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Efficacy of experimental inactivated and live *Rhodococcus equi* vaccines for thoroughbred Arabian mares in mice

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Abstract: The aim of this study was to determine the efficacy of inactive *Rhodococcus equi* vaccine candidates included that bacterin+aluminum hydroxide ($Al(OH)_3$), bacterin+VapA+($Al(OH)_3$), bacterin+Montanide IMS 3012 (IMS), bacterin+VapA+IMS, and live vaccine using mice as a model. The efficacy of vaccine was evaluated according to clinical findings, humoral and cellular immunity (levels of INF-g and IL-4), and results of microbiological culture from internal organs in dead or sacrificed mice. Inactive *R. equi* vaccines were subcutaneously administered to mice three times at 15-day intervals and live vaccine was intraperitoneally injected once. Fifteen days after the last vaccination, aerosol challenges were carried out with the pathogenic *R. equi* $VapA^+K2002$ strain in all groups. Two mice were sacrificed from each challenge groups on days 1, 3, 5, and 7. The antibody titers of vaccinated mice were found to be significantly higher than those of the controls. The largest number of INF-g positive samples were detected in the bacterin+VapA+IMS and bacterin+IMS groups. IL-4 positivity was determined only in live vaccine groups. The lowest reisolation rate of *R. equi* from internal organs was observed in the bacterin+VapA+IMS group. It was concluded that *R. equi* vaccines, and especially bacterin+VapA+IMS, are useful to protect mice against *R. equi* infection.

Key words: *Rhodococcus equi*, vaccine, mice model, VapA, Montanide IMS 3012, $Al(OH)_3$

1. Introduction

Rhodococcus equi is a gram-positive, nonmotile, obligate aerobic, intracellular microorganism. This organism is a facultative pathogen that causes pneumonia in foals. This pathogen also causes ulcerative enterocolitis, osteomyelitis, and septic arthritis in rare cases. *R. equi* is present in the soil and in horse feces. Foals are thought to become infected when they ingest or breathe in soil, dust, or fecal particles harboring the bacteria within the first few days of life (1,2). Inhalation of aerosolized virulent *R. equi* from the environment and intracellular replication within alveolar macrophages are essential components of the pathogenesis of *R. equi* pneumonia in foals (3). Virulence of *R. equi* is associated with the presence of plasmids of 80–90 kb that encode the 15–17 kDa lipoprotein virulence-associated protein A (VapA) (4). Clinical symptoms typically appear in less than 6 months. However, protection of the newborn foal is most important during the first week (5). In addition, *R. equi* may cause infections in the lungs of horses (6).

R. equi pneumonia significantly impacts the equine industry by causing financial losses because foals that recover from the disease are less likely to race as adults (7). The strategies used to protect against *R. equi* infection remain unsuccessful in foals. Although the use of a combination of antibiotics, such as rifamycin and erythromycin, is preferred in the treatment of this infection, the therapeutic effects are inconsistent due to bacteria that grow within cells and create granulomatous abscesses. In addition, the long-term use of antibiotics has potential risks, such as the development of resistance to antibiotics. The immunization of mares has been suggested by several researchers to prevent *R. equi* infection in foals (8–10).

In this study, the efficacy of *R. equi* vaccines was determined in mice for the protection of Arabian foals against *R. equi* infection. To achieve this aim, 4 inactive vaccine candidates and a live vaccine were prepared using the $VapA^+K2002$ strain of *R. equi*.

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2. Materials and methods

This research was approved (15/02/2008 and 2008/010) by the Ethics Committee of the Faculty of Veterinary Medicine of Selçuk University in Konya, Turkey.

2.1. Inactive vaccines

R. equi of the VapA$^+$K2002 strain, which was isolated from a dead foal at the Karacabey Arabian Horse Breeding Farms in Turkey, was cultured in brain-heart infusion (BHI) (Oxoid, Basingstoke, UK) supplemented with 1% yeast extract (Oxoid) at 37 °C for 48–60 h. The bacterial pellet was harvested by centrifugation at 6000 × *g* and washed three times with TBS (Tris, 19 nM, pH 7.4; NaCl, 150 mM). The bacterial concentrations were adjusted to 5 × 10^9 CFU/dose in 3 mL and inactivated using formalin (0.5% v/v).

The supernatant of the bacterial culture was filtered and concentrated for the VapA antigen using a 10 kDa Sartacon Slice 200 cassette filter (Sartorius AG, Göttingen, Germany). The VapA protein content was measured using a commercially protein assay kit (Protein Assay, Lowry, Bio-Rad, Richmond, CA, USA).

The inactive bacterin and bacterin+VapA vaccines included 5 × 10^9 CFU of *R. equi*/dose and 5 × 10^9 CFU of *R. equi* plus 0.5 mg of VapA/dose, respectively. The inactive vaccines were absorbed using 4% $Al(OH)_3$ gel (Vetal A/S, Adıyaman, Turkey) (11–13) or homogenized with an equal volume of mineral oil adjuvant (Montanide IMS 3012, Seppic, Castres, France) (14).

2.2. Live vaccine

Attenuated *R. equi* of the VapA$^+$K2002 strain was cultured into BHI supplemented with 1% yeast extract at 37 °C for 48 h. After washing three times, the bacterial concentration was adjusted to 1.2 × 10^9 CFU/dose. Antigen and stabilizer (10% skim milk powder) were mixed equally and the vaccines were lyophilized into small vials as a single dosage.

2.3. Vaccination and challenge

All vaccine doses were designed to be 3 mL for mare vaccination (15), and mice were vaccinated with 0.2 mL of vaccine. The challenge and seropotency groups included 108 and 48 mice, respectively (Table 1). Inactive vaccines (0.2 mL) were administered subcutaneously three times at 15-day intervals to the challenge and seropotency groups. The live vaccine (0.2 mL) was injected intramuscularly one time. After vaccination, adverse reactions were recorded after the observation of animal behavior and palpation of the injection site. Twenty-six mice were maintained as controls for the challenge (18 mice) and seropotency (8 mice) groups.

A total of 15 days after the third vaccination, the mice in the challenge groups were challenged with an aerosol administration of 1 × 10^9 CFU of the *R. equi* VapA$^+$S2002 strain using a nebulizer (Omron, Kyoto, Japan) in a special cabin. The mice were observed for morbidity and mortality for 14 days (16). After the challenge, two mice in each group were sacrificed on each sampling day (i.e. the 1st, 3rd, 5th, 7th, and 14th days). The amount of clearance of *R. equi* bacteria from the lungs was determined. In addition, the internal organs (i.e. the liver, spleen, kidney, heart, and lungs) of the mice were cultured on sheep blood (10% v/v) agar (Oxoid) and incubated under aerobic conditions at 37 °C for 24 h to reisolate *R. equi*.

2.4. Measurement of INF-g and IL-4

Blood samples were obtained retroorbitally from mice from the challenge and seropotency groups on days 5 and 15. The levels of INF-g and IL-4 (pg/mL) were measured using the commercially available Mouse IL-4 ELISA Ready-Set-Go kit (code number: 88-7044), eBioscience, San Diego, CA, USA) and Mouse IFN-g 'Femto-HS' High Sensitivity ELISA Ready-Set-Go kit (code number: 88-8314), (eBioscience), respectively, according to the manufacturer's instructions.

Table 1. Numbers of mice in the challenge and seropotency groups.

	Challenge group		Seropotency group
Vaccine types	For antibody, IL-4, and INF-g measurement	Sacrificed for bacterial reisolation	For antibody, IL-4, and INF-g measurement
Bacterin+$Al(OH)_3$	8	10	8
Bacterin+VapA+$Al(OH)_3$	8	10	8
Bacterin+IMS	8	10	8
Bacterin+VapA+IMS	8	10	8
Live vaccine	8	10	8
Control	8	10	8
Total	48	60	48

2.5. Serological monitoring in the groups

To determine the antibody titers in the mice, a homemade ELISA tool was used. ELISA was performed according to the procedure described by Takai et al. (17). Blood samples were obtained from the challenge and seropotency groups to determine the presence of anti-*R. equi* specific antibodies using ELISA on vaccine days 0, 15, and 30. In addition, blood samples were obtained from challenged mice on days 5 and 14 after challenge and on days 15 and 25 after the last vaccination in the seropotency groups.

2.6. Statistical analysis

The significance of differences observed within the groups was assessed using analysis of variance (ANOVA).

3. Results

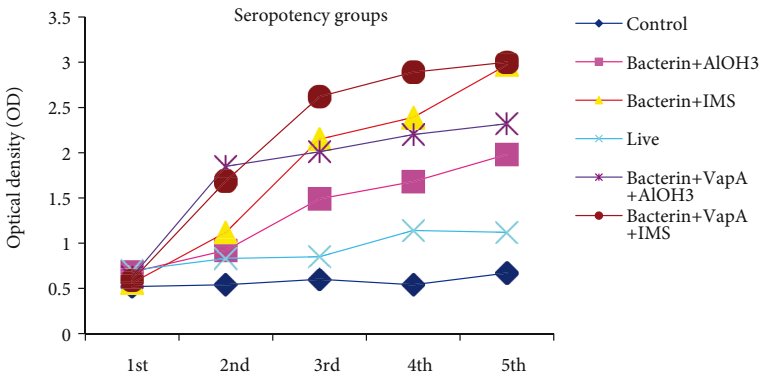
3.1. Seropotency

After the 1st, 2nd, and 3rd vaccinations, higher antibody titers were observed in blood samples from the vaccinated groups than in samples from the controls. A comparison of the vaccinated groups revealed that the highest antibody titers were observed in mice vaccinated with the bacterin+VapA+IMS and bacterin+IMS vaccine candidates. After the third vaccination, antibody titers were not increased in the live vaccine group, but antibody titers gradually increased in the other vaccine groups (Figure 1).

3.2. Challenge

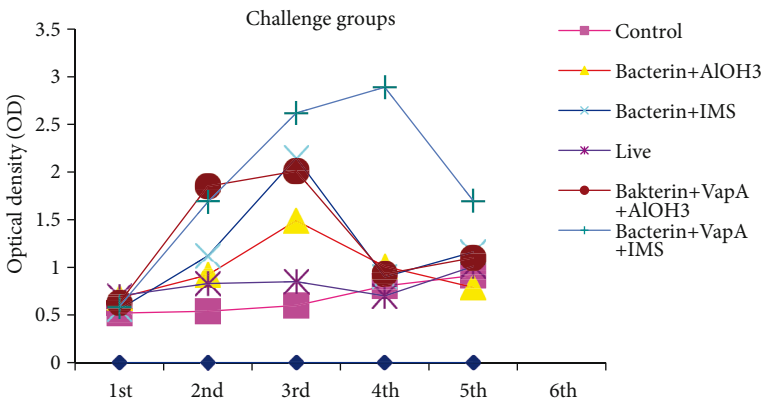
A decrease in antibody titers was observed in mice vaccinated with bacterin+IMS, bacterin+Al(OH)$_3$, and bacterin+VapA+Al(OH)$_3$ on the 5th day after challenge. However, an increase was observed in mice vaccinated with bacterin+VapA+IMS. While antibody titers were decreased in mice vaccinated with bacterin+VapA+IMS on the 15th day after challenge, titers increased in the other vaccine groups. In addition, an increase in antibody titers was observed in control mice on the 5th and 15th days after challenge (Figure 2).

The INF-g positivity rates of vaccinated mice in the challenge and seropotency groups were 0.344, 0.531, 0.687, 0.750, and 0.281 in the bacterin+Al(OH)$_3$,



Sampling (1st, 2nd, and 3rd sampling; the time of vaccination, 4th and 5th sampling; 15 days and 25 days after the last vaccination)

Figure 1. Antibody titers of seropotency groups.



Sampling (1st, 2nd, and 3rd sampling; the time of vaccination, 4th and 5th sampling; 5 days and 14 days after the last vaccination)

Figure 2. Antibody titers of challenge groups.

bacterin+VapA+Al(OH)$_3$, bacterin+IMS, bacterin+VapA+IMS, and live vaccine groups, respectively. These rates were higher in vaccinated mice than in controls, and the highest result was detected in the bacterin+VapA+IMS group (Table 2).

IL-4 positivity (0.468) was only determined in the live vaccine group; it was not determined in the controls or in the other vaccine groups (Table 3).

The *R. equi* numbers in the lungs of vaccinated mice were lower than those observed in the controls. The *R. equi* numbers were 77.7×10^4, 8.6×10^4, 8.4×10^4, 5.1×10^4, 2×10^4, and 9.6×10^4 CFU/mL in the control, bacterin+Al(OH)$_3$, bacterin+VapA+Al(OH)$_3$, bacterin+IMS, bacterin+VapA+IMS, and live vaccine groups, respectively (Table 4).

The numbers of mice with *R. equi*-positive internal organs (except lungs) in the vaccinated groups were significantly lower than in the controls. The rates (R values) of *R. equi*-positive lungs were 1.0 in the controls and 9.03, 9.25, 15.23, 38.85, and 8.09 in the bacterin+Al(OH)$_3$, bacterin+VapA+Al(OH)$_3$, bacterin+IMS, bacterin+VapA+IMS, and live vaccine groups, respectively (Table 4). The rates of other *R. equi*-positive internal organs were 0.775 in the controls and 0.200, 0.250, 0.075, and 0.300 in the bacterin+Al(OH)$_3$, bacterin+VapA+Al(OH)$_3$, bacterin+IMS, bacterin+VapA+IMS, and live vaccine groups, respectively (Table 5).

4. Discussion

R. equi infection is widespread around the world and leads to death in 3% of foals (9). To date, no licensed vaccine is available for the prevention of *R. equi*. In this study, the efficacy of inactive and live *R. equi* vaccine candidates were determined for the protection of Arabian foals using mice as a model. To achieve this aim, 4 inactive vaccines and a live vaccine were prepared using the VapA$^+$K2002 strain of *R. equi*, which was isolated from a foal infected with *R. equi*.

VapA is important in immunity against *R. equi* and stimulates Th1 immunity. Although the use of an

Table 2. IFN-g results obtained in mice.

Groups	Challenge		Seropotency		General	
	5th day	15th day	5th day	15th day	Positive/total	Rate
Control	1*/8**	2/8	1/8	0/8	4/32	0.125
Bacterin+Al(OH)$_3$	3/8	4/8	2/8	2/8	11/32	0.344
Bacterin+VapA+Al(OH)$_3$	2/8	4/8	4/8	6/8	17/32	0.531
Bacterin+IMS	6/8	8/8	4/8	4/8	22/32	0.687
Bacterin+VapA+IMS	7/8	8/8	4/8	5/8	24/32	0.750
Live vaccine	2/8	3/8	4/8	2/8	9/32	0.281

*IFN-g-positive mice number/**Total mice number.

Table 3. IL-4 results obtained in mice.

Groups	Challenge		Seropotency		General	
	5th day	15th day	5th day	15th day	Positive/total	Rate
Control	0*/8**	0/8	0/8	0/8	0/32	0
Bacterin+Al(OH)$_3$	0/8	0/8	0/8	0/8	0/32	0
Bacterin+VapA+Al(OH)$_3$	0/8	0/8	0/8	0/8	0/32	0
Bacterin+IMS	0/8	0/8	0/8	0/8	0/32	0
Bacterin+VapA+IMS	0/8	0/8	0/8	0/8	0/32	0
Live vaccine	4/8	4/8	5/8	2/8	15/32	0.468

*IL-4-positive mice number/**Total mice number.

Table 4. *R. equi* numbers in the lungs of vaccinated and control mice after challenge ($\times 10^4$).

Groups	Days					Total	Rate
	1	3	5	7	14		*CG/ VG
Control	7.0	11.9	31	17.9	9.9	77.7	1.00
Bacterin+$Al(OH)_3$	2.5	6.0	0	0.1	0	8.6	9.03
Bacterin+VapA+$Al(OH)_3$	1.5	6.5	0.3	0.1	0	8.4	9.25
Bacterin+IMS	0.6	2.8	0.8	0.7	0.2	5.1	15.23
Bacterin+VapA+IMS	0.4	1.4	0	0.1	0.1	2.0	38.85
Live	3.1	2.0	1.1	3.4	0	9.6	8.09

*CG: control group/VG: vaccine group

Table 5. Reisolation rate of *R. equi* from internal organs (i.e. heart, liver, spleen, and kidneys).

Groups	Days after challenge					Total	Rate
	1	3	5	7	14		
Control	2*/8**	7/8	6/8	8/8	8/8	31/40	0.775
Bacterin+$Al(OH)_3$	1/8	0/8	2/8	4/8	1/8	8/40	0.200
Bacterin+VapA+$Al(OH)_3$	0/8	0/8	2/8	6/8	2/8	10/40	0.250
Bacterin+IMS	4/8	0/8	2/8	4/8	2/8	12/40	0.300
Bacterin+VapA+IMS	0/8	0/8	0/8	2/8	1/8	3/40	0.075
Live	1/8	0/8	3/8	6/8	2/8	12/40	0.300

*Isolation number/**Total organ number.

adjuvant did not affect the antibody or DTH responses, a Th2 response directly and significantly reduced or removed the immune efficacy. Although the formation of a cytokine response to antigens was developed as an indirect assessment, evaluations of potential immunity against antigens should be direct in immunocompetent mice. In the present study, to demonstrate the efficacy of the VapA virulence factor during vaccination, this protein was added to bacterin vaccines (18).

Murine models of *R. equi* infection demonstrated that a cell-mediated immune response is essential for the clearance of the organism (19). Vanniasinkam et al. (20) reported that Th1 responses increased after the coadministration of a DNA vaccine and IL-2 plasmid expression in a model in BALB/c mice; however, protection was not observed in vaccinated mice when the mice were challenged with 1×10^7 bacteria/mL of virulent *R. equi*, except in mice vaccinated with a live vaccine. In addition, those authors stated that vaccines developed based on VapA and recombinant protein in BALB/c mice failed to prevent bacterial replication after challenge at high doses.

Both humoral and cellular immunity have been reported to be necessary for the disposal of *R. equi*. In experimental trials with hyperimmune plasma, CD4+ and CD8+ T cells may reduce the number of bacteria in the lungs. While clearance was accomplished via type 1 immune responses, type 2 responses may fail to clear the bacteria, leading to the development of lesions (21). The relationship between protection and a high anti-VapA-IgG antibody titer was emphasized in vaccinated mice (22,23). Haghighi and Prescott (24) reported high IgG2 antibody titers in mice vaccinated with a VapA-DNA vaccine compared to control mice and compared to mice vaccinated with a VapA-pcDNA vaccine and an IL-12-plasmid vaccine.

In this study, the highest antibody titer was observed in mice vaccinated with the bacterin+VapA+IMS and bacterin+IMS vaccines. In addition, antibody titers

began to rise and stabilize earlier in mice immunized with a bacterin+VapA+IMS vaccine than in control mice. However, a significant reduction in the antibody titers of vaccinated mice was observed after challenge. The explanation for this situation was considered to be the neutralization of challenge antigen with antibody.

Lopez et al. (25) reported that an attenuated riboflavin autotrophic *R. equi* strain was capable of INF-g expression in immune-suppressed BALB/c mice. In addition, protection against challenge with *R. equi* was observed in mice. IFN-g positivity was significantly higher in the vaccine group than in the control group in the challenge and seropotency tests of our study. The maximum numbers of IFN-g-positive samples were observed in the bacterin+VapA+IMS (0.750) and bacterin+IMS (0.687) vaccine groups. Oliveira et al. (23) vaccinated mice twice with *Salmonella* Typhimurium containing cloned VapA antigen and challenged the mice intravenously with *R. equi* 2 weeks after the last vaccination. Those authors reported high IL-12 levels in tissue homogenates of vaccinated mice, as indicated by ELISA; however, low TNF and IL-4 levels were observed.

In the challenge and seropotency tests of our study, IL-4-positive samples were detected only in mice vaccinated with live vaccines. IL-4 positivity could not be determined in control mice or in the other vaccine groups. Thus, we concluded that the determination of IL-4 was not an appropriate criterion for studies assessing vaccine efficacy.

Prescott et al. (18) reported that the bacterial clearance rate increased in the internal organs of CD1 mice vaccinated with the nonadjuvant VapA vaccine. However, no changes were reported in BALB/c mice. In addition, the use of adjuvants (i.e. $Al(OH)_3$ and immune-stimulating complexes (ISCOMs)) significantly increases the immunogenicity of the VapA antigen; ISCOMs were nonimmunogenic, and Th2 immunity was better stimulated by the $Al(OH)_3$ adjuvant. Phumona et al. (16) reported that DNA vaccines have specific advantages compared to classical vaccines. The combined effect of DNA vaccine candidates (i.e. pcDNA 3-Re-1 and pcDNA-3-Re-3 vs. pcDNA) with the *R. equi* heat shock protein GroEL2 was demonstrated to protect C3H/He mice against *R. equi* infection. Additionally, 7 days after aerosol challenge, a significant reduction of *R. equi* numbers in the lungs was observed in immunized mice. The absence of significant pathological changes, which reflects complete protection against *R. equi* infection, in mouse models may not accurately reflect the effectiveness of potential vaccine candidates. Thus, mouse models may not be appropriate in such vaccine studies. Conversely, Gonzalez-Iglesias et al. (26) reported that the murine lung infection model provides a useful tool for both *R. equi* virulence and vaccine studies. Oliveira et al. (23) cloned the *R. equi* VapA gene into *S.* Typhimurium because *Salmonella* spp. colonize and persist in mouse lymphoid tissues. In addition, 2 days after challenge, a 3- to 7-fold increase in *R. equi* clearance was observed. A reduction of the number of bacteria continued during infection in orally immunized mice. Additionally, bacterial growth was 50-fold lower in this group than in controls. Severe inflammation and necrosis occurred in nonimmunized mice, but these symptoms were 2-fold lower and leukocyte infiltration was temporary in vaccinated mice. No deaths were reported in vaccinated mice, and all unvaccinated mice died. In addition, high anti-VapA IgG antibody levels were reported to be associated with protection in immunized mice. We demonstrated that the bacterin+$Al(OH)_3$ vaccine, the bacterin+VapA+$Al(OH)_3$ vaccine, the bacterin+IMS vaccine, the bacterin+VapA+IMS vaccine, and the live vaccine provided 9.03-fold, 9.25-fold, 15.23-fold, 38.85-fold, and 8.09-fold more bacterial clearance from the lungs, respectively, compared to control mice. The immune responses that occur in vaccinated mice reduce bacterial growth in the lungs.

Haghighi and Prescott (24) immunized C57BL/6 and BALB/c mice with a DNA vaccine constructed by incorporating VapA into pcDNA3.1. Those authors stated that the immunization of mice resulted in enhanced clearance of *R. equi* from the livers of intravenously challenged mice compared to controls. In conclusion, DNA immunization with VapA enhances mouse immune responses against *R. equi* infection.

In this study, the reisolation rates of *R. equi* from the internal organs were significantly lower in vaccinated mice than in control mice after challenge. In addition, the smallest number of *R. equi* was isolated from mice vaccinated with the bacterin+VapA+IMS vaccine.

Whitehead et al. (19) reported that mice inoculated with *R. equi* 103-103-/pAWVapA and 103-/pNBV1 completely cleared infection, whereas strain 103-/pAW48A persisted in 47% of mice.

Consequently, the bacterin+VapA+IMS vaccine is the most effective vaccine against *R. equi* infection in a mouse model. The vaccination of pregnant mares with this vaccine for hyperimmune colostrum and plasma production may be useful for protecting foals against *R. equi* infection.

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