Safety of a live attenuated *Erysipelothrix rhusiopathiae* vaccine for swine

Eric J. Neumann a,*, Alex Grinberg a, Kathryn N. Bonistalli a, Hamish J. Mack a, Philip R. Lehrbach b, Nicole Gibson c

a Institute of Veterinary, Animal and Biomedical Sciences, College of Sciences, Massey University, Tennent Drive, Palmerston North 4442, New Zealand
b Fort Dodge Animal Health, 1 Maitland Place, Baulkham Hills, NSW 2153, Australia
c Fort Dodge Animal Health, 800 5th St NW, Fort Dodge, IA 50501, USA

1. Introduction

Swine erysipelas is an important bacterial disease of pigs caused by infection with *Erysipelothrix rhusiopathiae*; the clinical and pathological features of the disease have been well-described (Wood and Henderson, 2006). The disease is characterized by acute onset of systemic illness related to a bacteraemia. During the acute phase of the disease, lethargy, inappetence, lameness, and pyrexia are common. The appearance of characteristic rhomboidal-shaped urticarial lesions on the skin gives swine erysipelas its common name of ‘diamond-skin disease’. In its chronic form, swine erysipelas presents most frequently as persistent lameness.

*E. rhusiopathiae* is a Gram-positive bacillus. Its morphology can be variable appearing as slender, straight, or slightly curved rods. Most strains grow slowly on blood agar and produce a zone of partial hemolysis (Wood and Henderson, 2006). The genus *Erysipelothrix* contains at least two species (*E. rhusiopathiae* and *E. tonsillarum*) and over 20 serovars (To and Nagai, 2007); *E. rhusiopathiae* serovars 1 and 2 are the most frequently isolated serovars from pigs with swine erysipelas and are represented in most commercial swine erysipelas vaccines (Opriessnig et al., 2004; Takahashi et al., 1996).

Immunizing pigs against swine erysipelas using live or killed vaccines has been a common practice for many years and commercial products are generally considered to be
2. Materials and methods

2.1. Experimental design

The experimental design of this vaccine safety study, including features such as the number of pigs per replicate, number of back-passage replicates, vaccine route of administration, and study termination criteria, was planned to achieve compliance with guidelines provided by VICH for examination of live vaccines in target animals for absence of reversion to virulence (Anon., 2006). Six study groups (the first ‘vaccination replicate’ plus five serial ‘back-passage replicates’) were intended for this study. The first group consisted of seven, 8.5-week-old pigs determined to be bacteriologically and serologically negative for *E. rhusiopathiae* using methods described below.

Clinical observations and rectal temperatures were recorded individually on Days – 2 through Day 0 in order to establish normal baseline values. On Day 0, five pigs in the first vaccination replicate were inoculated with $2.8 \times 10^8$ CFU of the *E. rhusiopathiae* vaccine strain (approximately 10 times the label-dose). The dose was delivered 5.5 cm deep into the nasal cavity using a multi-port central venous catheter device (Hydrocath™; Seldinger central venous catheter kit, three lumen catheter 14 gauge 20 cm; Ohmeda, Swindon, UK). The total dose of organism was delivered in a 2 ml volume with separate 1 ml aliquots delivered into both the right and left nasal cavities. Delivery of each aliquot was followed by a 1 ml flush of diluent. Swabs were collected by inserting a dry cotton-tipped bacterial culture swab (Transwab®; MW171 transport medium charcoal; Medical Wire & Equipment, Corsham, England) 4–6 cm deep into each nostril; the same swab was used in both right and left nostrils. Swabs were transported to the laboratory within 2 h of collection. All pigs were observed for clinical signs consistent with swine erysipelas (lethargy, anorexia, lameness, cyanosis, urticaria, and morbidity) and rectal temperatures were recorded daily, for 14 days following vaccination.

The study intention was to utilize a pooled sample of *E. rhusiopathiae* organisms recovered from the nasal swabs of the first vaccination replicate as the source of an inoculum for 8.5-week-old pigs that would be used in the subsequent first back-passage replicate. This back-passage was to continue through all five serial back-passage replicates or until the first replicate occurred in which the organism could no longer be recovered. At that point, the group size would be increased (10 vaccinated and 2 negative control pigs) and an inoculum identical to that used for the previous replicate would be created and used for the final replicate.

Blood samples from pigs in each group were collected on Day 0 and Day 14 for detection of anti-*Erysipelatos* antibodies. Seroconversion post-vaccination would confirm exposure to the vaccine and provide some indication that an immune response had been generated.

2.2. *E. rhusiopathiae* inoculation strain

The *E. rhusiopathiae* strain used in this study was obtained from a commercial preparation of swine erysipelas bacterin (Suvaxyn E-oral; Fort Dodge Animal Health) that was previously characterized as genotype 1a (Opiressnig et al., 2004). Lyophilized preparations of the bacterin were rehydrated using the manufacturer’s propriety blend of sterile water and flavouring (Uptake Enhancer/Diluent; Fort Dodge Animal Health). The product is marketed for mass-immunization of susceptible pigs 6 weeks of age or older and is designed for oral delivery through drinking water.

The inoculum for the first passage was created from a frozen working seed X+2 (master seed plus two passages) of an *E. rhusiopathiae* commercial vaccine strain. The working seed was thawed, inoculated into Feist medium then incubated for approximately 13.5 h at 37 °C, mixing constantly. The culture was then mixed with stabilizer and dispensed into 50 ml glass vials filling 20 ml in each vial. The filled vials were frozen and lyophilized. To quantify the number of live organisms in each vial after lyophilization, vials were rehydrated with water and 10-fold serial dilutions prepared. Each dilution was plated on blood agar plates in triplicate and incubated at 37 °C for 48 h. The *E. rhusiopathiae* colonies in the dilution showing between 30 and 300 bacterial colonies were counted to calculate the number of colony forming units (CFU) per ml of suspension. Inoculums for all replicates were standardized to contain a total dose of $2.8 \times 10^8$ CFU of the *E. rhusiopathiae* in 2 ml; 1 ml was delivered into each nostril as described above.

2.3. Isolation of *E. rhusiopathiae* from nasal swabs

Four isolation protocols (Groshup and Timoney, 1990; Harrington and Hulse, 1971; Quinn et al., 1994) were used to ensure a high likelihood of recovering any vaccine or wild strains of *E. rhusiopathiae* from the nasal swabs. As the nasal swabs were expected to contain a polymicrobial flora, techniques that included use of some selective media were employed. After collection, nasal swabs were streaked onto Columbia sheep blood agar (CSBA; Fort Richards Labs, Auckland, New Zealand), CSBA supplemented with colistin sulphate (10.0 mg/l) and nalidixic acid (15.0 mg/l) agar (CNA; Fort Richards Labs), and 5% sheep blood agar supplemented with sodium azide (0.2 g/l) (ABA; Fort Richards Labs) plates. These plates were incubated at 37 °C then examined at 24 h intervals over 72 h; ABA plates...
were incubated in 10% CO₂. Swabs were also placed into Feist medium (Groschup and Timoney, 1990), which was incubated for 24 h at 37 °C followed by plating onto CSBA agar; the CSBA plates were incubated for 48 h at 37 °C and then examined for growth. Representative alpha-haemolytic colonies were picked from the plates, Gram-stained, then evaluated microscopically for the presence of pleomorphic Gram-positive rods. Any suspect colonies meeting this description were tested for a negative catalase reaction followed by biochemical testing (hydrogen sulfide production in the butt of triple sugar iron agar slants and indole negative).

The investigators evaluated the growth characteristics of the pig inoculum (vaccine strain) on each of the media used in the study. The vaccine strain grew well on all media, except ABA. On both CSBA and CNA, the vaccine strain colonies were of Type 1 (S-form) morphology having small, convex colonies comprised of short, nearly coccoid, Gram-positive rods (Dunbar and Clarridge, 2000). The vaccine strain had been previously adapted to Feist medium by the vaccine manufacturer. In an effort to evaluate the potential for New Zealand E. rhusiopathiae isolates to grow on the study media, three porcine-origin isolates identified by the investigators from previous New Zealand swine erysipelas cases were assessed and found to grow on all media used in the study.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Prior to ELISA testing, all samples were diluted 1:160 in a 0.01 M phosphate buffered saline (PBS) diluent containing 0.3% Tween 20 and 1.15% Non-Fat Dry Milk (NFDM). The ELISA was carried out as follows: each well in the plates (96-well MaxiSorp F98 plates; NUNC, Roskilde, Denmark) received 100 μl of coating buffer (0.159% Na₂CO₃ plus 0.293% NaHCO₃, in ultrapure water that exceeded ISO 3696 Grade 1, ASTM D1193-91 Type 1, and NCCLS Type 1 purity standards, pH 9.6) plus antigen (1.4 μg inactivated whole-cell preparation of E. rhusiopathiae X + 2 vaccine master seed, per well) and was incubated overnight at 4 °C. The day of the assay, plates were removed from 4 °C and manually washed six times with a 0.01 M PBS wash reagent containing 0.3% Tween 20. Following washing, each well was blocked with 200 μl of 1.15% NFDM in 0.01 M PBS containing 0.1% non-ionic detergent (Igepal CA-630; Sigma–Aldrich, MO, USA) and 0.01% antifoaming agent (Antifoam Y-30 Emulsion; Sigma–Aldrich) then incubated for 60 min at 37 °C. After incubation, plates were washed six times with wash reagent then 100 μl of the diluted serum sample was applied to each well; 100 μl of positive control serum (1:2560, serum:diluent) and negative control serum (1:80 serum:diluent) were applied in duplicate to each plate. Plates were incubated at 37 °C for 30 min, samples were removed, and plates washed six times with wash reagent. Following washing, 100 μl of horseradish peroxidase-conjugated goat anti-swine immunoglobulin G (IgG 0.5 mg/ml, heavy and light chains; KPL, Kirkegaard & Perry Laboratories, Maryland, USA) at a 1:2000 dilution was added to each well. The plates were incubated for 30 min at 37 °C and washed six times, then developed by adding 100 μl of 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) substrate (ABTS; KPL) to each well. After incubating for 30 min at 37 °C, the reaction was stopped by adding 100 μl of 1% sodium dodecyl sulfate. Optical density (OD) was measured immediately at dual wavelengths of 405 and 490 nm (OD 405/490); OD 405/490 of ≥0.200 was considered positive.

Previously, the investigators collected sera from 10 pigs each at 6, 7, 8, 9, 10 and 11 weeks of age from the farm that was to contribute pigs for the vaccine safety study. The presence of anti-E. rhusiopathiae antibodies in each sample was determined using the ELISA protocol described above. The results showed that OD values declined until 7–8 weeks of age at which point no further decay occurred and indicating that maternally derived antibodies were no longer present. Based on this data, OD values less than 0.200, when measured at dual wavelengths of 405–490 nm, were considered negative.

Serum samples collected in the vaccine safety study on Day 0 and Day 14 were assayed simultaneously (within each replicate) for the presence of anti-E. rhusiopathiae antibodies. All samples (Day 0 and Day 14 from each pig) were run in duplicate on a single plate for each replicate in order to minimize the impact of plate-to-plate variation.

2.5. Experimental subject animals

Pigs for this study were of Landrace-Large White-Duroc hybrid genetics sourced from a local commercial swine farm. The herd had not used any swine erysipelas vaccine in the 8 months prior, and had no clinical history of swine erysipelas for the 24-month period prior, to selection of pigs for this study. At Day –7 for each study replicate, 14–22, 7-week-old pigs were conveniently selected, identified by eartag, and a jugular blood sample and nasal swab were collected. Amongst sampled pigs, seven or 12 determined to be E. rhusiopathiae-negative by ELISA and culture were randomly selected, allocated to ‘control’ or ‘vaccinated’ treatment groups, then transported to the Massey University Pig Research Unit on Day –3.

Pigs within treatments were co-mingled throughout the trial; control and vaccinated groups were housed in separate rooms. Pigs were fed a commercial diet ad libitum formulated to meet the nutritional needs of 8–12-week-old pigs. Pen flooring was partially perforated and room temperature was kept at a constant 22 °C. Study personnel were confined to moving uni-directionally from control to vaccinated study rooms; this movement occurred only once daily, and only after outerwear and footwear had been changed and hands had been thoroughly washed and disinfected. This study protocol was approved by the Massey University Animal Ethics Committee (approval 06/124).

2.6. Statistical analysis

The rectal temperature of each pig was measured daily from Day –2 to Day 14. Temperature data collected on Days –2, –1, and 0 were averaged for each pig to establish its normal baseline temperature. All data was evaluated for the presence of time-related serial correlation (PROC
3. Results

3.1. Bacterial culture

A polymicrobial flora was cultured from nasal swabs of all seven pigs collected from Days 1 to 5 in the first vaccination replicate; no *Erysipelothrix* was identified in any sample. As was dictated by the protocol, when no *E. rhusiopathiae* could be recovered from inoculated pigs in an experimental replicate, the subsequent replicate (the second study group in this instance) was initiated but comprised of twice as many pigs (10 vaccinates and two controls) in order to improve the likelihood for organism recovery. In this second replicate, no *Erysipelothrix* was isolated from any pig. As no vaccine organism could be recovered from either the first or second replicate, no further attempt to create serial back-passage replicates was undertaken and the experiment was concluded.

3.2. Clinical parameters

All pigs in both replicates remained clinically normal and without pyrexia throughout the trial period. Temperature data from both replicates were combined for analysis and no serial correlation was present in either the control (\(D = 1.9956, p = 0.8250\)) or vaccinated (\(D = 1.8805, p = 0.9878\)) groups. These results indicated that temporal trends in body temperature were unlikely to be present and as no body temperature measurements exceeded the upper range of normal for healthy pigs, no further analysis was conducted. Average daily body temperatures for pigs each day post-vaccination are shown in Fig. 1.

3.3. Daily weight gain

Pigs in replicate one (mean bodyweight of 21.9 kg, \(n = 7\)) were slightly smaller on Day 0 than in replicate two (mean bodyweight of 26.7 kg, \(n = 12\)). However, average gain during the 14-day period was similar between the two replicates (replicate one mean gain = 12.3 kg/pig, replicate two mean gain = 11.4 kg/pig). After accounting for the effects of replicate and starting weight, the regression modelling indicated that vaccinated pigs did not gain weight at a rate that was significantly different than the control pigs. Vaccinated pigs gained an average 11.37 kg while control pigs gained 12.98 kg (\(t = 0.9082, p = 0.42\)).

3.4. Serological results

In the first replicate, two vaccinated pigs were found to have titers above the 0.200 cut-off value despite being serologically negative at Day –7. These two pigs remained culture negative before, and during the experiment and no clinical evidence of swine erysipelas was apparent on the source farm during this period. All pigs in the second replicate were serologically negative at Day 0.

At Day 14, four of five vaccinated pigs in replicate one demonstrated strong increases in OD value while the two control pigs showed only modest changes. Pigs in replicate two showed a similar pattern with nine of 10 vaccinated pigs producing increased OD values. The control pigs showed minimal increases in OD value. The overall magnitude of OD values in the second replicate was lower than in replicate one for unexplained reasons although the serological patterns were very similar to that of the first replicate.

McNemar’s test for the proportion of pigs with increasing OD values in the vaccinated as compared to the control group was not significant (\(\chi^2 = 1.3333, p = 0.25\)) but given the small number of control pigs in the study, this result should be interpreted cautiously. However, when evaluating the magnitude of titer changes using the Wilcoxon rank sum test, the vaccinated pigs showed highly significant increases in OD value between Day 0 and Day 14 as compared to the control pigs (vaccinated pigs 200% increase, control pigs 14% increase, \(p = 0.000516\)). Individual pig serological data is presented in Fig. 2.
4. Discussion

Vaccines against swine erysipelas are in common use around the world and previous researchers have commented on the need for safety studies on available vaccines (Opriessnig et al., 2002). While one can assume that some safety data has been generated in support of product registration in certain countries, no studies could be identified in the peer-reviewed literature that assessed the safety of any commercially available live attenuated swine erysipelas vaccine. Recent field occurrences of *E. rhusiopathiae* strains that may not be represented in current vaccines (Eamens et al., 2006) suggest the epidemiology of the disease may be changing and the need to ensure attenuated live are not contributing to the rise of new strains through reversion is increased. Additionally, the obligation to safely control diseases such as swine erysipelas that have a known zoonotic potential remains.

The current study was designed to evaluate the safety, as represented by persistence in pigs and potential for reversion to virulence, of Suvaxyn Oral-E vaccine. All vaccinated pigs remained free of clinical signs associated with acute or chronic forms of swine erysipelas during each of the 14-day replicates. It was surprising that no *E. rhusiopathiae* was recovered from either replicate. Both the vaccine strain and porcine origin strains of *E. rhusiopathiae* from New Zealand cases of swine erysipelas were evaluated in our laboratory prior to initiating the experiment in order to become familiar with their growth and phenotypic characteristics. Bacterial culture prior to inoculation demonstrated that the inoculum was viable, and except on ABA both the vaccine strain and a wild strains in our collection grew well on the media used in this study. The failure to isolate *E. rhusiopathiae* from the pigs could either indicate the presence of fast growing bacteria in the nasal flora that overwhelmed our ability to recover *E. rhusiopathiae* through standard culture techniques or that the vaccine strain of *E. rhusiopathiae* was rapidly cleared from the nasal cavity during the first 24 h post-vaccination. Despite the ease with which the organism can be maintained in culture, other authors have reported difficulty in recovering the organism from infected pigs unless they are in the acute stage of the disease (Wood and Henderson, 2006). Similar findings have been reported from cases of human erysipelas (Brooke and Riley, 1999).

Work done in pigs to examine potential routes of elimination of *E. rhusiopathiae* after experimental parental inoculation has been reported (Wood, 1967). Using virulent isolates, the organism could be recovered from various tissues in 17 of 18 pigs that were evaluated; blood, tonsils, feces, urine, mouth, and nasal passages were found to be the most reliable tissues for recovery. Specifically, 11 of 18 pigs had positive nasal samples with a median time to first recovery of 3 days (minimum = 1, maximum = 5). In the current study, intranasal inoculation followed by culture of 5 daily nasal swabs presented an efficient model that best reproduced the labelled vaccination recommendations and permitted a convenient sampling protocol supported by prior published findings. In the context of normal field use of the vaccine, nasal colonization would present an excellent opportunity both for transmission of the vaccine strain between pigs and for co-colonization with any wild-type strains in the pig’s environment, both features that could contribute to the likelihood of a vaccine’s reversion to virulence. In the current study, the attenuated nature of the vaccine was thought to make it less likely to be recovered from oro-pharyngeal or fecal samples as compared to nasal samples.

![Graph](image-url)
A control group of pigs inoculated with virulent *E. rhusiopathiae* was not included in this study. While inclusion of this group would not have strengthened any evaluation of the safety of the vaccine strain, it could have provided more certainty that the pigs were susceptible to infection. While differences in virulence between *E. rhusiopathiae* isolates have been reported (Wood, 1967), a host-genetic basis for resistance to infection with the organism has not been shown. Sporadic individual cases of swine erysipelas have been reported on the farm from which pigs used for the current study originated. While these cases were infrequent and occurred more than 24 months prior to selection of the experimental pigs, it did suggest that pigs from this population were genetically susceptible to infection if not vaccinated or previously exposed. Serologic data from this study demonstrated OD values in inoculated pigs increased by over 200% during the 14 days monitoring period while non-vaccinates had decreasing or negligible increases in OD value (14% increase overall). If pigs were not susceptible to the vaccine (blocking maternal antibodies or other non-specific passive mechanisms), no differences in serological results would have been expected. It should be noted that several individual pigs did present outlying data. Two vaccinated pigs in replicate one were above the positive cut-off level at Day 0. One of these pigs’ OD values at Day 14 increased in a manner consistent with the overall pattern in the overall dataset while the second pig appeared to show only a minimal response to vaccination. All pigs in replicate two were serologically negative at Day 0. While OD levels did not reliably increase above the 0.200 cut-off level by Day 14, 9 of 10 vaccinated pigs did show an increased level of antibody. For unknown reasons, antibody levels in both control pigs of replicate two also increased by Day 14. However, the statistical analysis showed that vaccination had a strong positive effect on OD values and supported the conclusion that the pigs did recognize the *E. rhusiopathiae* antigen in the vaccine. The ELISA used in this study was controlled to the best extent possible by running all samples in duplicate and ensuring that all pigs (Day 0 and Day 14) in a given replicate were included on the same microtiter plate. This process increased the level of confidence in the serological data for comparisons within a replicate but limited the extent to which data could be compared between replicates.

Given the negative culture results in both replicates, any tissue persistence beyond 14 days was thought to be unlikely. For this reason and the absence of any clinical indicators that disease had occurred, pigs were not sacrificed for the purpose of tissue collection at trial termination.

5. Conclusion

Suvaxyn Oral-E is a live attenuated vaccine for immunization of healthy swine against swine erysipelas. The strain of *E. rhusiopathiae* from which the vaccine was produced persisted for less than 24 h when placed deep into the nasopharynx of young swine. Pigs inoculated intranasally with a dose 10 times greater than the label indication remained healthy and did not show any clinical signs that were consistent with the occurrence of acute or chronic swine erysipelas. Furthermore, there was a lack of evidence for extensive nasal colonization by the vaccine strain as indicated by a failure to isolate it from the pigs. Serological results of the overall dataset showed that vaccine exposure resulted in a significant rise in anti-*E. rhusiopathiae* antibody levels. This suggested recognition of the antigen by the vaccinated pigs and evidence that an immune response had been initiated. The vaccine appeared safe and very unlikely to revert to a virulent state.

Conflict of interest statement

Neumann is currently being funded by Fort Dodge Animal Health to conduct a field-based vaccine efficacy project in New Zealand that is unrelated to the current work. Lehrbach and Gibson are employees of Fort Dodge Animal Health, the sponsor of the current work.

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