An evaluation of the role of antibodies to *Actinobacillus pleuropneumoniae* serovar 1 and 15 in the protection provided by sub-unit and live streptomycin-dependent pleuropneumonia vaccines

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Objective To evaluate the serological response of pigs receiving either the Porcilis APP vaccine or a modified live vaccine based on a streptomycin-dependent (SD) strain of *Actinobacillus pleuropneumoniae*, and then challenged with an Australian isolate of *A pleuropneumoniae* of either serovar 1 or 15 as a means of understanding the protection provided by both vaccines against serovar 1 but not against serovar 15.

Design The serological tests evaluated were serovar-specific polysaccharide ELISA tests (for serovar 1 and 15), ELISA tests for antibodies to three *A pleuropneumoniae* toxins (ApxI, ApxII and ApxIII) as well as to a 42 kDa outer membrane protein (OMP), a haemolysin neutralisation (HN) assay and immunoblotting. The tests were used to detect antibodies in vaccinated pigs that had been shown to be protected against serovar 1 but not serovar 15.

Results In the polysaccharide antigen ELISA assays, both vaccines resulted in a significant rise in the titre in the serovar 1 ELISA but not the serovar 15 ELISA. The Porcilis APP vaccinated pigs showed a significant response in the ApxI, ApxIII and 42 kDa OMP ELISA. In the ApxII ELISA, all pigs tested (ie the Porcilis APP vaccinates and the controls) were positive on entry to the trial. In the HN assay, the Porcilis APP vaccinated pigs showed a significant response after one dose while the SD vaccinated pigs required two doses of vaccine before a marked rise in titre was induced. Immunoblotting revealed that neither vaccine generated antibodies that recognised the ApxIII produced by serovar 15.

Conclusions The failure of these vaccines to provide protection against serovar 15 may be due to novel virulence factors possessed by serovar 15, significant differences between the ApxIII toxin of serovar 15 and those present in the Porcilis APP vaccine and the live vaccine or failure to induce antibodies to the serovar 15 specific polysaccharide.

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Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a severe respiratory disease of pigs that is of economic importance wherever pigs are raised.[1] A comprehensive serotyping scheme for *A pleuropneumoniae* which recognises serovars 1 to 14 has been proposed.[2] An additional new serovar – serovar 15 (previously regarded as serovar 12) – has been recognised very recently in Australia[3] and is the most common serovar isolated from Australian pigs, with serovar 1 being the next most common serovar.[4]

Overseas studies have reported that the protection provided by vaccines that consist of killed whole cells of *A pleuropneumoniae* is variable[5] and, at best, serovar specific.[6] Killed whole cell vaccines can reduce the mortality rate but fail to prevent infection or the development of lesions.[7, 8]

There is now a body of evidence that extracellular soluble proteins known as the *A pleuropneumoniae* RTX toxins, commonly called Apx toxins, are key protective antigens.[9] There are four different Apx toxins currently recognised - the strongly haemolytic and cytotoxic ApxI, the weakly haemolytic and moderately cytotoxic ApxII, the non-haemolytic but strongly cytolytic ApxIII[10] and the weakly haemolytic and in vivo expressed ApxIV.[11] There is now a general acceptance that effective pleuropneumonia vaccines must contain a range of these key protective antigens.

We have recently evaluated two new generation vaccines, Porcilis APP vaccine (a commercial sub-unit vaccine, Intervet Pty Ltd) and an experimental modified live streptomycin-dependent (SD) vaccine, for their ability to protect against the two dominant serovars of *A pleuropneumoniae* in Australian pigs.[12] This evaluation demonstrated that both vaccines provided significant protection against serovar 1 but not against serovar 15.[12]

In the current study, we use a range of serological assays to examine sera collected from the pigs in these vaccine trials.[12] The study was performed to compare and contrast the responses in pigs vaccinated with either vaccine and subsequently challenged with either serovar 1 or serovar 15 and to identify, if possible, the important antigens responsible for the protection provided by these vaccines against *A pleuropneumoniae* serovar 1 and determine why these vaccines failed to provide protection against *A pleuropneumoniae* serovar 15.

Materials and methods

Bacteria

The Australian *A pleuropneumoniae* strains used in this study were HS 54, the prototype Australian serovar 1 reference strain, and HS 143, the reference strain for serovar 15.[12]

Experimental design

Pig sera collected from a vaccine trial[12] were used in this study. The treatment groups in the vaccine trial were as follows: vaccinated with Porcilis APP vaccine and challenged with serovar 1, vaccinated with the SD vaccine and challenged with serovar 1, unvaccinated and challenged with serovar 1, vaccinated with Porcilis APP vaccine and challenged with serovar 15, vaccinated with the SD vaccine and challenged with serovar 15 and unvaccinated and challenged with serovar 15. The pigs were bled four times,: before the first vaccination (day 0), before the second vaccination (day 21), before challenge (day 35) and at necropsy, 7 days after challenge (day 42). The pigs were 6 weeks old at day 0.

Polysaccharide-based ELISA

The polysaccharide-based ELISAs used in this study were developed using the prototype Australian isolates of serovars 1 and 15 – HS 54 and HS 143 respectively. The ELISAs were based on the methodology described by Bossé et al.[13]

The antigens used in the ELISAs were prepared as previously described for serovar 1,[13] except that the bacterial cells were harvested from an overnight culture grown at 37^0C on Test Medium Agar containing 0.0025% (v/v) nicotinamide adenine dinucleotide and 1% (v/v) chicken serum (TM/SN).[14] The antigens were stored at -20^0C as the stock ELISA antigen solution.

The ELISA was performed as previously described,[15] except that the antigen was adsorbed onto the plates for 3 h at 37^{0}C. The results of the ELISA were expressed as an optical density (OD) reading at 405 nm. ELISA scores were calculated as the antilog of the OD multiplied by 1000.

Apx and OMP ELISA

The ELISAs for antibodies to ApxI, ApxII and ApxIII were performed as previously described.[16] The ELISA for antibodies to the 42 kDa OMP was performed using the same methodology as the Apx ELISA, with OMP prepared from a serovar 2 isolate being used as the coating antigen. The results of the ELISAs were expressed as $\log_2$ values and termed ELISA scores.

Haemolysin neutralisation titre determination

The *A pleuropneumoniae* serovar 1 reference strain, strain 4074, was used as the source of haemolysin for the haemolysin neutralisation (HN) test. The strain was grown in RPMI 1640 (Gibco BRL Life Technologies) supplemented with 2.5% foetal calf serum until an optical density of around 0.2 was reached. The culture was then centrifuged (3500 g, 10 min), the supernatant harvested and filtered (0.2 μm). The filtered culture supernatant was dispensed in 20 mL volumes and stored at -70^{0}C.

The serum samples used in the HN assays were heat inactivated for 1 h at 56^{0}C. The sheep red blood cells (RBC) used in the assay were washed in a citrate-phosphate-dextrose (CPD) solution (dextrose 140 mM, sodium citrate 89 mM, citric acid 16.9 mM, monobasic sodium phosphate 16.1 mM, pH 5.6) and initially suspended to 12.5% in CPD. The 12.5% suspension was diluted to 1% RBCs in 10 mM Tris/saline immediately prior to use.

To prepare a standard for complete RBC lysis, 50 μL of the 1% RBC suspension was mixed with 150 μL of distilled water in 24 wells of a microtitre plate. The plate was covered and incubated for 2 h at 37^{0}C. To pellet unlysed RBCs the plate was centrifuged at 120 x *g* for 10 min. A 100 μL sample of the supernatant from each well was transferred to a corresponding well of a flat bottomed microtitre plate. The haemoglobin content of each well was determined by measuring the OD at 410

nm using a microplate reader (Bioclone Vmax). The average OD value was obtained and used in subsequent computations as the maximal releasable haemoglobin content.

The haemolytic activity of the culture supernatant was determined as follows. Culture supernatant (0, 0.5, 1, 2, 5, 10, 20, 30, 50, 70, 90 and 100 μL volumes) was placed in the wells of round bottomed 96-well microtitre plates. The final volume of each well was adjusted to 100 μL RPMI 1640 medium containing 2.5% calf serum and 100 μL of 1% RBC suspension was added to each well. The plates were covered and incubated for 2 h at 37^0C. The unlysed RBCs were pelleted and the haemoglobin content was determined as above. This was repeated six times. The mean OD value was obtained and used in the computations.

The serum samples were heat inactivated for 1 h at 56^0C and their haemolytic activity was determined as follows. For each serum sample, 0, 50, 75 and 100 μL volumes of culture supernatant were placed in the wells of round bottomed microtitre plates. The final volume of each well was adjusted to 100 μL by the addition of RPMI 1640 medium containing 2.5% calf serum. To each well, 5 μL of heat inactivated test serum was added and the plate was incubated for 1 h at 37^0C. A 100 μL volume of a 1% RBC suspension was added to each well and the plates incubated for 2 h at 37^0C. The unlysed RBCs were pelleted and the haemoglobin content was determined as above. The serum samples were tested in triplicate and the mean OD value for each serum was used in all subsequent computations.

The results of the HN tests were expressed as neutralisation titres. The neutralisation titre was the number of haemolytic units that 1 mL of serum neutralised. One haemolytic unit was the volume in microlitres of culture supernatant required to lyse 50% of the RBCs in 100 μL of a 1% suspension. The calculation of the neutralisation titre for each serum was performed using the ELISA plate reader software, Softmax (Molecular Devices Corp., Sunnyvale, CA, USA). A standard curve was generated using the culture supernatant and then compared with the curve generated for each test serum. The neutralisation titres were calculated by dividing the volume of culture supernatant required to lyse 50% of the RBCs when 5 μL of test serum was added by the volume of culture supernatant necessary to lyse

50% of the RBCs (one haemolytic unit). This value was then multiplied by 200 to determine the haemolysin neutralisation activity per 1 mL of serum.

When calculating the mean HN titre, titres >50,000 were considered to be 50,000. If there were any titres >50,000 included in the calculations of mean titres, the actual mean was considered to be greater than that calculated.

Immunoblot analysis

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described.[17] *A pleuropneumoniae* strains HS 54 and HS 143 were grown in RPMI medium (Gibco BRL Life Technologies) supplemented with 2.5% foetal bovine serum and 0.0025% nicotinamide adenine dinucleotide (reduced form) for 6 h in a 37^0C water-bath. The culture was centrifuged (10,000 *g*, 10 min) and the supernatant harvested and concentrated 10X (Centricon centrifugal filter device YM 10, Amicon Bioseparations, Millipore). The concentrated sample was stored at -20^0C. Immunoblotting was performed as previously described.[18] A 5% skim milk powder (SMP) suspension was used as a blocking agent in the immunoblotting. The pig sera tested in the immunoblotting work were diluted 1 in 25 with 5% SMP. The secondary antibody used was a goat anti-swine IgG (H+L) horse radish peroxidase conjugate (Biorad) (diluted 1 in 200 in 5% SMP).

All three treatment groups challenged with serovar 1 were tested using culture supernatant of *A pleuropneumoniae* serovar 1 (strain HS 54) while the groups challenged with serovar 15 were tested with *A pleuropneumoniae* serovar 15 (strain HS 143).

Statistical analysis

The polysaccharide ELISA scores were analysed using the non parametric Kruskal-Wallis one way analysis of variance.

In computing the mean ELISA scores for ApxI, ApxII, ApxIII and 42 kDa OMP antibodies, values of <7 (indicating that the antibody concentration was below the detection limit of the assays) were treated as 7. As there were missing sera at some time points, the mean ELISA score was calculated for these time points using only the results of the available sera. At day 42, the mean ELISA score of the control groups challenged with serovar 1 were not calculated as sera were available for only 4 pigs (euthanasia of some pigs was necessary after challenge due to animal experimentation ethical constraints). Means that included ELISA scores of less than 7 were considered to be less than the calculated mean. The proportion of pigs with ELISA scores >7 was also determined and analysed together with the mean ELISA values. Fishers exact test was used to compare the number of positive pigs (that is, pigs with a ELISA score > 7) in the vaccinated and control groups. The analysis was performed within a challenge type (that is, the vaccinated pigs that were challenged with serovar 1 were compared with the controls that were challenged with serovar 1 whereas the vaccinates challenged with serovar 15 were compared with the controls challenged with serovar 15).

The difference between the HN titre at day 0 and day 21 was analysed using the non parametric Kruskal-Wallis one way analysis of variance. The differences between the treatment titres at day 35 and day 42 were not analysed because most of the titres in the treatment groups were >50,000.

The level of significance for all the statistical tests was $P \leq 0.05$. Statistix software was used for the analysis.

Results

Polysaccharide-based ELISAs

The summary of the polysaccharide ELISA results is presented in Table 1.

On day 0, prior to vaccination, the **median ELISA scores** of the vaccinated and unvaccinated pigs, whether in the serovar 1 or 15 ELISA, were not significantly different from each other.

At day 35, all groups of vaccinated pigs had a significantly higher median ELISA score in the serovar 1 ELISA than the control group (Table 1). The pigs given the SD vaccine had a median ELISA score lower than the Porcilis APP vaccinated pigs at day 21 and day 35 within both the serovar 1 and serovar 15 challenge groups (Table 1). However, a statistically significant difference was detected only at day 21 (serovar 15 challenge, serovar 1 ELISA). After challenge, there was a considerable increase in the median ELISA score of the control group given the serovar 1 challenge **(for example, from 1,745 at day 35 to 15,231 at day 42 in the serovar 1 challenge, serovar 1 ELISA - Table 1).** This indicates that the pigs were infected and responded by producing antibodies against serovar 1.

Prior vaccination with either the SD or the Porcilis APP vaccine had, with one exception, no observable effect on the **median** concentrations of antibodies to serovar 15 polysaccharide (Table 1) from day 0 to day 35. The one exception was that there was a significant difference between the pigs given the SD vaccine and both the controls and the Porcilis APP vaccinated pigs in the serovar 15 challenge groups at day 35 (Table 1).

A substantial increase in the median serovar 15 ELISA score was observed after challenge with serovar 15 for all the treatment groups (Table 1), indicating that all pigs were infected and responded by producing antibodies against serovar 15.

Apx and OMP ELISA assays

The results for the three Apx ELISAs as well as the OMP ELISA are summarised in Table 2. These ELISAs were performed only on the sera from Porcilis APP vaccinated pigs as only the Porcilis vaccinated pigs were exposed to all three Apx toxins and the OMP antigen. The controls were also tested to understand the antibody ELISA scores associated with the vaccination. Hence, a total of four treatment groups were tested (two Porcilis APP vaccinated and control groups each, with one of each group being challenged with serovar 1 and the other with serovar 15).

The mean ApxI antibody ELISA scores of the four groups at day 0 (6 weeks of age) were similar and ranged from <7.4 to <7.7. Compared with their respective control groups, the Porcilis APP vaccinated groups had significantly more positive pigs (pigs with a ELISA score > 7) after two vaccinations. Both control groups had a decreasing ApxI antibody ELISA scores from day 0 to day 21 and a decreasing percentage of pigs with an ELISA score of >7 from day 0 to day 35. An abrupt increase in the ApxI ELISA score of the control groups was observed 1 week after challenge.

The mean pre-vaccination ELISA score of all four treatment groups against ApxII at 6 weeks of age (day 0) were in the majority of cases already above the minimum detectable value, with 28 out of the 31 sera tested having a score of ≥9 and 18 of these being scores of >10. After two vaccinations, at day 35 (11 weeks of age), the mean ApxII antibody ELISA scores of the Porcilis APP vaccinated pigs were still lower than the mean pre-vaccination titres (day 0) and only slightly higher than the mean ELISA scores of the control groups. After challenge with either serovar, the mean ApxII antibody ELISA score of three of the four treatment groups rose. The percentage of pigs with an ELISA score of >7 for both control and vaccinated pigs was never less than 88% throughout the time period of the trial. There were only a few pigs over the period of the trial with an ELISA score below the detection minimum. The vaccinated and control groups never showed a significant difference in the number of positive pigs at any stage of the trial.

The mean pre-vaccination antibody ELISA scores against ApxIII ranged from <8.0 to 8.4. The number of positive pigs in Porcilis APP vaccinated groups was significantly higher than the control groups at day 35 (2 weeks after booster vaccination). A similar significant difference was present at day 21 but only for the Porcilis APP vaccinated pigs that were subsequently challenged with serovar 1. There was no such significant difference for the groups that were subsequently challenged with serovar 15.

The mean pre-vaccination OMP antibody ELISA scores of all pigs at 6 weeks of age (day 0) ranged from <7.3 to <8.1. From day 0 to day 35, the mean OMP antibody ELISA scores of the Porcilis APP vaccinated pigs increased, whereas the mean score of the control pigs decreased. The number of positive pigs in the Porcilis APP vaccinated groups was significantly higher than the control groups at

both day 21 and day 35. Seven days after challenge, the control group for the serovar 15 challenge still had lower OMP antibody ELISA scores than the vaccinated groups with the control pigs for the serovar 1 challenge still having a significantly lower number of positive pigs than the Porcilis APP vaccinated group.

HN Assay

The HN assay was used only on sera from pigs that were eventually challenged with serovar 1. This was done as the antibodies detected in this assay are produced against Apx I, a toxin that is produced by serovar 1 but not by serovar 15.[3] The median ApxI neutralisation titres of the Porcilis APP vaccinated, SD vaccinated and control pigs that were challenged with serovar 1 are presented in Table 3.

The median HN titre of pigs in the three treatment groups at day 0 were low and were not significantly different from each other. At day 21, the Porcilis APP vaccinated pigs had significantly higher neutralisation titres than the SD vaccinated and control pigs. Both vaccinated groups (Porcilis APP vaccine and SD vaccine) had increased ApxI neutralisation titres at day 35, after two vaccinations. The HN titre of the control group increased only after challenge.

Immunoblotting

Serovar 1 challenged pigs. Some representative immunoblots are shown in Figure 1.

At day 0, for all the groups, protein bands were observed in the area where reactions to ApxI and ApxII toxins could be expected, which was in the area between 97.4 and 116 kDa. ApxI has a molecular weight of about 105 kDa while ApxII has a molecular weight of about 103 kDa.[19] Immunoblot reactions to ApxI and ApxII were inseparable because of this close identity in molecular weight.

At day 35, which was after two vaccinations with either the SD vaccine or Porcilis APP vaccine, bands of the same size and colour intensity in the region of 103-105 kDa (as in day 0) were

observed in serum samples of pigs from both vaccinated groups while fainter and thinner bands of the same molecular weight were seen in the sera of pigs from the unvaccinated control group. This is best seen by contrasting lanes 4 (Porcilis APP vaccinated) and 7 (SD vaccinated) with lane 10 (control) in Figure 1 in the 103-105 kDa region.

At day 42, 7 days after challenge, the strong bands that apparently correlate with ApxI and ApxII were present in all serum samples from all the treatment groups.

Serovar 15 challenged pigs. Some representative immunoblots are shown in Figure 2.

At day 0, two bands approximating the size of ApxII and ApxIII toxins were observed in all sera from all three treatment groups. ApxII toxin is reported to be about 103 kDa while ApxIII toxin is about 120 kDa.[19]

At day 35, the band apparently corresponding to ApxII was still visible in sera from pigs in the two vaccinated groups and just slightly visible in the sera of control pigs. The bands that apparently correspond to ApxIII were barely visible in the sera of any pig in all three groups (see lanes 4, 7 and 10 of Figure 2).

At day 42, bands that apparently corresponded to ApxII and ApxIII toxins were distinctly present in sera from pigs in all three treatment groups.

Bands with a molecular weight of greater than 120 kDa were consistently found in the sera of pigs of all three treatment groups at days 0 and 42. The same band was also present in the sera of all pigs at day 35 but was very faint.

Discussion

The results from this study have confirmed that the serovar 1 and 15 polysaccharide ELISA assays are serovar-specific and do not detect antibodies to the heterologous serovar, matching findings

previously reported for this type of assay.[15] The serovar-specificity was most evident in the control groups after challenge. Control pigs challenged with serovar 1 showed an increase **ELISA score** in the serovar 1 ELISA but not in the serovar 15 ELISA. Similarly, control pigs challenged with serovar 15 had an increased **score** in the serovar 15 ELISA but not in the serovar 1 ELISA.

The polysaccharide ELISAs were not only capable of detecting antibody response to challenge but also to vaccination; again a finding reported previously.[20] We also found serovar-specificity with these ELISAs; all treatment groups given either vaccine had significantly higher **scores** in the serovar 1 ELISA, but not the serovar 15 ELISA, as compared with the control groups. Hence, the polysaccharide ELISAs clearly indicated that both vaccines were producing significant amounts of antibodies to serovar 1 but not to serovar 15. This strong response to serovar 1 but not serovar 15 is matched by the outcomes of the challenge trial. Based on average daily gain, mortality, clinical signs, lung lesions and re-isolation scores, both the Porcilis APP and the SD vaccines provided protection against serovar 1 challenge and little or no protection against the serovar 15 challenge.[12]

The results obtained in the current work even suggest a linkage between ELISA score and the degree of protection, that is, that a higher polysaccharide ELISA score corresponds with a higher level of protection in vaccinated pigs. The Porcilis APP vaccine provided better protection than the SD vaccine against serovar 1 challenge.[12] In a similar fashion, the serovar 1 ELISA scores of both Porcilis APP vaccinated pigs were significantly higher than those of both SD vaccinated pigs (at the bleed before challenge – see Table 1). In addition, unlike the SD vaccinated pigs which needed in some cases two doses to achieve a significantly higher score than the controls (Table 1), the Porcilis APP vaccinated pigs only required one dose to have a significantly higher polysaccharide ELISA score to serovar 1 than the control pigs (Table 1). Whereas the second SD vaccine dose resulted in an increase polysaccharide ELISA score to serovar 1, this score was significantly lower than that seen in the pigs given the Porcilis APP vaccine (Table 1). This matches with the lower level of protection induced by the SD vaccine as compared with the Porcilis APP vaccine.[12] It is important to note that our results are not suggesting that there is a causal relationship between protection and the level of antibodies detected in the polysaccharide ELISA. It is highly likely that other antibodies are involved in protection and hence our results are simply showing a correlation and not a relationship.

Both vaccines failed to elicit serovar 15 ELISA scores that were significantly above those recorded in the unvaccinated controls prior to challenge. The ELISA score to serovar 15 increased significantly only after challenge (Table 1). This lack of antibodies matches the limited protection against the serovar 15 challenge provided by both the SD vaccine and the Porcilis APP vaccine.[12] Again, our results are evidence of a correlation and not of a causal relationship. Indeed while there have been suggestions that antibodies to polysaccharides may play a role in partial protection against porcine pleuropneumonia[21] there are also reports that definitely conclude that there is no link between protection and antibodies to capsular polysaccharide.[22]

We used ELISAs to detect antibodies to ApxI, ApxII, ApxIII and the 42 kDa OMP. The three toxins are regarded as important virulence factors for *A pleuropneumoniae* while the OMP is a common surface exposed antigen.[9] These same four antigens are constituents of the Porcilis APP vaccine. We attempted statistical analysis of the actual ELISA scores but were unable to proceed due to the fact that there were a number of sera with results below the minimum cut-off of the tests, that is < 7. Hence, our analysis of these tests is based on a statistical analysis of the number of positive pigs (that is with an ELISA score of > 7), rather than the actual titres.

Our results show that the Porcilis APP vaccinated pigs developed an obvious antibody response to ApxI, ApxIII and OMP that resulted in significantly more positive pigs as compared with the control groups after two vaccinations (prior to challenge). The antibody response of the vaccinated and unvaccinated pigs to ApxII showed no such significant difference at any time point in the trial. The elevated levels of ApxII antibodies present in all pigs prior to vaccination would seem to be a key difference. It is noteworthy that the elevated pre-vaccination ApxII ELISA scores had no apparent effect on the efficacy of the Porcilis APP vaccine against a serovar 1 challenge.

As the pigs used in the vaccination trial came from an *A pleuropneumoniae* free herd, the pre-vaccination ApxII ELISA scores we detected were not due to infection with *A pleuropneumoniae*. The reactions are presumably in response to similar toxins produced by other *A pleuropneumoniae*-related

bacteria such as has been already reported for *A suis,*[23] *A rossi* and *Pasteurella aerogenes*[24] Indeed, it has been reported that antibodies to ApxII can be often found in the sera of healthy swine.[24] Moreover, in a study done by Bak et al.[25] using the same ELISAs for ApxI, ApxII, ApxIII and 42 kDa OMP, the mean ApxII ELISA scores of pigs from both vaccinated and unvaccinated sows were higher than the score to the other three antigens.

HN assays have been used previously to detect antibodies in pigs given pleuropneumonia vaccines.[26] Whereas methodological differences do exist, the HN assay we have used is similar to that used in an earlier study.[26] Using the HN assay, we found both vaccines induced elevated HN antibody titres in pigs after two vaccinations. Even without statistical analysis, it was obvious that the vaccinated pigs had a higher neutralisation titre compared with the pigs in the unvaccinated control group before challenge (day 35) (Table 3). Since these vaccinated pigs were protected against serovar 1 challenge,[12] it can be concluded that these HN antibodies were probably involved in the protective response. This is in agreement with the findings of other researchers.[26, 27] Indeed, it has been suggested that a HN titre of > 10,000 is protective.[26]

The Porcilis APP vaccinated pigs gave a significantly higher HN titre than the controls after one vaccination – reaching the protective level suggested by Devenish et al.[26] of > 10,000. The SD vaccinated pigs did not show a significant difference from the controls at this stage of the trial. As the SD vaccine, by virtue of being a live vaccine, probably multiples to different levels in different pigs, it is not possible to directly compare the antigenic masses of the two vaccines. All that can be concluded is that, under the conditions of the current trial, there was a significant difference in the ability of the two vaccines to stimulate HN antibodies after one vaccination.

The immunoblot system we have used is based on antigens present in culture supernatant. Hence, we have confined the analysis of the results of this assay to those antigens that are well recognised as being released into the culture supernatant ie the Apx toxins which range in size from 103 to 120 kDa. While antibodies to other lower molecular weight antigens were clearly detected in our work (for example, bands of approximately 45 kDa were present in some pigs after challenge), our system was not necessarily optimised for the detection of these antibodies.

The immunoblot results illustrated that bands which were of a very similar size to those associated with the specific antigen-antibody interactions for ApxI, ApxII and ApxIII were evident in both the pre-vaccination and the negative control sera of all the pigs. The pre-vaccination sera were taken at the start of the trial when the pigs were only 6 week old while the negative control sera came from 8 week old pigs. Since the colour intensity and thickness of the bands in the unvaccinated control groups decreased from day 0 to day 35, these bands were presumably associated with maternal antibodies. Schaller et al.[24] have also observed immunoblot reactions that indicate the presence of antibodies to Apx toxins in the sera of healthy and *A pleuropneumoniae* negative pigs.

The immunoblot results suggested that antibodies against ApxI and ApxII were probably important in the protection against the *A pleuropneumoniae* serovar 1 challenge. Unlike the bands in the control group, the ApxI and ApxII bands produced by the serum samples from the SD and the Porcilis APP vaccinated pigs were still very distinct at day 35 (immediately before challenge). This demonstrates that both vaccines elicited the production of antibodies against ApxI and ApxII. It would seem that this production of neutralising antibodies contributed to the protection of the vaccinated pigs against the serovar 1 challenge. This was in agreement with the findings of other researchers.[5, 26]

The immunoblot results also illustrate a possible reason why both the SD and subunit vaccines failed to provide protection against the serovar 15 challenge. Both vaccines failed to elicit, in vaccinated pigs, detectable immunoblot reactions, using the serovar 15 antigen, to ApxIII. ApxII and ApxIII are both secreted by HS 143,[3] the serovar 15 challenge strain, and are likely to be important virulence factors for this organism. The failure to detect a strong response in Porcilis APP vaccinated pigs in the serovar 15 immunoblot to ApxIII contrasts with the ApxIII ELISA where Porcilis APP vaccinated pigs did show a response, although the response was lower than that seen for ApxI and ApxII. This difference may be due to the fact that the immunoblotting was performed using serovar 15 antigen. In contrast, the ApxIII ELISA was performed using ApxIII extracted from a serovar 2 strain.[16] This suggests that the ApxIII produced by serovar 15 is antigenically different from the ApxIII produced by serovar 2.

The overall results of this serological study showed that the Porcilis APP vaccine stimulated antibodies against the serovar 1 specific polysaccharide (in ELISA assay), the 42 kDa OMP (in ELISA assay), ApxI (in HN, ELISA and immunoblot assays), and ApxII (in immunoblot and ELISA assays). High levels of maternal antibodies to ApxII, as detected in both the ELISA and immunoblot systems, did not appear to interfere with the efficacy of this vaccine. The SD vaccine elicited a marked antibody response to ApxI (as detected by the HN and immunoblot assays), and the serovar 1 specific polysaccharide. The immunoblotting results provide evidence that the two vaccines did not induce antibodies that recognised the serovar 15 ApxIII. Our results suggest that the failure of both vaccines to protect against a serovar 15 challenge may be due to novel virulence factors possessed by serovar 15; or significant differences in the immunologic nature of the ApxIII toxin of serovar 15 compared with those present in the Porcilis APP vaccine or the live vaccine, or the absence of antibodies to the serovar 15 specific polysaccharide.

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Table 1 Results of the polysaccharide ELISA tests to measure the response of pigs vaccinated and then challenged with either a serovar 1 or a serovar 15 organism.[a]

Treatment	Challenge	ELISA	Day 0	Day 21	Day 35	Day 42
			Median Score	Median Score	Median Score	Median Score
SD Vaccine	Serovar 1	Serovar 1	1,154	1,889[A]	8,808 [A]	17,292
Porcilis APP Vaccine			1,200	11,258 [A]	19,532 [A]	18,780
Unvaccinated Control			1,155	1,328[B]	1,745 [B]	15,231
SD Vaccine	Serovar 1	Serovar 15	1,157	1,274	1,255	1,172
Porcilis APP Vaccine			1,171	1,245	1,412	1,298
Unvaccinated Control			1,190	1,211	1,299	1,236
SD Vaccine	Serovar 15	Serovar 1	1,171	1,946 [B]	8,795 [A]	7,058 [AB]
Porcilis APP Vaccine			1,174	12,678 [A]	19,281 [A]	17,847 [A]
Unvaccinated Control			1,168	1,270 [B]	1,738 [B]	1,637 [B]
SD Vaccine	Serovar 15	Serovar 15	1,141	1,191	1,193 [B]	10,559 [B]
Porcilis App Vaccine			1,144	1,187	1,311 [A]	14,656 [A]
Unvaccinated Control			1,162	1,206	1,354 [A]	10,612 [AB]

[a] Day 0 = Day of first vaccination (at 6 wks of age); Day 21 = Day of booster vaccination (3 wks afters first vaccination); Day 35 = Day of challenge (2 wks after booster vaccination); Day 42 = One week after challenge

[b] Median score within a column that have a different superscript were significantly different ($p \leq 0.05$). This comparison has been done only within groups that have the same challenge organism and same ELISA antigen.

Table 2 Results of the Apx and OMP ELISA tests to measure the response of pigs vaccinated (two doses of the Porcilis APP vaccine) and then challenged with either a serovar 1 or a serovar 15 organism.[a]

Treatment	Challenge	ELISA	Day 0		Day 21		Day 35		Day 42	
			Mean Score[b]	% Positive[c]	Mean Score[b]	% Positive[c]	Mean Score[b]	% Positive[c]	Mean Score[b]	% Positive[c]
Porcilis App Vaccine	Serovar 1	ApxI	<7.4	50	<7.6	80^{A}	11.2	100^{A}	11.2	100
Unvaccinated Control	Serovar 1		<7.7	60	<7.0	11.1^{B}	<7.0	10^{B}	ND[d]	100
Porcilis App Vaccine	Serovar 15		<7.4	50	<7.3	40	10.0	100^{1}	<11.0	90
Unvaccinated Control	Serovar 15		<7.7	85.7	<7.0	20	<7.0	11.1^{2}	8.1	100
Porcilis App Vaccine	Serovar 1	ApxII	9.9	100	<8.2	90	9.0	100	9.3	100
Unvaccinated Control	Serovar 1		10.2	100	<7.8	88.9	<8.2	90	ND[d]	100
Porcilis App Vaccine	Serovar 15		10.4	100	<8.1	90	8.3	100	10.8	100
Unvaccinated Control	Serovar 15		11.6	100	8.4	100	<8.2	88.9	9.0	100
Porcilis App Vaccine	Serovar 1	ApxIII	<8.1	83.3	8.1	100^{A}	8.8	100^{A}	8.7	100
Unvaccinated Control	Serovar 1		<8.3	90	<7.2	22.2^{B}	<7.1	10^{B}	ND[d]	50
Porcilis App Vaccine	Serovar 15		<8.0	75	<7.8	90	8.9	100^{1}	9.5	100
Unvaccinated Control	Serovar 15		8.4	100	<7.1	40	<7.0	11.1^{2}	<7.5	60
Porcilis App Vaccine	Serovar 1	42 kDa OMP	<7.3	50	8.2	100^{A}	9.3	100^{A}	9.2	100^{A}
Unvaccinated Control	Serovar 1		<7.7	60	<7.1	22.2^{B}	<7.1	20^{B}	ND[d]	25^{B}
Porcilis App Vaccine	Serovar 15		<7.4	50	8.5	100^{1}	9.7	100^{1}	10.7	100
Unvaccinated Control	Serovar 15		<8.1	85.7	<7.2	40^{2}	<7.2	55.6^{2}	<7.7	80

[a] Day 0 = Day of first vaccination (at 6 wks of age); Day 21 = Day of booster vaccination (3 wks afters first vaccination); Day 35 = Day of challenge (2 wks after booster vaccination); Day 42 = One week after challenge.

[b] Means that have involved sera with a value of $\leq$7 have been calculated by assigning the value of 7 to these sera. These means are marked by a < sign.

[c]% Positive = % of pigs with a $\geq$ 7 titre. Statistical analysis was performed using the Fisher Exact test. The analysis was done on the number of positive pigs in the unvaccinated group as compared with the vaccinated group. Comparisons were performed only for treatment groups that received the same challenge. Results with a different superscript are significantly different.

[d] ELISA mean score not calculated as sera was available from only four pigs.

Table 3. Results of the haemolysin neutralisation assay to measure the response of pigs vaccinated (two doses of either of two different vaccines - Porcilis APP and a live streptomycin-dependent vaccine) and then challenged with a serovar 1 organism.[a]

Treatment	Median ApxI Neutralisation Titre[a] at			
	Day 0	Day 21	Day 35	Day 42
SD Vaccine	4,697	5,814[b]	>50,000	>50,000
Porcilis APP Vaccine	5,153	>8,095[a]	>50,000	>50,000
Unvaccinated Controls	4,960	5,607[b]	5,287	>20,934

[a] Day 0 = Day of first vaccination (at 6 wks of age); Day 21 = Day of booster vaccination (3 wks after first vaccination); Day 35 = Day of challenge (2 wks after booster vaccination); Day 42 = One week after challenge.

[b]For Day 0 and Day 21 HN titres , a different superscript means that a significantly different mean ($p \leq 0.05$). For Day 35 and Day 42 HN titres, statistical analysis was not possible due to the number of sera exceeding the maximum reading.

Figure 1. Immunoblot of the serological responses of serovar 1 challenged pigs using *A pleuropneumoniae* strain HS 54 (serovar 1) culture supernatant as the antigen. Lane 1 is a broad range MW marker. Lane 2 is the negative control serum while lane 12 is the positive control serum. Lanes 3, 4 and 5 - serum samples from SD vaccinated pig taken at Day 0, 35 and 42 respectively. Lanes 6, 7 and 8 - serum samples from a Porcilis APP vaccinated pig taken at Day 0, 35 and 42 respectively. Lanes 9, 10 and 11 - serum samples from a control pig taken at Day 0, 35 and 42 respectively.

Figure 2. Immunoblot of the serological responses of serovar 15 challenged pigs using *A pleuropneumoniae* strain HS 143 (serovar 15) culture supernatant as the antigen. Lane 1 is a broad range MW marker. Lane 2 is the negative control serum while lane 12 is the positive control serum. Lanes 3, 4 and 5 - serum samples from a SD vaccinated pig taken at Day 0, 35 and 42 respectively. Lanes 6, 7 and 8 - serum samples from a Porcilis APP vaccinated pig taken at Day 0, 35 and 42 respectively. Lanes 9, 10 and 11 - serum samples from a control pig taken at Day 0, 35 and 42 respectively.