temperature ≥40 °C; N^{G} S^{G}—Viral RNA detected in nasal and saliva swabs respectively by RT-PCR; N^{V} S^{V}—virus detected in nasal and saliva swabs respectively by virus isolation on cell culture; N^{GV} S^{GV}—both RNA and live virus detected in the nasal and saliva swabs, respectively.

pig no. 2 was positive at 3 dpc, pig no. 3 at 7 dpc, with only pig no. 4 showing RNA on two consecutive days (4–5 dpc). One pig in O-V4 tested positive at 7 dpc. The levels of RNA were variable (8.7×10^3–2.7×10^6 copy numbers). Surprisingly, one pig in O-UVC had 7.9×10^5 copy numbers in the blood at 14 dpc (results not shown).

3.4. Detection of FMDV by RT-qPCR and virus isolation from nasal and saliva swab samples

Samples were deemed positive when either RNA was detected or virus isolated. Saliva swabs from all surviving pigs in O-UV were positive from 1 to 10 dpc, while pig no. 4 tested positive for viral RNA until 14 dpc (Table 1). Nasal swabs were positive between 1 and 7 dpc, while pigs nos. 2 and 4 tested positive until 14 dpc. Pigs in O-UVC tested positive for virus in the saliva swabs from 2 dpc. Before the breach at 9 dpc, three of the five pigs had live virus isolated from the saliva at one or more time points.

All pigs in O-V7 tested positive in both oral and nasal swabs at 1 dpc. From 3 dpc, the recovery from swabs was intermittent but present in at least three pigs daily until 14 dpc. Live virus was isolated from the nasal swab of one pig until 9 dpc. Viral RNA was detected in the nasal and saliva swabs of three of the contact pigs (O-UVC7) between 1 and 3 dpc but not subsequently.

Virus was detected in either the nasal or saliva swabs of pigs nos. 11 and 13 in O-V4 at 1 dpc. At 2 dpc, all samples were positive. From 6 dpc the detection was intermittent, but RNA was still detected in swabs at 14 dpc. Contact pigs (O-UVC4) were also positive from 1 dpc, with live virus found in nasal and saliva swabs of three pigs on that day. Live virus was also recovered from the saliva of one pig at 14 dpc (Table 1).

3.5. Antibody responses in the different groups of pigs

Only two pigs that were vaccinated seven days prior to challenge (O-V7: nos. 23 and 24) were sero-positive to the structural proteins on the day of challenge (Table 2). At 5 dpc, all five pigs in O-V7 and O-V4 had sero-converted including one pig (no. 2) in O-UV that had not been vaccinated, but challenged. By 10 dpc, all the challenged pigs were sero-positive. In the contact groups, sero-conversion was detected in a single pig (no. 7) in O-UVC between 10 and 14 dpc.

None of the pigs had detectable antibodies to FMDV NSP until 10 dpc when all pigs in O-UV were positive and remained so until 14 dpc. One of the contact pigs (no. 7) in O-UVC had antibodies to the NSP at 14 dpc. One pig (no. 12) in O-V4 was sero-positive on both 10 and 14 dpc, while two pigs in O-V7 (nos. 24 and 25) were sero-positive only on 10 dpc (Table 2).

3.6. Comparison of the viral RNA recovered from samples across the different groups

The amount of viral RNA detected from the saliva swabs was statistically different among all treatment groups when evaluated for all sampling times (Table 3; $P < 0.001$). Furthermore, both vaccinated groups had significantly less RNA compared to the unvaccinated challenged O-UV; however there was no significant difference between O-V4 and O-V7. Pigs in O-UV had significantly more detectable RNA than O-V7 at 4, 6, 9, 10, and 14 dpi. There were no significant differences observed between O-UV

Table 2
Results of cELISA for antibodies against structural proteins against FMDV and NSP antibody ELISA.

Group	Pig ID	cELISA					NSP antibody ELISA				
		0 dpc	5 dpc	7 dpc	10 dpc	14 dpc	0 dpc	5 dpc	7 dpc	10 dpc	14 dpc
O-UV	1	Neg	Neg		Pos	Pos	Neg	Neg		Pos	Pos
	2	Neg	Pos		Pos	Pos	Neg	Neg		Pos	Pos
	3	Neg	Neg		Pos	Pos	Neg	Neg		Pos	Pos
	4	Neg	Neg		Pos	Pos	Neg	Neg		Pos	Pos
	5	Neg	–	–	–	–	Neg	–	–	–	–
O-UVC	6	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	7	Neg		Neg	Pos	Pos	Neg		Neg	Neg	Pos
	8	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	9	Neg		Neg	Neg	Neg	Neg		Neg	Neg	–
	10	Neg		Neg		Neg	Neg		Neg		Neg
O-V4	11	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	12	Neg	Pos		Pos	Pos	Neg	Neg		Pos	Pos
	13	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	14	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	15	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
O-UVC4	16	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	17	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	18	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	19	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	20	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
O-V7	21	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	22	Neg	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	23	Pos	Pos		Pos	Pos	Neg	Neg		Neg	Neg
	24	Pos	Pos		Pos	Pos	Neg	Neg		Pos	Neg
	25	Neg	Pos		Pos	Pos	Neg	Neg		Pos	Neg
O-UVC7	26	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	27	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	28	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	29	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg
	30	Neg		Neg	Neg	Neg	Neg		Neg	Neg	Neg

Pos: positive; Neg: negative; – animal removed.

and O-V4 or between O-V4 and O-V7 at any individual sampling day.

In nasal swabs, the amount of viral RNA detected was statistically different among all treatment groups when evaluated over all days (Table 4; $P < 0.001$). Furthermore, O-V7 group had significantly less detectable RNA compared to both O-UV and O-V4. The amount of detectable RNA was not different between O-UV and O-V4. The amount of detectable RNA was different between challenged and contact groups for multiple experimental days but there were no detectable differences among O-UV, O-V4 and O-V7 at any individual experimental day.

Levels of RNA detected in faecal swabs were statistically different between all treatment groups when evaluated over all sampling times (Supplementary Table 2; $P < 0.001$). Furthermore, O-UV had significantly more detectable RNA compared to O-V4 but not O-V7.

When adjusting for the observed barrier breakage in a multivariable model, the amount of RNA detected was different among the treatment groups (Table 5; $P < 0.001$). Pigs within both vaccinated groups had less detectable RNA than O-UV. O-V7 had less detectable RNA compared to O-V4 but the overlapping confidence intervals suggested that the difference was not statistically different. The amount of FMDV RNA detected varied by specimen type with the lowest levels in blood and the highest in nasal swabs. Overlapping confidence intervals for nasal and oral swabs suggested that the difference between these two specimen types was not statistically significant.

The amount of FMDV RNA detected in the blood was positively correlated with the presence of clinical lesions (rho = 0.211, $P < 0.001$).

4. Discussion

Most vaccine efficacy studies are performed with homologous challenge and there is a dearth of information on protection when there are genetic and antigenic differences between the vaccine strain and challenge virus. Antigenic matching results between the challenge virus and the O_1 Manisa vaccine are not available, however, comparisons between another virus that was isolated during 2010 in Vietnam indicated an acceptable match (Annual reports of the World Reference Laboratory 2010, 2011). In this study, there was 80% and 60% protection in pigs vaccinated seven and four days prior to a heterologous challenge, respectively, indicating that vaccination can provide some protection at early time points. Similar results were obtained when pigs were challenged by exposure to aerosols generated by infected pigs [4], a method that is seen as less traumatic and a more natural route of exposure compared to the coronary band route used in our study. However, pigs vaccinated four and seven days prior to exposure could infect cohorts with which they had direct contact [4,28,29] indicating that vaccination does not fully prevent disease transmission within a pen soon after vaccination whereas those vaccinated 14 days prior to infection did not infect their cohorts [28].

More importantly, vaccination lowered the amount of virus excreted compared to the unvaccinated challenged controls, although there was not a statistically significant difference between the two vaccine groups. This is in agreement with other vaccine experiments [28–32] although in at least one experiment the pigs vaccinated seven days before challenge excreted more virus than the infected controls on some occasions [28]. Therefore although vaccinated pigs may still become infected, the overall viral load

Table 3
Median (range) viral RNA copy numbers recovered from saliva swabs via PCR. All contact animals were unvaccinated.

Day PC	Unvaccinated		Exposed 4 days post vaccination		Exposed 7 days post vaccination		*P* value*
	Challenged (O-UV)	Contact (O-UVC)	Challenged (O-V4)	Contact (O-UV4)	Challenged (O-V7)	Contact (O-UV7)	
1	NGD[a,b] (NGD, 1.3E+2)	NGD[b]	NGD[b]	NGD[b]	2.8E+4[a] (NGD, 9.2E+4)	NGD[b]	0.002
2	6.7E+5[a] (6.1E+3, 2.5E+6)	NGD[b]	1.8E+4[a,b] (NGD, 1.2E+5)	NGD[b]	1.0E+5[a] (6.0E+3, 1.0E+7)	NGD[b]	<0.001
3	6.7E+5[a] (2.9E+5, 2.3E+6)	NGD[b]	4.0E+5[a,b] (7.4E+4, 1.8E+6)	NGD[b]	1.2E+4[a] (NGD, 2.4E+7)	NGD[b]	0.001
4	7.9E+5[a] (4.0E+5, 1.2E+6)	NGD[b] (NGD, 1.0E+3)	5.2E+3[a,b] (5.3E+2, 7.5E+4)	NGD[b] (NGD, 4.3E+4)	NGD[b] (NGD, 2.2E+5)	NGD[b]	0.001
5	7.5E+5[a] (1.5E+5, 5.8E+6)	NGD[a,b] (NGD, 8.9E+3)	8.6E+2[a,b] (NGD, 5.7E+3)	NGD[b]	NGD[a,b] (NGD, 3.5E+4)	NGD[b]	0.004
6	7.8E+4[a] (3.2E+4, 2.4E+5)	NGD[a,b] (NGD, 1.0E+7)	5.1E+2[a,b] (NGD, 2.1E+4)	NGD[b]	NGD[b]	NGD[b]	0.003
7	9.4E+4 (NGD, 7.2E+6)	NGD (NGD, 2.5E+6)	1.2E+3 (NGD, 1.9E+4)	NGD	NGD (NGD, 4.7E+3)	NGD	0.131
8	3.4E+3[a] (NGD, 2.4E+4)	NGD[a] (NGD, 4.0E+5)	1.4E+3[a] (4.2E+2, 4.4E+3)	NGD[a]	NGD[a] (NGD, 2.8E+3)	NGD[a]	0.018
9	1.3E+3[a] (1.6E+2, 1.2E+4)	NGD[b]	NGD[b]	NGD[b]	NGD[b] (NGD, 3.5E+3)	NGD[b]	0.001
10	1.1E+4[a] (1.2E+3, 1.4E+4)	NGD[b]	3.7E+2[a,b] (NGD, 9.5E+3)	NGD[a,b] (NGD, 2.0E+3)	NGD[b]	NGD[b]	0.002
11	NGD (NGD, 1.9E+2)	NGD (NGD, 5.3E+2)	NGD (NGD, 7.6E+2)	1.7E+3 (NGD, 2.9E+3)	NGD (NGD, 1.7E+3)	NGD	0.237
12	NGD (NGD, 2.2E+2)	1.3E+3 (NGD, 1.5E+5)	NGD (NGD, 1.9E+3)	NGD	NGD	NGD	0.087
13	ND	4.4E+4 (4.4E+4, 4.4E+4)	NGD (NGD, 3.5E+2)	NGD (NGD, 2.6E+3)	NGD (NGD, 5.2E+3)	NGD	0.148
14	NGD[a] (NGD, 2.0E+2)	1.6E+4[a,b] (2.4E+3, 2.5E+4)	NGD[a,b] (NGD, 6.2E+4)	NGD[a,b] (NGD, 2.6E+3)	NGD[b]	NGD[b]	0.010
Overall	1.3E+3[a] (NGD, 7.2E+6)	NGD[b,d] (NGD, 1.0E+7)	NGD[c] (NGD, 1.8E+6)	NGD[b] (NGD, 4.3E+4)	NGD[c,d] (NGD, 2.4E+7)	NGD[b]	<0.001†

PC = post-challenge. NGD = no genome detected. ND = no testing done.
* Based on Kruskal–Wallis tests for a difference among the 6 treatment groups. Medians without superscripts in common are significantly different based on pairwise Mann–Whitney *U* tests after Bonferroni correction of *P* values.
† Based on mixed-effects linear regression over all days. Medians without superscripts in common are significantly different based on model results after Bonferroni correction of *P* values.

and infectious period may be significantly reduced in a facility [28,32,33] thereby assisting in disease control.

Live virus and/or viral RNA could be detected in the excretions intermittently until the study was terminated at 14 dpc, similar to other short term experiments where detection lasted until 11 dpc [28–30]. In one long term study, RNA was detected up to 28 dpc in the saliva and probang of a vaccinated pig [31]. The implications of these findings are not clear, as in most instances no live virus could be isolated.

The study furthermore aimed to determine if transmission would occur to unvaccinated pigs when kept in close, but indirect contact with vaccinated and infected pigs. The pigs kept next to the unvaccinated challenged controls only showed disease at 13 and 14 dpc. This followed a break in the wall that allowed pigs to have direct contact from 9 dpc. However, live virus and viral RNA were detected in these pigs from 2 dpc indicating that despite the absence of clinical signs and lack of sero-conversion, these pigs were exposed to live virus. Van Roermund [32] showed that walls between groups of pigs reduced transmission 10- to 20-fold compared to within pen transmission. In addition, when pigs were separated by a wooden wall, transmission was delayed to three days after exposure, compared to within pen transmission that usually occurred within one day. Airborne transmission seems to be relatively rare when pigs don't have direct contact [34] and it is speculated that faeces and urine may also cause transmission [32]. In our experiment it was similarly possible that these excretions may have run between the pens, and this was certainly observed with groups O-UV and O-UVC. The delayed infection could therefore be due to the lower amount of RNA found in faeces compared to nasal and saliva samples.

Temperature was positively correlated with the presence of clinical lesions (rho = 0.141, $P = 0.003$) but not viraemia (rho = 0.081, $P = 0.193$). Three pigs had elevated temperatures on 0 dpc, possibly due to stress. Viral RNA was detected only in the blood of needle infected pigs on very few occasions between 3 and 7 dpc and not in any of the pigs in group O-V7. The contact pig in O-UVC that had RNA in the blood at 14 dpc, also showed evidence of heart lesions and viral RNA in the saliva on that day. All pigs in O-UV had antibodies to the NSP from 10 to 14 dpc indicating that sufficient virus replication had occurred to stimulate antibodies.

The detection of antibodies to the NSP is an important tool during post-outbreak surveillance. Two pigs in O-V7 (nos. 24 and

Table 4
Median (range) viral RNA copy numbers recovered from nasal swabs via PCR. All contact animals were unvaccinated.

Day PC	Unvaccinated		Exposed 4 days post vaccination		Exposed 7 days post vaccination		*P* value*
	Challenged (O-UV)	Contact (O-UVC)	Challenged (O-V4)	Contact (O-UV4)	Challenged (O-V7)	Contact (O-UV7)	
1	NGD[a,b] (NGD, 1.2E+4)	NGD[a]	NGD[a]	NGD[a] (NGD, 1.3E+3)	2.7E+5[b] (1.1E+4, 4.9E+6)	NGD[a]	0.001
2	1.8E+5[a] (4.2E+4, 2.1E+5)	NGD[b]	1.7E+3[a,b] (3.4E+2, 8.6E+4)	NGD[b]	5.5E+5[a] (2.8E+4, 6.9E+5)	NGD[b]	<0.001
3	1.4E+5[a] (3.4E+3, 6.1E+5)	NGD[b]	4.9E+4[a] (1.1E+3, 6.9E+5)	NGD[a,b] (NGD, 1.4E+3)	3.9E+3[a,b] (2.1E+3, 1.5E+5)	NGD[b]	<0.001
4	1.2E+5[a] (7.4E+3, 3.8E+5)	NGD[b]	1.1E+4[a,b] (NGD, 1.0E+5)	NGD[a,b] (NGD, 3.5E+3)	2.4E+3[a,b] (NGD, 1.1E+4)	NGD[b]	0.001
5	2.8E+5[a] (5.4E+4, 3.1E+5)	NGD[b]	1.2E+3[a,b] (6.4E+2, 2.0E+5)	4.8E+3[a,b] (NGD, 5.6E+4)	1.2E+4[a,b] (NGD, 4.1E+4)	NGD[b]	0.002
6	5.8E+5[a] (1.7E+5, 7.8E+5)	NGD[b]	5.1E+2[a,b] (3.1E+2, 1.5E+4)	NGD[b] (NGD, 5.2E+3)	1.2E+3[a,b] (NGD, 4.6E+4)	NGD[b]	0.001
7	1.3E+3 (NGD, 1.4E+5)	NGD	2.6E+3 (NGD, 1.0E+5)	3.7E+3 (NGD, 1.2E+4)	NGD (NGD, 3.7E+4)	NGD	0.071
8	2.9E+3[a] (7.2E+2, 4.4E+5)	NGD[a]	7.4E+2[a] (2.9E+2, 3.1E+4)	2.8E+3[a] (NGD, 1.1E+4)	4.2E+3[a] (NGD, 3.8E+5)	NGD[a]	0.005
9	8.5E+3[a] (5.6E+3, 2.9E+4)	NGD[b]	NGD[a,b] (NGD, 2.5E+2)	NGD[b]	9.2E+2[a,b] (NGD, 4.8E+3)	NGD[b]	0.001
10	4.4E+3[a] (1.9E+3, 1.2E+4)	NGD[b]	2.8E+3[a,b] (NGD, 1.5E+7)	NGD[a,b] (NGD, 2.1E+3)	NGD[a,b] (NGD, 4.3E+3)	NGD[b]	0.003
11	7.8E+1 (NGD, 1.4E+3)	NGD	2.4E+2 (NGD, 4.1E+3)	NGD	NGD (NGD, 1.5E+4)	NGD	0.084
12	NGD (NGD, 3.8E+2)	NGD	NGD (NGD, 4.5E+2)	NGD (NGD, 2.4E+4)	NGD (NGD, 1.2E+4)	NGD	0.611
13	ND	NGD	8.7E+2 (NGD, 6.8E+3)	NGD (NGD, 5.8E+3)	6.0E+3 (NGD, 5.0E+4)	NGD	0.054
14	1.0E+2 (NGD, 3.4E+2)	NGD	2.5E+2 (NGD, 2.8E+3)	NGD (NGD, 2.2E+3)	NGD (NGD, 1.6E+3)	NGD	0.380
Overall	4.1E+3[a] (NGD, 7.8E+5)	NGD[b]	5.2E+2[a,c] (NGD, 1.5E+7)	NGD[d] (NGD, 5.6E+4)	2.1E+3[c] (NGD, 4.9E+6)	NGD[b]	<0.001†

PI = post-challenge. NGD = no genome detected. ND = no testing done.
* Based on Kruskal–Wallis tests for a difference among the 6 treatment groups. Medians without superscripts in common are significantly different based on pairwise Mann–Whitney *U* tests after Bonferroni correction of *P* values.
† Based on mixed-effects linear regression over all days. Medians without superscripts in common are significantly different based on model results after Bonferroni correction of *P* values.

Table 5
Multivariable model results including viraemia.

Variable	Estimate (95%CI)	*t* Statistic	*P* value
Experimental group			<0.001
Contact with unvaccinated (O-UVC)	−6.95 (−8.06, −5.85)	−12.353	<0.001
Contact with vaccinated pigs challenge 4 dpv (O-UVC4)	−5.38 (−6.31, −4.44)	−11.309	<0.001
Contact with vaccinated pigs challenged 7 dpv (O-UVC7)	−7.13 (−8.06, −6.20)	−14.996	<0.001
Vaccinated 4 days prior to challenge (O-V4)	−1.99 (−2.90, −1.09)	−4.321	<0.001
Vaccinated 7 days prior to challenge (O-V7)	−2.79 (−3.69, −1.88)	−6.049	<0.001
Unvaccinated challenged (O-UV)	Referent		
Barrier			
Breakage	3.30 (1.82, 4.77)	4.392	<0.001
Intact	Referent		
			<0.001
Sample type			
Nasal swab	5.22 (4.50, 5.95)	14.092	<0.001
Oral swab	4.38 (3.66, 5.11)	11.825	<0.001
Whole blood	Referent		

CI = confidence interval. Model estimates for the variables associated with days post-inoculation are not presented in the table. Pig sex was not significant ($P = 0.198$) so it was removed from the final model. All treatment groups were significantly different ($P < 0.05$) except for the comparisons of O-UVC and O-UVC4 ($P = 0.07$). Nasal and oral swab values were significantly different ($P = 0.018$).

25) were sero-positive for NSP antibodies on only 10 dpc but not on 14 dpc, probably indicating a low level of virus replication, confirming findings from other studies that showed vaccination resulted in a lower and shorter duration of responses to NSP [29]. Both these pigs had lesions only at the site of inoculation. One pig in O-V4 that had generalised disease, sero-converted on 10 dpc and remained positive until 14 dpc whilst one other pig in that group that had lesions on the inoculated foot away from the inoculation site, remained negative. None of the indirect contact pigs in O-UVC4 and O-UVC7 sero-converted to NSP antibodies. However, viral RNA and on occasion live virus, could be detected in saliva and nasal swabs of most of these pigs. It is therefore possible that the NSP tests may fail to identify all infected pigs during an outbreak.

Although vaccination did not protect all the challenged pigs, it decreased the amount of virus excreted and remains an important tool for control during an outbreak.

Conflict of interest statement

All the authors have read and approved the manuscript and there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.04.045

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Appendix 12: A Malaysia 97 monovalent foot-and-mouth disease vaccine (>6PD50/dose) protects pigs against challenge with a variant FMDV A SEA-97 lineage virus, 4 and 7 days post vaccination

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A Malaysia 97 monovalent foot-and-mouth disease vaccine (>6PD$_{50}$/dose) protects pigs against challenge with a variant FMDV A SEA-97 lineage virus, 4 and 7 days post vaccination

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ABSTRACT

Pigs play a significant role during outbreaks of foot-and-mouth disease (FMD) due to their ability to amplify the virus. It is therefore essential to determine what role vaccination could play to prevent clinical disease and lower virus excretion into the environment. In this study we investigated the efficacy of the double oil emulsion A Malaysia 97 vaccine (>6PD$_{50}$/dose) against heterologous challenge with an isolate belonging to the A SEA-97 lineage at 4 and 7 days post vaccination (dpv). In addition, we determined whether physical separation of pigs in the same room could prevent virus transmission. Statistically there was no difference in the level of protection offered by 4 and 7 dpv. However, no clinical disease or viral RNA was detected in the blood of pigs challenged 4 dpv, although three of the pigs had antibodies to the non-structural proteins (NSPs), indicating viral replication. Viral RNA was also detected in nasal and saliva swabs, but on very few occasions. Two of the pigs vaccinated seven days prior to challenge had vesicles distal from the injection site, but on the inoculated foot, and two pigs had viral RNA detected in the blood. One pig sero-converted to the NSPs. In contrast, all unvaccinated and inoculated pigs had evidence of infection. No infection occurred in any of the susceptible pigs in the same room, but separated from the infected pigs, indicating that strict biosecurity measures were sufficient under these experimental conditions to prevent virus transmission. However, viral RNA was detected in the nasal swabs of one group of pigs, but apparently not at sufficient levels to cause clinical disease. Vaccination led to a significant decrease in viral RNA in vaccinated pigs compared to unvaccinated and infected pigs, even with this heterologous challenge, and could therefore be considered as a control option during outbreaks.

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1. Introduction

Foot-and-mouth disease (FMD) directly impacts livestock production due to loss in productivity and usually affects the economy further due to quarantine and import restrictions on live animals and their products. Vaccination has been used successfully in a number of previously endemic countries to control the disease and most countries free from FMD will consider emergency vaccination if an outbreak should occur.

Susceptible domestic species include cattle, sheep, goats, pigs and water buffalo. All may demonstrate lesions on areas of friction such as the mouth, feet and teats in lactating animals, but subclinical infections can also occur, especially in sheep and goats [1]. Pigs are the amplifier hosts of the disease and excrete large amounts of virus in all secretions and excretions [2,3]. For this reason it is imperative to prevent them from becoming infected or to decrease viral shedding using vaccination.

There are seven serotypes of FMD virus (FMDV—A, O, C, Asia-1, SAT 1, SAT 2 and SAT 3) and large numbers of variants exist

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within each. Since cross protection between serotypes does not exist [4,5], vaccines need to contain multiple strains to ensure immunity to more than one serotype. Even within serotypes, protection is not complete [6,7]. FMDV serotype A viruses have always been considered to be antigenically the most diverse [8,9], and have genetically been classified under three broad genotypes [10]. The Asian genotype consists of several lineages and sub-lineages with viruses belonging to the lineage A SEA-97 being endemic to South East Asia (SEA) and new clusters emerging in the region [10,11]. These viruses have recently spread beyond the SEA region to cause outbreaks in countries that were previously free of serotype A [12].

FMD is endemic in many parts of the world and occurs in most countries in SEA. Through their proximity and the amount of trade and travel, these countries pose the biggest perceived risk to Australia's livestock industries and agricultural economy. Australia's last suspected outbreak was in 1872 [13] and having FMD-free status, together with the absence of several other diseases, has provided the country with a significant trade advantage. The local pork industry is small compared to high producing countries such as China, South Korea and Japan, but the potential overall losses due to a large outbreak of FMD could reach 50 billion Australian dollars over a 10 year period [14]. For this reason it is important to determine whether the strains in the Australian vaccine bank will provide early protection in pigs against the serotype A viruses that are currently circulating in SEA.

2. Materials and methods

2.1. Cell lines, viruses and vaccine

Baby hamster kidney-21 (BHK-21) cells were used for all virus culture. The challenge virus (A/VIT/08/2005) belongs to the FMDV A SEA-97 topotype, circulating in Vietnam and other SEA countries, and has a relative homology (r1) of 0.51 to the A Malaysia 97 (A/MAY/97) vaccine strain (WRL Report 2006; http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO%20FMD%20Ref%20Lab%20Network%20Report%202006.pdf). The virus was passaged three times in BHK-21 cells before preparation of the pig-derived challenge virus.

A monovalent double oil emulsion A/MAY/97 vaccine (>6PD_{50}/dose) was prepared by Merial, United Kingdom.

2.2. Animal ethics and pigs used in the study

The animal studies were performed according to the Australian code of practice for the care and use of animals for scientific purposes (AEC1514 and 1571). Sero-negative three-month-old cross-bred Landrace pigs were obtained from a commercial piggery in Vietnam.

2.3. Preparation of challenge virus

Five healthy pigs were used to prepare pig-derived challenge virus. Two pigs were administered 1 ml of A/VIT/08/2005 intravenously into the ear vein, 1 ml intramuscularly on the dorsal aspect just behind the left ear and 2 ml intradermally into the foot-pad of the left-hind limb at multiple sites (0.1 ml/site in each digit). The animals were monitored for the appearance of lesions for three days. A 10% (w/v) suspension of tissue homogenate was prepared in phosphate buffered saline using the epithelial tissue from the coronary band and foot lesions and three more pigs were inoculated intradermally with 0.1–0.2 ml of a 10% (w/v) suspension in the foot pad of the left-fore limb. Epithelial tissue from the coronary band and foot lesions was collected and a 10% (w/v) suspension of tissue homogenate was prepared and stored at −80 °C.

2.4. Titration of A/VIT/08/2005 pig-derived virus

Four healthy pigs were used for titrating the pig-derived virus at $\log_{10}$ dilutions (10^{-1} to 10^{-8}) in basal medium eagles (BME) cell culture medium supplemented with 1% foetal calf serum (FCS). Two pigs received 0.1–0.2 ml of inoculum dilutions −2, −3, −4 and −5, whereas two other pigs were administered dilutions −4, −5, −6 and −7, intradermally in the footpad. Each dilution was administered to two feet. Lesions at the inoculation sites were scored at 24, 36, 48, 60 and 72 h post inoculation. The 50% pig infective dose per ml (PID_{50}/ml) was calculated using the Spearman-Kärber method [15].

2.5. Pig immunisation and challenge

The experiment consisted of three groups of eight pigs each in separate rooms. One group was vaccinated intramuscularly in the mid neck region with 2 ml of vaccine (0.82 mm × 38.1 mm) seven days prior to challenge (A-V7), another four days before challenge (A-V4) and the last group was left unvaccinated and was challenged on day 0 (A-UV). Vaccinations were staggered so that the virus challenge occurred on the same day. For each of these groups, five additional non-vaccinated pigs were kept in the same room (comprising groups A-UVC7, A-UVC4 and A-UVC), but were separated by a waist-high steel wall that prevented direct contact with the challenged animals.

Groups A-V7, A-V4 and A-UV were challenged with $10^{5.0}$ PID_{50} of the pig-derived virus by inoculation in two sites in the left-hind foot pad (0.2 ml/site). The animals were observed and sampled daily for 14 days, and rectal temperatures recorded. Clinical scores were determined by giving each site of lesion development, except the inoculation site, one point (four feet, tongue, mouth and snout); the maximum score was therefore seven. Nasal secretions, saliva and faeces were collected in duplicate using cotton swabs (diameter: 2.7 mm; length: 150 mm); one swab was used for virus isolation (0.5 ml of BME with 10% FCS and antibiotics) and the other for viral genome detection (0.5 ml of lysis buffer with carrier RNA and proteinase K; Startec Biomedical AG, Germany). Swabs were submersed in the buffer and stored at −80 °C. Clotted blood for serum was collected on −7, −4, 0, 5, 7, 10 and 14 days post-challenge (dpc). Whole blood was collected in EDTA tubes on 0, 1–7, 9, 10 and 14 dpc.

The animals in groups A-UVC7, A-UVC4 and A-UVC were observed and sampled as described above. Clotted blood for serum was collected on days 0, 5, 7, 10 and 14 dpc. Whole blood was collected in EDTA tubes on 0, 3, 5, 7, 9, 10 and 14 dpc.

2.6. Quantitative real-time reverse transcriptase PCR (RT-qPCR) for detection of FMD viral RNA

Total RNA from samples was extracted using the InviMag Virus RNA Mini kit/KF96 (Stratec Molecular, Germany) on an automated nucleic acid extraction system (KingFisher Flex Magnetic Particle Processor, ThermoFisher Scientific, USA) following the manufacturer's protocol. RT-qPCR was carried out using Ambion AgPath-ID MasterMix (Life Technologies, USA) using the assay previously described by [16].

In vitro transcribed RNA was prepared using the Megascript T7 kit (Ambion, USA) from a pBluescript KS+ plasmid containing the FMDV IRES region [17]. The RNA was purified and checked for integrity by RT-PCR using the specific primers that would be used for the RT-qPCR [18], and by sequencing. RNA standards were prepared to determine a standard curve for each RT-qPCR run.

2.7. Serology to detect antibodies to FMDV structural proteins

A liquid phase blocking ELISA (LPBE) was performed as described by Hamblin et al. [19] using A/MAY/97-specific reagents. Antibody titres were expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera resulted in an optical density equal to 50% inhibition of the mean optical density of the reaction (antigen) control wells [15]. Sera showing a titre of log 1.20 were considered positive.

2.8. Serology to detect antibodies to FMDV non-structural proteins

A competitive ELISA (c-ELISA) was performed on serum samples at 1:5 dilutions [20]. The final OD values were expressed as percentage inhibition relative to the mean OD of the OD Max control wells representing the no serum controls i.e. $100 - (100 \times (\text{OD test serum mean/OD Max control mean}))$ where a positive result was recorded for samples that were ≥50% inhibition of the OD max control.

2.9. Statistical analysis

Quantitative data were assessed for normality by calculating descriptive statistics, plotting histograms, and performing the Anderson–Darling test for normality using commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). Data were transformed using the natural logarithm when necessary to improve the distributional form prior to statistical analysis. Data were descriptively presented as the median and range. A linear mixed models approach was used to estimate the effect of treatment group on viral genome quantity determined using RT-qPCR. Independent models were fitted for the four types of PCR specimen (whole blood and oral, nasal, and faecal swabs) in addition to a combined analysis of all sample types. All models included a random effect term for pig identification to account for the repeated measurements and also included fixed effect terms for treatment group, and experimental day. Bonferroni correction was used to adjust *P* values for multiple post-hoc comparisons. Statistical analyses were performed in commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and results interpreted at the 5% level of significance.

3. Results

3.1. Adaptation and titration of A/VIT/08/2005 in pigs

The challenge virus was passed through pigs twice and had a titre of approximately 10^6 PID_{50}/ml when titrated in pigs.

3.2. Vaccine efficacy study

All eight pigs in group A-UV developed generalized disease between 2 and 5 dpc with lesions on all four feet, snout, lower lip and tongue. In total five out of eight pigs had temperatures ≥40 °C between 3 and 5 dpc on one or more days. Pig #5, which had lesions at all sites, had an elevated temperature also at 8 dpc (Table 1).

In group A-V4, none of the eight pigs showed generalized disease with secondary lesions or increased temperatures, but four of the pigs developed vesicles at the site of inoculation between 2 and 5 dpc (Table 1).

Two of the pigs in group A-V7 (#29 and #31) developed vesicles at the site of inoculation while pigs #28 and #29 showed secondary lesions on the coronary band of the inoculated feet 7 and 5 dpc, respectively (Table 1). No other lesions were detected. None of the contact pigs (groups A-UVC, A-UVC4 and A-UVC7) developed any lesions or elevated temperatures (data not shown).

3.3. Detection of viral RNA in swabs

All eight pigs in group A-UV had viral RNA in nasal and saliva swabs by 2 dpc that was detected in most pigs up to 5 dpc (Table 2; Supplementary Tables 1 and 2). By 6 dpc, only four pigs tested

Table 1
Summary of the clinical outcome in pigs after challenge with A/VIT/08/2005. The in-contact groups did not show any signs of clinical disease or temperature.

Group	Animal ID	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc	9–13 dpc	14 dpc[a]
A-UV	1	–	♦	6	6	6	–	–	–	–	–
	2	–	4	4	4	4	–	–	–	–	–
	3	–	–	3	4	4	–	–	–	–	–
	4	–	2	2	2	2	–	–	–	–	–
	5	♦	4	4	6	6	–	–	–	–	–
	6	–	4	4	6	6	–	–	–	–	–
	7	–	–	–	6	6	6	6	–	–	–
	8	–	–	–	–	2	4	4	–	–	–
A-V4	14	–	–	–	–	♦	♦	–	–	–	–
	15	–	♦	♦	♦	♦	–	–	–	–	–
	16	–	–	–	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–	–	–	–
	18	–	♦	♦	♦	–	–	–	–	–	–
	19	–	–	–	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–	–	–	–
	21	–	♦	♦	♦	–	–	–	–	–	–
A-V7	27	–	–	–	–	–	–	–	–	–	–
	28	–	–	–	–	–	–	1	1	–	–
	29	–	♦	♦	♦	♦1	–	–	–	–	–
	30	–	–	–	–	–	–	–	–	–	–
	31	–	♦	♦	♦	–	–	–	–	–	–
	32	–	–	–	–	–	–	–	–	–	–
	33	–	–	–	–	–	–	–	–	–	–
	34	–	–	–	–	–	–	–	–	–	–

♦ Inoculation site positive; Clinical score was determined as follows: 1 for each affected foot (lesion at the site of inoculation was not counted), tongue, mouth and snout. The maximum score is therefore 7; shaded boxes indicate temp ≥40 °C.

[a] All pigs were euthanized.

Table 2
Viral RNA detected in nasal, saliva and faecal swabs as well as EDTA blood using RT-qPCR.

		Days post challenge														
Groups	Pig ID	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A-UV	**1**	–	–	SP	SP,NP	SP,NP	SP,NP	NP	–	–	–	–	–	–	–	–
	2	–	–	SP,NP	SP,NP	SP	–	–	–	–	–	–	–	–	–	–
	3	–	–	NP,FP	SP,NP	SP	SP	–	–	–	NP	–	–	–	–	–
	4	–	–	SP,NP,FP	SP,NP	SP,NP	NP	–	–	–	–	–	–	–	–	–
	5	–	–	SP,NP	SP,NP	NP	NP	–	–	–	–	–	–	–	–	–
	6	–	–	SP,NP	SP,NP	SP	SP	SP	–	–	NP	–	–	–	–	–
	7	–	–	SP	–	SP	SP,NP	NP	–	–	–	–	–	–	–	–
	8	–	–	NP	–	SP,NP	SP	SP	–	SP,FP	NP	–	–	–	–	–
A-UVC	**9**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A-V4	**14**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	16	–	–	–	SP	–	–	–	–	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	19	–	–	–	–	NP	–	–	–	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–	SP	–	–	–	–	–	–	–
	21	–	–	–	NP	–	–	–	–	–	–	–	–	–	–	–
A-UVC4	**22**	–	–	–	NP	–	–	–	–	NP	–	–	–	–	–	–
	23	–	–	–	–	NP	NP	–	–		–	–	–	–	–	–
	24	–	–	–	NP	NP	–	–	NP	NP	–	–	–	–	–	–
	25	–	–	–	NP	–	–	–	–	–	–	NP	–	–	–	–
	26	–	–	–	–	NP	NP	–	–	–	–	–	–	–	–	–
A-V7	**27**	–	–	–	SP,NP	SP,FP	SP,NP	NP	–	NP	–	–	–	–	–	–
	28	–	–	–	SP,NP,FP	NP	–	–	–	NP	–	–	–	–	–	–
	29	–	–	–	SP,NP	–	SP	–	–	–	–	–	–	–	–	–
	30	–	–	–	SP,NP,FP	SP,FP	SP	–	–	–	–	–	–	–	–	–
	31	–	–	–	SP,NP	SP	SP	–	SP	–	–	–	–	–	–	–
	32	–	–	–	SP,NP	SP	SP,NP	–	SP	–	–	–	–	–	–	–
	33	–	–	–	SP,FP	–	SP,FP	–	–	–	–	–	–	–	–	–
	34	–	–	–	NP	SP	SP	–	NP	–	–	–	–	–	–	–
A-UVC7	**35**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	39	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

SP—saliva swab positive by PCR, NP—nasal swab positive by PCR, FP—faecal swab positive by PCR.
Shaded boxes indicate viral RNA detected in blood (animals in groups A-UV, A-V4 and A-V7 were tested on 0-7, 9, 10 and 14 dpc; animals in groups A-UVC, A-UVC4 and A-UVC7 were tested on 1, 3, 5, 7, 9, 10 and 14 dpc). The actual values are indicated in Supplementary Tables 1–4).

positive with three of the nasal swabs positive at 9 dpc. In contrast, viral RNA was only detected in faecal swabs in three samples (in two pigs at 2 dpc and in one pig at 8 dpc; Table 2; Supplementary Table 3). No RNA was found in samples collected from pigs in group A-UVC.

In group A-V4, four of the eight pigs had positive samples: one nasal and one saliva sample tested positive at 3 dpc, followed by another nasal swab at 4 dpc and, finally, a saliva sample at 7 dpc. No RNA was detected in the faecal swabs. In contrast, RNA was detected in only the nasal swabs of all five indirect contact pigs (group A-UVC4) between 3 and 10 dpc (Table 2; Supplementary Table 1).

Viral RNA was present in all pigs of group A-V7 at 3 dpc, and two pigs had RNA in their nasal swabs at 8 dpc. Four pigs had low levels of viral RNA in their faecal swabs 3–5 dpc (Table 2, Supplementary Tables 1–3). All swabs collected from the contact pigs in group A-UVC7 were negative.

3.4. Detection of viral RNA in the blood

Viral RNA was detected only in pigs of groups A-UV and A-V7 (Table 2; Supplementary Table 4). In seven of the eight unvaccinated and infected pigs (A-UV), RNA was detected between 2 and 10 dpc, while pig #1 tested negative, although it had clinical disease (Table 1). In group A-V7 viral RNA was detected in six of the pigs between 1 and 10 dpc with only two animals having a number of consecutive days positive. The infected pigs in group A-V4 and those in contact groups A-UVC, A-UVC4 and A-UVC7 did not have detectable RNA in the blood.

3.5. Serological response in the study animals

All the pigs were sero-negative on the day of challenge as determined by the LPBE except three of the eight animals that were vaccinated seven days prior to challenge (Group A-V7; Fig. 1). By 5 dpc, seven of the unvaccinated pigs had antibodies, five of the pigs in group A-V4 and all eight in group A-V7. All the infected animals were positive by 10 dpc. The indirect contact animals did not have any detectable antibodies (results not shown).

None of the pigs had antibodies to the non-structural proteins using the c-ELISA until 7 dpc when five of the pigs in group A-UV and one in A-V4 sero-converted (Fig. 1). By 14 dpc six of those in group A-UV were sero-positive, and two more in A-V4 demonstrated antibodies as well as one in group A-V7.

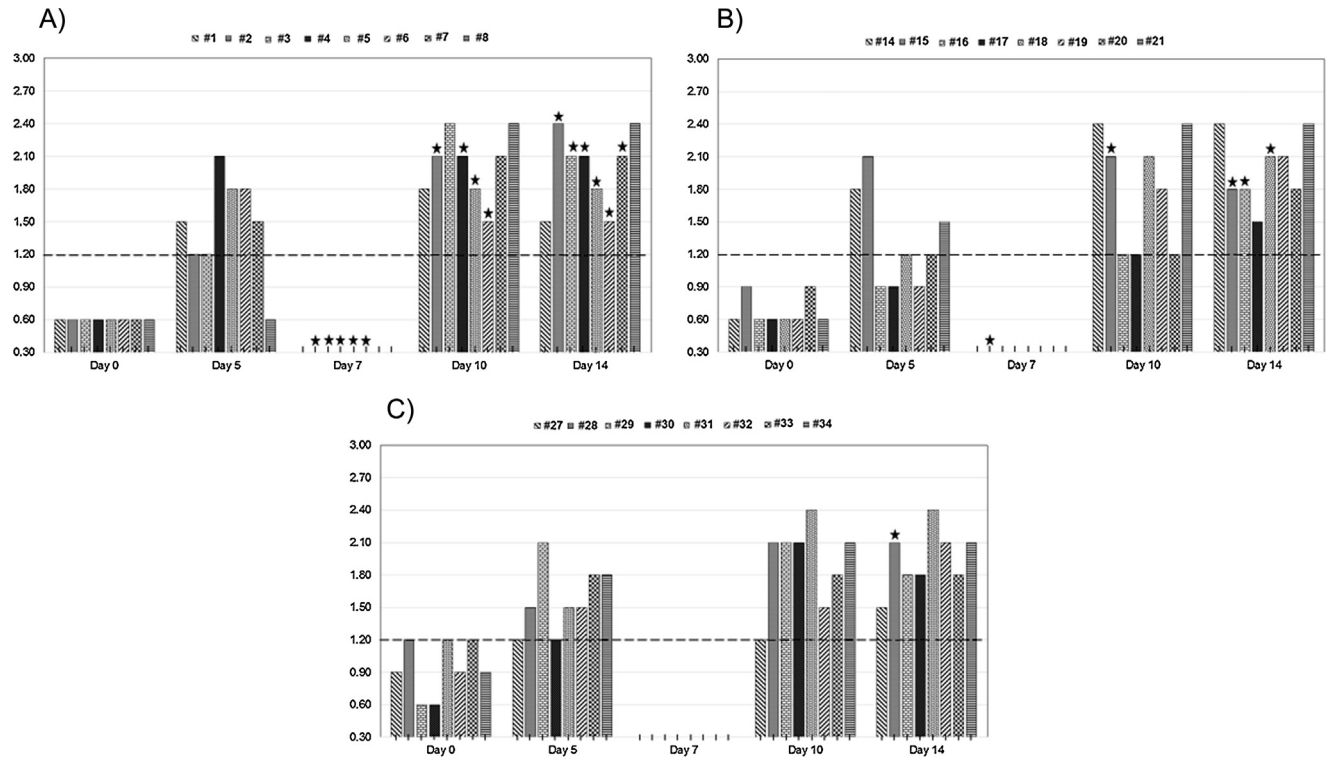

Fig. 1. Serological results showing the antibody titres determined by LPBE for pigs that were (a) unvaccinated and challenged (A-UV); (b) vaccinated and challenged 4 dpv (A-V4); and (c) vaccinated and challenged 7 dpv (A-V7). The broken line indicates the cut-off value for declaring positive samples according to the guidelines in the OIE Manual (2012). No antibodies were detected in pigs from groups A-UVC, A-VC4 and A-VC7. Stars indicate animals that that had antibodies to the non-structural proteins. LPBE was not performed on Day 7 samples.

3.6. Comparison of the amount of viral RNA in various excretions

Pigs in group A-UV had significantly more virus than all other groups in nasal ($p < 0.05$) and saliva ($p < 0.05$) swabs. Viral RNA in blood was infrequently identified, but group A-UV had significantly more compared to A-V4 ($p < 0.05$). Group A-V7 also had significantly more RNA in the blood compared to all groups, except A-UV ($p < 0.05$). There were no significant differences among groups in the amount of viral RNA detected in faecal samples. Group A-UV had significantly more RNA compared to the other groups when evaluated over all specimen types (Table 3). Faecal samples had significantly less viral RNA compared to saliva and nasal swabs when evaluated over all groups.

4. Discussion

In the present study, a >6 PD_{50}/dose A/MAY/97 vaccine was used to determine its protective ability in pigs 4 and 7 dpv against the variant A SEA-97 strain, A/VIT/08/2005. Vaccine efficacy against this variant virus had not been tested previously. Generalisation to other sites, such as the uninoculated feet, or mouth, was not observed in any of the vaccinated pigs, indicating that the vaccine protected against clinical disease. Two of the pigs vaccinated 7 days prior to infection had lesions on the coronary band of the inoculated feet at 5 and 7 dpc, respectively, but no other lesions were noted. The convention when reading protection during vaccine challenge studies is to only score lesions on un-inoculated feet, or the mouth. Therefore, it is uncertain whether the lesions on the coronary band, away from the inoculation site, represented generalised disease. Only one of these pigs (#29) had viral RNA in the blood. The clinical scores for both these two pigs were 1, compared to the unvaccinated and challenged pigs where the scores ranged from 2 to 6. Six of the pigs in group A-V7 had viral RNA in the blood. Three of the pigs only had detectable RNA in the blood at 5–7 dpc, suggesting infection by cohorts, rather than by direct inoculation. None of the pigs vaccinated four days prior to challenge had any viral RNA in the blood.

Only three of the pigs vaccinated seven days prior to challenge had detectable antibodies using the LPBE at the time of challenge. All the other pigs were sero-negative. Protection has been observed in other cases with low or undetectable antibody levels [21–24]. Barnett et al. [22] argued that in the absence of specific antibodies, innate immune responses could be the first line of defence against viral intrusion since the majority of viral infections preferentially induce the production of Type 1 interferons. However, there is also evidence that the swine innate response is inhibited by FMDV infection [25–27]. This study did not investigate the role of innate and early adaptive immune responses post vaccination and infection, but these could explain why pigs that were vaccinated four days prior to challenge were protected in the absence of antibodies [23,28,29]. Guzman et al. [30] suggested that such animals may be protected because of cell-mediated immune responses. Levels of several cytokines (IL-6, IL-8 and occasionally IL-12) increase soon after a single application of a high potency vaccine in pigs [31,28] and some evidence exists that IL-6 might increase the odds of protection against challenge [32]. In addition, it was previously shown that IgM peaked in vaccinated pigs by 7 dpv [33], and it is therefore possible that the protection we observed could in part have been contributed to this low specificity and high avidity arm of the adaptive immune response.

High levels of virus excretion in unvaccinated pigs infected by intra-dermal injection with a high challenge dose, causing severe clinical signs, are common in challenge infections in pigs [34]. Whereas pigs vaccinated with a regular vaccine (3 μg/dose payload of O Taiwan 146S antigen) were protected by 14 dpv [35]. In our study there was no clinical or serological evidence to

Table 3
Multivariable model results evaluating the effect of treatment group on the quantity of viral RNA recovered from whole blood and saliva, nasal, and faecal swabs.

Variable	Estimate (95% CI)	*t* statistic	*P* value
Experimental group			<0.001*
Contact with unvaccinated (A-UVC)	−1.41 (−1.70, −1.11)	−9.401	<0.001
Contact with vaccinated after 4 days (A-UVC4)	−1.23 (−1.53, −0.94)	−8.238	<0.001
Contact with vaccinated after 7 days (A-UVC7)	−1.41 (−1.70, −1.11)	−9.401	<0.001
Vaccinated 4 days prior to exposure (A-V4)	−1.40 (−1.65, −1.15)	−10.934	<0.001
Vaccinated 7 days prior to exposure (A-V7)	−0.59 (−0.84, −0.34)	−4.622	<0.001
Unvaccinated exposed (A-UV)	Referent		
Sample type			<0.001*
Nasal swab	−0.05 (−0.31, 0.21)	−0.378	0.706
Saliva swab	0.07 (−0.19, 0.33)	0.528	0.598
Faecal swab	−0.54 (−0.80, −0.28)	−4.129	<0.001
Whole blood	Referent		

CI = confidence interval.
* Overall test for a difference among all treatment groups. Other *P* values represent the comparison of individual groups to the referent.

suggest that transmission occurred between the infected pigs and the unvaccinated indirect contact pigs in the same room. The challenged animals in groups A-UV, A-V4 and A-V7 were excreting viral RNA, but at significantly different levels ($P < 0.05$) between 2 and 9 dpc. No detectable RNA could be found in pigs of groups A-UVC or A-UVC7. On only four occasions were low levels of RNA found in oral and nasal swabs of group A-V4 between 3 and 7 dpc, while all five contact pigs had very low levels of RNA in their nasal swabs between 3 and 10 dpc in the absence of clinical disease or sero-conversion (Supplementary Table 1). This could indicate that the pigs were inhaling virus, but not sufficient quantities to cause infection.

Pigs are known to be refractory to infection via the respiratory route and an infectious aerosol dose of 2,500 $TCID_{50}$ is required to establish disease in close to 100% of experimentally infected pigs [36]. Recently, Gonzales [37] concluded that an infectious aerosol dose of 3300 and 3900 $TCID_{50}$ is needed to establish infection and disease, respectively, in pigs. Transmission is therefore not expected to occur if physical barriers are used to prevent infected pigs from making direct contact with susceptible pigs, and if measures are taken to prevent the mechanical transfer of virus [36]. In our study, vaccinated pigs excreted 100-fold less viral RNA for a short duration (3–6 dpc) when compared to the unvaccinated pigs (2–9 dpc), and there was no transmission to the indirect contact pigs, probably because of the physical separation and strict biosecurity measures regarding personnel movements and fomite transmission. Similar observations were made with pigs that were vaccinated with O_1 Manisa vaccine and challenged with an O Mya-98 lineage virus, where vaccinated animals did not transmit disease to neighbouring pigs despite RNA being detected in the oral and nasal swabs of the in-contact pigs [38]. Eble et al. [39] also found that separation of pigs lowered the transmission rate. This clearly shows that vaccination, along with efficient biosecurity measures, should prevent transmission of FMD between pens if the animals are not in direct contact.

The results of the statistical model suggested that the amount of viral RNA detected in all in-contact groups was independent of vaccination status. However, vaccination was effective at reducing the amount of viral RNA detected relative to unvaccinated controls. Therefore, this is further evidence that the aerosol route of infection might not be important for FMDV spreading among pigs. The model also suggested that the amount of viral RNA shedding in A-V4 was significantly less than A-V7 (based on non-overlapping 95% confidence intervals). The reasons for this observation are difficult to explain and might have been due to individual variability among the susceptibility of pigs, challenge dose administration, or vaccination. This relative effect was observed in all specimen types suggesting that data management or laboratory errors were unlikely the reason for this finding. The statistical model also suggested that whole blood, oral swabs and nasal swabs contained similar levels of viral RNA, which was higher than what was detected in faecal swabs. All specimen types, other than faecal swabs, would therefore appear to be suitable for monitoring the infection status of pigs.

Extrapolation of experimental results to the field is always difficult and the efficacy of a given vaccine may differ from what is observed experimentally [40]. Considering this, when used in conjunction with biosecurity and movement restrictions, a single vaccination with A/MAY/97 may be effective under field conditions in pigs challenged with the A SEA-97 lineage to lower virus excretion and assist in eradication of the virus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.07.014

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Appendix 17: Collection of Oral Fluids Using Cotton Ropes as a Sampling Method to Detect Foot-and-Mouth Disease Virus Infection in Pigs

Transboundary and Emerging Diseases

SHORT COMMUNICATION

Collection of Oral Fluids Using Cotton Ropes as a Sampling Method to Detect Foot-and-Mouth Disease Virus Infection in Pigs

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Keywords:
foot-and-mouth disease virus; pigs; rope; diagnosis

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Summary

In high-density farming practices, it is important to constantly monitor for infectious diseases, especially diseases that have the potential to spread rapidly between holdings. Pigs are known to amplify foot-and-mouth disease (FMD) by excreting large amounts of virus, and it is therefore important to detect the virus quickly and accurately to minimize the spread of disease. Ropes were used to collect oral fluid samples from pigs, and each sample was compared to saliva samples collected from individual animals by detecting FMD virus RNA using real-time PCR. Two different experiments are described where groups of pigs were infected with different serotypes of FMD virus, either with or without vaccination, and unvaccinated pigs were kept in aerosol contact. The sensitivity of the rope sampling varied between 0.67 and 0.92, and the statistical agreement between this method and individual sampling ranged from substantial to moderate for the two different serotypes. The ease of collecting oral fluids using ropes together with the high sensitivity of subsequent FMD detection through PCR indicates that this could be a useful method to monitor pig populations for FMD virus infection. With further validation of the sensitivity of detection of FMD virus RNA, this can be a cost-effective, non-invasive diagnostic tool.

Introduction

Infectious diseases constituting a threat to both livestock health and farmer's livelihoods have the potential to spread rapidly where high-density farming practices occur. To minimize the rate of spread, it is important to regularly monitor for the introduction of various diseases. Rope sampling is a non-invasive method of oral fluid collection (saliva and oral mucosa) from pigs, which provides diagnostic material that may be tested for different infectious agents and can assist with disease surveillance. This sampling method has previously been used for the detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and influenza A virus in pig and boar populations (Prickett et al., 2008a,b; Kittawornrat et al., 2010; Detmer et al., 2011).

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals caused by a single-stranded RNA virus belonging to the *Picornaviridae*. Foot-and-mouth disease virus has a wide host range, but importantly impacts negatively on high-intensity farming systems, especially pigs and dairy cattle, causing major economic losses in countries that have been free of the disease. Pigs are known to amplify FMD virus by excreting large amounts of virus, even before the onset of clinical signs (reviewed in Thomson and Bastos, 2004). It is therefore important to detect the virus early using a diagnostic test with high sensitivity, especially if pigs are maintained in an

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intensive rearing system with the potential for rapid and widespread disease dissemination. This paper reports on the effectiveness of sampling for FMD virus RNA in oral fluid using ropes by comparison with individually collected saliva swab samples using two infection means. Some pigs were infected with FMD virus, and others were in aerosol contact.

Materials and Methods

Experimental design

All the animal experiments were performed in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes (CSIRO AAHL Animal Ethics Committee protocols 1497 and 1571). Only animals that tested serologically negative were included in these studies where the efficacy of two vaccines (O1 Manisa and A Malaysia 97) was investigated. The results of these studies will be published separately. Three groups of 5 pigs each (groups O-V7, O-V4 and O-UV) were challenged with a pig-adapted O/VIT/2010 (O Mya98 topotype) strain of FMDV via the heel bulb route in the first experiment (Experiment 1). Groups O-V7 and O-V4 were vaccinated 7 and 4 days, respectively, prior to challenge with a monovalent O_1 Manisa double oil emulsion vaccine (at least 6 PD_{50}), while group O-UV remained unvaccinated. An additional group of 5 unvaccinated pigs was kept as indirect contact controls separated by a 1.5-m-tall solid steel partition from each vaccinated and infected group (groups O-UVC7, O-UVC4 and O-UVC). In another experiment (Experiment 2), two of three groups (A-V7 and A-V4) of 8 pigs each were vaccinated 7 and 4 days, respectively, prior to challenge with a monovalent A Malaysia 97 double oil emulsion vaccine (at least 6 PD_{50}). The third group of 8 pigs, A-UV, was not vaccinated. All pigs were challenged with a pig-adapted A/VIT/2005 (A SEA-97 topotype) strain of FMDV via the heel bulb route. Three additional groups of 5 unvaccinated pigs (A-UVC7, A-UVC4 and A-UVC) were kept as indirect contact controls as described in Experiment 1.

Clinical observation and sampling

Pigs were examined daily for generalized disease. Cotton ropes were prepared by washing with clean tap water to remove any foreign matter and then air-dried. Pigs were able to access 30 cm lengths of roughly 5-mm-thick rope tied at pig shoulder height for 15–30 min per day for 2 weeks after challenge. Oral fluids were wrung from ropes into sterile plastic bags, and approximately 2 ml of each sample was added to 2 ml viral transportation medium (basal Eagle's medium, 10 mM HEPES, 10% tryptose phosphate broth, 10% foetal calf serum) and stored at −80°C until tested. Saliva samples were collected daily from the buccal cavity of each pig using sterile cotton swabs and stored in a lysis solution (Stratec Molecular, Birkenfeld, Germany) with carrier RNA and proteinase-K at −80°C until tested.

Sample analysis

Total RNA was extracted from oral fluid samples using the InviMag Virus RNA Mini kit/KF96 (Stratec Molecular) on an automated nucleic acid extraction system (KingFisher Flex Magnetic Particle Processor; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The extracted RNA was tested immediately or stored at −80°C until required. Real-time RT-PCR (qRT-PCR) was carried out using Ambion AgPath-ID MasterMix (Life Technologies, Carlsbad, CA, USA) as described previously (Shaw et al., 2007). An internal 18S RNA control was used to validate the RNA extraction procedure, determine the integrity of the RNA sample and confirm the absence of significant levels of PCR inhibitors for the rope samples. Quantification of the PCR on the saliva samples was carried out with RNA standards prepared by *in vitro* transcription of 550 bases of the IRES region cloned into pBluescript KS+ (Boyle et al., 2004) using a Megascript T7 kit (Ambion, Foster City, CA, USA). A cycle threshold (C_t) value of <40 was considered positive.

Statistical analysis

The results of the rope samples for each group were compared with the combined daily results of the saliva swabs for the same group. A group of animals were deemed positive for virus RNA in the saliva swabs if at least one of the animals in the group was positive. The group estimates for the rope and saliva samples were used to calculate prevalence of disease, test sensitivity and specificity, predictive values (probabilities for true positive, true negative, false positive and false negative) and positive and negative likelihood ratios and their 95% confidence intervals using a 2 × 2 contingency table (Altman and Bland, 1994). The agreement between the two sampling procedures was compared using Kappa statistics (κ) (Cohen, 1960).

Results

Generalized FMD infection was observed in all five infected pigs in group O-UV by 2 days post-challenge (dpc), in two pigs of group O-V4 by 3 dpc, two pigs in group O-V7 by 2 dpc, all eight pigs in group A-UV between 2 and 5 dpc and finally 2 of 8 pigs in group A-V7 between 5 and 7 dpc. One of the pigs in group O-UVC also showed lesions consistent with FMD on 13 dpc of the

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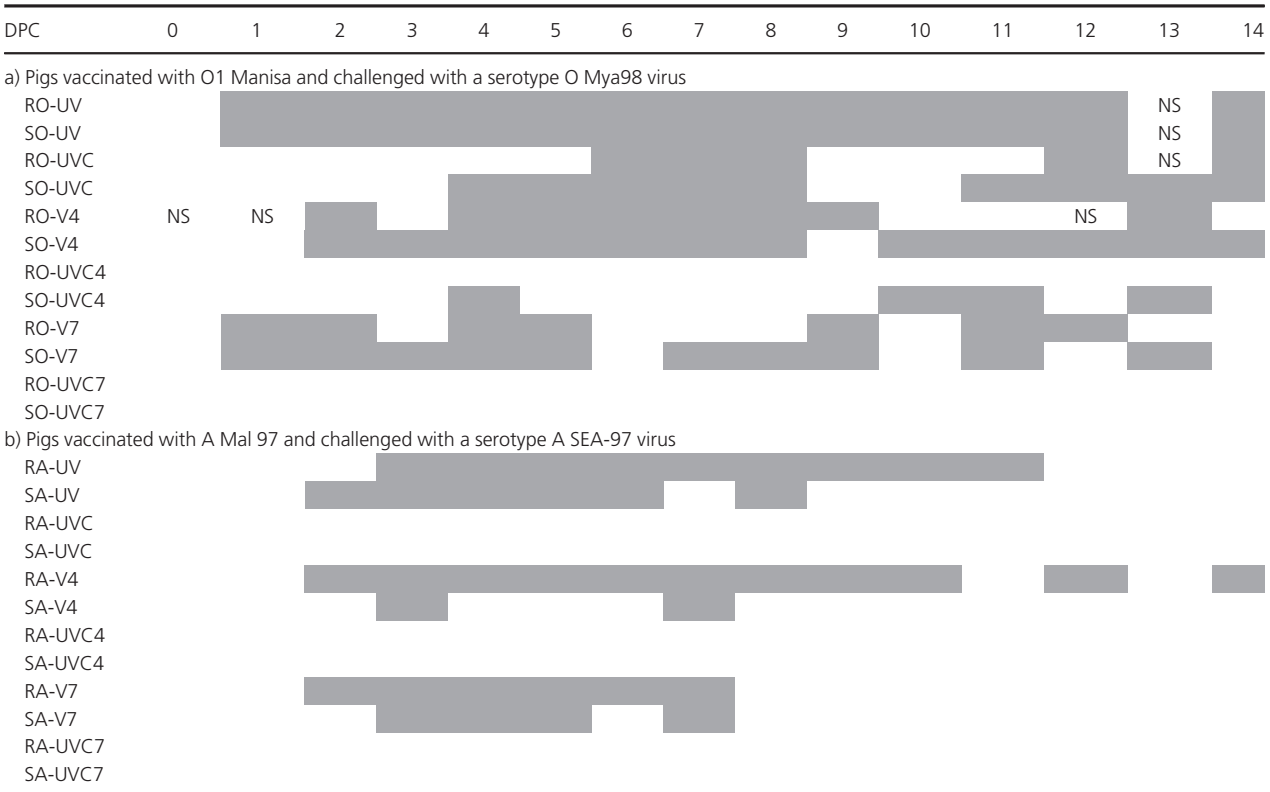
experiment after breaching the partition on day 9. Groups O-UVC4, O-UVC7, A-V4, A-UVC, A-UVC4 and A-UVC7 did not show any clinical signs of FMD until 14 dpc when the experiment was terminated (W. Vosloo, T.T.H. Nguyen, G.T. Fosgate, J. Morris, V.P. Kim, V.N. Quach, T.T.P. Le, H. Dang, X.H. Tran, P.V. Pham, V.H. Vo, T.Q.A Le, T.M.T. Mai, T.V.Q. Le, T.L. Ngo, and B.N. Singanallur, in preparation).

18S RNA was consistently detected in all the rope samples, and the 'C_t' values ranged between 20 and 30 (results not shown). Viral RNA was found in rope samples in all the groups where the animals were exhibiting clinical signs of FMD. In group O-UV, viral RNA was present as early as 1 dpc with C_t values mostly <30 and was detected in each daily sample until termination of the experiment at 14 dpc (Table 1a). Pigs in group O-V4 initially did not chew the ropes and only individual saliva samples were collected on days 0 and 1; RNA was detected intermediately in rope samples from 2 dpc until 13 dpc. Viral RNA was detected intermittently from group O-V7 from 1 dpc until 12 dpc. At 6 dpc, RNA was also detected in the contact group, O-UVC, 3 days before they breached the partition. Viral RNA was again detected in this group at 13 dpc when the first clinical signs were observed. None of the animals in groups O-UVC4 and O-UVC7 tested positive for viral RNA.

Pigs infected with the A/VIT/2005 virus deposited RNA in the rope samples from 2 to 3 dpc (A-UV, A-V4 and A-V7) with the shortest duration in group A-V7 where the pigs were vaccinated 7 days before challenge (2–7 dpc; Table 1b). In groups A-UV and A-V4, viral RNA was detected up to 11 and 14 dpc, respectively. No RNA was detected in rope samples exposed to the contact groups (A-UVC, A-UV7 and A-UV4).

Each pig was individually sampled daily using cotton swabs to collect saliva for comparison with the results from the rope samples. Groups O-UV and O-V7 showed RNA in saliva swabs at 1 dpc, followed by group O-V4 at 2 dpc (Table 1a). At least 1 pig was positive in group O-UV for the duration of the experiment, while RNA was also detected in the vaccinated and challenged pigs in groups O-V4 and O-V7 on most days using swab sampling. At 4 dpc and on most days after that, RNA could be detected in swab samples from the contact group O-UVC. Although group

Table 1. Comparison of daily results when sampling pigs using ropes and individual saliva swabs. A group of animals were deemed positive for saliva swabs if at least one of the animals in the group was positive on any given day



Blank: negative; shaded: positive; DPC, day post-challenge; NS, not sampled; UV, unvaccinated; UVC, unvaccinated contact group for UV; V4, vaccinated 4 days prior to challenge; UVC4, unvaccinated contact group for V4; V7, vaccinated 7 days prior to challenge; UVC7, unvaccinated contact group for V7; R, rope; S, saliva.

O-UVC4 had 1 pig that was positive for RNA 4 dpc, RNA was only detected in swab samples from this group again from 10 dpc. No FMDV RNA was detected in group O-UVC7 swab samples throughout the experiment.

Group A-UV had FMDV RNA-positive samples from 2 to 8 dpc, with 7 of 8 pigs being positive at 4 dpc, while the vaccinated and challenged group A-V4 only had RNA-positive samples on 3 and 7 dpc, with 1 of 8 pigs being positive. Group A-V7 showed RNA-positive swab samples from pigs between 3 and 7 dpc. Although high numbers of pigs in the latter group were positive on most days, the average RNA copy number was $<10^3$. Groups A-UVC, A-UVC7 and A-UVC4 did not show FMDV RNA in saliva swabs (Table 1b).

Discussion

Viral RNA was detected in both rope and individual saliva samples from all groups of infected animals. There was a strong correlation (99–100%) between the results obtained from both rope and swab samples collected from infected animals in groups O-UV and A-V7. Similarly, in the contact groups O-UVC7, A-UVC4 and A-UVC7, all tested negative for viral RNA using both rope and swab samples. Low levels of RNA and variation in days where RNA was detected led to poorer correlation for groups O-V7 (73.3%), O-V4 (58.3%), O-UVC (78.6%), O-UVC4 (73.3%), A-V4 (40.0%) and A-UV (66.7%). For example, in group O-UVC4, RNA was detected in saliva swabs on 4 occasions (4, 10, 11 and 13 dpc), but was not detected in the rope samples (Table 1a). Given that the pigs in this group never showed clinical disease, it probably indicates that virus excretion was very low. It is therefore possible that when low levels of virus are present, factors that influence RNA recovery such as time at room temperature (the ropes were available to the pigs for up to 30 min), proteases and other enzymes could destroy the virus and RNA prior to testing. In contrast, viral RNA was detected in the rope samples of group A-V4, where the individual saliva samples were negative (Table 1b). This could be a result of the small amount of material collected with the swabs compared to the rope that was available to pigs for a longer period where more saliva was collected as a result. More experiments are needed to validate the sensitivity of FMDV RNA detection as well as volumes when using swabs for collection.

The comparative statistical results for sampling performance of the ropes against saliva swabs in detecting the presence of FMDV RNA per group of animals are shown in Table 2. The group was considered positive if one animal was positive by saliva and the sensitivity and specificity estimates were calculated using a 2 × 2 table. The sensitivity of viral RNA detection in the rope samples ranged from 0.67 for Experiment 1 to 0.92 for Experiment 2 when compared with the results of the saliva samples. The positive predictive values, a measure of true positives, were 0.94 for Experiment 1 compared to 0.42 for Experiment 2, the latter being lower due to the difference in the number of positive animals between the two experiments. In both the experiments, the accuracy was >80% (81%; 75–87%), indicating that rope samples are a good determinant for FMD detection. The kappa values indicated a moderate level of agreement between the two methods (0.61 for serotype O and 0.48 for serotype A).

Compared to saliva swabbing and clinical examinations, rope sampling is a less laborious approach to sample collection that is further stress-free for animals. The ease with which oral fluid samples can be collected makes rope

Table 2. Comparative statistics of results obtained from rope samples and individual saliva swabs. A group of animals was deemed positive for saliva swabs if at least one of the animals in the group was positive on any given day

Parameters	O1 Manisa versus O Mya98 (Experiment 1)	A Malaysia 97 versus A SEA-97 (Experiment 2)	Overall (Experiment 1 + Experiment 2)
Positive in oral swabs & rope samples	31	11	42
Positive in oral swabs only	15	1	16
Positive in rope samples only	2	15	17
Negative in oral swabs & rope samples	37	63	100
Prevalence	0.54 (0.44–0.65)	0.13 (0.06–0.20)	0.33 (0.26–0.40)
Sensitivity	0.67 (0.57–0.77)	0.92 (0.86–0.97)	0.72(0.66–0.79)
Specificity	0.95 (0.90–1.00)	0.81 (0.73–0.89)	0.85 (0.80–0.91)
Accuracy	0.80 (0.71–0.89)	0.82 (0.74–0.90)	0.81 (0.75–0.87)
Positive predictive value	0.94 (0.89–0.99)	0.42 (0.32–0.53)	0.71 (0.64–0.78)
Negative predictive value	0.71 (0.62–0.81)	0.98 (0.96–1.01)	0.86 (0.81–0.91)
Kappa (SE=0.085)	0.61 (0.44–0.77)	0.48 (0.26–0.71)	0.58 (0.45–0.71)
Level of agreement	Moderate	Moderate	Moderate
Odds ratio	38.23 (8.11–180.26)	46.2 (5.53–386.13)	15.44 (7.14–33.42)

Values in parentheses are 95% confidence intervals.

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sampling an attractive sampling method. On most days, copious amounts of oral fluids were collected from the ropes; however, this method is not without its limitations and the amount of oral fluid sample collected is greatly dependent on animal behaviour. Clinically affected pigs exhibited less interest in the ropes possibly due to the effects of the disease. In the experiments described here, all pigs had access to the ropes even during periods of disease and diseased pigs often chewed the ropes once their healthy counterparts became bored and lost interest in the ropes. One group of pigs had to be encouraged to accept the rope, but once familiar their interest was maintained throughout the experiment. The ropes were used to distract the pigs while swab sampling was performed. It is therefore possible that saliva swabs could be positive due to cross-contamination of the mouth when pigs were chewing on the ropes. However, because there was a significant number of individual swabs negative on days the ropes were positive, this was not likely to be a problem, but in future experiments, ropes should only be available to the pigs after sampling to avoid this uncertainty.

Overall, cotton rope sampling of oral fluids can be considered a successful method to detect FMD virus RNA from pig populations. With further validation of the specificity and sensitivity of detection, this may be a cost-effective, non-invasive, sampling tool to detect FMD in a pen considering that susceptible, unvaccinated pigs will rapidly infect each other due to direct contact.

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