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EXPERIMENTALLY INDUCED DISEASE

An Experimental Dermal Oedema Model for Apx Toxins of *Actinobacillus pleuropneumoniae*

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Summary

In-vivo models of *Actinobacillus pleuropneumoniae* (App) infection in pigs are required for the development of vaccines and investigations of pathogenicity. Existing models cause severe respiratory disease with pulmonary oedema, dyspnoea and severe thoracic pain, and careful monitoring and early intervention with euthanasia is, therefore, needed to avoid unnecessary suffering in experimental animals. As a potential replacement for the existing respiratory infection model, an in-vivo protocol was evaluated using intradermal or subcutaneous injection of different App strains and Apx toxins into the abdominal skin of pigs. High concentrations of serovar 1 and serovar 10 App induced diffuse visible dermal oedema and inflammation. Injection of Apx toxins alone did not adequately produce macroscopic lesions, although an influx of inflammatory cells was seen on histopathology. ApxI-producing strains of App induced more inflammation than ApxII- and ApxIII-producing strains. Induction of skin lesions by injection of App or Apx toxins was not sufficiently repeatable or discrete for a robust experimental model that could be used for assessment of novel interventions.

Keywords: *Actinobacillus pleuropneumoniae*; animal model; skin; toxin

Actinobacillus pleuropneumoniae (App) causes contagious pleuropneumonia in pigs. Acute disease can result in pyrexia and dyspnoea and can be fatal. Less severe respiratory disease is also described but impacts on weight gain and food conversion efficiency (Macinnes and Rosendal, 1988). Pathology associated with the acute disease is described as a necrotizing, fibrinohaemorrhagic pneumonia with pleurisy (Liggett *et al*, 1987; Bertram, 1988). The Apx toxins produced by App are considered to be key determinants of virulence and largely responsible for the lesions observed (Kamp *et al*, 1997; Bossé *et al*, 2002). Four Apx toxins are recognized (ApxI–IV). ApxI is strongly haemolytic and cytotoxic, ApxII is weakly haemolytic and moderately cytotoxic and ApxIII is strongly cytotoxic but does not cause haemolysis (Frey *et al*, 1993). ApxIV is genetically related to ApxI–III and expressed *in vivo* but confers no toxin action or contribution to the pathogenicity of *A. pleuropneumoniae* (Dreyfus *et al*, 2004; Assavacheep, 2009). Eighteen distinct serovars of App are recognized (Bossé *et al*, 2018), based on their capsular polysaccharide antigen. Each serotype generally expresses the same combination of Apx toxins, although there are exceptions (Sassu *et al*, 2018).

In-vivo models for reproducing contagious pleuropneumonia in pigs and testing novel vaccines and antibiotics are well established (Rycroft *et al*, 1991b; Oldfield *et al*, 2009). Infection with live App organisms by aerosol, endobronchial or intratracheal inoculation is commonly used and causes severe clinical signs within 24–48 h (van Leengoed and Kamp, 1989; Inzana *et al*, 1993; Jacobsen *et al*, 1996; Antenucci *et al*, 2018). Other species such as mice and the wax moth (*Galleria mellonella*) have been

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trialled as alternative, more ethical animal models but are not truly representative of the disease seen in swine (Pereira *et al*, 2015; Bao *et al*, 2019). In this study, an alternative skin model of App infection was evaluated in pigs. Since Apx pore-forming toxins are known to cause endothelial changes and oedema, our hypothesis was that injection of App organisms and/or Apx toxins could be used to induce repeatable, yet mild, lesions in the dermis representative of the pulmonary lesions in pigs. Such a localized visible indicator of pathogenesis could be used to test novel interventions without causing respiratory disease.

This study was performed under a UK Home Office Licence according to the Animals in Scientific Procedures Act 1986. Procedures were approved by the Royal Veterinary College Animal Welfare Ethical Review Body. App serotype 1 strain 4074, serotype 10 strain 13,039, serotype 7 strain WF83, serotype 2 strain HK361 and serotype 2 mutants HK361e and HK361h (Rycroft *et al*, 1991a) (Table 1) were grown overnight on chocolate agar (Oxoid, www.oxoid.com) followed by overnight culture in Columbia broth (Oxoid) supplemented with 5 μg/ml β-NAD and 3mM $CaCl_2$ at 37°C with agitation at 180 rpm. Subcultures (1/10) were made in Columbia/NAD/$CaCl_2$ and grown to an OD_{600} of between 0.5 and 0.7 and centrifuged three times at 13,000 rpm. Culture supernatants were removed by pipette and whole organisms were resuspended in phosphate buffered saline (PBS). Culture supernatants were also prepared from subcultures grown to an OD_{600} of approximately 1.0 and from overnight cultures. In pigs 3 and 4, subcultures grown to OD_{600} of approximately 1.0 were also diluted 1:2 in Columbia/NAD/$CaCl_2$ for use at some skin sites.

Eight 6-week-old commercial cross-bred pigs were anaesthetized with 4 mg/kg alfaxalone (Alfaxan; Jurox Animal Health, www.jurox.com) by slow intravenous injection and injected subcutaneously or intradermally with App organisms resuspended in PBS or with App culture supernatants on up to 10 marked sites on the ventral abdomen (Supplementary Fig. 1). Injection of PBS only (negative control site for organisms) or Columbia/NAD/$CaCl_2$ only (negative control site for supernatants) was also performed. Pigs were subsequently monitored regularly for evidence of a gross skin reaction or systemic signs of App infection. At 48 h post injection, pigs were euthanized and skin samples fixed in 10% formalin. After paraffin wax embedding, tissue sections were stained with haematoxylin and eosin (HE) and examined microscopically.

Initially, injection of whole organisms was performed in an attempt to induce dermal oedema. Two pigs (nos. 1 and 2) were injected subcutaneously at each of eight sites on the ventral abdomen with

Table 1
***Actinobacillus pleuropneumoniae* strains and formulations administered to pig abdominal skin**

Pigs	*Strain (serotype)*	*Formulation*	*Route of administration*	*Toxin profile*		
1, 2		Whole organisms (10^3–10^6 CFU/ml)	Subcutaneous			
	4074 (1)			ApxI	ApxII	
	13039 (10)			ApxI		
3, 4		Culture supernatant	Subcutaneous			
	4074 (1)			ApxI	ApxII	
	13039 (10)			ApxI		
5, 6		Culture supernatant	Subcutaneous			
	WF83 (7)				ApxII	
	HK361 (2)				ApxII	ApxIII
	HK361e (2)					ApxIII
	HK361h (2)			–	–	–
7		Culture supernatant	Intradermal			
	13039 (10)			ApxI		
	HK361 (2)				ApxII	ApxIII
	HK361e (2)					ApxIII
	HK361h (2)			–	–	–
8		Whole organisms (10^3–10^4 CFU/ml)	Intradermal			
	13039 (10)			ApxI		
	HK361 (2)				ApxII	ApxIII
	HK361e (2)					ApxIII
	HK361h (2)			–	–	–

CFU, colony forming units; –, no Apx toxins produced.

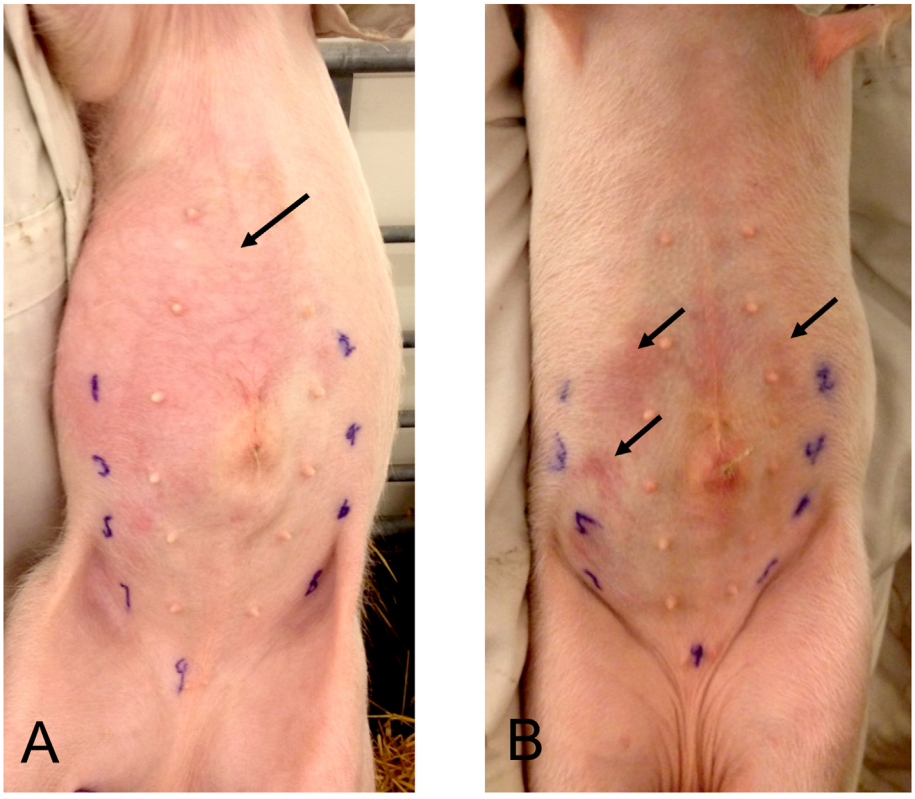

Fig. 1. Erythema, skin, pigs. Subcutaneous inoculation with *A. pleuropneumoniae* organisms 6 h post injection. (A) Pig 1 has erythematous and bruised skin around injection sites 1 (strain 4074, 10^6 CFU/ml) and 2 (strain 13039, 10^6 CFU/ml) extending craniolaterally. (B) Pig 2 has more focal bruising and erythema around injection sites 1 (strain 4074, 10^6 CFU/ml), 2 (strain 13039, 10^6 CFU/ml) and 3 (strain 4074, 10^5 CFU/ml).

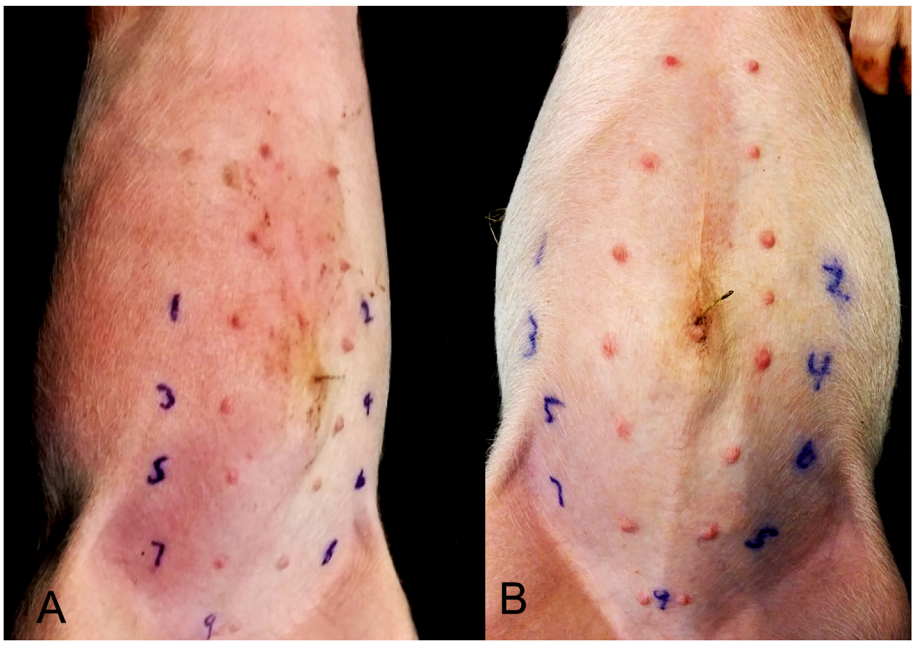

Fig. 2. Erythema, skin, pigs. Subcutaneous inoculation with *A. pleuropneumoniae* organisms 48 h post injection. (A) Pig 1 has erythema and subcutaneous oedema extending laterally from injection sites 1 (strain 4074, 10^6 CFU/ml) and 3 (strain 4074, 10^5 CFU/ml). (B) Pig 2 has more subtle oedema extending cranially from site 1 (strain 4074, 10^6 CFU/ml).

different concentrations, estimated on the basis of OD_{600} values and subsequently confirmed by plating serial dilutions (1×10^3, 1×10^4, 1×10^5 and 1×10^6 colony forming units [CFU]) of whole App organisms (Table 1) that express ApxI and ApxII (strain 4074, serotype 1) or ApxI only (strain 13039, serotype 10). These pigs developed visible erythema and bruising of the ventral abdominal skin within 6 h of subcutaneous injection of the organisms, especially at the injection sites, where the highest concentrations of both organisms had been administered (Fig. 1). At 24 h post injection, gross subcutaneous oedema was visible in pig 1. By 48 h post injection, the erythema and oedema, although diffuse, were more focussed around the App 4074 injection sites, particularly in pig 1 (Fig. 2). Histological examination of the ventral abdominal skin at injection site 1 (1×10^6 CFU of App 4074) of pig 1 revealed focally extensive, severe necrosuppurative panniculitis and fasciitis with abscessation. A large abscess, composed of mostly degenerate neutrophils and fewer macrophages, lymphocytes and plasma cells, expanded the deep panniculus muscle. Moderate oedema expanded the deep fascia, and degeneration and early necrosis of skeletal muscle myofibres was observed. The superficial, mid- and deep dermis were markedly expanded by oedema.

To assess whether the dermal oedema was a result of Apx toxins, two pigs (pigs 3 and 4) were injected subcutaneously with cell-free culture supernatants, containing Apx toxins, from the same organisms (App strains 4074 and 13039) (Table 1). Erythema developed at injection sites within 6 h of injection. A more pronounced reaction was observed at sites inoculated with App 4074 culture supernatant compared with App 13039 sites and in pig 3 compared with pig 4 (Supplementary Fig. 2). By 24 h post injection, erythema had reduced and one pig had no visible lesions, while the other had mild bruising at one site (log phase culture supernatant OD_{600} 0.5, App strain 4074) and a palpable increase in skin thickness at another site (overnight culture supernatant App strain 4074). By 48 h, pea-sized lumps were palpable at both sites. Histological examination of one site (log phase culture supernatant OD_{600} 0.5, App strain 4074) revealed focally extensive, minimal lymphoplasmacytic and neutrophilic perivascular dermatitis. Low numbers of perivascular lymphocytes, plasma cells and viable neutrophils were visible within the superficial to mid-dermis. There was also mild atrophy of dermal adipocytes (Fig. 3). At the other site (overnight culture supernatant App strain 4074) multifocal accumulations of mixed inflammatory cells, including neutrophils, macrophages and to a lesser extent lymphocytes and plasma cells, were observed, often

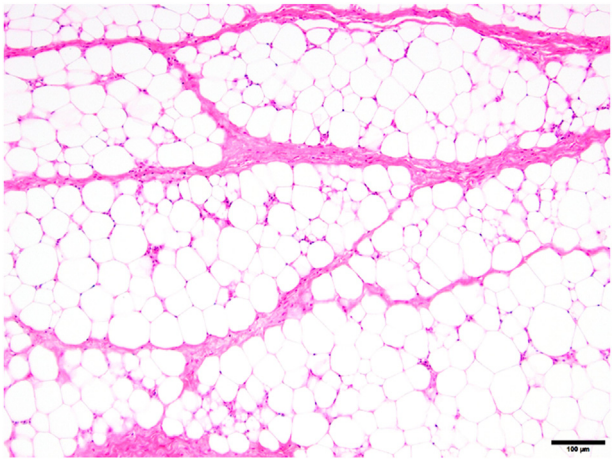

Fig. 3. Inflammation, subcutaneous tissue, pig. Rare individual lymphocytes and plasma cells within subcutaneous adipose tissue 48 h post inoculation with log phase culture supernatant (*A. pleuropneumoniae* strain 4074, OD_{600} 0.5). Skin biopsy. HE. Bar, 100 µm.

around blood vessels and extending to the adjacent dermis. Dermal adipose tissue was mostly atrophic or degenerate. The underlying panniculus muscle was degenerate or necrotic with infiltration of mixed inflammatory cells (Fig. 4).

To determine whether different Apx toxins would induce similar gross and histopathological lesions, two pigs (nos. 5 and 6) were each injected subcutaneously with culture supernatants from App strain WF83 (ApxII only), App strain HK361 (ApxII and ApxIII) and App mutants HK361e (ApxIII only) and HK361h (no Apx toxins) at eight sites on the

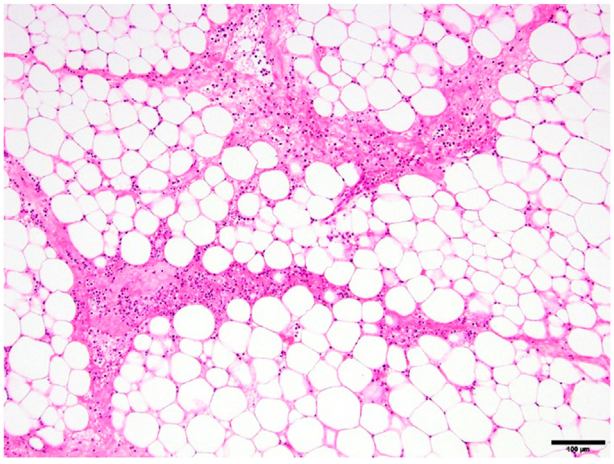

Fig. 4. Inflammation, subcutaneous tissue, pig. Multifocal necrosis of subcutaneous adipose tissue with fibrin exudation and moderate infiltrates of neutrophils, lymphocytes and plasma cells 48 h post inoculation with overnight culture supernatant of *A. pleuropneumoniae* strain 4074. Skin biopsy. HE. Bar, 100 µm.

ventral abdomen (Table 1). There was little evidence of any lesions at the sites injected with culture supernatants from log phase growth, when peak ApxII and ApxIII activity is observed *in vitro*. Mild swelling was seen at sites injected with stationary phase culture supernatants from strains WF83 and HK361 after 6 h but had disappeared by 24 h post injection. Histological examination revealed minimal inflammation at the site injected with log phase HK361 culture supernatant but low numbers of mixed inflammatory cells, mainly viable neutrophils but also low numbers of lymphocytes, plasma cells and macrophages, were observed at the site injected with overnight HK361 (Supplementary Fig. 3) culture supernatant when in-vitro ApxII and ApxIII activity is low, suggesting that the changes observed are unlikely to have been caused by Apx toxin activity alone. There were similar histopathological changes at skin sites injected with culture supernatant of the mutant HK361e, which produces only ApxIII toxin, with minimal inflammation observed at the site injected with log phase culture supernatant. Mild to moderate lymphoplasmacytic and neutrophilic perivascular dermatitis was seen at the site injected with the overnight culture supernatant. At sites injected with culture supernatant of the mutant HK361h strain, which does not produce Apx toxins, minimal inflammation was observed for both the log phase and overnight culture supernatants with low to moderate numbers of lymphocytes and plasma cells and fewer neutrophils within the deep dermis and subcutaneous adipose tissue (Supplementary Fig. 4).

One pig (no. 7) was injected with the same culture supernatants intradermally rather than subcutaneously to determine whether this route would be more effective at inducing skin lesions. As with subcutaneous injection of toxin, erythema and skin induration was observed 6 h post injection with mild erythema still visible after 24 h. Another pig (no. 8), injected intradermally with whole organisms of wild type HK361 and HK361e and h mutants, had no visible lesions post injection. There was a mild increase in neutrophils, lymphocytes and plasma cells at the site injected with HK361e but no histological changes were seen at the sites injected with HK361 and HK361h.

Development of a skin model that could mimic the impact of Apx toxin without causing severe disease and distress to experimental pigs would be a valuable tool for assessment of novel interventions. It was anticipated that this method could enable researchers to assess either neutralizing serum *in vivo* by simultaneous injection of toxins and sera into the dermis or the immune status of vaccinated pigs by their ability to limit lesion formation. For this method to be feasible, induction of repeatable discrete compact lesions or induration, visible to the eye or measurable by skin calipers, would be needed. We found that injection of a high concentration (10^6 CFU) of App serotype 1 organisms induced visible diffuse oedema within 6 h of subcutaneous injection. However, this reaction was not consistent between the two pigs receiving the same treatment and was too severe in one pig to be considered as a suitable, ethical replacement of current methods. The differences observed between the two pigs are difficult to interpret and it is uncertain whether they represent a genuine variation in host response to injection of APP or to other confounding factors. Intradermal injection of serotype 2 App organisms did not produce any visible lesions, although high concentrations were not delivered in order to avoid the severe reactions observed in pig 1 and maintain animal welfare. It is possible that high concentrations of organisms are necessary to induce lesions in the skin. Injection of higher (2×10^9 CFU) but not lower concentrations (8×10^7 and 4×10^8 CFU) of serotype 5 unencapsulated mutants has been shown to induce small abscesses after subcutaneous injection (Inzana *et al*, 1993).

Further objectives of this research were to determine whether different Apx toxins or combinations of toxins alone could induce gross and histopathological lesions and whether or not there would be observable differences in lesions between the toxins administered. Although only a small number of pigs were studied for ethical reasons, it appeared that there were some differences between strain 4074 supernatants containing ApxI and ApxII and strain 13039 supernatants containing ApxI only, as an influx of inflammatory cells was visible at sites injected with strain 4074 supernatants in one pig, while there were no visible signs of inflammation at sites injected with strain 13039 supernatant. Overnight culture supernatants were more effective at inducing inflammation, suggesting that this inflammatory reaction is potentiated by lipopolysaccharide (LPS) rather than ApxI or Apx II alone, for which gene expression and activity peaks earlier at the late exponential/early stationary growth phase (MacDonald and Rycroft, 1992; Jarma and Regassa, 2004). However, LPS alone has been demonstrated not to induce haemolysis or cytotoxicity *in vitro* when ApxI-negative mutants were compared with wild type serotype 10 isolates (Chang *et al*, 2014). The impact of injection of supernatants from serotype 7 WF83 and serotype 2 HK361 strains and mutants were much less distinct, with minimal gross lesions and only mild histopathological evidence of inflammation. There did not appear to be any difference in the lesions produced between strains

producing both ApxII and ApxIII and mutants lacking either ApxII or both toxins. This was possibly because ApxII and ApxIII induce less severe dermal inflammation than ApxI, and any differences may therefore have been imperceptible. In a previous study, pigs developed minimal clinical signs when inoculated endobronchially with recombinant ApxII toxin alone and the ability of ApxIII toxin to induce necrosis was less than ApxI (Kamp *et al*, 1997).

In conclusion, this study has demonstrated that skin lesions can be elicited by injection of whole App organisms or Apx toxins, but this response is not sufficiently repeatable or representative to be a useful model for assessment of novel interventions against porcine pleuropneumonia. Further exploration of alternative models of App infection or refinement of current models is warranted to both improve animal welfare and facilitate high-quality studies with relevant outcomes to clinical infection and disease control.

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Author CRediT Statement

A N Rycroft, B Catchpole, F Soutter: Conceptualization. **A Rycroft, F Soutter**: Methodology. **A N Rycroft, F Soutter**: Validation. **F Soutter, S L Priestnall**: Formal analysis. **F Soutter, A N Rycroft**: Investigation. **F Soutter**: Writing – original draft preparation; **A N Rycroft, B Catchpole, S L Priestnall**: Writing – review and editing. **F Soutter**: Visualization. **A N Rycroft, B Catchpole**: Supervision. **F Soutter**: Project administration. **A N Rycroft, B Catchpole**: Funding acquisition.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship or publication of this article. All authors have read and agreed to the published version of the manuscript.

Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcpa.2022.04.004.

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