

Comparison of the efficacy of a subunit and a live streptomycin-dependent porcine pleuropneumonia vaccine

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Objective To evaluate the efficacy of two new-generation porcine pleuropneumonia vaccines when challenged with Australian isolates of *Actinobacillus pleuropneumoniae* of serovars 1 and 15.

Design The Porcilis APP vaccine and an experimental streptomycin-dependent strain of *A pleuropneumoniae* were evaluated in a standardised pen trial. Each vaccine/challenge group consisted of 10 pigs.

Results With the serovar 1 challenge, the Porcilis APP vaccine and the live vaccine, compared with the control group, gave significant protection in terms of clinical signs, lung lesions, re-isolation scores and average daily gain (ADG) postchallenge. Only the Porcilis APP vaccine provided significant protection against mortality. In the serovar 15 challenged pigs, the only significant difference detected was that the Porcilis APP vaccinated pigs had a better postchallenge ADG than the controls. None of the Porcilis APP vaccinated pigs showed signs of depression postvaccination and none were euthanased after challenge with either serovar 1 or 15. The pigs vaccinated with the live vaccine showed obvious depression after each vaccination and a total of 3 pigs were euthanased after challenge (one with serovar 1 and two with serovar 15).

Conclusions Both of the vaccines provided significant protection against a severe challenge with serovar 1 *A pleuropneumoniae*. Neither vaccine was effective against a serovar 15 *A pleuropneumoniae* challenge. There was evidence that the Porcilis APP vaccine did provide some protection against the serovar 15 challenge because the ADG, after challenge of pigs given this vaccine, was greater than the control pigs.

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Porcine pleuropneumonia, a severe respiratory disease of pigs that is of economic importance wherever pigs are raised, is caused by *Actinobacillus pleuropneumoniae*.¹ Two different biovars are recognised within the species. Biovar 1 requires nicotinamide adenine dinucleotide (NAD), or V factor, for growth in vitro while biovar 2 is NAD-independent.¹ Nielsen et al² proposed a comprehensive serotyping scheme for *A pleuropneumoniae*, which integrates the two biovars and recognises serovars 1 to 14. Very recently, a new serovar, serovar 15, has been recognised in Australia.³ This newly recognised serovar was previously thought to belong to serovar 12.³ Serovar 15 is the most common serovar isolated from Australian pigs, with serovar 1 being the next most common.⁴

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Inactivated bacterins have been widely used in Australia, with the first reported evaluation of such a vaccine being the work of Mason et al.⁵ Overseas studies have reported that the protection provided by these bacterins was inconsistent⁶ and, at the most, only serovar specific or homologous protection was achieved.⁷ When used in the field, these bacterins usually reduced the mortality rate, as reported by Mason et al⁶, but often did not prevent infection or the development of lesions.⁸

During the 1990s, there was a great improvement in our understanding of the components of *A pleuropneumoniae* that were important in both pathogenesis and immunity. In particular, the central role of the extracellular soluble proteins known as the *A pleuropneumoniae* RTX toxins, commonly called Apx toxins, became clear. The four different Apx toxins are the strongly haemolytic and cytotoxic ApxI, the weakly haemolytic and moderately cytotoxic ApxII, the non-haemolytic but strongly cytolytic ApxIII⁹, and the weakly haemolytic and in vivo expressed ApxIV.¹⁰

This improvement in knowledge about the key antigens in immunity has resulted in development of an *A pleuropneumoniae* subunit vaccine, which is commercially available overseas under the name of Porcilis APP. The Porcilis APP vaccine is composed of ApxI, II, III and a 42 kDa outer membrane protein (OMP).^{11,12} Field trials of the Porcilis APP vaccine have been reported in Croatia,¹³ France,^{14,15} Italy,¹⁶ the Netherlands¹⁷ and Sweden.¹⁸ These field trials have generally reported that the Porcilis APP vaccine results in a significantly lower mortality rate^{16,17} and fewer lung lesions.^{14,16} Significantly improved average daily weight gain was also reported in one of the French studies.¹⁴

Live attenuated vaccines have also received considerable attention. As an example, Prideaux et al¹⁹ described a live vaccine based on a mutant with an inactivated *apxIIC* gene. Within the family *Pasteurellaceae*, which contains *A pleuropneumoniae*, streptomycin-dependent vaccines have been evaluated to control disease caused by *Mannheimia (Pasteurella) haemolytica*^{20, 21} and *P multocida*.^{21, 22}

In the current study, we report on an evaluation of the ability of both the Porcilis APP vaccine and an experimental streptomycin-dependent live *A pleuropneumoniae* vaccine to protect pigs against a challenge from either serovar 1 or serovar 15, the two dominant serovars of *A pleuropneumoniae* in Australian pigs.

Materials and methods

Pigs and treatment groups

The pigs used in this study were sourced from a closed commercial piggery, which is known to be free of antibodies to *A pleuropneumoniae* serovars 1 and 15 based on the ELISA described by Bowles et al.²³ Sixty pigs aged 6 weeks of age, allocated into six groups, were used. Groups 1 and 2 were vaccinated with the live

streptomycin dependent vaccine, groups 3 and 4 with the Porcilis APP vaccine while groups 5 and 6 were the controls.

Streptomycin-dependent live vaccine

The live streptomycin-dependent vaccine strain was *A pleuropneumoniae* serovar 1 strain SD 4074. The organism was made streptomycin-dependent by serial passage of the serovar 1 reference strain of *A pleuropneumoniae* (strain 4074) on chocolate agar plates containing increasing levels of streptomycin. The organism was grown overnight on chocolate agar containing streptomycin at 400 µg/mL. A loopful of bacteria from this plate was inoculated into 20 mL of RPMI (Gibco) supplemented with streptomycin at 400 µg/mL. After overnight incubation, this broth was then used to inoculate 500 mL of RPMI (Gibco) supplemented with streptomycin at 400 µg/mL and the culture was incubated for 8 h in a 37°C water-bath with constant stirring. The culture was harvested by centrifugation (5000 g, 10 min) and the pellet resuspended in 50 mL of RPMI (Gibco) supplemented with streptomycin at 400 µg/mL.

Preparation of challenge bacteria

The *A pleuropneumoniae* strains used in the challenge procedure were HS 54, the prototype Australian serovar 1 reference strain, and HS 143, the reference strain for serovar 15.³

The challenge was prepared by a standard method used by the Bacteriology Research Laboratory at Animal Research Institute, Yeerongpilly, Queensland. In brief, the *A pleuropneumoniae* strain was grown overnight on TM/SN agar.²⁴ A heavy suspension was prepared in RPMI and adjusted to an optical density equivalent to that of a MacFarland No. 5 tube. The viable cell count of this suspension was determined by a spread plate technique using TM/SN agar. The suspension was stored at 4°C overnight. The next day, the viable count plates were examined, the count determined and the suspension adjusted to 1 x 10⁹ colony forming units (cfu) per ml. Each pig within a treatment group received a 1 mL challenge of the relevant suspension. The challenge was delivered by spraying 1 mL of the inoculum and 1 mL of air from a 3 mL syringe fitted with a soft plastic tube that was inserted into the left nostril of the pig.

Trial protocol

For the first 5 weeks of the trial, groups 1 and 2 were housed in one isolation shed and groups 3 to 6 in another shed. This was done to prevent any possibility of spread of the live vaccines to groups 3 to 6. On the day of the challenge, groups 1, 3 and 5 were moved together into one isolation shed and groups 2, 4 and 6 were moved together into the second isolation shed.

The pigs were vaccinated on day 0. The Porcilis APP vaccine was administered as per the manufacturer's recommendation – a 2 mL dose by the intramuscular (IM) route. The live vaccine was given as a 2 mL dose by the subcutaneous route. The control pigs received a 2 mL dose of saline via the IM route. Three weeks later (day 21), all pigs were revaccinated as described above.

On day 35, all pigs were challenged – groups 1, 3 and 5 with HS 143 and groups 2, 4 and 6 with HS 54.

For the 7 days following challenge, the pigs were checked every 6 h and clinical signs in every pig were scored as follows: 0 = no signs; 1 = increased respiration; 2 = abdominal breathing; 3 = cough; 4 = dyspnoea and 5 = euthanasia.

At 7 days after challenge (or at euthanasia following challenge), a necropsy was performed. At necropsy, the trachea and lungs were

cultured for the presence of the challenge organism. The percentage of lungs affected by pleuropneumonia was scored based on the method described by Hannan et al.²⁵ Each lung lobe was scored and lobe scores were added to yield a lung score for each pig. The scoring scheme was as follows: 0 = 0% of lobe with lesions; 1 = 1-20% affected; 2 = 21-40% affected; 3 = 41-60% affected; 4 = 61-80% affected and 5 = 81-100% affected.

The vaccination site was examined and checked for any adverse reaction to the injections.

Culture for *A pleuropneumoniae* from the trachea and lung lesions was performed on sheep blood agar with a feeder streak of *Staphylococcus hyicus*.

The growth of *A pleuropneumoniae* obtained on the isolation plates was scored as follows: 0 = no growth; 1 = growth limited to primary inoculation area; 2 = growth occurring in the first streak; 3 = growth in the second streak; 4 = growth in the third streak.

Other bacterial colonies observed and believed to be of possible significance were identified using API 20 NE (BioMerieux SA, Marcy-l'Etoile, France) and Microbact 24E (Disposable Products Pty Ltd, Adelaide, South Australia). For both identification systems, the manufacturers' instructions were followed.

Blood samples were collected from the pigs immediately before both vaccinations, immediately before challenge and at necropsy. Weights were recorded for all pigs each week. Two weighing scales were used, one for each shed. Calibration of the scales was done at the start of each weighing process.

Statistical analysis

The vaccinated pigs that were challenged with serovar 1 were compared with controls that were challenged with serovar 1, while the vaccinates challenged with serovar 15 were compared with controls challenged with serovar 15.

Mortality rates were analysed using Fisher's exact test. The lung lesion, clinical sign and re-isolation scores were considered categorical variables and the differences between treatments were analysed using the nonparametric Kruskal Wallis analysis of variance. The daily weight gains (both pre- and post-challenge), which were continuous variables, were assumed to be normally distributed and the differences between treatments were analysed using the analysis of variance. The level of significance for all statistical tests was $P \leq 0.05$. Statistix software by Analytical Software was used for all the analyses.

Results

Vaccine safety

No adverse reaction to the Porcilis APP vaccine was observed in any of the vaccinated pigs during the period of the trial. The vaccinated pigs did not show any depression during the day immediately after the first or second vaccinations. No injection site reactions were observed. Furthermore, no lesions were observed at the vaccination site during necropsy in any of the Porcilis APP vaccinated pigs.

The pigs vaccinated with the live streptomycin dependent vaccine, on the other hand, showed obvious depression for about 5 days after the first vaccination and about a day after the second vaccination. Loss of appetite was also observed after the first vaccination but this did not have any significant effect on prechallenge average daily weight gain (ADG). One pig had an abscess at the vaccination site detected at necropsy. None of the other live vaccinated pigs had any lesions at the vaccination site at the time of necropsy.



Before challenge, the mean ADG of the Porcilis APP and the live vaccinated pigs were not significantly different from that of the matching unvaccinated controls.

Results of serovar 1 challenge

The summary results of the serovar 1 challenge are presented in Table 1. Two pigs from the live vaccinated group were removed from the trial before challenge for animal welfare reasons unconnected with the trial.

The mean ADG of the pigs in the 3 treatment groups from 6 weeks old (day 0) to challenge (at day 42) were not significantly different from each other. All the pigs that survived up to the time of challenge gained weight. At 7 days postchallenge, the mean ADGs were significantly different from each other (Table 1). Porcilis APP vaccinated pigs had the highest mean ADG. The mean ADG of the live vaccinated pigs, postchallenge, was also significantly higher than the mean ADG of the unvaccinated pigs. The 4 surviving pigs in the unvaccinated group still gained weight after challenge but the mean ADG was significantly lower than the mean ADG of both groups of vaccinated pigs (Table 1). In both vaccinated groups (groups 2 and 4), pigs without lung lesions had higher ADG.

Six pigs from the control group and 1 pig from the live vaccinated group were euthanased post challenge (Table 1). Except for 1 pig in the control group, all euthanasia was performed 48 h postchallenge. The control pig that lived beyond 48 h was euthanased at 72 h postchallenge. All the Porcilis APP vaccinated pigs survived up to the time of necropsy. The euthanased live vaccinated pig had an abscess at the vaccination site. The number of deaths in the Porcilis APP vaccinated group was significantly different from the control group ($P < 0.05$), while the number of deaths in the live vaccinated group was not ($P = 0.07$).

The median clinical scores of both vaccinated groups were significantly different from the unvaccinated group but there was no significant difference between the vaccinated groups (Table 1). Only three pigs in the Porcilis APP vaccinated group showed respiratory signs after challenge. One pig coughed while the other two had abdominal respiration. Half of the live vaccinated pigs coughed. Increased respiration and abdominal respiration were also observed. Only 3 live vaccinated pigs did not show any clinical signs after challenge. For the unvaccinated group, almost all the pigs showed signs of both abdominal respiration and coughing. All the pigs in the unvaccinated group showed some form of respiratory disease after challenge.

The median lung lesion score of the unvaccinated group was significantly higher compared with the median

lung lesion scores of both vaccinated groups (Table 1). Although the median lung lesion score of the Porcilis APP vaccinated pigs was lower than the median lung lesion score of the live vaccinated pigs, it was not significantly different. Six of the 10 pigs in the Porcilis APP vaccinated group and 4 of the 8 pigs in the live vaccinated group had normal lungs after challenge. All pigs in the control group had lung lesions at necropsy. The pigs that were euthanased shortly after challenge had the highest lung lesion scores. In all groups, the diaphragmatic lobes of the lungs were the most severely affected.

The median re-isolation score for the unvaccinated group was significantly higher than that of both vaccinated groups (Table 1). *A. pleuropneumoniae* was re-isolated from 9 of the 10 pigs in the unvaccinated group, 2 of the 8 pigs in the live vaccinated group and 3 of the 10 pigs in the Porcilis APP vaccinated group. Higher re-isolation scores were obtained from lung swabs than from tracheal swabs.

Results of serovar 15 challenge

The summary results of the serovar 15 challenge are presented in Table 2.

The mean ADG of both vaccinated groups from 6 weeks old (day 0) to challenge (at day 42) were not significantly different from the mean ADG of the control group. The Porcilis APP vaccinated group had a significantly lower prechallenge ADG than the live vaccinated group. The mean ADG of the Porcilis APP vaccinated pigs, postchallenge, was significantly higher compared with the

Table 1. A pleuropneumoniae serovar 1 challenge of pigs previously vaccinated with a live vaccine, a commercial subunit vaccine or unvaccinated.

Observation	Live vaccinates	Porcilis APP vaccinates	Control pigs
Prechallenge ADG (g) ^a	727.16 ^A (99.06)	654.88 ^A (65.31)	679.29 ^A (83.28)
Post challenge ADG (g) ^a	677.08 ^B (150.09)	906.25 ^A (200.37)	312.5 ^C (288.68)
No. of euthanasias performed	1 ^{AB}	0 ^A	6 ^B
Median clinical signs score ^b	0.415 ^A (0-5)	0 ^A (0-0.5)	4.75 ^B (1.67-14)
Median lung lesion score ^b	0.5 ^A (0-20)	0 ^A (0-7)	15 ^B (7-25)
Median re-isolation score ^b	0 ^A (0-8)	0 ^A (0-3)	7.5 ^B (0-8)
No. of pigs ^c	8	10	10

^aStandard deviation of ADG shown in parentheses.

^bRange in clinical signs, lung lesion and re-isolation scores within each treatment group shown in parentheses.

^cOriginally all groups consisted of 10 pigs. However, two pigs from Group 2 were removed from the trial prior to challenge for animal welfare reasons.

^{A,B,C}Values with different superscripts (within a row) are significantly different $P \leq 0.05$.

Table 2. A pleuropneumoniae serovar 15 challenge of pigs previously vaccinated with a live vaccine, a commercial subunit vaccine or unvaccinated.

Observation	Live vaccinates	Porcilis APP vaccinates	Control pigs
Prechallenge ADG (g) ^a	758.75 ^A (87.61)	683.75 ^B (89.76)	717.5 ^{AB} (59.86)
Post challenge ADG (g) ^a	-98.21 ^B (250.18)	357.14 ^A (328.19)	78.57 ^B (208.79)
No. of euthanasias performed	2 ^A	0 ^A	0 ^A
Median clinical signs score ^b	1.25 ^A (0-8)	1 ^A (0-2.17)	0.5 ^A (0-2)
Median lung lesion score ^b	11 ^A (3-25)	7.5 ^A (0-23)	11.5 ^A (5-20)
Median re-isolation score ^b	4.5 ^A (0-7)	3 ^A (0-7)	2.5 ^A (0-8)
No. of pigs	10	10	10

^aStandard deviation of ADG shown in parentheses

^bRange in clinical signs, lung lesion and re-isolation scores within each treatment group shown in parentheses.

^{A,B}Values with different superscripts (within a row) are significantly different $P \leq 0.05$.

mean ADG of the surviving pigs in both the live vaccinated and unvaccinated control groups. Even though not statistically significant, the mean ADG of the live vaccinated pigs, postchallenge, was lower than the unvaccinated group. Four of 8 pigs in the live vaccinated group lost weight after challenge, with 1 pig losing 500 g per day for the 7 days postchallenge. Three of the 10 pigs in the unvaccinated group also lost weight but to a lesser extent than the pigs in the live vaccinated group. On the other hand, only 1 pig in the Porcilis APP vaccinated group lost weight in the postchallenge period.

Two pigs in the live vaccinated group were euthanased 48 h after challenge. All pigs in Porcilis APP vaccinated and unvaccinated groups survived up to the time of necropsy. The euthanasia rates of the treatment groups were not statistically different from each other (Table 2).

The median clinical scores of the 3 treatment groups were not significantly different from each other (Table 2). Almost all pigs challenged with serovar 15, whether they were vaccinated or not, showed some clinical signs. Coughing was the main clinical sign observed.

The median lung lesion scores of the 3 treatment groups were not significantly different from each other (Table 2). Except for 1 pig in the Porcilis APP vaccinated group, all pigs in the 3 treatment groups had some lung lesions. Most of the lesions were found in the diaphragmatic lobes of the lungs.

The median re-isolation scores of the 3 treatment groups were not statistically different (Table 2). Lung swabs resulted in higher re-isolation scores than the tracheal swabs for all 3 treatment groups.

Identity of other bacteria isolated from the tracheal swabs

Enterobacter agglomerans was isolated from the trachea of a pig vaccinated with the Porcilis APP vaccine and challenged with serovar 15. *Bordetella bronchiseptica* was isolated from the tracheas of 3 pigs (2 pigs that were Porcilis APP vaccinated and challenged with serovar 1 and 1 unvaccinated pig challenged with serovar 15).

Discussion

The aim of this trial was to test the efficacy of new generation *A pleuropneumoniae* vaccines against Australian *A pleuropneumoniae* isolates of serovars 1 and 15, the dominant serovars in Australia. These vaccines, the Porcilis APP commercial product and an experimental live streptomycin-dependent strain, have been developed in an attempt to provide protection against all *A pleuropneumoniae* serovars. Since the Porcilis APP vaccine contains ApxI, ApxII and ApxIII toxins and the 42 kDa OMP, it was expected to elicit protective immunity against these important virulence factors. On the other hand, the live streptomycin-dependent vaccine was hypothesised to undergo limited replication in the pig, closely mimicking a natural infection and providing the necessary antigens to stimulate a protective immune response.

The results of this study showed that both vaccines were able to provide significant protection, as assessed by ADG, clinical signs, lung lesions and re-isolation scores, against the serovar 1 challenge. Only the Porcilis APP vaccine provided significant protection against mortality. For serovar 15 challenge, no significant protection was afforded by the live vaccine while the Porcilis APP vaccine only resulted in improved ADG.

The other bacteria, aside from *A pleuropneumoniae*, which were isolated from tracheal swabs of various pigs in this trial are not

likely to have had any major effect on the outcome of the trial. These bacteria have been reported as normal inhabitants of the respiratory tract of healthy pigs^{26,27} (*B bronchiseptica*) or as airborne contaminants (*E agglomerans*).^{28,29}

The overall results indicate that, for both serovar 1 and serovar 15 challenge, the Porcilis APP vaccine was more efficacious than the live vaccine. This was evident in terms of the mean postchallenge ADG. Moreover, although the differences were not statistically significant, the Porcilis APP vaccinated pigs had lower clinical scores, lesion scores and re-isolation scores than those of the live vaccinated pigs.

The efficacy of both the Porcilis APP vaccine and the live vaccine for the serovar 1 challenge was predictable. The Porcilis APP vaccine contains 2 of the major virulence factors, ApxI and ApxII, known to be present in serovar 1 isolates of *A pleuropneumoniae*, as well as a common immunogenic protein, the 42 kDa OMP. As serovar 1 of *A pleuropneumoniae* is known to be clonal,³⁰ the antigenic make-up of the live vaccine strain must be very similar to the Australian serovar 1 challenge strain HS54.

The failure of the Porcilis APP vaccine to protect against a serovar 15 challenge might be due to the presence of important virulence factor(s) in this newly discovered *A pleuropneumoniae* serovar that were not present in the subunit vaccine. It is also possible that the important virulence factors like the Apx II and III toxins secreted by serovar 15³ are different from those incorporated in the subunit vaccine. According to Jansen et al,³¹ the *apxIIICABD* genes are very similar among biovar 1 serovars, with the exception of serovar 2 which has 32 differences in the amino acid sequence of the structural toxin protein, ApxIIIA, when compared with the other ApxIIIA of biovar 1 serovars.

ApxII toxins with the exception of those of serovar 6, all have very similar sequences.³² Hence, it would seem unlikely that the lack of protection by the Porcilis APP vaccine against serovar 15 could be associated with differences in the ApxII toxin component of the vaccine.

The exact structures of the *apxII* and *apxIII* operons of serovar 15 have not been determined. Hence, it is difficult to speculate about the failure of these vaccines to protect against this serovar. Further work in this area is necessary.

The failure of the live vaccine to protect against the serovar 15 challenge was possibly due to the fact that this serovar possesses virulence factors against which the live vaccine was unable to elicit antibody. An example could be antibody to the ApxIII toxin, as the vaccine strain was serovar 1, a serovar that does not secrete ApxIII toxin. It is worth noting that ApxIII toxin, which is secreted by serovar 15, by itself can cause lesions of porcine pleuropneumonia.^{33, 34}

The live vaccine only provided homologous, and not heterologous serovar protection. Since the vaccine was able to provide protection against HS 54 (serovar 1), there was probably limited replication of the vaccine strain in the pigs, which allowed them to respond effectively against the serovar 1 challenge. However, in spite of this, the live vaccine failed to protect against HS 143, the serovar 15 challenge. This outcome supports the findings of Crujisen et al³⁵, who detected complete protection in convalescent pigs against infection with the homologous serovar and variable protection against a heterologous serovar. However, it is contrary to the finding of Nielsen³⁶ who found a lasting immunity against reinfection from all other serovars after infection with



one serovar. Recently, cross protection between serovars 5 and 1A was achieved using a genetically defined attenuated mutant of *A pleuropneumoniae* as a live vaccine.³⁷ Unlike serovars 1 and 15, serovars 1 and 5 are highly related in terms of secreted Apx toxins.^{38,39} Although serovar 1 and 15 both secrete ApxII and possibly other common virulence factors, such similarities were not enough to elicit cross protection by the live serovar 1 vaccine.

Overall, both the Porcilis APP vaccine and the live vaccine afforded significant protection against the HS 54 (serovar 1) challenge but failed against the HS 143 (serovar 15) challenge.

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