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Comparison of the efficacies of *Rhodococcus equi* recombinant vaccine in mice

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Abstract

Rhodococcus equi is an important bacterial pathogen and causes severe chronic granulomatous pneumonia in foals below 6 months of age. It has also become an opportunistic and emerging pathogen in immunocompromised humans. Vaccination is the most cost-effective strategy for controlling and preventing this infection. Although several potential virulence genes and candidate immunogens have been identified over the years, no effective vaccine is currently available to prevent *R. equi* disease in horses. Recently, bacterial vector vaccines have been shown to be promising for *R. equi.* In this study, the *virulence-associated protein A (VapA)* gene of *R. equi* was cloned into Protein Expression System small ubiquitin-related modifier (pET-SUMO) expression vectors and transferred into *Escherichia coli* BL21 (DE3). Also, adjuvant significantly affects the efficacy of recombinant vaccines. Therefore, native VapA and recombinant VapA were formulated with Immunostimuling Microparticle System (IMS 3012) or PetGel A (recommended for horses) and subcutaneously administered to mice. The immunization effect of four different vaccines was determined by assaying antibody titers and survival rates. The antibody response was slightly higher in the PetGel A formulations than IMS 3012. Survival rates were lower in the PetGel A formulations than IMS 3012. Given these results, recombinant VapA adjuvanted with PetGel A represents a promising formulation for developing new-generation *R. equi* vaccines.

Introduction

Rhodococcus equi is a Gram-positive, non-motile, obligate aerobe, and intra-cellular pathogen for macrophages, and soil saprophytic bacterium. The *R. equi* causes severe pyogranulomatous bronchopneumonia, polysynovitis, and sudden death in foals below six months of age.[1,2] Due to the high morbidity and mortality, it is a significant problem of global importance for the equine industry.[3] Additionally, foals recovering from *R. equi* infection are less likely be suitable for race when become adult. The *R. equi* significantly impacts the equine industry through the cost of medicine and death of foals. Also, long-term antibiotic treatment for *R. equi* infection can be ineffective.[4,5] The *R. equi* is also considered an emerging and opportunistic infection in immunocompromised humans. Immunocompromised (80.00%) and immunocompetent (30.00%) individuals having *R. equi* often present with pulmonary infections.[6] Notably, it can cause various complications in humans, primarily cavitary pneumonia, pulmonary infections, lung abscess, lung tumors, lung infections, bacteremia, infective endocarditis, and meningitis.[7] The *R. equi* infection in humans was first reported in 1967 in a man with hepatitis receiving immunosuppressant medications.[8] For these reasons, it is now emerging as an important opportunistic pathogen of humans.

The pathogenicity of *R. equi* is associated with plasmid-encoded virulence-associated protein A (VapA). The pVapA is a virulent type associated intimately with pulmonary infections in foals and humans. [9] Since rapid diagnosis in foals is often quite difficult, the antibiotic treatment for rhodococcal pneumonia is delayed and long, or results may not be obtained.[10]

Morbidity and mortality rates vary among farms, and this difference is reported to be due to the combination of environmental and management conditions, and the virulent strains being present in each farm. Virulent isolates carry a *VapA* containing 15.00 to 17.00 kDa lipoprotein A, and have been isolated from farms with endemic situations.[11,12]

The *R. equi* vaccines efficiently preventing illness would be commonly used and have a significant impact on morbidity and mortality. However, *R. equi* conventional vaccines (killed, live, and attenuated vaccines) have proved to be ineffective due to the immaturity of the immune system of the foals.[13] Recently, bacterial vector

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vaccines cloning the *VapA* gene have been shown to be promising for *R. equi* in the mouse model.

Escherichia coli vector vaccines produce the highest levels of soluble protein, and can induce an immune response.[14] This study aimed to develop a method to express recombinant VapA (rVapA) in a soluble and active form (using Protein Expression System small ubiquitin-related modifier (pET-SUMO) vector system in *E. coli*) containing the *R. equi VapA* gene. In addition, the adjuvant significantly affects the efficacy of vaccines. The PetGel A and Montanide Immunostimuling Microparticle System (IMS 3012) are different water-based and recommended adjuvants for sensitive animals, including horses. Both adjuvants induced a suitable antibody response for resistance to *R. equi* infection in horses. In addition, PetGel is recommended for horses, and it has been proven to have the best immunopotentiation capacity.[15,16] Therefore, the rVapA protein was formulated with PetGel A and Montanide IMS 3012 adjuvants in the current study.

Consequently, the aim of this study was to investigate the *E. coli* vector system expressing the VapA as an immunogen in a mouse model. The immunization effect of VapA was determined by assaying antibody titers, and morbidity and mortality rates. Recombinant vaccine candidates were developed for use as horse vaccines and tested for effectiveness in a mouse model.

Materials and Methods

Bacterial strain and validation of *VapA* gene by polymerase chain reaction (PCR). In this study, Veterinary Medicine, Selcuk University Culture Colletion (SUVF 185) strain of *R. equi*, an isolate from a pneumonic foal, was obtained from the culture collection of the Veterinary Faculty of Selcuk University, Konya, Türkiye. The *R. equi* genomic DNA extraction was purified using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer.

Amplification and cloning of the *VapA* gene. The VAPA-F (TCACCAGCTACCAGAGCC) and VAPA-R (ATGAAG ACGGTTTCTAAGGCGAT) primer pairs were designed upon the available genome sequence in the National Center for Biotechnology Information (NCBI) GenBank Database (Accession No. KT443895) and used to synthesize 543-bp VapA open reading frame region. The reactions for PCR amplification used DNA purified from the *R. equi* as a DNA template. Each PCR reaction included 2.50 µL PCR buffer (with KCl), 3.50 mM $MgCl_2$, 0.20 mM dNTP, 1.00 mM of each primer, 1.00 U of Taq DNA polymerase, and 1.00 U 5.00 ng μL^{-1} template DNA; 14.90 µL ultra-pure water was added to obtain a final volume of 25.00 µL. The PCR amplification was performed under the following conditions: A primary denaturation step at 94.00 ˚C for 2 min, 40 repeated cycles starting with denaturation step at 94.00 ˚C for 90 sec, annealing at 57.00 ˚C for 60 sec, and 2 min at 72.00 ˚C as an extension step followed by a final extension step at 72.00 ˚C for 7 min. The samples were subjected to electrophoresis in a 1.50% (w/v) agarose gel (Promega, Madison, USA) in the 1.00 X tris-borate-ethylene-diaminetetraacetic acid (EDTA) buffer (40.00 mM Tris, 20.00 mM boric acid, and 1.00 mM EDTA; pH: 8.30), stained with 6.00 X loading dye, and visualized under ultra-violet illumination using a Gel Image Analysis System with a 100-bp DNA ladder (Sigma, Deisenhofen, Germany). The PCR product was then purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). The purified product was then ligated to the pET-SUMO vector and transformed by One Shot®Mach1™-T1R (Invitrogen, Waltham, USA) and *E. coli* BL21 (DE3) competent *E. coli* cells using a heat shock method.

Expression and production of rVapA. The recombinant colony from the overnight culture was inoculated and incubated overnight into the 25.00 mL of tryptone-yeast medium with 50.00 µg mL^{-1} kanamycin at 37.00 ˚C; after that, 0.50 mL of overnight culture was incubated in tryptone-yeast medium supplemented with kanamycin (50.00 µg mL^{-1}) at 37.00 ˚C and at 250 rpm until the optical density reached 600 (approximately 2 hr). The protein expression was induced with 1.00 M isopropyl ß-D-1-thiogalactopyranoside to a final concentration of 0.10 mM. After induction by isopropyl β-D-1-thiogalacto-pyranoside, the recombinant bacterial cells were collected by centrifugation, and the rVapA was purified using Ni-NTA Spin Kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. The rVapA and native VapA (nVapA) were concentrated using a Membrane Cassette System (Polyethersulfone, 10022213, Sartorius Stedim Slice200).[17]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of VapA. The rVapA was evaluated by SDS-PAGE.[14,15] The recombinant protein samples were separated by running on a 12.00% SDS-PAGE. For SDS-PAGE, one gel fixed (40.00% (v/v) methanol and 7.00% (v/v) acetic acid), and stained with 0.25% (w/v) Coomassie Blue. The other gel was transferred to a 0.20 µm cellulose nitrate membrane in a Semi-dry Transblot System (Bio-Rad Mini Trans-Blot® Cell, Hercules, USA) for the immunoblot assay. The VapA antibody (provided from horses vaccinated with nVapA) was used as a primary antibody, and the anti-horse immunoglobulin G horseradish peroxidase-linked (Sigma, St. Louis, USA.) diluted 1/5,000 times was used as a secondary antibody. After adding δ-phenylenediamine dihydrochloride substrate (Sigma), the image was acquired with colorimetric detection using Carestream (Gel Logic 212 PRO; Bruker, Billerica, USA).

Immunization of a mouse model. This study was approved by the Ethics Committee of Selcuk University Experimental Medicine Research and Application Center, Konya, Türkiye (No. 2018/44; Date: 28.12.2018). In this

study, the rVapA of *R. equi* was cloned and expressed using the pET-SUMO system. The mice were intra-peritoneally immunized at the base of the tail twice at 21-day intervals with rVapA or nVapA (5.00 µg protein in total for each vaccine dose) formulated in IMS 3012 and PetGel A adjuvants.[18] The administration was performed in the mice groups, including the rVapA vaccine, nVapA vaccine, and negative control groups. Each of the four VapA-treated groups of six Swiss albino mice (weighing 15.00 - 18.00 g) was used to elicit an antibody response. Group 1 was injected with rVapA + IMS 3012, group 2 was injected with nVapA + IMS 3012, group 3 was injected with rVapA + PetGel A, and group 4 was injected with nVapA + PetGel A. The antibody responses in these groups of mice were investigated using the enzyme-linked immunosorbent assay (ELISA). For the challenge assay, the other four groups were vaccinated with rVapA + IMS 3012, nVapA + IMS 3012, rVapA + PetGel A, and nVapA + PetGel A, respectively. Ten days after the injection, all the mice were intraperitoneally challenged with the median lethal dose of active *R. equi*.[18,19] The negative control group was intraperitoneally injected with phosphate-buffered saline. Then, the survival rate in each group was determined.

Statistical analysis. The data were analyzed using the SPSS Software (version 20.0, IBM Corp., Armonk, USA) with ANOVA followed by Tukey test for the statistical significance analyses of the differences between vaccinated and control groups. A significance threshold of $p < 0.01$ was applied to establish statistical significance.

Results

Verification and sequencing of pET-SUMO-VapA cloning vector. A DNA band of 450 bp was observed as a result of the PCR performed at the species level according to the *16S rRNA* of the *R. equi* isolate. After the transformation, the presence of the *rVapA* gene was confirmed using VAPA-R and VAPA-F primers and detecting bands of 543 bp (Fig. 1). As a consequence of the comparison of the results of the sequence analysis of the *VapA* gene with the accession number of KT443895 of *R. equi* in the NCBI gene bank and the *VapA* gene (*rVapA*) obtained after the cloning of the *rVapA* gene, a 99.40% of similarity was found between the two genes.

SDS-PAGE and western blot analyses. In the supernatant of the medium containing *E. coli* BL21 (DE3), soluble rVapA weighing approximately 28.00 kDa (nVapA of 17.00 kDa and pET-SUMO fusion protein of 11.00 kDa) was determined by SDS-PAGE. The result of the western blot analysis showed a 28.00 kDa band associated with rVapA on the nitrocellulose membrane (Fig. 2).

rVapA humoral immune responses and challenge assay results. The presence of antibodies was calculated according to the ELISA results after the mice were immunized with vaccines prepared with nVapA and rVapA antigens and different adjuvants. The negative cutoff values were calculated as optical densities of 0.18 and 0.199 for rVapA and nVapA, respectively (Table 1). The presence of antibodies in mice sera according to the results of ELISA was compared between control and vaccine groups, and according to the results of the *t*-test, a statistically significant difference was found ($p < 0.01$). While the statistical analysis was evaluated with an average of ELISA values of four different vaccinations, differences between these groups were significant. The antibody response was slightly higher in PetGel A formulations than IMS 3012. Among vaccine groups, the averages of rVapA + PetGel A were determined to be the highest, while the averages of nVapA + IMS 3012 were the lowest (Table 1).

The morbidity and mortality observed in the mice of the challenge assay are given in the Table 2. No wound and inflammation at the injection site were observed in any group vaccinated with the vaccines prepared with two different adjuvants.

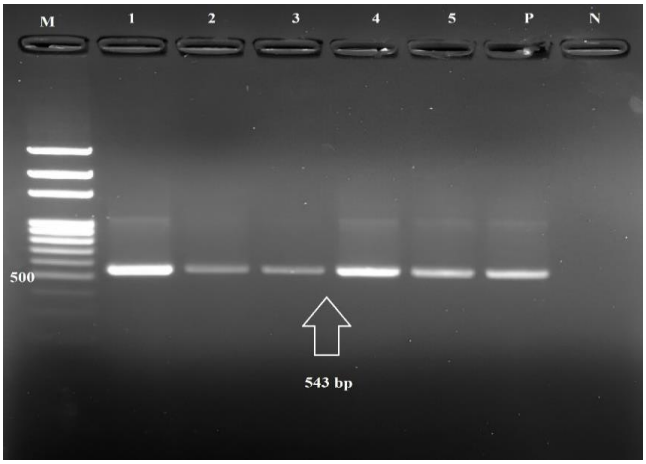

Fig. 1. Agarose gel image of the polymerase chain reaction products. M: Marker; P: Positive control; N: Negative control; Lanes 1-5: Validation of the *recombinant virulence-associated protein A* gene.

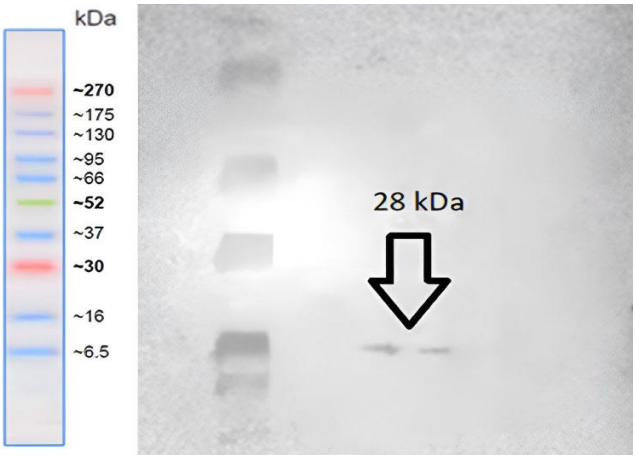

Fig. 2. Recombinant virulence-associated protein A detected by western blot analysis.

Table 1. Comparative results of the enzyme-linked immunosorbent assay.

Antigens	Negative control	PetGel A		IMS 3012	
		rVapA	nVapA	rVapA	nVapA
nVapA	0.012	0.756 ± 0.00163	0.727 ± 0.00122	0.719 ± 0.00163	0.612 ± 0.001
rVapA	0.015	0.759 ± 0.00156	0.643 ± 0.00118	0.688 ± 0.00166	0.598 ± 0.001

rVapA: Recombinant virulence-associated protein A; nVapA: Native virulence-associated protein A.

Table 2. The morbidity and mortality rates in the challenged mice.

Parameters	Negative control	nVapA + IMS 3012	rVapA + IMS 3012	rVapA + PetGel A	nVapA + PetGel A
Morbidity	5/6	2/6	2/6	1/6	2/6
Mortality	5/6	0/6	0/6	0/6	0/6

rVapA: Recombinant virulence-associated protein A; nVapA: Native virulence-associated protein A.

Discussion

The *R. equi* is especially transmitted to foals during the neonatal period through fecal, oral, and respiratory routes, and causes serious pneumonia.[20] Although several control strategies have been applied to prevent *R. equi* infection in foals, complete success has not been achieved. Although antibiotics, such as rifampin and erythromycin, are used for treatment, their therapeutic effects are inconsistent and require long-term treatment due to the intra-cellular growth of the agent. This situation causes high costs and potential risks, such as the development of resistant *R. equi*.[18-20] Since the *R. equi* infection is intra-cellular, and the immune system of neonatal foals is immature, vaccination is quite complex. Although the application of live vaccines in foals generates a strong immune response, it is considered objectionable due to the infection and environmental contamination caused.[17,18] Therefore, the traditional inactivated vaccines, compared to the current modern vaccines, are ineffective.[21]

The VapA as an important virulence factor in the replication of *R. equi* in host macrophages has led to the development of VapA-based vaccines to prevent *R. equi* in recent years.[22] There is an evidence that the VapA protein may be an important antigen involved in the protective immunity against *R. equi* infections in foals. For this reason, some researchers have attempted to develop VapA vaccines against *R. equi*. Vanniasinkam *et al.* have prepared a vaccine by transferring the *VapA* gene to the pIMVS-Re3 vector, and vaccinated BALB/c and C57BL/6 mice intra-muscularly; it was determined that, while the BALB/c mice vaccinated with the *R. equi* VapA vaccine presented a high antibody response, a low antibody response was found in C57BL/6 mice.[19] However, no protection was observed when 1.00×10^7 bacteria per mL virulent *R. equi* was injected into the mice in the challenge assay. Besides, the VapA vaccine efficacy studies in mice were conducted on the heat shock protein GroEL2 rather than VapA.[19]

Potential vaccine candidates, such as VapA, VapD, VapF, and VapG have also been studied[23] and shown to increase the T helper (Th)1 and Th2 responses in mice, adult mares, and foals.

Fernandez *et al.* have reported that BALB/c mice are completely protected from intra-peritoneal infection with *R. equi* due to the sera from horses vaccinated with VapA antigen and Triton X-114 as an adjuvant.[24] Reportedly, in the CD1 mouse model, the immune system is effectively stimulated, and protection is ensured if the VapA antigen is used without adjuvant.[25] Erganiş *et al.* have reported that the antibody titers induced by the vaccine formulated with bacterin, VapA, and IMS 3012 as an adjuvant start to increase earlier than those induced by the vaccine containing only bacterin and IMS 3012 as an adjuvant.[17]

Oliveira *et al.* have observed that in the mice inoculated 14 days apart twice by the intra-gastric route with 1.00×10^7 bacteria per mL of *Salmonella Typhimurium* in which the *VapA* gene region was transferred, and the challenge trial with *R. equi* was performed two weeks after the last vaccination, the interleukin 12 levels in the lymphoid tissue homogenates have risen. In addition, it was reported that while there was no death in vaccinated mice, death was observed in all unvaccinated mice, along with severe inflammation and necrosis.[26]

When the supernatant containing *R. equi* VapA was administered to adult horses, it was observed that the protection against virulent *R. equi* was due to an increased Th1 secretion.[27] Cauchard *et al.* have reported that when comparing the vaccine they have prepared with the VapA + IMS 3012 formulation and the bacterin vaccine, high titers of immunoglobulin G are synthesized in the serum of vaccinated pregnant mares and their foals, and no *R. equi* infection is observed in the foals, while in four foals (26.00%) of the 15 unvaccinated mares, *R. equi* pneumonia is developed.[28]

According to the results obtained after the vaccination of the mice with the four different candidate vaccines, it was observed that in this study, in accordance with the other studies, all the candidate vaccines were protective in terms of antibody response, back-isolation, and death rates. It was determined that the groups with high antibody results, according to the ELISA analysis, yielded proportional results in the challenge trials. It was observed that, except for the control group, an antibody response developed; the infection rate from the organs was low in the rVapA + PetGel A group, which was the vaccine group

with the highest antibody titer average according to the ELISA results, with a value of 0.759. However, in the control group, in which antibody was not formed, a high rate of death was observed.

To increase the adaptive immune response against the antigen, an appropriate adjuvant selection is important for vaccine formulations.[29] However, the effect of the adjuvant generally depends on the interactions of the adjuvant and antigen. The side effects and efficiency of the adjuvant may vary depending on the vaccine used;[28,29] for this reason, in candidate vaccine trials, the reliability of the vaccine, as well as its efficiency should be supported.

In a study on mares,[23] it was observed that there was no significant difference in skin thickness in the safety tests of IMS 3012 and PetGel A adjuvants. Erganiş *et al.*[18] prepared two inactive candidate vaccines using *R. equi* (K2002 strain), and Montanide IMS 3012 and Montanide PetGel A as adjuvants (bacteria + nVapA + IMS 3012 and bacterial nVapA + PetGel A). The antibody titers of the vaccinated pregnant mares were determined by coated ELISA, and it was reported that the *R. equi* vaccine adjuvanted with PetGel A was more effective in increasing the antibody response than with IMS 3012. In the immunological findings of the study, both adjuvants could induce immunity against *R. equi* infection, but PetGel A presented the best immuno-enhancing capacity.

In this study, it was observed that antibody response developed in all vaccinated groups, and PetGel A and IMS 3012 effectively stimulated the immune system. Both adjuvants were considered to be safe as no wounds or inflammation were observed in the vaccination area in all mouse groups. In addition, injection site necrosis did not occur in the groups of vaccines formulated with PetGel A; it was concluded that, although IMS 3012 and PetGel A induce comparable antibody levels, PetGel A is relatively superior for these reasons.

As a result, in this study, the expression of *R. equi* VapA in *E. coli* was obtained by combining rVapA with two different adjuvants, developing candidate vaccines protecting against infection in a mouse model. It was determined that, among the four different candidate vaccines, the most immunogenic and safest vaccine formulation was the combination of rVapA + PetGel A. It is thought that the antigenic power of this protein determined in a mouse model may be a model for vaccination in foals, and it is also thought that the rVapA may help in the development of diagnostic kits.

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Conflict of interest

The authors declare no conflict of interest.

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