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AUSTRALIAN Pork™

Testing the Antibody Response of Pigs to Foot-and-Mouth Disease Vaccines

Final Report
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Executive Summary

Foot-and-mouth disease (FMD) is one of the most infectious diseases affecting cloven hoofed ruminants and pigs. Although it does not cause mortality in adult animals, the production losses and economic impacts due to trade embargoes can be severe. Australia has been free of FMD since 1872 and the current projections are that a widespread outbreak can cause direct and indirect losses of up to $12 billion. For this reason the country has strict quarantine measures in place to protect its lucrative export markets.

However, in the case of an outbreak, it is possible that emergency vaccination using high potency vaccines will be used to control the disease. For this reason Australia has invested in a vaccine bank that contains 9 antigens stored at ultra deep temperatures. These antigens can be used to formulate vaccine at short notice. It was necessary to test these vaccines in Australian pigs to ensure they provide a rapid and strong immune response should they be used during an outbreak. Since live FMD virus is not allowed in the country, the animals could not be challenged, the ultimate proof that vaccines are efficacious. This aspect will be addressed in the FMD Risk Management Project.

Three different formulations each containing two or three different antigens from different serotypes were injected into 6 pigs each. The pigs were bled on a regular basis and given a booster vaccination at day 21. The final bleed occurred at day 35 at which time large volumes of sera were collected for storage as bulk positive controls.

All sera were tested for antibodies to the non-structural proteins (NSPs) using an in-house developed assay. These antibodies are an indication of infection and are used to distinguish between vaccinated and infected animals. However, when vaccines are contaminated with NSPs, the animals can sero-convert, making the use of the discriminatory assays impossible. None of the sera tested positive, indicating that the vaccines were not grossly contaminated with NSPs as indicated after two injections.

The sera were furthermore tested for antibodies to the structural proteins using 2 different ELISAs. Most animals (75%) sero-converted between days 7-9 as measured by the IpELISA, with only one animal vaccinated with O1 Manisa sero-converting at day 6. These results are in agreement with other studies where high potency vaccines were used in pigs. An anamnestic response was observed in the majority of pigs after the boost they received at day 21.

There was a statistically significant difference in the serological responses between the various vaccine strains ($p=0.002$) impacted mostly by the significant differences in the last 3 bleeds (days 14, 21, and 35). The two O vaccines, O1 Manisa and O Campos did not differ as determined by a repeated measures 2-way ANOVA, whilst there was a significant difference between the 3 A vaccines ($P<0.0001$). This was due to the increased serological reaction observed with the A22 Iraq vaccine strain. Of most concern was the response to A Malaysia 97 and especially A24 Cruzeiro, where one animal only sero-converted after the boost. These differences in reaction are most probably not due to the reagents used in the ELISAs, as the A24 Cruzeiro reagents are homologous to the vaccine strain. No antibodies could be detected to SAT-2 which is currently under investigation.

Vaccine 1, that contained O1 Manisa and A22 Iraq, had the highest antibody titres compared to the other formulations, and it is unknown whether this observation was due to some inherent factor of the vaccine or the improved reaction to these 2 strains. The ELISAs provided anomalous results

with pigs vaccinated with vaccine 3, consisting of Asia-1 Shamir and A Malaysia 97. One pig sero-converted as early as 3 days post vaccination against Asia-1 while 2 more had one day each of early responses as determined by the lpELISA. These results are regarded with suspicion, but were found upon repeat of the assay. One pig also had one positive titre at day 3 against A Malaysia 97 when using the cELISA that measured negative again until day 8. The reason for these non-specific reactions is not clear.

Of the 2 assays used to measure antibodies, the lpELISA consistently showed sero-conversion 1-4 days before the cELISA. However, if the cut-off value for the cELISA is lowered, the tests compare better for all serotypes although there was not a statistically significant difference in Kappa statistics between the lpELISA and the cELISA at 50% or 30% cut-off for most strains, except O1 Manisa and Asia-1 Shamir.

The high potency vaccines provided from Merial, drawn from the Australian vaccine bank, induce antibodies in Australian pigs. Sero-conversion was comparable to that of other studies. Since the animals could not be challenged, it is not known whether they will be protected against clinical disease. The experiments planned under the FMD Risk Management Project, jointly funded by Industry and Government will provide a more accurate estimate of the potency of these vaccines.

Background to Research

Foot-and-mouth disease (FMD) virus infects over 70 species of cloven hoofed animals including cattle, sheep and pigs (Hedger, 1981). It is the most infectious animal disease known and of primary concern to the livestock industry in Australia. In the event of an outbreak of FMD a decision will be made on whether to vaccinate livestock to prevent the disease spreading.

Most countries that are free of FMD take stringent measures to protect their livestock industries and have contingency plans to stamp out an outbreak within the shortest period of time. Previously destruction of animals (culling and 'stamping out') was used to eradicate the disease, but due to ethical reasons and public demand, most free countries now accept that vaccination will play an important role during control measures. Australia has established a commercially produced FMD vaccine bank maintained by Merial at Pirbright in the United Kingdom for a 5 year period from 2004-2009 and has recently replaced that with a new bank for the period 2010-2015.

There is a specific need to demonstrate that these vaccines will induce the required immune responses in Australian livestock as measured by antibody levels following vaccination. There is also a need demonstrate that diagnostic assays developed at AAHL will detect FMD vaccine induced antibodies in Australian cattle, pigs and sheep should the decision to vaccinate Australian livestock be made.

Vaccination response in pigs, in particular, has shown that the antibody produced in pigs following vaccination is not as high as in cattle and sheep. In addition, vaccine manufacturers require that vaccines be administered as two doses, 4-6 weeks apart, followed by regular revaccination every 4-6 months for prophylactic use. However, it is generally accepted that only one high potency dose will be administered in an outbreak in a free area that should provide protection to disease. Vaccine trials at AAHL will give a good indication of the serological response in Australian pigs after one or two inoculations using the FMD vaccines from the Australian FMD vaccine bank. These results would be invaluable in addressing control options in AUSVETPLAN as well as ensuring that the vaccines delivered by the manufacturer are of high quality.

Objectives of the Research Project

- Import sufficient doses of foot and mouth disease (FMD) vaccine by arrangement with Animal Health Australia and Merial.
- Vaccinate pigs in the AAHL secure Large Animal Facility with three combinations of FMD vaccines and collect serum samples for testing.
- Investigate antibody responses of Australian pigs to these vaccines.
- Evaluate the performance of FMD test procedures, including conventional tests and tests to detect non-structural proteins, in terms of their ability to detect, or not, antibody responses to vaccination with these vaccines.
- Using data from this and other trial work, report on the ability of tests available for use at AAHL to differentiate between vaccinated animals and unvaccinated animals after a single vaccination and after a boost.
- Develop a pool of reagents for use in future research work or surveillance testing.
- Provide a final report covering all of the above services.

Introductory Technical Information

Although pigs are more resistant to infection by the airborne route than cattle and sheep, they are known as amplifier hosts due to their high level of airborne virus excretion (Alexandersen et al., 2003; Sellers and Parker, 1969). They can be a source of infection to other species over large distances if conditions are favourable (Alexandersen and Donaldson, 2002; Donaldson and Alexandersen, 2002). Pigs can also be exposed to infection via the oral route when fed untreated swill illegally, making them a risk for introduction of disease to other livestock.

It has previously been shown that high potency emergency FMD vaccines provide protection (Doel et al., 1994; Salt et al., 1994; Cox et al., 1999; Salt et al., 1998) even when FMD virus specific antibodies have not been detected or were present at levels not usually considered protective (Cox et al., 1999; Salt et al., 1998; Mackowiak et al., 1962; Sutmoller and Vieira 1980; Black et al., 1984; Pay and Hingley, 1987; Van Maanen and Terpstra, 1989; McCullough et al., 1992). However, it is still generally accepted that humoral antibodies directed to the structural proteins correlate to protection although novel approaches such as measuring the IgA responses (Eble et al., 2007) and stimulation of cytokines (Barnett et al., 2002) may play a role in prediction of protection in future.

Currently there are two ELISAs available at the Australian Animal Health Laboratory (AAHL) to measure antibodies to the structural proteins of FMD virus. The liquid phase blocking ELISA (lpELISA; Hamblin et al., 1986a,b) was previously the test prescribed by the World Organisation for Animal Health (OIE) for trade in animals. However, this test was not particularly robust and lead to false positive reactions depending on the population of animals studied (Chenard et al., 2003). Haas (1994) demonstrated that 4% false positive reactors could be found in normal unvaccinated animals that could rise to 18% in stressed animals. Furthermore, it is recommended that low positive animals be retested using the virus neutralisation test (VNT) which cannot be used at AAHL since it requires the use of live virus. It is therefore essential for AAHL to also utilise the solid phase competition ELISA (cELISA; Mackay et al., 2001) as an alternative or even preferred test. The latter has been shown to be more robust and easier to use, whilst it has improved specificity.

Research Methodology

Materials

Vaccines Used in the Study

Three combinations of FMD vaccine at $6PD_{50}$ containing various strains of the Australian vaccine bank (2004-2009) were imported from Merial, Pirbright, United Kingdom (Vaccine 1: O1 Manisa, A22 Iraq; Vaccine 2: O Campos, SAT 2 Eritrea, A 24 Cruzeiro; Vaccine 3: A Malaysia 97, Asia 1 Shamir).

Method/Process

The experiment was conducted according to the AAHL animal ethics committee regulations (AEC 3 – 1318). Six 4-6 week old pigs that tested negative to all serotypes of FMD virus were vaccinated intramuscularly with 2ml of each vaccine formulation according to the instructions provided by the manufacturer. The animals were brought into the Large Animal Facility at AAHL one week prior to the start of the experiment to allow them time to adjust. Their temperatures were monitored on a daily basis using a BioTherm Microchip that was implanted subcutaneously above the left shoulder. A reader was used to record temperatures daily.

At day 0 they were bled and vaccinated and sampled thereafter at days 1-10, 14, 21, 35. At day 21 they were boosted as previously described. The animals were examined for possible FMD lesions on days 1-10.

Bleeding was performed by using a 20G 1.5 inch vacutainer needle from the jugular vein. The sera was removed from the clot after centrifugation for 10 minutes at 3000rpm and stored in 96 well deep well plates (Fisher Biotec Pty Ltd) at -20°C until the end of the experiment. Sera were tested with each animal's sequential bleeds on the same plate.

Serological Assays Used

The sera were all tested for antibodies to the non-structural proteins (NSPs) using the in-house developed 3ABC competitive (c)ELISA (Foord et al., 2007) with the Sigma anti-chicken IgY-HRP conjugate at 1/3000 and normal bovine serum as negative control. Antibodies to the structural proteins were measured using the lpELISA (Hamblin et al, 1986a) and cELISA (Mackay et al., 2001) all with the Dako anti GP HRPO conjugate at 1/2000 and normal pig serum as negative control. The antigens in the assays are indicated in Table 1.

	Coat antibody	**Antigen**	**Detection**	**Pos control**
3ABC		baculo expressed 3ABC at 1/400 (0804-10-1301)	Chicken egg antibody (IgY) at 1/100 (0405-07-2000)	3ABC FMD positive bovine serum - Animal 8603 9/9/97; 4WPI C1 Detwold; 8WPI Asia1 Shamir; 12WPI A22 Iraq (0804-11-1643)
		lpELISA		
O1 Manisa	Rabbit anti O1 BFS1860 at 1/5000 (8511-15-3600)	O 1 MANISA (TUR/78) at 1/1000 (0604-21-0100)	Guinea pig O1 BFS1860 at 1/1000 (0607-29-1510)	FMD Vac Exp. Cow 81-89 Pooled Type 'O' Day 28 Pilot 2 (1001-04-0000)
Asia 1	Rabbit anti Asia 1 Pakistan at 1/5000 (8511-15-3609)	ASIA 1 ISR/89) at 1/1000 (0604-21-0900)	Guinea pig anti Asia 1 Pak at 1/2000	FMD Vac. Exp. Cow 1-6 Pooled type 'Asia 1' Day 21 Pilot 1 (1001-04-0001)

			(1003151422)	
A24 Cruzeiro	Rabbit anti A24 Cruzeiro at 1/5000 (8511-15-3113)	A24 Cruzeiro at 1/250 (0703-19-1400)	A5/22/24 COMB at 1/1000 (8511-15-3314)	Bovine anti-FMDV sera from Winnipeg Lab, Canada A24 Cruzeiro C-140 28dpi (0601-30-0002)
A22 Iraq	Rabbit anti A22 Iraq at 1/5000 (8511-15-3112)	A22 Iraq Antigen at 1/1000 (0312-09-1310)	Guinea pig anti-A5/A22/A24 Comb at 1/1000 (8511-15-3314)	Type A22 Cow pool 1-6 Day 21 (0610-10-1400)
O Campos	Rabbit anti O at 1/5000 (8511-15-3110)	O Campos Antigen at 1/1000 (0508-19-1500)	Guinea pig anti O blocked at 1/2000 (0607-29-1510)	Type O FMD Vac. Exp. Cow pool (1001-04-0000)
SAT 2 ERITREA	Rabbit anti SAT 2 at 1/5000 (9006-15-3607)	SAT 2 ZIM/83 Antigen at 1/100 (0208-16-0800)	Guinea pig anti-SAT 2Ken at 1/500 (8511-15-3217)	Bovine positive serum for SAT 2 (8710-23-1558)
A/Malaysia/97	Rabbit anti-A5/A22/A24 comb at 1/5000 (8511-15-3114)	A/Malaysia/97 Antigen at 1/250 (0208-16-0500)	Guinea pig anti-A5/A22/A24 Comb at 1/1000 (8511-15-3704)	Type A22 Cow pool 1-6 Day 21 was unsuitable. Used 10-01211-0424 as positive serum (0610-10-1400)
cELISA				
O Manisa	Rabbit anti O1 BFS1860 at 1/5000 (8511-15-3600)	O 1 MANISA (TUR/78) at 1/800 (0604-21-0100)	Guinea pig O1 BFS1860 at 1/1000 (0607-29-1510)	FMD Vac Exp. Cow 81-89 Pooled Type 'O' Day 28 Pilot 2 (1001-04-0000)
Asia 1	Rabbit anti Asia 1 Pakistan at 1/5000 (8511-15-3609)	ASIA 1 ISR/89) at 1/1000 (0604-21-0900)	Guinea pig anti Asia 1 Pak at 1/2000 (1003151422)	FMD Vac. Exp. Cow 1-6 Pooled type 'Asia 1' Day 21 Pilot 1 (1001-04-0001)
A24 Cruzeiro	Rabbit anti A24 Cruzeiro at 1/5000 (8511-15-3113)	A24 Cruzeiro at 1/500 (0703-19-1400)	Guinea pig antiserum to A5/22/24 COMB at 1/1000 (8511-15-3314)	Bovine anti-FMDV sera from Winnipeg Lab, Canada A24 Cruzeiro C-140 28dpi (0601-30-0002)
A22 Iraq	Rabbit anti A22 Iraq at 1/5000 (8511-15-3112)	A22 Iraq Antigen at 1/1000 (0312-09-1310)	Guinea pig anti-A5/A22/A24 Comb at 1/1000 (8511-15-3314)	Type A22 Cow pool 1-6 Day 21 (0610-10-1400)
O Campos	Rabbit anti O at 1/5000 (8511-15-3110)	O Campos Antigen at 1/1000 (0508-19-1500)	Guinea pig anti O blocked at 1/1000 (0607-29-1510)	Type O FMD Vac. Exp. Cow pool (1001-04-0000)
SAT 2 ERITREA	Rabbit anti SAT 2 at 1/5000 (9006-15-3607)	SAT 2 ZIM/83 Antigen at 1/200 (0208-16-0800)	Guinea pig anti-SAT 2Ken blocked at 1/200 (8511-15-3217)	Bovine positive serum for SAT 2 (8710-23-1558)
A/Malaysia/97	Rabbit anti-A5/A22/A24 comb at 1/5000 (8511-15-3114)	A/Malaysia/97 Antigen at 1/250 (0208-16-0500)	Guinea pig anti-A5/A22/A24 Comb at 1/2000 (8511-15-3704)	Type A22 Cow pool 1-6 Day 21 (0610-10-1400)

Statistical Analysis
The results were statistically analysed using various options in the GraphPad Prism version 5.02 (2008) package. Kappa values were determined using online services.

Discussion of Results

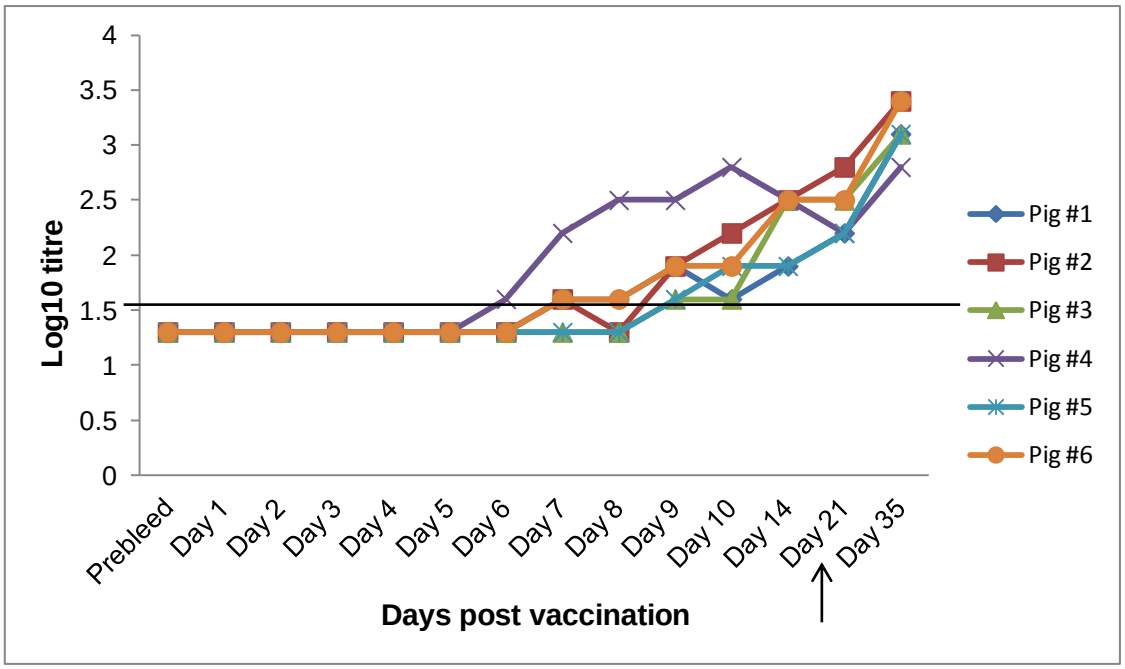
Vaccine Reactions
None of the pigs showed any local reactions to the vaccine or any clinical signs indicative of FMD. Only one pig (#17) that received Vaccine 3 had a slight temperature reaction (40.3°C) at days 10 and 12 post vaccination (results not shown).

Serological Results of Assay to Determine Antibodies to the NSPs
None of the pigs sero-converted to the NSPs after either one or two vaccinations (results not shown).

Serological Results of Pigs Vaccinated with Vaccine 1
Vaccine 1 contained antigens to O1 Manisa and A22 Iraq. Antibodies to O were determined using both the lpELISA and cELISA. Only 1 pig (#4) out of the 6 had sero-converted to serotype O as measured by the lpELISA by day 6 with titres that peaked at day 10, after which it started declining. By day 7 pigs #1, #2 and #6 had low titres, whilst pigs #3 and #5 sero-converted by day 8. All 6 pigs demonstrated an increase in titres at day 35, post the boost they had received at day 21 (Fig. 1a).

Sero-conversion rates as determined using the cELISA similarly indicated that pig #4 was the first animal to sero-convert, however only with doubtful results (inhibition of between 40-49%) at days 7 and 8. Pigs #3 and #6 had measurable antibodies by day 9 (>50% inhibition) while #2 was doubtful. Pig #5 only sero-converted by day 14. All pigs demonstrated an increase in titres at day 35 (Fig. 1b).

A)



B)

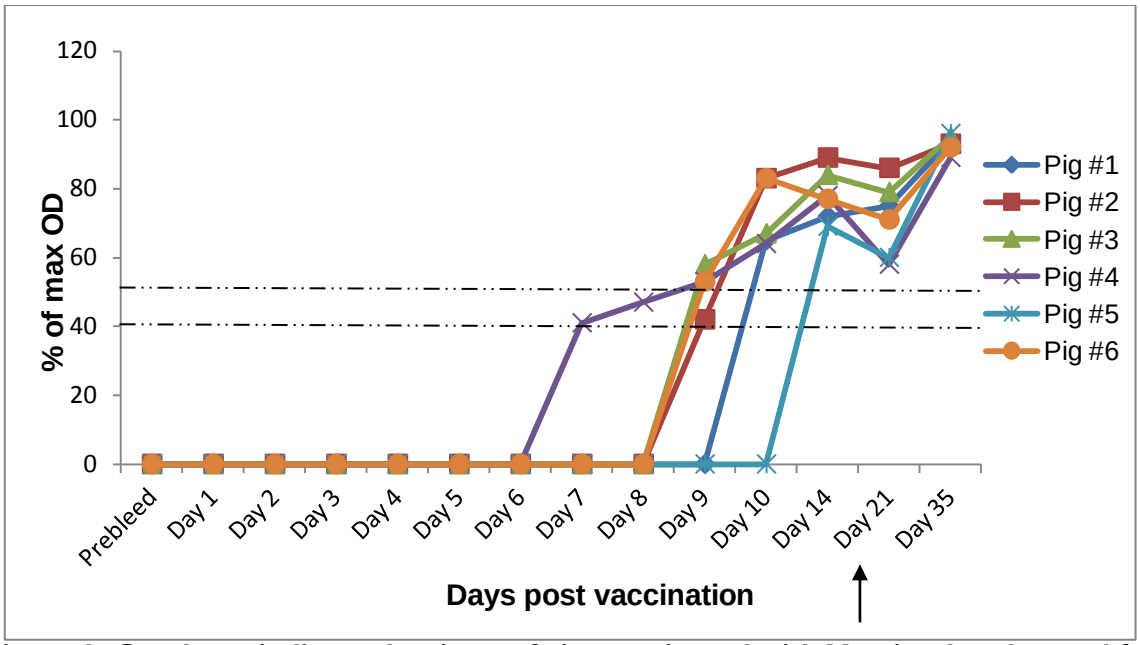
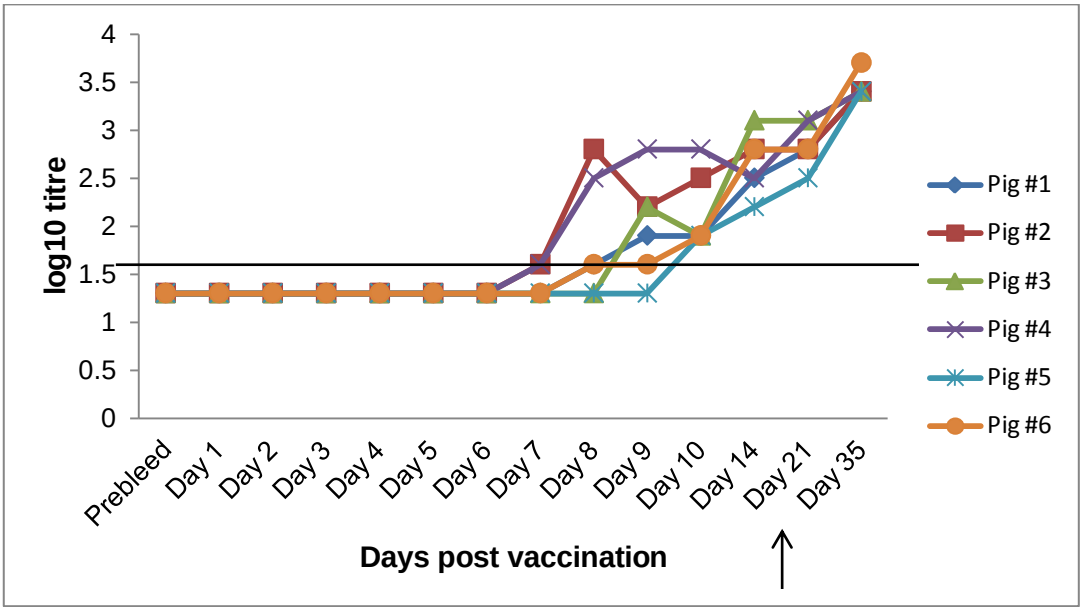


Figure 1: Graphs to indicate the titres of pigs vaccinated with Vaccine 1 and tested for antibodies to serotype O1 Manisa using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of log_{10}=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive.

Sera were also tested for antibodies to A22 Iraq using the lpELISA. Pigs #2 and #4 demonstrated low levels of sero-conversion by day 7 with significant increases in titres by day 8. Two more pigs had sero-converted by day 8 (#1 and #6), while #5 only showed detectable antibodies by day 10. All pigs had increased titres by day 35 (Fig. 2a).

Sero-conversion rates as determined by the cELISA were delayed compared to the lpELISA (Fig. 2b). Pig #4 was marginally positive by day 8, while pig #1 and #2 had doubtful results by day 9 and pig #5 by day 10. All 6 pigs were sero-positive by day 14 with a similar increase in reaction seen at day 35.

A)



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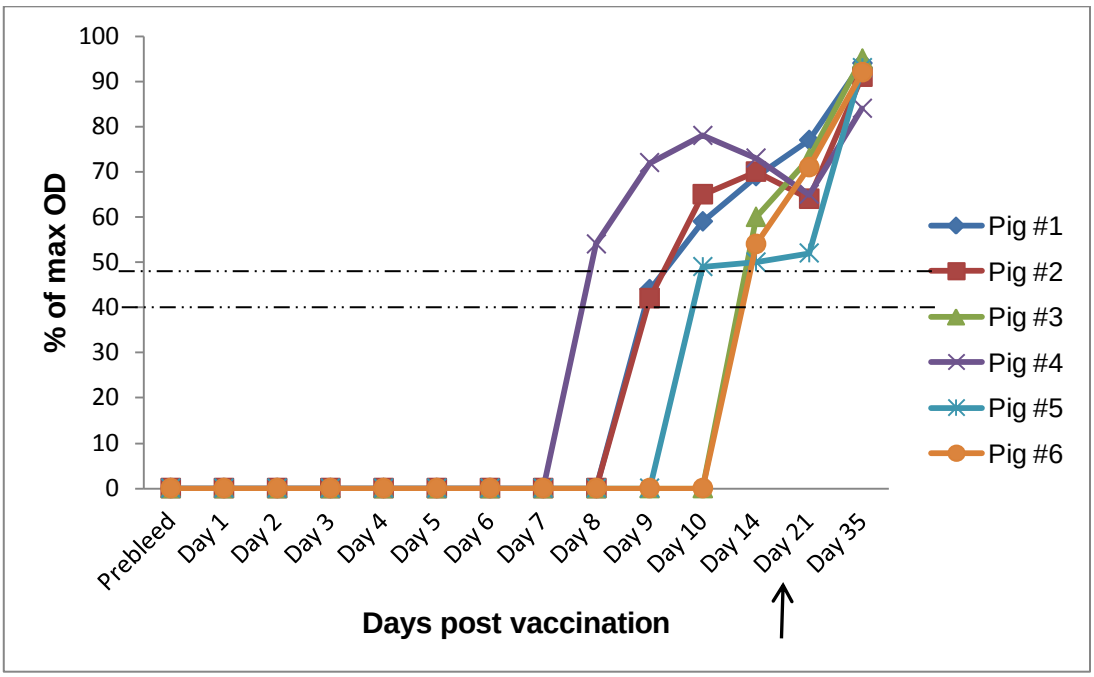


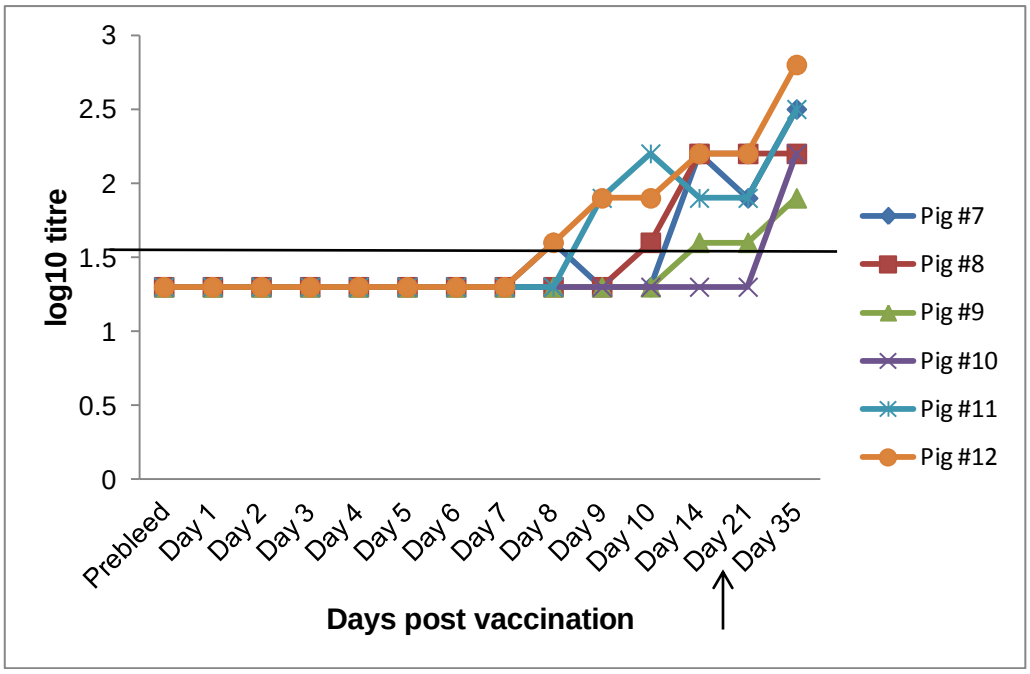
Figure 2: Graphs to indicate the titres of pigs vaccinated with Vaccine 1 and tested for antibodies to serotype A22 Iraq using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of $\log_{10}$=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive.

Serological Results of Pigs Vaccinated with Vaccine 2

Vaccine 2 contained antigens to A 24 Cruzeiro, O Campos and SAT 2 Eritrea and antibodies to A24 were determined with the 2 different assays. Using the lpELISA, pig #11 and #12 demonstrated antibodies at days 9 and 8 respectively, followed by pig #8 at day 10. Pigs #7 and #9 had measurable antibodies at day 14 whilst #10 only showed antibodies at day 35. All the pigs demonstrated an increase in antibody levels at day 35 (Fig. 3a).

With the cELISA pigs #8 and #12 showed doubtful positive results that were confirmed at day 14 as positive. All the pigs had antibodies at day 21 (pig #10 had doubtful results), followed by an increase at day 35 (Fig. 3b).

A)



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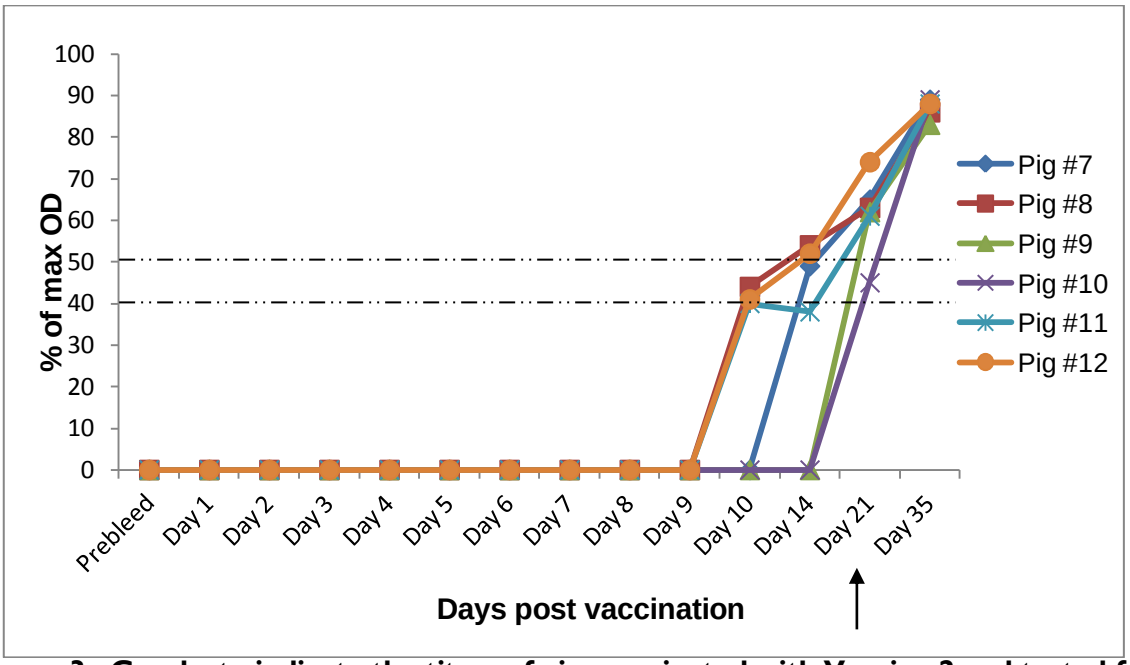
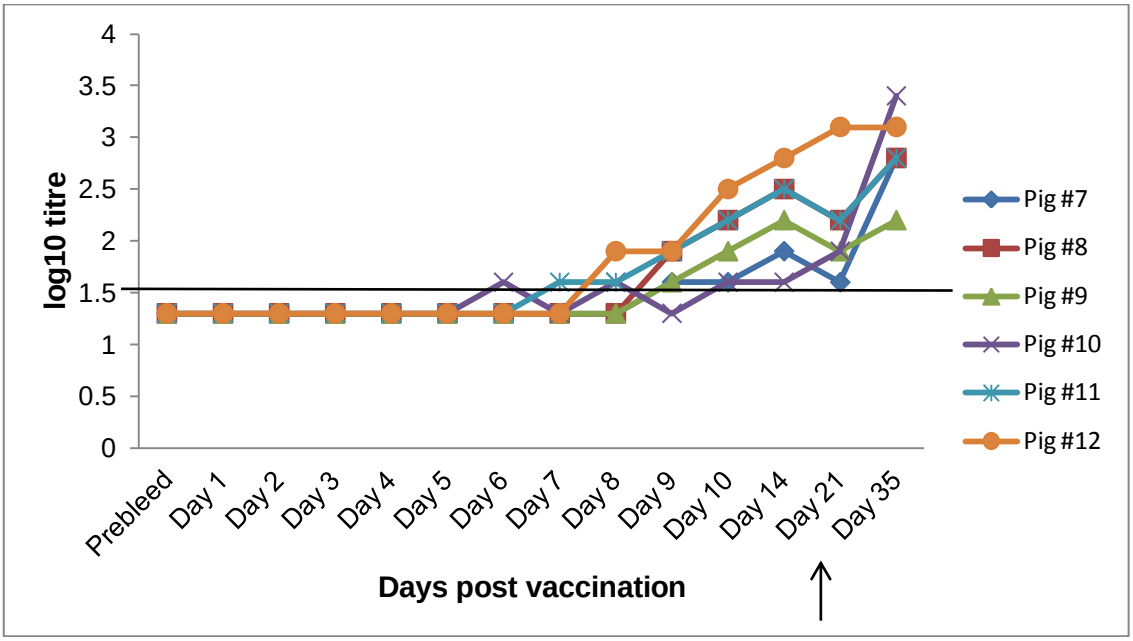


Figure 3: Graphs to indicate the titres of pigs vaccinated with Vaccine 2 and tested for antibodies to serotype A 24 Cruzeiro using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of $\log_{10}$=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive.

Antibodies to O Campos were detectable in pig #11 at 7 days post vaccination, followed by pigs #10 and #12 at day 8 and pigs #7-9 at day 9 using the lpELISA. However, pig #10 had low level of antibodies up to day 21 and at day 9 the results were negative. All pigs had a significant increase in antibodies post-boost, expect pig #9 that had only a small increase (Fig. 4a).

Pig #10 was also doubtful or negative until day 21 when using the cELISA. Pigs #7-10 were doubtful or positive on day 7, at least 2 days earlier than when using the lpELISA. All animals showed an increase in antibody levels at day 35 (Fig. 4b).

A)



B)

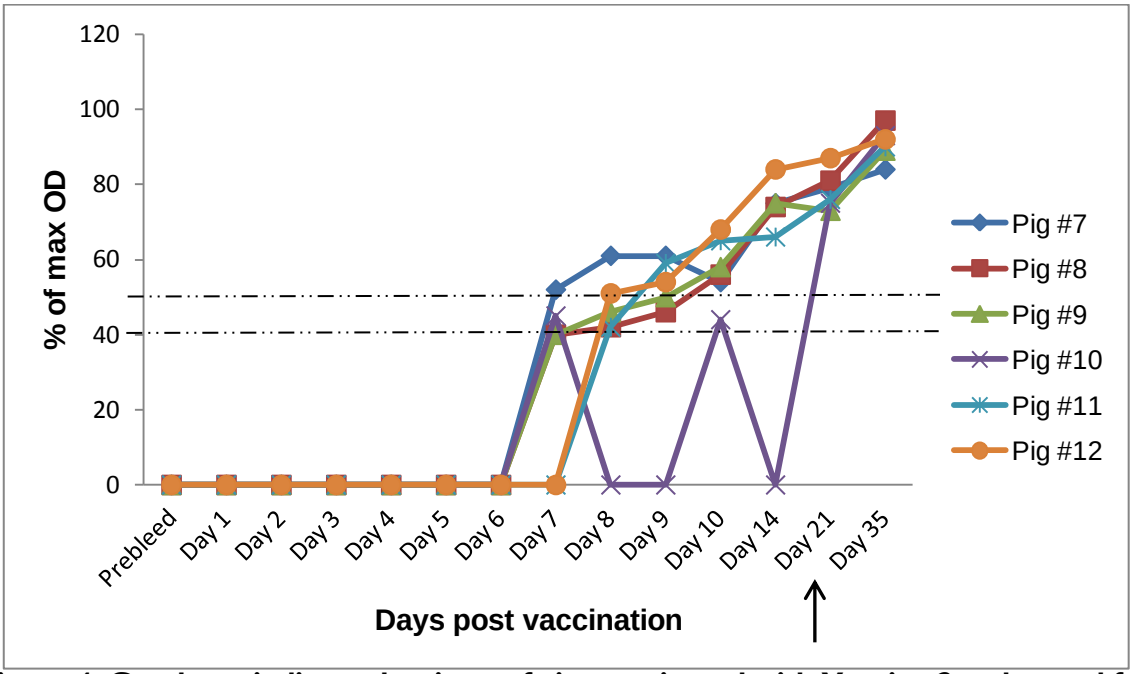


Figure 4: Graphs to indicate the titres of pigs vaccinated with Vaccine 2 and tested for antibodies to serotype O1 Campos using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of $\log_{10}$=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive.

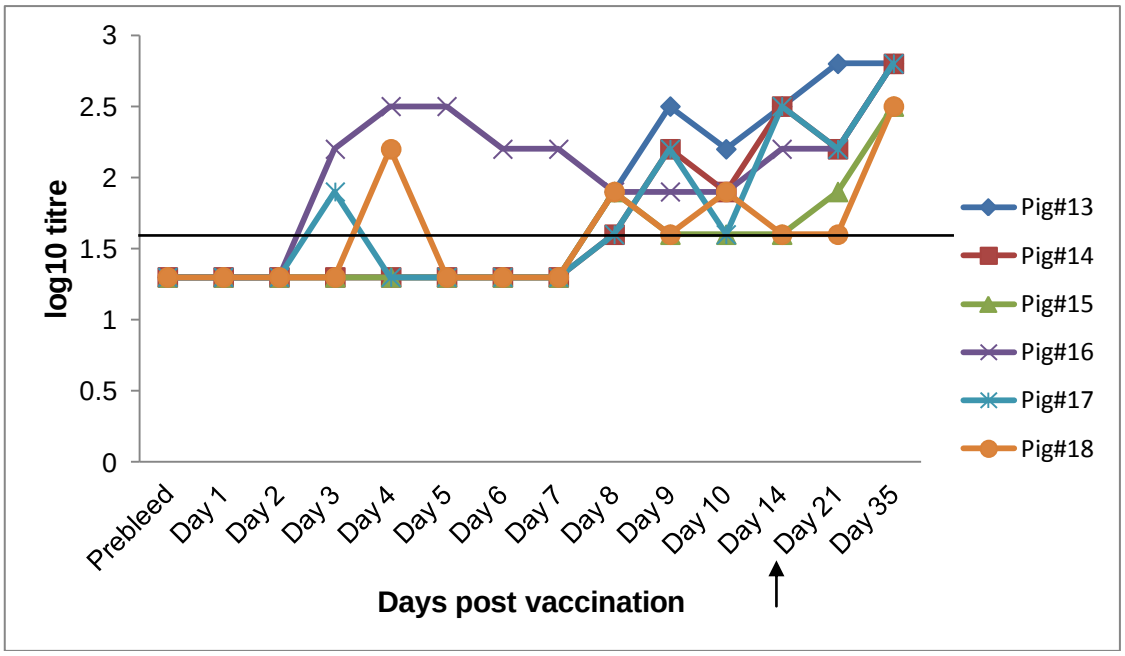
All sera tested negative to SAT-2 on the lpELISA, whilst only 2 animals (pigs #8 and #12) were sero-positive on day 35 with the cELISA (results not shown). The outcome of the VNT results will indicate whether the vaccine was ineffective or our tests are not working.

Serological Results of Pigs Vaccinated with Vaccine 3

Antibodies to serotypes A and Asia 1 were measured in Vaccine 3, consisting of antigens to A Malaysia 97 and Asia 1 Shamir. Sera collected from pigs #16, #17 and #18 between days 3 and 7 gave significant reactions in the Asia1 lpELISA. The result appears to be sample associated, rather than a test anomaly since the samples presented the same results upon retest. However the early appearance of antibody from day 3 is regarded with suspicion, and the pigs are not regarded as sero-positive so early after vaccination (Fig. 5a). Without these results all 6 pigs had sero-converted to Asia 1 on day 8 and showed variable levels of antibodies until day 35, where all had an increase in titre (Fig. 5a).

The ambiguous results obtained with the lpELISA for pigs #16-18 were not observed with the cELISA. The cELISA performed poorly and indicated only pig #13 as sero-positive at day 8. Pigs # 14-16 had doubtful positive results, followed by pig # 17 at day 14. Pig # 18 only sero-converted at day 35 (Fig. 5b).

A)



B)

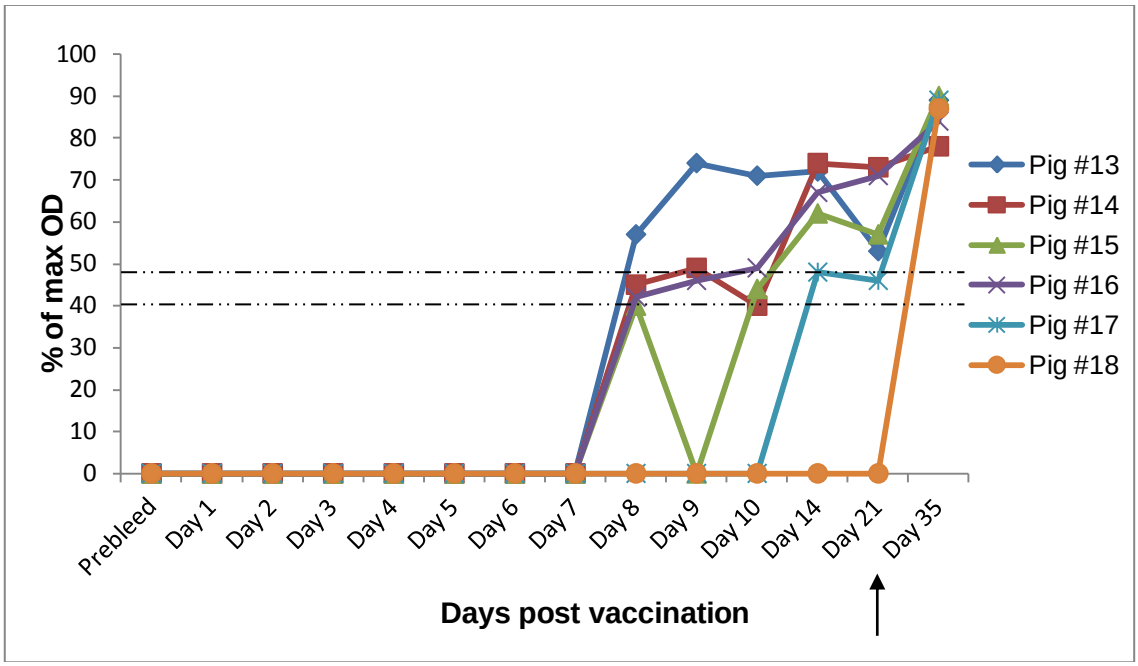


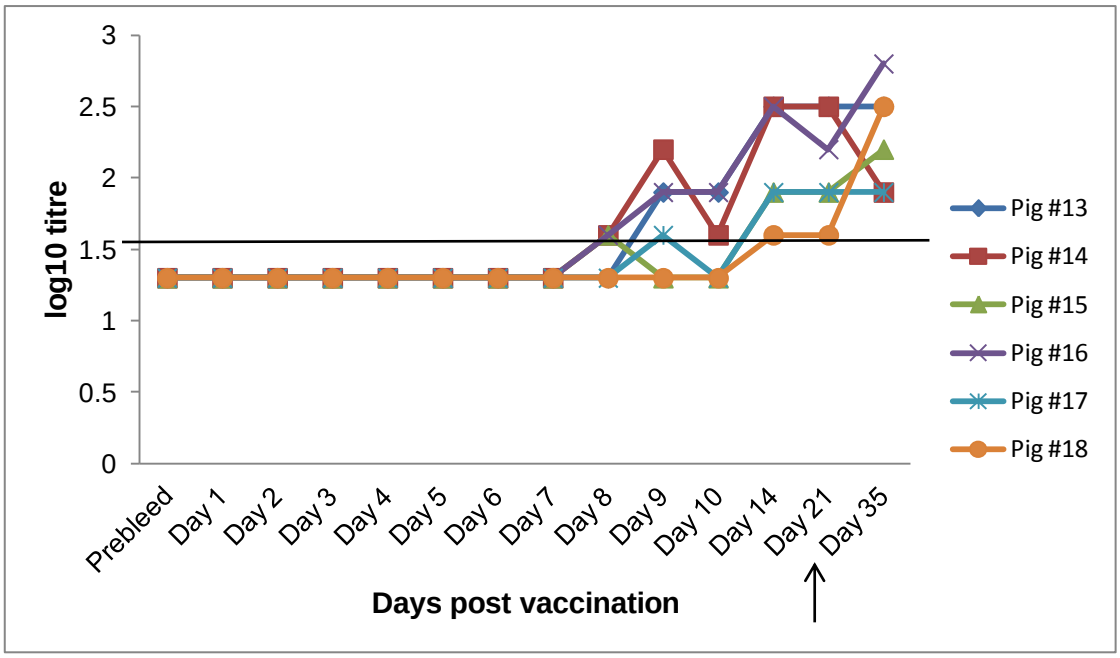
Figure 5: Graphs to indicate the titres of pigs vaccinated with Vaccine 3 and tested for antibodies to serotype Asia-1 using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of $\log_{10}$=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive.

Three pigs (#14 – 16) had detectable antibodies to A Malaysia 97 by day 8 on the lpELISA (Fig. 6a). However, pig #15 was again negative on days 9 and 10 adn was therefore considered truly positive only by day 14. A similar result was observed for #17 that was positive on day 9 and negative at day 10. Pig 18 only sero-converted at day 14. Only 3 pigs demonstrated an increase in titre at day 35

(#15, #16 and #18), 2 maintained the same titre (#13 and #17) and pig #14 had a decrease in titre (Fig. 6a).

Using the cELISA, pig #15 showed a reaction of 70% at day 3, similar as what was observed for Asia-1. However, it was then negative until days 8-10 where it had doubtful results and the first true positive result was only observed at day 14. Pig #18 had a doubtful result at day 14 and a positive result at day 21. All the other pigs had doubtful to positive results from days 7, 8 and 9 (Fig. 6b).

A)



B)

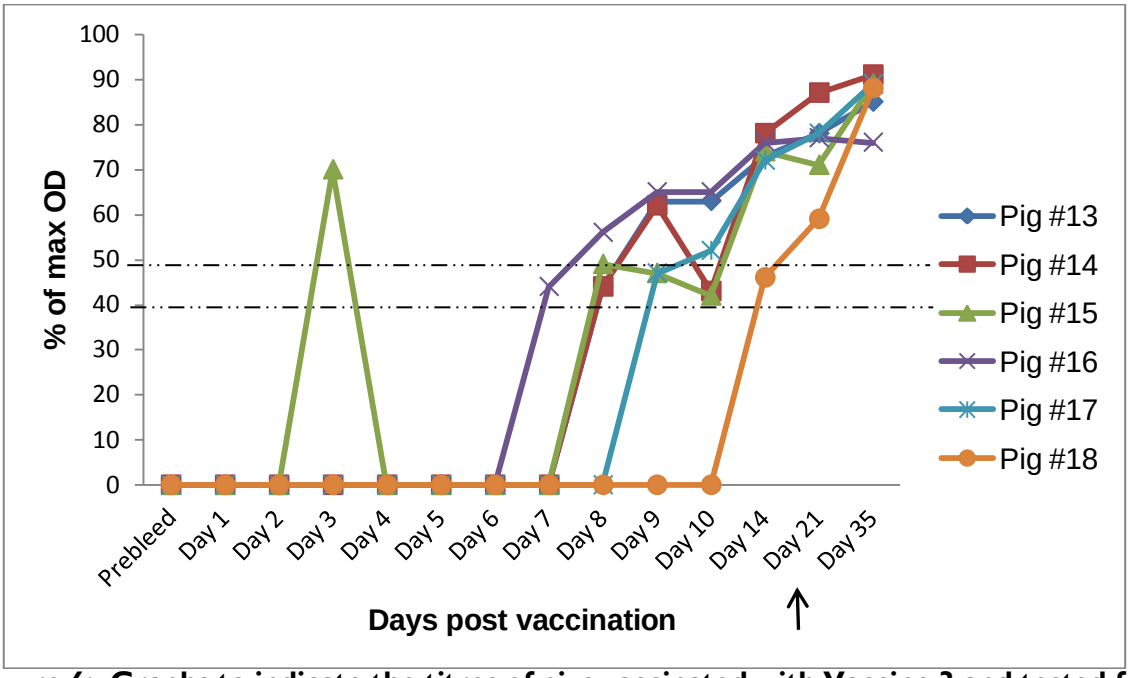


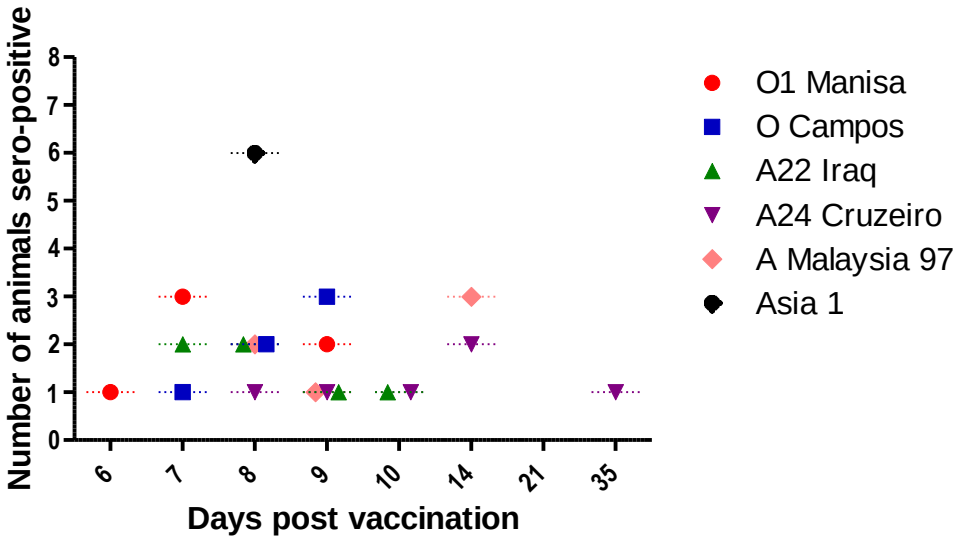
Figure 6: Graphs to indicate the titres of pigs vaccinated with Vaccine 3 and tested for antibodies to serotype A Malaysia 97 using A) the lpELISA and B) the cELISA. The arrow indicates Day 21 where the animals received a boost. The solid line indicates the positive cut-off of $\log_{10}$=1.6 (1/40) for the lpELISA and the dotted lines indicate the borderline values of 40-49% for the cELISA. Values >50% inhibition are deemed positive

Summary of the Day of Sero-Conversion

Figure 7 provides a summary of how many animals sero-converted at each day post vaccination as determined by the lpELISA. One animal sero-converted at day 6 with the O1 Manisa vaccine, 3 at day 7 and 2 at day 9. All 6 pigs sero-converted to Asia-1 at day 8 (when discarding the anomalous results), while the poor response to A24 Cruzeiro is evident by the one animal sero-positive only at day 14 and another only after the boost at day 35. The A Malaysia 97 similarly had 3 sero-positives

only at 14 days post vaccination (Fig 7a). Most animals sero-converted between days 7-9 (27/36), with 13 of those at day 8 (Fig. 7b).

A)



B)

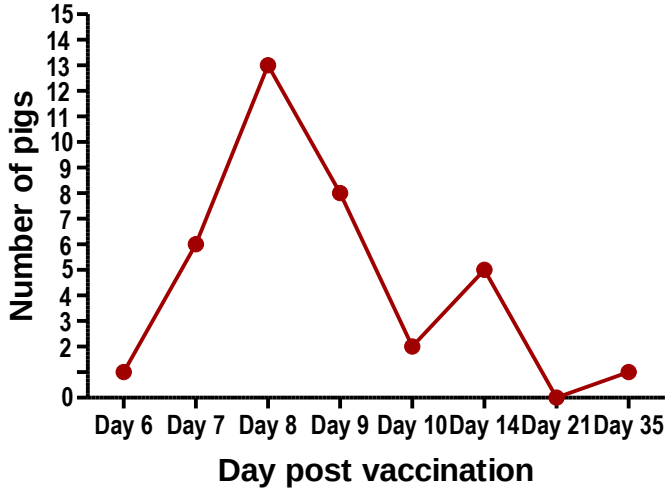


Figure 7: Graphs to summarise the number of animals that sero-converted for the first time each day post vaccination. A) Scatterplot to indicate the number of per vaccine strain. B) Graph to show the total number of animals per day.

Comparison of the Responses to the O and A Vaccine Strains

The serological responses of the animals vaccinated with O1 Manisa and O Campos and the 3 serotype A vaccines (A22 Iraq, A24 Cruzeiro and A Malaysia 97) were compared respectively using two-way repeated measures ANOVA with the Bonferroni post tests comparing each vaccine with the other. There was not a significant difference in the serological responses between the O1 Manisa and O Campos vaccines (Fig. 8; P=0.26).

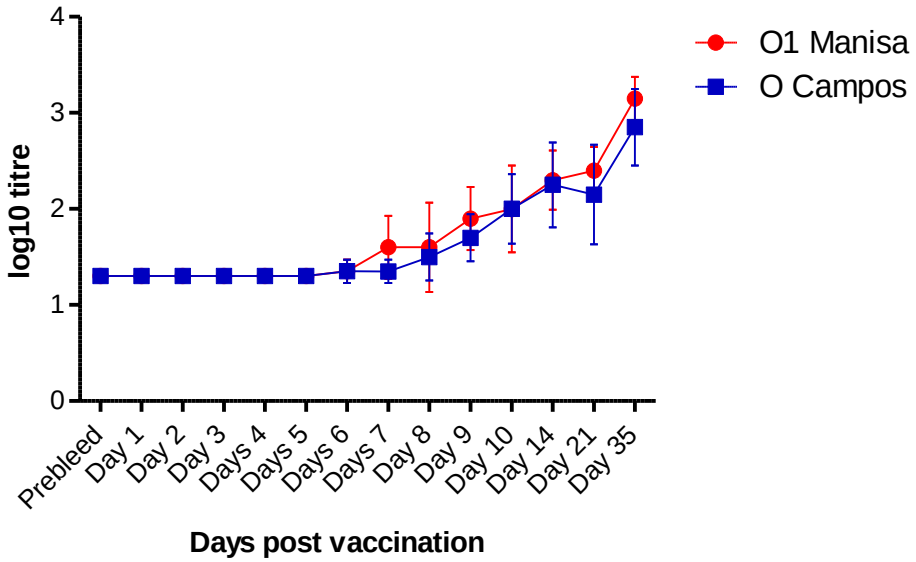


Figure 8: Comparison of the average titres with SD of the 6 pigs vaccinated with the O1 Manisa and O Campos vaccines respectively. Vertical bars indicate the SD.

In contrast, there was a significant difference in the responses elicited by the 3 different A type vaccines (P<0.0001). This was mainly as a result of significant differences on days 10, 14, 21 and 35. There was not a significant difference between any of the time points with animals vaccinated with A24 Cruzeiro and A Malaysia 97 (Figure 9).

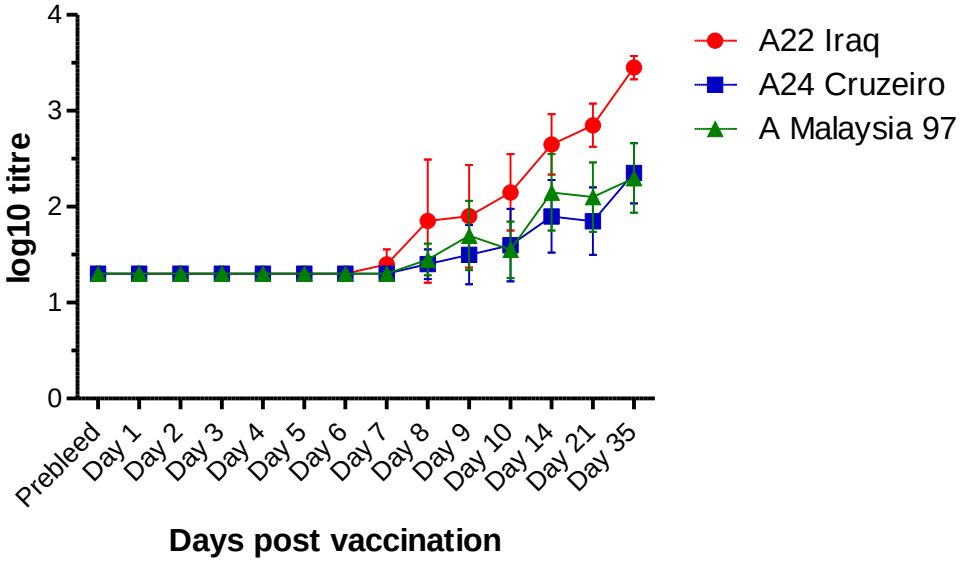


Figure 9: Comparison of the average titres with SD of the 6 pigs vaccinated with the A 22 Iraq, A24 Cruzeiro and A Malaysia 97 vaccines respectively. Vertical bars indicate the SD.

When comparing the responses of all 7 vaccine strains in a similar manner, there were significant differences (p=0.002), mostly due to significant differences in the last 3 bleeds (days 14, 21 and 35), but these were not significantly different between all the vaccines (results not shown).

Comparison between the lpELISA and cELISA at Different Cut-Off Values

The cELISA cut-off used at AAHL is 50% with values between 40-49% given as indeterminate. However, the initial test was used at 30% cut-off (Mackay et al, 2001). The lpELISA were compared to the cELISA at either a 50% or 30% cut-off using Kappa statistics to determine whether the difference in cut-off point will improve the test performance (Table 2). The Kappa statistic for O1Manisa increased from 0.403 (95% CI 0.212-0.593) to 0.789 (95%CI 0.634-0.960) when the cut-off was lowered to 30% (p=0.03). The only other statistically significant difference was with Asia-1 Shamir (p=0.02). However, the lpELISA had positive results for Pig #16 from day 3 onwards, as well as for pigs #17 and #18 on days 3 and 4 respectively, results that are regarded with suspicion. None of the other tests had significant differences with a change of cut-off value (Table 2).

Table 2: Summary of the Kappa value comparisons using two different cut-off values for the cELISA compared with the lpELISA

	O1 Manisa 50%	O1 Manisa 30%	O Campos 50%	O Campos 30%	A22 Iraq 50%	A22 Iraq 30%	A24 Cruzeiro 50%	A24 Cruzeiro 30%	A Malaysia 50%	A Malaysia 30%	Asia 1 50%	Asia 1 30%
Mean of Kappa values for 6 pigs per vaccine	0.403	0.797	0.788	0.762	0.687	0.789	0.692	0.875	0.792	0.811	0.415	0.699
SD	0.181	0.155	0.205	0.196	0.128	0.166	0.201	0.152	0.166	0.206	0.345	0.343
Std error	0.074	0.063	0.084	0.080	0.052	0.068	0.082	0.062	0.068	0.084	0.141	0.140
Lower 95% CI of mean	0.212	0.634	0.573	0.556	0.553	0.615	0.480	0.716	0.618	0.595	0.0524	0.339
Upper 95% CI of mean	0.593	0.960	1.003	0.967	0.822	0.964	0.903	1.034	0.966	1.026	0.777	1.059
P value	0.027		0.794		0.0772		0.226		0.841		0.017	

Discussion

Antibodies to the NSPs are mostly a result of active infection where virus replication occurs and NSPs are produced. Inactivated and purified FMD vaccines should not have NSPs present. Therefore the presence of antibodies to the NSPs is an indication of infection and can be used to distinguish between vaccinated and infected animals (Bruderer et al, 2004). However, if the vaccine is not sufficiently purified, NSP antibodies can be generated in animals vaccinated numerous times. In this experiment, the pigs were vaccinated twice and none of the serum samples tested positive for antibodies to the NSPs after the first or second vaccination indicating that the vaccines were not grossly contaminated with NSPs.

In contrast to the test measuring antibodies to the NSPs, most other assays measure antibodies against the structural, capsid proteins of FMD virus. The presence of these antibodies is an indication of vaccination and/or infection. Since AAHL is not allowed to work with live virus, it was not possible to measure the neutralising antibodies using a VNT, the preferred method for measuring potential vaccine protection and the gold standard. However, the two ELISAs used gave a good estimation of the time of sero-conversion post vaccination using the high potency vaccines provided by Merial.

The performance of the lpELISA was consistently superior to the cELISA when detecting post vaccinal antibodies to the different vaccine strains in pigs. The former scored animals as positive generally 1-4 days before the cELISA. The cELISA cut-off is currently set at 50% at AAHL across the different serotypes and strains, whereas a cut-off of 30% was used initially when the test was developed (Mackay et al., 2001). However, the cut-off made a significant difference when comparing the lpELISA with the cELISA only for O1 Manisa and Asia 1, indicating that strain specific cut-off values may be needed. Mackay et al (2002) found that both ELISAs performed very similarly when taking the day of sero-conversion as the measure, and also found that positive results could vary by one day. When testing sera with high titres, the two tests presented with comparable results, much as was found in our study. In a study comparing the cELISA and VNT, the former detected sero-conversion 5-9 days post infection, but it was more rapidly detected by VNT (Paiba et al., 2004). During a validation exercise for the cELISA after the outbreak in the United Kingdom in 2001, a large number of pig, cattle and sheep sera were tested (Paiba et al., 2004). Importantly for this validation, the cut-off value was adjusted according to the application of the test. When wanting to prove freedom from infection, the authors raised the cut-off to 60% so as to ensure an increase in test specificity (Paiba et al., 2004).

Although we know that all the vaccines used in this study contain antigens at $6PD_{50}$ (the factor by which the antigen dose may be reduced to still protect 50% of the vaccinated animals against challenge with 10 000 cattle infectious doses administered intra-dermalingually), Merial does not inform clients of the actual amount of antigen (in µg) in the formulations. It is therefore difficult to compare results with other published studies where the amount of antigen is known. In an attempt to compare our results to that of other studies, the day of sero-conversion as determined by the lpELISA was used. Most animals sero-converted between days 6-9 to serotype O1 Manisa and days 7-10 to A22 Iraq. In a previous study cattle and sheep vaccinated with O1 Manisa and A22 Iraq from the Australian vaccine bank on average sero-converted at day 7 (Hammond, 2005). In our study the poorest rate of sero-conversion was observed with A24 Cruzeiro, where one animal only showed titres at day 35, after the boost at day 21.

In another study, pigs vaccinated with a vaccine containing 6.1µg 146S antigen had detectable neutralising antibody levels 4-6 days post vaccination when measured using VNT (Barnett et al, 2002) and from 7 days onwards in another study where the payload was 2.9ug per dose, equalling a PD_{50} >112 in cattle (Barnard et al, 2005). However, whilst pigs showed antibodies from days 4-6 when using the VNT, at least 2 animals had no detectable antibody levels (one at day 5 and the other at day 6) when using an indirect sandwich ELISA (Barnett et al, 2002). It is also possible that the slower rate of response with some vaccine strains in our study was due to the test being more specific for IgG antibodies as Barnett et al (2002) showed that the IgM response in vaccinated pigs started at 5 days post vaccination and peaked at 7-14 days, whilst the IgG response started 9-14 days post vaccination. In contrast, the sero-conversion rate in infected pigs seems to be much better as pigs infected with type O/Taiwan/97 showed antibodies as early as day 3 using a neutralisation assay (Chen et al., 2007). The titres in most pigs peaked at day 5 post infection where after it declined and remained constant over 180 days. Rates of sero-conversion are therefore enhanced during infection compared to vaccination.

From these comparisons it seems that pigs vaccinated with the Merial vaccine had comparable reactions to previous studies except for serotype A24 Cruzeiro, which warrants further investigation. Of the 36 measurements in the study, 75% sero-converted by day 8, with ~17% on day 7, ~36% on day 8 and ~22% on day 9. The rate of sero-conversion will be confirmed once the VNT results are obtained from Merial.

Differences in the observed rate of sero-conversion could be influenced by the reagents used for the ELISAs in our study. It is preferable to use homologous reagents in the ELISAs where the capture and typing antisera were raised against the antigen used in the test. Due to the limitations of not being able to work with live FMD virus in Australia, AAHL has difficulty in building up a homologous bank of reagents. However, the A24 reagents are homologous (Table 1) and it is therefore possible that poor response was due to the vaccine and not the test. Surprisingly, the SAT-2 tests failed to measure any antibodies in either test which could be a reflection of the non-homologous reagents. These matters should be resolved once the VNT results are available.

The antibody titres to A22 Iraq and O1 Manisa were the highest after 35 days compared to the other vaccine strains. These 2 strains were included in the same vaccine formulation (vaccine 1) and it is therefore not clear whether the response is due to an inherent factor in the vaccine such as adjuvant or quantity of antigen. It is unlikely that the results were due to anomalies in the ELISAs. The cause of the anomalous results observed for Vaccine 3 containing A Malaysia 97 and Asia-1 Shamir where some animals had high titres that then apparently disappeared is unknown but seemed to be due to the sera as repeats of the tests presented the same results and both serotypes indicated these results.

Although it is known that each FMD virus strain has different characteristics, the vaccines contained an equal PD_{50} for each strain and we therefore compared the responses of the various O and A isolates respectively. There was not a statistically supported difference between the serological reactions to O1 Manisa and O Campos, but A22 Iraq had a very strongly supported difference ($p<0.0001$) with A24 Cruziero and A Malaysia 97. The reason is not clear and once the VNT results can be included in the analysis, this aspect will be further investigated.

These results provide an indication of whether Australian pigs respond to the vaccine antigens in the Australian vaccine bank. Since the animals were not challenged with live virus, it is not possible to know whether they will be protected against clinical disease. However, it is important to consider

previous findings that indicated protection can be provided as early as 3-5 days post vaccination when using a high potency vaccine even in the absence of high neutralising titres (Mackowiak et al., 1962; Sutmoller and Vieira 1980; Black et al., 1984; Pay and Hingley, 1987; Van Maanen and Terpstra, 1989; McCullough et al., 1992; Salt et al., 1998; Cox et al., 1999;). The experiments planned under the FMD Risk Management Project, jointly funded by industry and government will provide a more accurate estimate of the potency of these vaccines.

Implications and Recommendations

The high potency vaccines provided from Merial, drawn from the Australian vaccine bank, induce antibodies in Australian pigs. Sero-conversion was observed from day 7-9 with most vaccine strains. The rate was slower when serotype A24 Cruzeiro and A Malaysia 97 antibodies were detected. Since the animals could not be challenged, it is not known whether they will be protected against clinical disease. The experiments planned under the FMD Risk Management Project, jointly funded by Industry and Government will provide a more accurate estimate of the potency of these vaccines.

Aliquots of particular samples have been sent to Merial for homologous testing using VNT. These results will be useful in determining the sensitivity of the AAHL assays and will be used as benchmark for test development and improvement, if needed.

The experiment also provided valuable reagents for test validation to ensure AAHL can provide an excellent diagnostic service to the livestock industries in Australia.

Intellectual Property

Commercially available vaccines were tested in pigs and no intellectual property has emanated from this study.

Technical Summary

This project has demonstrated that the antigens kept in the Australian vaccine bank can be formulated into successful vaccines that elicit immune responses in Australian pigs. The diagnostic assays at AAHL can measure sero-conversion, but establishing a bank of homologous reagents is needed as well as further validation of the assays once the reagents are available.

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Publications Arising

List publications and where possible append copies of published articles.
Note that all publications arising from the project, either during or after completion, must be approved by APL on the standard *APL Request for Disclosure* form before release.

Vosloo W, Lunt R, Morrissy C, Jeggo M, Colling A, Singanallur N. Serological monitoring of pigs using various formulations of foot and mouth disease vaccines at 6PD50. Manuscript to be submitted to the Australian Veterinary Journal.

Colling A, Morrissy C, Barr J, Meehan G, Wright L, Goff W, Gleeson LJ, van der Heide B, Riddel S, Yu M, Eagles D, Doughty, Daniels P W, Khounsy S, Thanlong N, Vu PP, Nguyen TP, Nguyen T, Linchongsubongkoch W, Hammond J, Johnson M, Unger H and Crowther J. Development and validation of a 3ABC FMD antibody ELISA. To be submitted to New Zealand Veterinary Journal.

McNabb L, Lunt R, Morrissy C, Jeggo M, Colling A and Vosloo W. Testing the Antibody Response of Pigs to Foot-and-Mouth Disease Vaccines. Paper presented at the AAVLD Conference 25-26th November 2010.