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Chimeric \textit{vapA/groEL2} DNA vaccines enhance clearance of \textit{Rhodococcus equi} in aerosol challenged C3H/He mice

(with 2 Figures and 1 Table)

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Abstract

*Rhodococcus equi* remains a significant bacterial pathogen, causing severe pyogranulomatous pneumonia in foals aged 1 to 3 months. There is no effective vaccine currently available for the prevention of *R. equi* pneumonia. DNA vaccines are known to offer specific advantages over conventional vaccines. The aim of this study was to evaluate three recombinant DNA vaccine candidates, namely pcDNA3-Re1, pcDNA3-Re3 and pcDNA3-Re5, combining of the *R. equi* heat shock protein GroEL2 and VapA immunogenic antigens, using C3H/He mice as an experimental model. All vaccines elicited at least a doubling of the IgG2a/IgG1 ratio in comparison to the controls, indicating a bias to the Th1 response, which is postulated to be crucial for bacterial clearance and protective immunity against intracellular pathogens. In addition, the immunised mice had a significant reduction of *R. equi* from their lungs at 7 days after the aerosol challenge. However, examination of lung pathology 14 days after the challenge showed no gross differences in pathological changes between the unvaccinated and vaccinated animals. The inconsistency in histological findings suggests that the precise level of protection against *R. equi* pneumonia in the murine model of infection may not represent a true efficacy of the potential vaccine candidates, indicating the mouse may not be the ideal non-equine model for vaccine studies.

Key words: DNA vaccines, *Rhodococcus equi*, *groEL2* and *vapA*
1. Introduction

*Rhodococcus equi* is a facultative intracellular bacterial pathogen that persists and multiplies within macrophages [1]. Its intracellular survival is considered to be necessary for the development of disease, which is characterised by chronic and sometimes severe pyogranulomatous pneumonia and lung abscesses in foals aged 1 to 3 months. *R. equi* has also become an emerging and opportunistic pathogen in immuno-compromised humans, particularly AIDS patients [2,3]. Many vaccines developed over the years for the prevention of *R. equi* infection in horses have been unsuccessful [4-7]. Current approaches in prevention of *R. equi* disease are dependent on farm management and administration of hyperimmune (HI) plasma intravenously into young foals. Interestingly, a recent evaluation of risk factors for development of *R. equi* pneumonia in foals revealed that poor farm management or a lack of attention to preventative health practices is not related to development of the disease. However, housing foals in stalls with dirt floors and high density farming of foals and dams may increase the risk of development of *R. equi* pneumonia [8]. Administration of HI plasma containing *R. equi* antibodies to foals has been shown to assist in the prevention of *R. equi* pneumonia on some endemic farms [9]. Conversely, failure of HI plasma to prevent pneumonia caused by *R. equi* has also been reported [10]. In a recent study, foals were given either HI plasma or normal equine plasma and subsequently experimentally infected with *R. equi*. Each group showed a similar level of protection against *R. equi*, even though significantly higher antibody titres against *R. equi* were seen in foals given the HI plasma [11]. Even if HI plasma is considered of having some value, the precise components and mechanisms of the plasma required for protection remain unknown. Moreover, this passive immunisation is expensive and impractical for use on a large scale.
Many virulence-associated factors have been investigated for protective immunity against *R. equi* infection. Some of these factors include extracellular enzymes (“equi factors”), virulence-associated proteins (VapA, VapC –VapH) and lipoglycans. Among these antigens, the 15 – 17 kDa VapA is essential for survival within macrophages and for development of disease in foals [12,13]. VapA, a surface-expressed lipoprotein encoded by a virulence plasmid, is considered to be the major immunogenic antigen of *R. equi* [14] and it has been the most widely investigated in vaccine studies for the prevention of *R. equi* infection [15-17]. The VapA based DNA vaccine candidates have been shown to effectively stimulate Th1 cell-mediated immune responses in mice and adult horses [15,17,18]. However, further development and modification of these vaccine candidates is needed prior to immunisation of neonatal foals.

Another potential vaccine candidate against *R. equi* infection is the *R. equi* groEL2 gene encoding the 60 kDa heat shock protein (Hsp60), which belongs to a family of molecular chaperonins that are highly conserved in all organisms [19]. Based upon previous studies in *R. equi*-related organisms containing groEL1 and groEL2 genes, expression of the GroEL2 protein appears to be much higher than that of the GroEL1 protein when the organism is subjected to environmental stresses such as high temperature [20]. In addition, the GroEL2 protein has been found to be immunodominant target of both the humoral and cellular immune responses [21].

It has been demonstrated that the N-terminal B cell epitope TSLNLQKDEPNGASDTAGQ (amino acids 62 – 81) of the VapA protein is immunodominant in foals and is a possible target for diagnostic test development [22-24]. Within this region, the 11 amino acid epitope NLQKDEPNGRA (amino acids 65 – 75) was highly recognised by sera
from foals with R. equi pneumonia [24], and was subsequently used as vaccine candidates in previous studies [17,25]. Vanniasinkam and co-workers also showed that both vapA- and groEL2-based DNA vaccines elicited a predominant Th1 biased immune response in BALB/c mice. However, none of the vaccinated mice elicited an enhanced bacterial clearance from spleens and livers after 5 days post-challenge intravenously with 1.5 x 10^7 cfu of R. equi strain ATCC33701. The lack of enhanced clearance could be due to the imperfect nature of the BALB/c mouse model. Additional reasons for this unsuccessful outcome might be the dose size of bacterial challenge, the intravenous route of the challenge and (or) the incomplete immunogenic antigen of vapA based DNA vaccine constructs that inadequately mount a cell-mediated immune response against the R. equi infection. Interestingly, a genetic mapping of murine loci revealed that BALB/c mice tend to develop a Th2 type response controlling by genetic background of T cell [26].

In this study, immunogenicity of vaccine candidates was carried out with C3H/He mice. The immunised mice were challenged with R. equi by the aerosol route, which is likely to occur in the natural infection of horses. A chimeric groEL2/vapA vaccine construct was prepared by insertion of a portion of the vapA gene encoding the immunogenic 20 amino acid-epitope TSLNLQKDEPNGRASDTAGQ or the 11 amino acid-epitope NLQKDEPNGRA into a hydrophilic region of the R. equi heat shock protein (groEL2) gene.

2. Materials and methods

2.1. C3H/He mice

4 – 6 week old ‘pathogen-free’ female C3H/He inbred mice were purchased from the Animal Resources Centre (ARC) (Canning Vale, WA, Australia) and held under barrier
conditions in a Physical Containment, Level 3 (PC3) facility (IMVS, Adelaide, Australia). A minimum of 20 mice was required for each potential vaccine candidate and controls. The experimental animals were housed 5 – 6 mice in a filtered top cage. All animals had free access to water and standard mouse food.

2.2. Bacterial strains

*R. equi* ATCC 6939 was grown in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) and used for cloning and sequencing of *groEL2*. The *R. equi* ATCC 33701 strain was grown in Columbia horse blood agar (Oxoid, Adelaide, Australia) and used as live vaccine. *E. coli* DH5α was used as a host for recombinant plasmids for cloning and preparation of *groEL2* and chimeric *vapA/groEL2* DNA vaccines. The *E. coli* bacteria were grown in Luria-broth (LB) medium or Columbia agar containing 100 μg/ml of ampicillin for propagation of ampicillin resistant transformants.

2.3. Construction of pcDNA3-Re1

The 1.625 kb *groEL2* gene was amplified by polymerase chain reaction (PCR) from DNA plasmids of the previously developed pIMVS-Re1 construct [25], using primers 5′ - ACGTACATGGGCAAAGATCATCGC - 3′ (forward) and 5′ - CTTCTAGACGGCGATGCGAAATGC - 3′ (reverse). The forward and reverse primers contain *KpnI* and *XbaI* sites (underlined), respectively. The forward primer also have a start codon ATG (in bold) and a Kozak sequence CCATGG (italicised). PCR was performed using standard conditions at an annealing temperature of 65°C using DyNAzyme™ EXT DNA polymerase (Finnzymes, Finland). The PCR product was cloned into the plasmid vector pcDNA3
(Invitrogen, Abingdon, USA) and designated as pcDNA3-Re1. The recombinant plasmid pcDNA3-Re1 was transformed into E. coli DH5α for further preparation of DNA vaccine.

Construction of the plasmid pIMVS-Re1 was previously described [25]. Briefly, the groEL2 gene was PCR-amplified from chromosomal DNA of R. equi ATCC 6939, using primers 5’ - CAAGGAGGTCGAGAGACCAAGG - 3’ (forward) and 5’ - GTGCCCGGATCTTTGAC - 3’ (reverse). The 402 bp PCR product was used as a probe in a Southern hybridisation to identify a single SphI fragment of approximately 4.7 kb in size. Sequences of the 4.7 kb fragment was determined using ABI Prism DNA sequencing kit (Perkin Elmer, Foster City, CA). The fragment was purified and cloned into the plasmid pGEM-7zf (-) vector and designated as pIMVS-Re1.

2.4. Construction of pcDNA3-Re3

A portion of the vapA gene encoding the 11 amino acid-epitope NLQKDEPNGRA of the virulent R. equi ATCC 33701 VapA protein was the region of interest to be inserted into the R. equi heat shock protein groEL2 gene. Standard site-specific mutagenesis by overlap extension PCR as described by Sambrook and Russell [27] was used. Construct pcDNA3-Re1 was used as a template in the initial two PCR reactions.

The primers used in the initial PCR were 5’ - AACCTTCAGAAAGACGAACCGAACGGTCGAGCGAGCGTCAGGAAGCGGCTCTCG - 3’ designated GVIF (sequence corresponding to the VapA epitope to be inserted is underlined) and 5’ - CTATAGAATAGGGCCCTCTAGACGG - 3’ designated GVOR. Additional PCR was performed using primers GVIR with sequence 5’ -
TGCTCGACCCTCGGATTCTCAGTGTTCGAGTTCGCTCGGTCGCTCGAAGTACACGCG - 3’ (sequence corresponding to the VapA epitope to be inserted is underlined) and GVOF 5’ - GAGACCCAAGCTTGTGACCAGTGG - 3’. The PCR products obtained from both reactions were separated on a 1% agarose gel and purified using the QIAquick gel purification kit (Qiagen, GmbH, Germany). Approximately, 100 ng of each of the PCR products were used as the template in the final PCR reaction which was performed using the primers GVOF and GVOR containing restriction endonuclease KpnI and XbaI cleavage sites (italicised), respectively. All PCR reactions were performed using standard PCR conditions at an annealing temperature of 59°C. The final PCR product and pcDNA3 vector were digested separately with KpnI/XbaI and ligated together using method of Sambrook and Russell [27]. The construct was designated as pcDNA3-Re3 and was transformed into E. coli DH5α for vaccine preparation.

2.5. Construction of pcDNA3-Re5

Similarly to the construction of pcDNA3-Re3, the pcDNA3-Re5 candidate was constructed by inserting a portion of the vapA gene encoding the 20 amino acid-epitope TSLNLQKDEPNGRASDTAGQ into the R. equi heat shock protein groEL2 gene. To obtain this larger epitope, a pcDNA3-Re4 construct containing half (TSLNLQKDEP) of the entire epitope was developed first and subsequently used as a DNA template for further construction of the chimeric pcDNA3-Re5 containing the entire 20 amino acid epitope.
The *pcDNA3-Re4* construct was made by PCR-amplification of the purified plasmid *pcDNA3-Re1*, using two pairs of primers in two separate PCR reactions. The primers used in the first PCR were RMG/VN1 with sequence 5’-ACGCTCCGGTTCGTCTTTCTGAAGTTAAGGAATGGCCGTCGGTCGCG-3’ (sequence corresponding to the VapA epitope to be inserted is underlined) and FGD with sequence 5’-ACGGTACCAGCCAAGATCATCGC-3’ containing a start codon (in bold), Kozak sequence and a site recognised by restriction endonuclease *KpnI* (italicised). The second PCR was performed using primers FMG/VN1 with sequence 5’-GACGCCACTTCGTAAACCTTCAGAAAGACGAACCGGAGCGTCAGGAAGC-3’ (sequence corresponding to the VapA epitope to be inserted is underlined) and RGD with sequence 5’-CGTCCTAGAGAAGGATCCATGCAGCC-3’ containing a stop codon (in bold) within a site recognised by restriction endonuclease *XbaI* (italicised). In the final PCR, the forward FGD and reverse RGD primers were used to amplify the full-length mutant *groEL2*. An annealing temperature of 65°C was used in PCR amplifications for both *pcDNA3-Re4* and *pcDNA3-Re5* constructs. The final PCR product and pcDNA3 vectors were digested separately with *KpnI/XbaI* and ligated together as described previously. The construct designated as *pcDNA3-Re4* was transformed into *E. coli* DH5α for plasmid selection and DNA sequencing.

To construct the *pcDNA3-Re5*, the remaining portion of the epitope (NGRASD TAGQ) was PCR-amplified from a purified preparation of plasmid *pcDNA3-Re4* used as a template. Primers used for the construction of *pcDNA3-Re5* were the same as those for the construction of *pcDNA3-Re4* with the exception of the following primers. The primer RMG/VN2 with sequence 5’-ACGCTCTTGCCCGGCGGTATCGCTTGCTCGACCGTTCGGTTCGTCTTTCTG-3’ was used instead of RMG/VN1 in the first PCR. In the second PCR, the forward mutagenic
primer FMG/VN1 was replaced with primer FMG/VN2 5’-GAACCGAACGTCGAGCAAGCGATACCGCCGGCAGACGTCAGGAAGC-3’. The full-length mutant *groEL2* containing the portion of the *vapA* gene that corresponds to the epitope TSLNLQKDEPNGRASDTAGQ was amplified in the third PCR as described above. Restriction digests of the final PCR product and pcDNA3 vectors using *KpnI/XbaI* combination were ligated together and transformed into *E.coli* DH5α for plasmid selection and DNA sequencing. This DNA construct was designated as *pcDNA3-Re5* and subsequently purified and used for vaccine preparation.

2.6. **PCR amplification of chimeric groEL2/vapA for gene sequencing**

Fragments of restriction digests were purified and PCR-amplified for gene sequencing. The forward primer used was 5’ - GCTGCTCGACACCGCCAAGGAG - 3’ and reverse primer was 5’ - TGTGCCTTGCGGCGGTCACCGA - 3’. The amplification procedure was carried out using an annealing temperature of 60°C. A small amount of the amplified PCR product was analysed by electrophoresis on 1% agarose gels. The remaining PCR products were purified using QIAquick® PCR purification kits (Qiagen, GmbH, Germany) and stored at -20°C until required for gene sequencing and analysis.

2.7. **DNA sequencing and analyses of chimeric groEL2/vapA constructs**

Sequencing of PCR products and plasmid constructs was performed using ABI PRISM DNA sequencing kits (Perkin Elmer, Foster City, CA). A sequencing reaction was prepared in a total volume of 20 μl by addition of 4 μl of Big Dye Terminator Version 3 mix, 1 μl (3.3
pmoles) of a specific forward or reverse primer, 2 μl (10 ng) of purified DNA template and 13 μl of sterile water. Thermal cycle for sequencing consisted of 25 cycles of 30 sec at 96°C, 15 sec at 50°C and 4 mins at 60°C. A soak file of 4°C was used for maintaining the extension products after cycling. Prior to DNA sequencing, the extension PCR products were purified by the isopropanol method described by the ABI Prism® protocol (PE Biosystems, USA). Each extension reaction was precipitated with 80 μl of 75% isopropanol and an addition of 1 μl of glycogen (Roche, Mannheim, Germany) for forming a visible pellet. The precipitation was incubated at room temperature for 15 mins, followed by centrifugation at 12,000 × g (Eppendorf centrifuge 5415D, Germany) for 30 mins. The supernatant containing unincorporated dye terminators was discarded completely. The pellet was washed twice with 250 μl of 75% isopropanol by centrifugation for 5 mins and vacuum dried. The purified extension products were then sequenced on an ABI 373A DNA sequence analyser (Applied Biosystems, USA) in the Division of Molecular Pathology, IMVS, Adelaide. Analysis of nucleotide sequences was performed using GeneBase version 1.0 (Applied Maths, Kotrijk, Belgium) and nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.8. Preparation of DNA vaccines

The procedure of plasmid DNA preparation was carried out by following precisely the protocol of the “Endofree® Plasmid Mega Kit” (Qiagen, GmbH, Germany) and using the buffers provided by Qiagen. The DNA eluted from the column was precipitated with isopropanol, rinsed with endotoxin-free 70% ethanol and vacuum dried. The “endotoxin-free” plasmid DNA was redissolved in a suitable volume of endotoxin-free TE buffer prior to preparation of DNA vaccines. Endotoxin levels were measured using the QCL-1000 limulus
amoebocyte lysate kit (Bio Whittaker, MD, USA). According to the protocol supplied by Qiagen, 1 ng of LPS corresponds to 1 – 10 EU (endotoxin unit), and DNA purified using EndoFree Plasmid Kits that contained less than 0.1 EU/μg yielded over 100% of the transfection efficiency. Amount of endotoxins in DNA vaccines in this study was approximately 10 EU/mg, which was considered to be very low by the manufacturer.

Prior to vaccine use, the plasmid DNA was prepared for intramuscular injection using a standard method described by Lakritz and co-workers [28]. The purified plasmid DNA was added with NaCl to a final concentration of 0.1 M, followed by the addition of 2 volumes of absolute ethanol and incubation at –20°C for 30 mins. The precipitated DNA was harvested by centrifugation at 12,000 × g (Eppendorf centrifuge 5415D, Germany) for 20 mins. The DNA pellet was rinsed, dried as before and resuspended in the sterile endotoxin-free injectable PBS to a concentration of 1 mg/ml. The vaccine preparations were stored at –20°C until required for immunisation.

2.9. Preparation of R. equi for use as live vaccine

*R. equi* strain ATCC33701 was prepared for infection of mice using previously described method with some modifications [29]. The strain was grown on Columbia horse blood agar plates for 48 hrs at 37°C. The bacteria were suspended in sterile endotoxin-free PBS to obtain an OD of approximately 0.6 – 0.7 at 600 nm. This suspension contained approximately $1 \times 10^9$ *R. equi* /ml. The suspension was diluted 1 in 1000 in the PBS to obtain a final concentration of approximately $1 \times 10^6$ cells/ml. Approximate numbers of bacteria were confirmed in retrospect by plating an aliquot of the inoculum onto CHBA agar just prior to inoculating the mice. Counting of bacterial colonies was performed after 48-hrs incubation at 37°C.
2.10. **Vaccination of mice**

Groups of mice were vaccinated with vaccine candidates 3 times at 2-week intervals. Two weeks after the final vaccination, each group of mice was challenged with approximately $1\times 2 \times 10^9$ virulent *R. equi* ATCC 33701 via the aerosol route, using a nebuliser pump therapy kit (Ventalair forte II, Allersearch, Australia). One animal in each group was sacrificed to determine the retained dose of bacteria in the lungs immediately after the challenge. Bacterial clearance in the lungs of vaccinated mice ($n = 4$/group) was measured at days 3, 5 and 7 after challenge with *R. equi*. Isolates from these mice were identified and confirmed by DNA sequencing of the 16S rRNA gene. Serum samples were collected and pooled within each group prior to each immunisation, bacterial challenge and at day 5 after of the challenge. Sera were used in detection of antibody and cytokine responses. At 5 days post-challenge, three mice in each group were sacrificed for measurement the production of cytokines IFN-$\gamma$ and IL-4 in their sera, spleens and lungs. The remaining animals (minimum of 5 mice/group) in all groups were euthanased at day 14 after challenge for examination of lung pathology. Blood was collected from each mouse by retro–orbital bleeds using 75 × 1.2mm micro haematocrit tubes (Vitrex, Modulohm A/S, Denmark).

2.11. **DNA vaccination**

Each group of mice was vaccinated separately with pcDNA3–Re1, pcDNA3–Re3 and pcDNA3–Re5 DNA vaccine candidates. Each mouse was immunised intramuscularly with 100 $\mu$g (100 $\mu$l) of an appropriate DNA vaccine into hind quadriceps muscle.
2.12. Live *R. equi* vaccination

Groups of mice were vaccinated with sub-lethal doses of live *R. equi* for comparison with the vaccine candidates. Each animal was vaccinated with approximately $1 \times 10^5$ live *R. equi* strain ATCC33701 (100 µl of $1 \times 10^6$ *R. equi/ml) via intraperitoneal route.

2.13. Vaccination of control animals

Control groups of mice were vaccinated with pcDNA3 vector or endotoxin-free PBS. Each mouse in the PBS group was administered with 100 µl of PBS via intraperitoneal route. Animals in the other control group were vaccinated intramuscularly with 100 µl (100 µg) of purified preparation of pcDNA3.

2.14. Detection of total IgG and subclasses IgG1, IgG2a and IgG2b

Determination levels of IgG and its subclasses were performed using ELISA based upon His-tagged proteins. Briefly, Nunc™ maxisorp 96-well plates were coated with 100 µl per well of a purified His-tagged protein at concentration of 20 µg/ml in 50 mM coating buffer, pH 9.6 (16 mM Na$_2$CO$_3$ and 34 mM NaHCO$_3$). The plates were incubated overnight at 4°C and then blocked with 1% casein/PBST at room temperature for 1 hr. The plates were washed five times with PBS containing 0.05% Tween 20 (PBST) using an automatic ELISA washer (Ultrapwash Plus™, Dynex Technologies Inc., Chantilly, VI, USA). After the washing, excess buffer was removed from the plates by slapping the plates (well side down) on a clean towel. Serum samples were diluted 1 in 250 in PBST containing 0.20 mg/ml of *E. coli* extract (Promega, Madison, WI, USA) for background reduction and incubated at room temperature for 30 mins. A volume of 100 µl of the diluted serum was added into each well
and incubated overnight at 4°C. The washing step was repeated prior to addition of appropriate diluted horseradish peroxidase-conjugated anti-species antibody in 1% casein/PBST with a volume of 100 μl per well. The plates were incubated at room temperature for 1 hr, followed by the washing procedure and additional three washes with PBS alone. The presence of peroxidase was detected by addition 100 μl of freshly prepared chromogenic substrate solution (Tetramethylbenzidine (TMB) in phosphate-citrate buffer, pH 5.0) to each well and incubated for 15 mins at room temperature and protected from light. The resulting colour reaction was stopped by addition 100 μl of 0.5 N H2SO4 into each well. Optical density (OD) values were read on a MR7000 ELISA plate reader (Dynatech laboratories, USA) using a test wavelength of 450 nm and a reference wavelength of 630 nm.

2.15. Induction of IFN-γ and IL-4

Production of mouse interferon gamma (IFN-γ) and interleukin 4 (IL-4) in immunised mice was measured at day 5 after the challenge, using the Quantikine® mouse immunoassay kits (R&D Systems Inc., Minneapolis, MN, USA). The assay was based upon the quantitative sandwich ELISA technique and was performed according to the provided protocol. All reagents including standards, controls and antibodies were provided in the commercial kits. A monoclonal antibody specific for mouse IFN-γ or IL-4 has been pre-coated onto microtitre-plates. Sera, cell culture supernatants, controls and serial dilutions of standards were added into the wells. Any unbound substances were washed off prior to addition of a polyclonal antibody specific for mouse IFN-γ or IL-4. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction
was stopped by adding the stop solution as supplied with the kit. Optical density (OD) values were measured on a MR7000 ELISA plate reader (Dynatech laboratories, USA) using a test wavelength of 450 nm and a reference wavelength of 570 nm. The sample values were then read off the standard curve.

2.16. Statistical analysis

Statistical analyses were performed by using an interactive GraphPad Software (http://graphpad.com/quickcalcs/ttest1.cfm) developed by Associate Professor John C. Pezzullo, Department of Pharmacology and Biostatistics, Georgetown University, Washington DC, USA. Data were analysed using Unpaired Student’s t-test. Resulting $P$ values of less than 0.05 were considered significant.

3. Results

The vaccine candidates were evaluated for the effectiveness based upon their abilities in the clearance of $R.\ equi$ from the lungs in immunised C3H/He mice, induction of cell-mediated immune responses and prevention of $R.\ equi$ pneumonia by means of pathological changes of lung tissues. All of the chimeric DNA vaccine candidates were confirmed as correct by DNA sequencing as previously described.

3.1. Bacterial clearance in the lungs

All of the mice immunised with $pcDNA3-\text{Re1}$, $pcDNA3-\text{Re3}$, $pcDNA3-\text{Re5}$ and live $R.\ equi$ showed a significant clearance of $R.\ equi$ in the lungs at 7 days after the challenge with average bacterial counts of approximately $575 \pm 35.36$, $320 \pm 28.28$, $400 \pm 0.0$ and $100 \pm 0.0$,
respectively (Fig. 1). In contrast, average bacterial counts in the controls were 805 ± 7.07 (pcDNA3 vaccinated mice) and 1250 ± 212.13 (PBS treated mice). Only mice vaccinated with live *R. equi* were able to significantly eliminate the *R. equi* infection at day 5 after the challenge. No significant clearance was obtained in any of the vaccinated mice at 3 days post-challenge.

### 3.2. Induction of IgG, IgG1, IgG2a and IgG2b antibodies

Sera from four groups of mice vaccinated with the *pcDNA3-Re1* DNA vaccine construct produced the highest level of total IgG with an average OD reading of 0.787 ± 0.266 (Table 1). Whilst, mice immunised with the *pcDNA3-Re 3* and *pcDNA3-Re5* vaccines induced the total IgG with average OD readings of 0.494 ± 0.194 and 0.569 ± 0.295, respectively. However, moderately high levels of IgG2a in comparison to IgG1 and IgG2b antibodies were detected in all mice vaccinated with these promising vaccine candidates, indicating a bias towards a Th1 type response.

The ratio of IgG2a/IgG1 antibodies induced by both *pcDNA3-Re1* and *pcDNA3-Re5* vaccines was approximately fourfold higher than that generated by the pcDNA3 control. Nonetheless, there was no significant difference in immune responses between the three chimeric *groEL2/vapA* DNA vaccine candidates. In addition, all of the vaccinated mice significantly (*P < 0.05*) induced higher levels of antibodies than the control groups of mice vaccinated with pcDNA3 vector or treated with endotoxin-free PBS. These results suggest that the vaccine efficacy of *pcDNA3-Re1*, *pcDNA3-Re3* and *pcDNA3-Re5* should be further investigated.

### 3.3. Induction of murine IFN-γ and IL-4 cytokines
IFN-γ has been shown to promote the production of IgG2a (Th1 biased response) whereas IL-4 promotes the production of IgG1 and IgG2b (Th2 biased response) [30]. In this study, the IFN-γ levels measured in sera from mice immunised with pcDNA3-Re1, pcDNA3-Re3 and pcDNA3-Re5 DNA vaccines were twice higher than that of the control mice. However, splenocytes and lung cells from these vaccinated mice secreted very low levels of both IFN-γ and IL-4 (lower than limit of assay sensitivity) (data not shown). The moderate levels of IFN-γ in pooled sera (n = 14) of the immunised mice at 5 days after the challenge was correlated with higher levels of IgG2a than IgG1, indicating a bias towards a Th1 cell-mediated immune response. It was not surprising that IL-4 response was undetectable since production of the serum IgG1 and IgG2b were much lower than IgG2a in all of the immunised and control mice, indicating a weaker Th2 and stronger Th1 biased response.

3.4. Lung pathology

Examination of lung tissues at 14 days after infection with R. equi showed no gross differences in the mononuclear cell composition and distribution in the lung between the individual groups. Mice (n = 8/group) immunised with pcDNA3 vector and PBS presented with pathology similar to that of R. equi exposed lungs and characterised by multifocal hyperplasia of BALT and perivascular lymphocytic cuffing (Fig. 2.1) in 5 and 6 animals in each group, respectively. No pathological changes (apparently normal) (Fig. 2.2) were seen in the remaining animals. Mice (n = 13) that were vaccinated with pcDNA3-Rel showed normal lung tissues in five mice while eight mice showed mild hyperplasia of BALT and perivascular lymphocytic cuffing. Similarly, mice (n = 13) that were immunised with pcDNA3-Re3 showed no pathological changes in three mice whereas ten mice had mild hyperplasia of BALT and perivascular lymphocytic cuffing. Mice (n = 14) that were
vaccinated with pcDNA3-Re5 showed normal lungs in five mice and very mild perivascular lymphocytic cuffing in the remaining mice.

4. Discussion

The present study was supported by the previous finding [17] that only the live R. equi vaccinated mice was found to significantly eliminate the bacteria at day 5 after the challenge. After 7 days post-challenge, significant differences in R. equi clearance from the lungs were observed between the mice immunised with pcDNA3-Re1, pcDNA3-Re3, pcDNA3-Re5 and live R. equi in comparison to the controls which were vaccinated with pcDNA3 vector and PBS (Fig. 1). Among these vaccine candidates, pcDNA3-Re5 and live R. equi vaccines elicited the most significant difference ($P \leq 0.0005$) of clearance in comparison to the control mice vaccinated with pcDNA3. The pcDNA3-Re5 vaccine is the chimeric DNA vaccine containing the immunogenic 20-mer N-terminal B cell epitope TSLNLQKDEPNGRASDTAG of the R. equi VapA protein that has been shown to be highly recognised by sera from foals with R. equi pneumonia [22]. An important observation was that mice vaccinated with pcDNA3-Re1 and pcDNA3-Re5 elicited an almost four-fold higher ratio of IgG2a/IgG1 than the controls and live R. equi-vaccinated mice. Although mice vaccinated with pcDNA3-Re3 produced a lower ratio of IgG2a/IgG1 (approximately 2.54), moderate levels of IgG2a (0.386) and IFN-$\gamma$ were obtained in this group of animals. The enhanced production of IgG2a is considered to reflect the IFN-$\gamma$ response, which is associated with Th1 cell-mediated immunity [31]. A Th1 cell-mediated immune response is known to be crucial for a complete bacterial clearance and is able to provide protective immunity against intracellular bacterial pathogens including R. equi infection, whereas a Th2 response may result in disease development [32]. However, none of these DNA vaccines
(pcDNA3- Re1, pcDNA3-Re3 and pcDNA3-Re5) enhanced clearance of *R. equi* at 5 days after the aerosol challenge even though they appeared to elicit a response biased towards Th1. This could possibly be due to an insufficient humoral response generated by the DNA based vaccines during the initial infection since several studies suggest that both cellular and humoral immune responses are important in protective immunity against *R. equi* infections [33,34].

The IgG2a/IgG1 ratio of the live *R. equi* immunised C3H/He mice was approximately equal to one, indicating an almost equally mixed Th1/Th2 response. Surprisingly, the DNA vaccines appeared to produce a much stronger Th1 type response based upon the IgG subclasses than the live vaccine. However, the bacterial clearance was somewhat inferior in the group of DNA vaccines immunised mice than that of the live *R. equi* based vaccine (Fig. 1). Although the live *R. equi*-based vaccine showed an enhanced clearance on day 5 after the challenge, the clearance may be incomplete because the bacteria were able to eventually recolonise the lung and cause *R. equi* pneumonia at 14 days post-challenge when pathology studies on the lungs were carried out. These animals showed pathology similar to that of *R. equi* exposed lungs in foals characterised by hyperplasia bronchial-associated lymphoid tissue (BALT) with inflammatory of lymphocytes (Fig. 2.1). This may have resulted from persistent bacteria in tissues that may have been inaccessible to the antibodies. In this regard, this data may support some other studies that antibodies (humoral immunity) may only play a significant role in the elimination of the intracellular bacteria during the initial of infection. Whilst, cellular immunity (T cells) is required for a complete clearance and considered to be more crucial in host defences against intracellular pathogens [35,36].
Data from mouse studies has demonstrated that mice that develop CD4\(^+\) Th1 response with IFN-\(\gamma\) production are able to clear a virulent challenge whereas mice that generate CD4\(^+\) Th2 response with production of IL-4 develop pulmonary lesions [37,38]. In this study, all of the mice immunised with the \(pcDNA3-Re1\), \(pcDNA3-Re3\) and \(pcDNA3-Re5\) vaccines elicited higher levels of IgG2a antibodies than IgG1 and IgG2b, indicating a predominant Th1 response. However, these vaccinated mice only showed production of IFN-\(\gamma\) in sera but was not secreted by lung cell and splenocyte cultures. Some studies have also suggested that B6 and C3H mice generally tend to produce more of a Th1 response against infectious agents such as \(Toxoplasma gondii\) [39] or to peptide antigens [40] whereas BALB/c mice tend towards a Th2 response [41]. A Th1-biased response against \(R. equi\) infection shown in the immunised mice in this study that may support these studies is also a question.

Examination of lung pathology (14 days post-challenge) showed no gross differences in the mononuclear cell composition and distribution between the control animals vaccinated with \(pcDNA3\) vector or treated with PBS and the immunised mice with \(pcDNA3-Re1\), \(pcDNA3-Re3\) or \(pcDNA3-Re5\) vaccines. Although the presence of multifocal bronchial-associated lymphoid tissue (BALT) hyperplasia and perivascular lymphocytic cuffing were observed to be higher in the control mice, these results represented very small numbers of animals. Larger numbers of mice or a different animal model may be used in further evaluation of these genetic vaccines. It would also be interesting to find out if co-administration of IL-12 as adjuvants in the DNA immunisation improves the efficacy of these potential vaccine candidates since several studies have shown an enhanced Th1 immune response upon co-administration of an IL-12 expression plasmid with DNA vaccines [17,40,42]. IL-12 is an important cytokine involved in the initiation and maintenance of cellular responses, which is required for protection against \(R. equi\) [43].
Inconsistencies in the pathological changes of the control mice were possibly due to the natural resistance of mice to virulent \textit{R. equi} infection. In contrast, foals are known to be more susceptible to \textit{R. equi} infection. Thus, the immune responses associated with effective pulmonary clearance of \textit{R. equi} in mice may not mimic that in foals. In addition, the precise extent of protection against \textit{R. equi} pneumonia in mice using these vaccine candidates may not represent their true efficacy in foals.

In conclusion, the vaccine studies performed have raised a serious question about the suitability of the non-equine animal model for \textit{R. equi} infection. To obtain more reliable results of the vaccine efficacy, very large numbers of mice or a different animal model may be required to represent their true effectiveness as it may be in foals. Co-administration of an IL-12 expression plasmid with DNA vaccines should also be performed to enhance the cellular responses, which is required for protection against \textit{R. equi} infections.
Fig. 1. Average counts of *R. equi* in the lungs of immunised mice at days 3, 5 and 7 after the bacterial challenge. The values are shown as means ± SD.

Each coloured bar represents an average count of four mice from a specific group of vaccination. The asterisks indicate statistically significant differences: * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.0005$ in comparison to the control mice vaccinated with pcDNA3 vector.
Table 1

Production of immunoglobulin subclasses induced by mice vaccinated with the DNA vaccine candidates.

<table>
<thead>
<tr>
<th>Vaccines and controls</th>
<th>Ave. ELISA ODs of antibodies induced in pooled sera* (diluted 1:250) from immunised C3H/He mice</th>
<th>Ave. ratios of Th1/Th2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total IgG</td>
<td>IgG1</td>
</tr>
<tr>
<td>pcDNA3 vector</td>
<td>0.363 ± 0.208</td>
<td>0.198 ± 0.124</td>
</tr>
<tr>
<td>PBS</td>
<td>0.103 ± 0.013</td>
<td>0.089 ± 0.048</td>
</tr>
<tr>
<td>Live R. equi</td>
<td>1.813 ± NA</td>
<td>1.727 ± NA</td>
</tr>
<tr>
<td>pcDNA3-Re1</td>
<td>0.787 ± 0.266</td>
<td>0.346 ± 0.447</td>
</tr>
<tr>
<td>pcDNA3-Re3</td>
<td>0.495 ± 0.195</td>
<td>0.169 ± 0.097</td>
</tr>
<tr>
<td>pcDNA3-Re5</td>
<td>0.570 ± 0.295</td>
<td>0.225 ± 0.226</td>
</tr>
</tbody>
</table>

* Results represent mean OD ± SD of pooled sera from 4 separate experiments
Fig. 2. Haematoxylin and eosin (H & E) staining of murine lung tissue, using magnification × 580. (1): A similar R. equi exposed lung pathology as seen in horses characterised by focal pyogranulomas lesions characterised by aggregated epithelioid macrophages and multinucleated giant cells (indicated by arrows), admixed with numerous polymorphonuclear lymphocytes (PMN); (2): Normal lung tissue characterised by thin alveolar walls with dilated clear alveolar space.
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