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Vaccine development for *Prescottella equi*

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a

Abstract

Prescottella equi (formerly *Rhodococcus equi*) is an intracellular pathogen that causes pyogranulomatous pneumonia in Thoroughbred foals. There is currently no vaccine available for the prevention of this disease in foals despite years of research. Cell mediated immunity is considered crucial for overcoming an infection caused by this pathogen. The virulence associated protein (VapA) is a well characterized immunogenic protein associated with this pathogen and was used to develop DNA and recombinant protein vaccines in this study. Vaccine candidates and live *P. equi* based vaccine were tested in BALB/c mice. Mice were challenged with virulent *P.equi* 2 weeks following the last boost and IgG subtypes and bacterial clearance from spleen and liver determined. The DNA vaccine elicited a significant IgG2a response indicating a Th1 biased immune response. The IFN gamma response from DNA and recombinant VapA vaccinated mice was moderate. The results of the challenge study showed that neither the recombinant VapA protein nor DNA vaccine enhanced clearance of *P. equi* in this model.

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1. Introduction

Prescottella equi (formerly *Rhodococcus equi*) is a ubiquitous Gram-positive actinomycete and a significant pathogen in foals particularly Thoroughbred foals less than 6 months old. *P. equi* predominantly causes a pyogranulomatous bronchopneumonia in the host and *P. equi* disease is associated with significant morbidity and mortality in horse breeding farms in many parts of the world. [1-3] Virulent isolates of *P. equi* contain an 80–90 kb plasmid which encodes virulence-associated proteins such as the virulence associated protein VapA.[4] Over the years a number of vaccine candidates, some of these based upon VapA have been developed for the prevention of *P. equi* disease in foals. However, none of these vaccines have been transitioned to be commercially available.[5-10]Studies conducted using live, virulent *P.equi* have been shown to be the most effective at inducing significant protection against challenge while attenuated *P.equi* based vaccines have not been shown to be protective.[10,11] Due to safety issues however live virulent *P. equi* is not considered to be suitable for development as a vaccine. Studies have shown that while both Th1and Th2 immune responses are required to overcoming a *P. equi* infection, strong Th1 type cell mediated responses play an important role in clearing an infection caused by this pathogen .[12]

This study, describes the development of DNA and protein vaccines based upon VapA that were tested in the murine model and compared with a live virulent *P. equi* vaccine.

2. Materials and Methods

2.1 Construction of vaccines used in this study

2.1.1 DNA vaccine

The *vapA* gene was PCR amplified from *P. equi* ATCC 33701 using the forward primer 5′-GAGGATCCATGGAGACTCTTCACAAGACG-3′ and reverse primer 5′-GATGAATTCTAACAACCGAGGCTGAGCG-3′. The forward primer also contained a Kozak sequence CCATGG (start codon underlined). The PCR product was cloned into the pcDNA3 vector (Life Technologies, USA) to construct plasmid for use as a DNA vaccine. Large-scale plasmid preparations were prepared by standard methods and purified twice by CsCl gradient centrifugation and dialysed twice against TE buffer. Prior to vaccination. Endotoxin levels in the vaccine preparations were determined and shown to be less than 10 pg/ml by the QCL-1000 *Limulus* Amoebocyte Lysate Kit (BioWhittaker, USA)

2.1.2.Recombinant VapA vaccine

The *vapA* gene was amplified using PCR with the forward primer 5′-GACCATGGATGGAGACTCTTCACAAGACG-3′ and the reverse primer 5′-GCCTCGAGGGCGTTGTGCCAGCTACC-3′ for cloning into the pET-28a (+) (Novagen, USA) expression vector to create the construct for recombinant His tagged VapA vaccine preparation. VapA from this construct was expressed in *E.coli* and purified using a previously described method for the production of His-tagged proteins (Vanniasinkam et al., 2004).

2.2Vaccination of mice

Groups of five 6-8 week old BALB/c mice were injected with 50 μg of DNA vaccine (50 μl volume) into each quadriceps muscle, 50 μg DNA vaccine and 5 μg murine cytokine IL-12 expressing plasmid pORF-mIL12 (InvivoGen, USA), 50 μl aliquot of the VapA protein preparation containing a concentration of 100 μg protein, mixed with an equal volume of 1.3% aluminium hydroxide gel (Alhydrogel, Asia Pacific Specialty Chemicals Ltd, NSW, Australia), injected intraperitoneally or 10^5 live *P. equi* strain ATCC 33701 administered by the intraperitoneal route. Control group was injected with 50 μg of empty plasmid vector. This study was repeated on 3 separate occasions.

All mice were vaccinated on 3 occasions, 2 weeks apart and bled prior to every boost and just before challenge.

2.2 Detection of total IgG and subclasses IgG1, IgG2a, IgG2b

Levels of VapA specific total IgG and IgG subclasses in serum were performed using an ELISA on mouse serum diluted 1 in 250 in PBS/0.05% Tween-20 buffer using a previously described method using a His-tagged VapA antigen as the coating antigen in this assay.[9] Serum from individual mice were pooled prior to use in the assays.

2.3 Determination of DTH response

DTH response studies were performed using whole *P. equi* strain ATCC 33701 antigen preparations as described previously.[9]The right hind footpad of each mouse was injected with 20 µl of the antigen and the corresponding left hind footpad was injected with 20 µl PBS. Footpad thickness was measured at 24, 48 and 72 h intervals using Vernier calipers (the average of three readings was obtained). DTH at 24 h was used in all analyses as the reaction was most significant at this time compared with the control. Percentage swelling was calculated using the following formula: *P. equi* antigen footpad swelling (mm) – PBS footpad swelling/PBS footpad swelling×100. Experiments were repeated on three occasions.

2.4Challenge studies

Two weeks following the last immunisation, animals were challenged with an intravenous inoculation of 1.5×10^7 virulent *P. equi* ATCC 33701. Approximately 1.5 x 10^7 organisms produced mild symptoms of *P. equi* disease in the mice (ruffled coats and dull eyes). This dose was used in all the challenge studies. Bacterial clearance from the spleen and liver was determined 48 to 120 hours after challenge. Mice were euthanased by CO_2 inhalationfollowed by cervical dislocation and the spleen and liver removed. The spleen and liverwere homogenised in PBS using a sterile mortar and pestle, and a viable bacterial count was performed on dilutions of the homogenate in PBS (1 in 10^2 to 1 in 10^6 dilutions). The dilutions were plated on horse blood agar and grown at 37 °C for 48 h and the colonies were counted. Results were obtained from three separate experiments.

2.5Statistical analysis of data

Data were analysed using a Wilcoxon (rank sum) two-sample test with SAS software (SAS Institute, Inc., NC, USA).

3. Results

3.1 IgG response to vaccination

Bothe the DNA and recombinant VapA vaccines elicited a moderate total IgG response. The DNA vaccine unlike the recombinant VapA vaccine elicited a significant IgG2a response (and relatively low IgG2b and IgG1) (Table 1). This indicates a Th1 biased immune response. The immune response to the recombinant VapA vaccine was a much higher IgG1 response than with the DNA vaccine, possibly indicating a weaker Th1 type bias in immune response than that observed with the DNA vaccine. Co-administration of IL-12 did not make a significant difference to the immune response generated to the DNA vaccine. IgG1 to Ig2a ratio was greatest at 6 weeks (9.4, 2.5, 0.91, 10.8 for DNA, VapA, vector control, live *P. equi* respectively). This shows that the highest Th1 response was generated by the DNA vaccine.

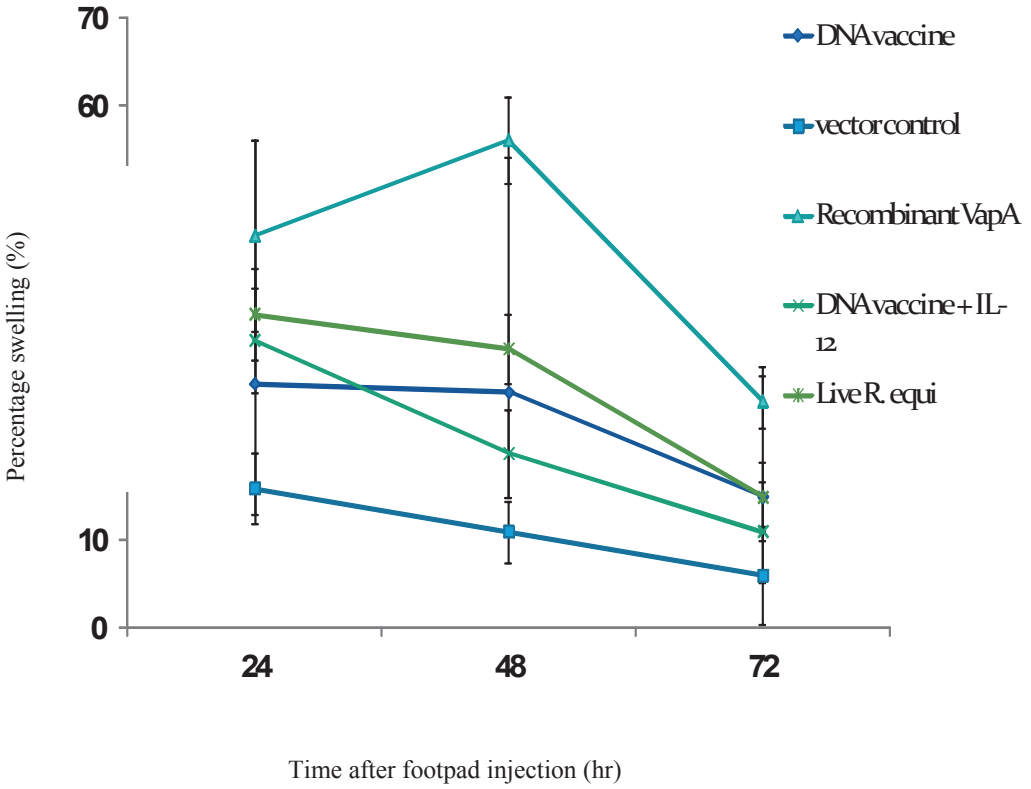
Table 1 IgG1, IgG2a and IgG2b response at 6 weeks following prime

Vaccine	IgG1 (OD 450nm) ± standard deviation	IgG2a (OD 450nm) ± standard deviation	IgG2b (OD 450nm) ± standard deviation
DNA vaccine (*vapA*)	**0.035 ± 0.02**	**0.332 ± 0.007**	**0.06 ± 0.005**
Plasmid ctrl	**0.013 ± .002**	**0.038 ± 0.02**	**0.017 ± 0.01**
Recombinant VapA	**0.586 ± .01**	**1.514 ± 0.08**	**1.35 ± 0.07**
DNA vaccine (*vapA*) + IL-12	**0.274 ± 0.05**	**0.251± 0.08**	**0.011 ± 0.003**
Live *P. equi*	**0.019 ± 0.001**	**0.206 ± 0.02**	**0.049 ± 0.01**

3.2 DTH response to vaccination

DTH responses were measured and showed that the highest DTH response was observed in mice vaccinated with the recombinant VapA vaccine (fig 1).

Fig. 1 DTH response 6 weeks after prime



3.3Challenge studies

Neither the protein nor DNA vaccine significantly enhanced clearance of *P. equi* following challenge unlike the live *P. equi*vaccine which showed a twofold reduction in *P.equi* isolated from the spleen and liver at 72 hours following challenge (data not shown).

4. Discussion and future directions

Based upon the results of this study, of the vaccine candidates tested, the *vapA*-based DNA vaccine elicited the highest Th1 response. However, while a significant Th1response was elicited by the DNA vaccine, mice that were administered this vaccine did not show enhance clearance of *P.equi* from the spleen and liver following challenge. Diasappointingly, the co-administration of the IL-12 encoding plasmid while increasing the Th1 bias of the immune response did not enhance clearance of *P. equi*. Plasmid encoded IL-12 has been used by researchers to enhance the efficacy of vaccines.[13,14] However, in this study IL-12 administration did not significantly

improve the efficacy of the vaccine. The lack of protection afforded by the vaccine candidates tested, as observed in the challenge studies, may be partly attributed to the model they were tested in. Mice have been used as the small model for vaccine studies by *P. equi* researchers for some years; various strains including BALB/c have been used.[6,10,11] However, in relation to *P.equi* vaccine research, even though they are still a widely used small animal model, there are clearly some challenges with using mice as they cannot easily be used for the evaluation of vaccines as mice are not a model that closely resembles the natural pulmonary *P. equi* infection in foals. DNA vaccines have been shown to be successful against a range of pathogens, however in this study the DNA vaccine candidate tested was not shown to be protective against challenge.[15-17]The levels of antibodies generated by the DNA and recombinant protein vaccines in this study show that these vaccine candidates can potentially be developed further as efficacious vaccines as researchers have shown that while cell mediated immune responses are important for protection against *P. equi* high titres of *P.equi* specific antibodies do play a role in providing immunity against *P. equi* induce disease.[18,19]
Overall, vaccine development for *P. equi* has not led to a commercially available vaccine to date and approaches such as viral vector based vaccine development are currently being investigated. The vaccine candidates developed in this study could potentially be used in heterologous prime boost vaccine regimes together with other vaccine modalities in future studies.

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