Herald of Science of S.Seifullin Kazakh Agrotechnical Research University: Veterinary Sciences. – Astana: S. Seifullin Kazakh Agrotechnical Research University, 2024. – № 4 (008). – P. 51-58. - ISSN 2958-5430, ISSN 2958-5449

doi.org/ 10.51452/kazatuvc.2024.4(008).1811
UDC 579.23'314:616-097.155.37 **Research article**

Antigenic Activity of Various *Rhodococcus equi* Strain Plasmids

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Received: 02-12-2024 **Accepted:** 20-12-2024 **Published:** 30-12-2024

Abstract

Background and Aim. *Rhodococcus equi (R. equi)* infection is a fatal cause of equine *rhodococcosis.* Infections have also been reported in other species and humans. This study evaluated the immune response of R. equi in rabbits.

Materials and Methods. Different strains of R. equi (with and without plasmids) were administered to rabbits. Blood samples were collected from the ear veins of the rabbits at 7, 14, 21, and 28 days after the initial administration of the antigen, and the serum was examined for specific antibodies against *R. equi* plasmid antigens using the complement fixation reaction.

Results. The minimum antibody level was recorded on day 7. The average antibody level throughout the study period was slightly above the median, indicating a small asymmetry toward higher values. By day 28, the antibody level had reached 75% of values ≤4.25.

Conclusion. All strains of *R. equi* exhibited antigenic properties to varying degrees. The antibody level upon plasmid pVapN administration was higher than that of the other cases.

Keywords: *Rhodococcus equ*i; plasmid pVapB; plasmid pVapA; plasmid pVapN; antigenic activity.

Introduction

Rhodococcus equi (R. equi) is a pleomorphic Gram – positive *coccobacillus bacterium* commonly found in the gastrointestinal tract of horses. *R. equi* is recognized as an important pathogen based on reports of 3% mortality in foals, which is widespread globally [1, 2, 3]. *R. equi* is a primary lung pathogen in foals aged 6 months with high mortality rates [4, 5]. Pneumonia caused by *R. equi* can also occur in adult horses, especially in immunocompromised individuals, who can develop systemic infections [6].

In infected animals, chronic and purulent bronchopneumonia is typically observed and is associated with high mortality, especially in foals that have not been subjected to specific antibacterial therapy [3, 7]. Numerous studies using virulent strains of *R. equi* isolated from horses in different geographical regions worldwide have demonstrated the diversity of plasmid sizes and the predominance of different types of plasmids [8, 9, 10, 11, 12].

It has been established that only virulent strains of *R. equi*, which express the virulence-associated protein (VapA) of 15-17 kDa and possess a large virulence plasmid of 85-90 kDa containing the VapA gene, are pathogenic to horses. To date, 12 types of plasmids have been identified in VapA-positive strains from horses [11, 13, 14, 15]. VapA and VapB, which are closely related at the amino acid level, are located on the cell surface, and their expression is regulated by temperature and pH [16].

In studies on the source of intermediate virulence, VapB-positive strains have been isolated from the submandibular lymph nodes of pigs; these isolates were found to be of intermediate virulence in mice and contained one of five large plasmids sized 79±95 kb [17]. Virulent strains of *Rhodococcus equi* expressing the virulence-associated protein of 15–17 kDa (VapA) and possessing a large virulence plasmid (pVAPA) of 85–90 kb, containing the vapA gene are pathogenic to horses. Over the last two decades, after pVAPA, two types of host-associated virulence plasmids of *R. equi* have been discovered: the circular plasmid pVAPB, associated with isolates from pigs in 1995, and the recently identified linear plasmid pVAPN, linked to isolates from cattle and goats.

Molecular epidemiological studies of R. equi infections in foals on equine breeding farms in Japan and many countries around the world have been conducted over the past three decades, and epidemiological studies using the digestion schemes of plasmid DNA from virulent isolates with restriction enzymes have shown 14 different subtypes of pVAPA and their geographical preferences [18, 19, 20].

Virulent strains of *R. equi* isolated from sick foals and horse breeding environments exhibit a uniform plasmid pattern [21]. Certain types of plasmids are characteristic of specific geographical regions, which can be used in epidemiological studies [10, 11].

The aim of our research was to study the antigenic activity of various plasmids of the *R. equi* strain.

Materials and Methods

To study the antigenic activity of the plasmids of the *R. equi* strain, rabbits were immunized. We used the following strains, provided by Kitasato University (Japan):

1) The *Yokkaichi_P* strain contains no plasmids.
2) S4 cells containing the plasmid pVapB.
3) ATCC33701 cells carrying the plasmid pVapA.
4) Yokkaichi strain containing the plasmid pVapN.
5) The *R. equi* strain was isolated by us in 2023 [22].

Cultivation of *Rhodococcus equi* Suspensions: For inoculation, a 2-day culture of the strain in tubes was washed with saline solution to achieve a microbial cell concentration of 2–5 billion cells in 1.0 cm^3, according to the optical turbidity standard. This suspension was used to inoculate Tartakovsky flasks with NANAT medium [23]. The inoculated flasks were supplemented with 4.0–5.0 cm^3 of suspension and placed in a thermostatic incubator at 37 °C for 2 days. After 2 days, the cultured material was visually inspected for purity and typical growth characteristics.

Preparation of the antigens: The culture was washed with a sterile 0.5% phenolize saline solution, pH 7.0–7.2, using 25–30 cm^3 per flask. The resulting suspension, containing approximately 20–30 billion microbial cells in 1.0 cm^3 according to the turbidity standard, was filtered through a double-layered gauze filter into bottles. The purity of the gram-stained smears. The mixture was then heated in a water bath at +70 °C for 60 min.

After cooling, the heated suspension was stored in a refrigerator at +2 °C–4 °C, where it was also checked for purity and sterility.

Sterility determination: From the antigen stock, the samples were inoculated onto MPA and NANAT media. The cultures were incubated in a thermostat for 3 days at a temperature of 37 °C–38 °C. The cultures remained sterile throughout the observation period.

In each group, three rabbits weighing 2.0–2.5 kg were selected and subcutaneously injected with the antigen at a dose of 2 cm^3 twice, with an interval of 14 days. Blood samples were collected from the ear veins of the rabbits at 7, 14, 21, and 28 days following the first antigen administration, and the serum was tested for specific antibodies against the plasmid antigens of *Rhodococcus equi* using the prolonged complement fixation test (PCFT).

PCFT was performed under cold conditions in tubes with 0.2 cm^3 volumes of serum, antigen, and complement, with the hemolytic system in a titrated working dose. Each antigen was titrated with the corresponding rabbit serum using the checkerboard method in PCFT. Serum samples were collected from rabbits on days 0, 7, 14, 21, and 28 of the experiment.

Statistical Analysis: Data analysis was performed using R-Studio software, applying the non-parametric Kruskal–Wallis test. Statistical significance was assessed at a threshold level of $P < 0.05$ [24].

The experiments and methodology used to conduct research on laboratory animals comply with the requirements of biological safety and the ethical principles of animal experimentation in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Conclusion of the Bioethics Commission of the Kazakh National Agrarian Research University dated November 2, 2022).

Results

The checkerboard titration of the antigen and rabbit serum before antigen administration yielded a negative result. The results of the studies, starting from the seventh day of the experiment, are presented in Table 1.

Table 1 – Antibody Levels in Rabbits Administered Different Strains (with no plasmids, plasmids) of *R. equi*

Days of the Experiment	Group number-Average antibody titer				
	1 - no plasmids	2 - pVapB	3 - pVapA	4 - pVapN	5 - *R.equi*
7	-	1:5	1:5	1:5	1:5
14	-	1:10	1:10	1:10	1:10
21	1:10	1:20	1:20	1:40	1:20
28	1:20	1:80	1:40	1:80	1:40

As can be seen from the table, when the antigen from the strain without plasmids was injected into the group of rabbits on days 7 and 14, no antibodies were detected in the PCFT, whereas in the groups of rabbits injected with pVapB, VapA, pVapN, and *R.equi*, the antibody titer was within 1:5 and 1:10, respectively.

From day 21 in the first group, antibody levels began to appear at 1:10 and peaked at 1:20 on day 28. On day 28 of the experiment, a significant increase in antibody titer was observed in rabbits in groups 2 and 4, i.e., when antigen from the strain carrying the pVapB and pVapN plasmids was administered.

Because of the studies in the first group, rabbit serum antigen had the highest titer at dilutions of 1:50, 1:100, and 1:150. In serum samples positive for the strain without plasmids, the indicator was 1:10. With a decrease or increase in antigen dilutions, a decrease or absence of antibody levels is observed. An increase or decrease in antibody titers is directly dependent on the dilution of the antigen.

The absence of anticomplementary properties was demonstrated in the reactions of antigen dilutions with physiological solutions.

The antigen dose with a high antibody response in the reactions was taken as the equivalent zone. In addition to the equivalent zone, a decrease in the reaction results was observed at other antigen dilutions.

Because of the reactions, the dilution of the antigen that gave the highest antibody titer in the serum was taken as the antigen unit (AU). This AU is the most effective antigen dilution.

It was established that 1 AU for an antigen without plasmids is 1:150, with a working dose of the antigen of 1:150 equivalent to 1 AU. Higher than 1 AU resulted in self-limiting reactions.

In the checkerboard titration of rabbit serum treated with the antigen from the plasmid pVapB, the following results were obtained: 1 AU was 1:250, and the working dose of the antigen was 1:200, which corresponds to 1.25 AU.

For the titration of rabbit sera and the antigen from the plasmid pVapA, 1 AU was 1:300, and the working dose was 1:250, resulting in 1.2 AU.

Regarding pVapN, 1 AU was 1:200, while the working dose was 1:250 (1.3 AU). The 1 AU of the antigen from the *R. equi s*train was 1:300, whereas the working dose was 1:250 (1.2 AU).

The minimum antibody level was recorded on day 7. 25% of the values were ≤2.0, which coincides with the level on day 14. The median value indicates the central tendency of the data. The average antibody level over the entire period was slightly above the median, indicating a small asymmetry toward higher values. By day 28, the antibody level had reached 75% of values ≤4.25.

A P value of >0.05 indicates the absence of significant differences between groups. Variations in antibody levels are more likely related to variability than to the action of the plasmids.

Plasmids did not have a significant effect on antibody levels. However, the antibody level upon the administration of the plasmid pVapN (3.0) was somewhat higher than that in the other cases.

A clear increase in antibody titers was observed in rabbits with increasing time after the administration of the strains. This indicates the formation of an immune response. The dynamics of antibody titers showed a significant increase in antibody levels as the observation time increased, indicating the activation of the immune response. The differences between days were statistically significant ($P < 0.01$).

Analysis of the impact of plasmids on antibody production indicated that the differences between groups with various plasmids and those without plasmids were not statistically significant ($P = 0.62$). Nevertheless, plasmid pVapN showed a slight trend toward increased antibody levels.

The values of the antibody titers exhibited a slight asymmetry toward an increase, suggesting a rise in the antibody levels in later days of the experiment.

Discussion and Conclusion

Pathogenic strains are classified as virulent or intermediate virulent based on the presence of plasmid genes encoding virulence-associated protein A (VapA) or B (VapB), respectively [25]. Strains lacking VapA or VapB are classified as avirulent. The highly conserved gene encoding the conjugative transfer protein, traA, is present in strains carrying the virulence plasmid.

The new linear virulence plasmid *R. equi* (pVapN) has been characterized in isolates from cattle [26] and identified in a single isolate from dogs [27], but it has not been described in other species. Epidemiological data indicate that strains carrying pVAPN are pathogenic to ruminants [19].

VapN-positive *R. equi* has been isolated from cattle in Japan. Although *R. equi* is generally considered to have low pathogenicity in cattle, the influence of certain predisposing factors can lead to widespread infection [28].

VapA is an important antigen involved in the humoral protective immune response to *R. equi* infections caused by virulent strains in foals [29].

In this study, specific *R. equi* strains that are known to be pathogenic in various animal species were used. In our experience with rabbits, they elicited an immune response characterized by a clear increase in antibody titers over time following the administration of the strains.

All strains of *R. equi* (with and without plasmids, as well as the strain itself) exhibited antigenic properties to varying degrees. The antigen from the R. equi strain without plasmids showed the lowest level of activity. The antibody level upon administration of the plasmid pVapN was somewhat higher than that in the other cases.

Statistical analysis showed that the differences between the means at each stage of the experiment were statistically significant ($P < 0.01$), confirming a progressive increase in the immune response and greater variability at later stages of follow-up.

“Antigenic unit” was used as a measure of antigenic activity in serological reactions. The highest antigen titer corresponding to the highest value of positive blood serum with antibodies of the corresponding type was used as the antigenic unit. The value of the antigenic unit is not affected by the activity of the specific blood serum used to determine it; thus, the antigenic unit can be determined with any positive blood serum by titration using the checkerboard method.

The results may help obtain hyperimmune (positive) serum for the development of diagnostic kits for *rhodococcosis*. This study should be considered as a pilot study for the further development of *R. equi* bioproducts in horses and goats.

Authors' Contributions

GI, EK, and BO: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the collected data, and prepared the manuscript. MZ, SK, and NM: Prepared materials for hyperimmunization’s and assessed antigen activity. UZ and BV: conducted the hyperimmunization of rabbits. All authors read, reviewed, and approved the final manuscript.

Acknowledgments

This work was supported by grant funding from the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan for 2023-2025, grant AP19680565 “Creation of an

experimental infection model in goats using virulent *Rhodococcus equi* containing pVAPN as a model for *Rhodococcus* infection in foals."

We express our gratitude to Sh. Takai (Kitasato University, Japan) for kindly providing the plasmids of the *Rhodococcus equi* strain, and to Yerlan Dutbayev (Department of Plant Protection, Kazakh National Agrarian Research University, Almaty, Kazakhstan) for conducting the statistical analysis of the obtained data.

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