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UDC 616.98:578.74:615.383:636.1 **Research article**

Rhodococcal antigen: Evaluation of the antigenic unit using horse blood serum in the prolonged complement fixation test

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Abstract

Background and Aim. *Rhodococcus equi* pneumonia occurs endemically on some farms, whereas on others it occurs sporadically or is not detected at all. On endemic farms, the prevalence and severity of the disease may also vary seasonally. This study aimed to assess the seroprevalence of R. equi infection in horses from selected regions of Kazakhstan.

Materials and Methods. A serological study was conducted to assess the prevalence of *Rhodococcus equi* infection in horses. A total of 260 serum samples collected from four administrative regions (Astana city, Erementau district, Shieli district, and Zhanakorgan district) were examined using the prolonged complement fixation test (PCFT). Statistical analysis included descriptive statistics, distribution assessment, and one-way analysis of variance.

Results. The overall seroprevalence was 19.6%. The highest seroprevalence was observed in the Erementau district (41.8%) and Astana city (36.0%), whereas the lowest was recorded in the Shieli district (12.0%), with an intermediate value in the Zhanakorgan district (17.1%). No statistically significant differences in mean seroprevalence were detected between districts ($p = 0.231$). However, analysis at the individual farm level revealed a highly heterogeneous, clustered distribution of the pathogen. The distribution of seropositivity was markedly right-skewed, with a median of 15%, and 25% of farms were completely free of infection. At the same time, localized outbreaks with infection rates of up to 66.7% were detected. The antibody titers were predominantly at the 1:10 level (56.9%), which, together with the focal distribution pattern, suggests endemic circulation of the pathogen.

Conclusion. The findings indicate that epidemiological surveillance should be shifted from territorial to farm-level monitoring in order to ensure more targeted detection and control of infection foci.

Keywords: clustered distribution; endemic circulation; epizootology; long-term complement fixation test; *Rhodococcus equi*; seroprevalence.

Introduction

Rhodococci are aerobic, gram-positive, pleomorphic, non-motile bacteria commonly found in soil and capable of proliferating on simple nutrients provided by herbivore manure. They also grow well in the intestines of grazing animals. *Rhodococcus equi* is the most frequently isolated species of the genus *Rhodococcus* and is recognized as an important veterinary pathogen. It causes bronchopneumonia,

particularly in foals [1]. *Rhodococcus equi* was first isolated from foals with bronchopneumonia in 1923 [2]. Currently, the genus *Rhodococcus* is distinguished from closely related acid-fast or partially acid-fast genera, such as Gordonia, Nocardia, and Mycobacterium [3].

Rhodococcus equi is a ubiquitous bacterium. The genus *Rhodococcus* is closely related to the genera *Mycobacterium* and *Corynebacterium*. These bacteria are aerobic, gram-positive coccobacilli capable of invading macrophages. *Rhodococcus equi* is widely recognized as the causative agent of purulent bronchopneumonia in foals, also known as rhodococcosis. The disease primarily affects foals during the first 3 months of life. Due to high morbidity, mortality, and treatment costs, the disease has a significant economic impact on horse breeding worldwide. However, our understanding of this disease remains limited, and many aspects remain unclear [4-9].

Rhodococcus equi is a soil organism that is widely distributed in the environment and colonizes the intestinal tract of many herbivores [1, 10, 11]. Virulent strains expressing 15-17-kDa antigens have frequently been isolated from horses and soil on horse-breeding farms [12-14], and human transmission from soil or animals has been documented [15-17].

Rhodococcus equi pneumonia occurs endemically on some farms, whereas on others it occurs sporadically or is absent on others. Prevalence and severity may vary seasonally [18, 19]. In Australia, 1%-10% of Thoroughbred foals are diagnosed with *R. equi* pneumonia annually, with mortality rates historically reaching 61%. Early diagnosis and improved antimicrobial therapy have contributed to reduced mortality [20].

In many endemically infected farms, morbidity reaches 20%, while mortality ranges from 5% to 100% [21, 22].

Regardless of the diagnostic methodology, *R. equi* pneumonia is often primarily a clinical diagnosis supported by laboratory tests. Early detection and treatment can significantly improve prognosis.

This study obtained a *rhodococcal* antigen for the diagnosis of *rhodococcosis* in horses and evaluated its performance in the prolonged complement fixation test.

Materials and Methods

Preparation of the Seed Culture A lyophilized culture of *Rhodococcus equi* was used as the inoculum source for antigen production.

Sterile physiological saline was added to an agar slant containing the culture to prepare the inoculum. After the microbial mass was completely dissolved, 10-fold serial dilutions were prepared (10^{-7}-10^{-9}) in sterile saline (4.5 cm3 per tube). From the last two dilutions, 0.1 cm^3 aliquots were inoculated onto 3-5 Petri dishes containing tryptone soy agar (TSA). The agar plates were pre-dried in an incubator at 37-38 °C for 24 h. The microbial suspension was evenly spread and incubated at 37 °C-38 °C for 48 h. Colonies were examined for purity and morphological characteristics. Typical colonies were subcultured in test tubes containing nutrient medium. The culture grown for 48 h at 37 °C was used as the working seed culture. It was stored on agar slants at 2-4 °C for no longer than 5 weeks.

Inoculation of *R. equi* suspension into flasks. The 48-h-old culture of the strain was washed off with physiological saline to obtain a suspension containing 2-5 × 10^9 microbial cells per 1.0 cm3, according to the optical turbidity standard. This suspension was used to inoculate flasks containing TSA. The inoculated flasks were moistened with 4.0-5.0 cm3 of the suspension and incubated for 3 days at 37 °C. After 24 h, the agar surface in the flasks was re-moistened to enhance growth. After a further 48 h, the purity and typical growth characteristics of the grown culture were visually assessed.

Antigen preparation. The culture was washed off using sterile 0.5% phenolized physiological saline (pH 7.0-7.2) at a volume of 25-30 cm^3 per flask. The resulting suspension, at an approximate concentration of 20-30 × 109 microbial bodies per 1.0 cm^3 (turbidity standard), was filtered through a double layer of gauze into bottles and heated in a water bath at +70 °C for 60 min.

The heated suspension was stored in a refrigerator at 2-4 °C for 7-10 days after cooling. In parallel, it was tested for purity and sterility by microscopy and culture.

Sterility testing. From five antigen vials, inoculations were performed using a sterile glass pipette as follows: 0.2-0.3 cm3 onto meat-peptone agar and Sabouraud agar in tubes, and 0.5-1.0 cm^3 onto meat-peptone broth and meat-peptone liver broth under a layer of petroleum jelly oil in vials.

The inoculated media were incubated for 10 days at 37 °C-38 °C; Sabouraud agar was incubated at 20-24 °C. All cultures were required to remain sterile throughout the observation period.

Antigen control in the prolonged complement fixation test (PCFT).

Titration of the indicator system. Reaction components (blood serum, antigen, and complement) were used at a volume of 0.2 cm^3 each, whereas the indicator system was used at the working dose determined by titration (Table 1).

Table 1. Determination of the indicator system titer in PCFT

Reaction components	Tube No.									
	1	2	3	4	5	6	7	8	9	10
Negative blood serum, 1:5	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Rhodococcosis antigen, 1:200	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Complement at working dilution	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Refrigerator at 2-6 °C for 16-18 h										
Indicator system	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Water bath at 37 °C for 20 min										
Result	CH	CH	CH	CH	CH	CH	PH	PH	PH	NH
Note: CH - complete hemolysis; PH - partial hemolysis; NH - no hemolysis										

To perform the titration, 10 tubes were placed in a rack. Negative blood serum diluted to 1:5 was added and inactivated in a water bath at 62-64 °C, after which the *rhodococcosis* antigen at the working titer (antigen unit) and complement at the working titer were added at 0.2 cm^3 each. The tubes were thoroughly mixed and incubated at 2-4 °C for 16-18 h. The rack was maintained at room temperature for 20 min. The indicator system (2% sheep erythrocyte suspension and a triple dose of hemolysin in equal volumes) was then added in increments from 0.1 to 1.0 cm^3 at 0.1 cm^3 intervals, followed by incubation in a water bath at 37 °C for 20 min. The working titer was defined as the close one interval below that which yielded complete erythrocyte hemolysis.

Determination of antigen activity in the PCFT. The antigen was diluted in physiological saline (pH 7.0-7.2) to 1:100, 1:150, 1:200, 1:250, 1:300, and 1:350 for chessboard titration. Positive anti - *R. equi* serum was preliminarily dispensed into tubes at dilutions of 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1,280 in a volume of 0.2 cm^3 and inactivated in a water bath at 62-64 °C for 30 min. For each dilution, 0.2 cm^3 of antigen and complement (with a 50% increase over the working titer) were added in a volume of 0.2 cm^3 each, and the tubes were incubated in a refrigerator at +2 to +4 °C for 16-18 h (Table 2).

Table 2. Scheme for performing the main PCFT experiment

Reaction components	Tubes for each dilution		
	1	2	3
Test blood serum	0,04	0,04	0,02
Physiological saline	0,16	0,16	0,18
Water bath (62-64 °C), 30 min			
Rhodococcosis antigen	-	0,2	0,2
Physiological saline	0,2	-	-
Complement at the working dilution	0,2	0,2	0,2
Refrigeration at 2-6 °C for 16-18 h			
Indicator system	0,4	0,4	0,4
Water bath at 37 °C for 20 min			

Anticomplementary activity was assessed in series containing all antigen dilutions and positive serum along with physiological saline (Table 3).

Table 3. Antigen titer determination using the chessboard method in the PCFT

Positive serum	Test antigen						
	1:100	1:150	1:200	1:250	1:300	1:350	PS
1:5	4+	4+	4+	4+	4+	4+	-
1:10	4+	4+	4+	4+	4+	2+	-
1:20	4+	4+	4+	4+	4+	-	-
1:40	4+	4+	4+	4+	3+	-	-
1:80	4+	4+	2+	-	-	-	-
1:160	4+	2+	-	-	-	-	-
1:320	4+	2+	-	-	-	-	-
1:640	4+	2+	-	-	-	-	-
1:1280	2+	-	-	-	-	-	-
PS	4+	3+	-	-	-	-	-
Note: PS. - physiological saline							

Reaction outcomes were recorded using crosses based on the degree of erythrocyte hemolysis.

The working antigen titer was defined as the dilution that produced a high antibody titer with positive serum and did not inhibit hemolysis in the physiological saline control (i.e., did not exhibit anticomplementary activity). As shown in Table 3, the dilutions of 1:200-1:300 met these criteria, yielding the highest antibody titer of 1:40. The 1:300 dilution was taken as 1 antigen unit (AU), the 1:250 dilution corresponded to 1.25 AU, and the 1:200 dilution corresponded to 1.5 AU. Thus, the working titer of the rhodococcosis antigen was selected as an AU within the range of 1.0-1.5.

Evaluation of the rhodococcosis antigen under field (production) conditions. Serological testing of equine sera for anti – *R.equi* antibodies. Blood samples (5-8 mL) were collected aseptically from the jugular vein of horses into sterile tubes. Serum was obtained from clotted blood by centrifugation (3,000 rpm for 10 min) and stored at −20 °C until use in serological assays.

Data were analyzed using RStudio software and the nonparametric Kruskal-Wallis test at a significance level of $p < 0.05$ [23, 24, 25].

Results and Discussion

Table 4 presents the results of testing equine blood sera for the presence of antibodies to *R. equi* using the PCFT.

Table 4. Results of equine blood serum testing using PCFT for antibodies against *R. equi*

No.	District/area	Number of samples	PCFT results					
			Positive (antibody titer)					Negative
			1:5	1:10	1:20	1:40	Total positive	
1	Astana city	25	1	8	0	0	9	16
2	Erementau District	55	3	15	3	2	23	22
	“Nurali” farm	55	3	15	3	2	23	22
3	Shieli District	100	3	3	4	2	12	88
	Kurbanbekov N.	20	0	1	1	0	2	18
	“Aliyev” farm	20	1	0	1	1	3	17
	“Kozhakhmet” (IE)	20	0	1	0	1	2	18
	“Kanagat” farm	20	1	1	1	0	3	17

Continuation of Table 4

	“Bibi” farm	20	1	0	1	0	2	18
4	Zhanakorgan District	80	3	6	5	2	16	64
1	“Qosqozha” farm	6	1	1	1	1	4	2
	“Damu” farm	4	0	1	0	0	1	3
2	“Aidarhan” farm	2	0	0	0	0	0	2
	“Barys-4” farm	4	0	0	0	0	0	4
	“Miras” farm	2	0	0	0	0	0	2
	Nurmaganbek E.	2	0	0	0	0	0	2
3	“Nur-Abyl” farm	10	0	1	1	0	2	8
4	Alimbetov A.	10	1	1	1	0	3	7
5	“Damir-S” farm	10	0	1	1	1	3	7
6	“Aksarai” farm	10	0	0	0	0	0	10
7	“Kobeldes” farm	10	1	1	1	0	3	7
8	“Nur” farm	10	0	0	0	0	0	10
	Total	260	9	29	8	5	51	209

Serological testing of 260 equine serum samples by PCFT revealed that antibodies to *Rhodococcus equi* were detected in 51 cases, yielding an overall seroprevalence of 19.6%. The district-level seroprevalence is presented in Table 5.

Table 5. Seroprevalence of *R. equi* by district

District/area	Number of samples	Positive	Seroprevalence, %
Astana city	25	9	36,0
Erementau District	55	23	41,8
Shieli District	100	12	12,0
Zhanakorgan District	80	16	20,0
		P-value	0.2311

Table 5 summarizes the mean seroprevalence values in the four administrative units under investigation. The highest seroprevalence was recorded in the Erementau District (41.82%), whereas the lowest was observed in the Shieli District (12.00%). The result of a one-way analysis of variance, expressed as $p = 0.2311$, exceeds the commonly accepted threshold for statistical significance ($\alpha = 0.05$), indicating that there were no statistically significant differences among the mean seroprevalence levels in the compared groups at the time of the study. Therefore, the observed variation may be attributed to random intra-sample variability. The distribution of antibody titers among the 51 samples that tested positive for *rhodococcosis* is shown in Table 6 and Figure 1.

Table 6. Distribution of the antibody titers

Titer	Number of samples	Proportion, %
1:5	9	17,6
1:10	29	56,9
1:20	8	15,7
1:40	5	9,8

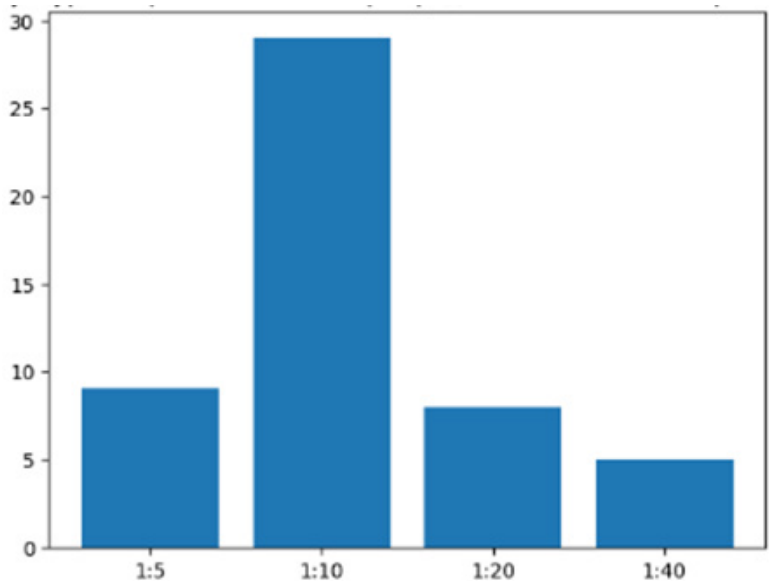
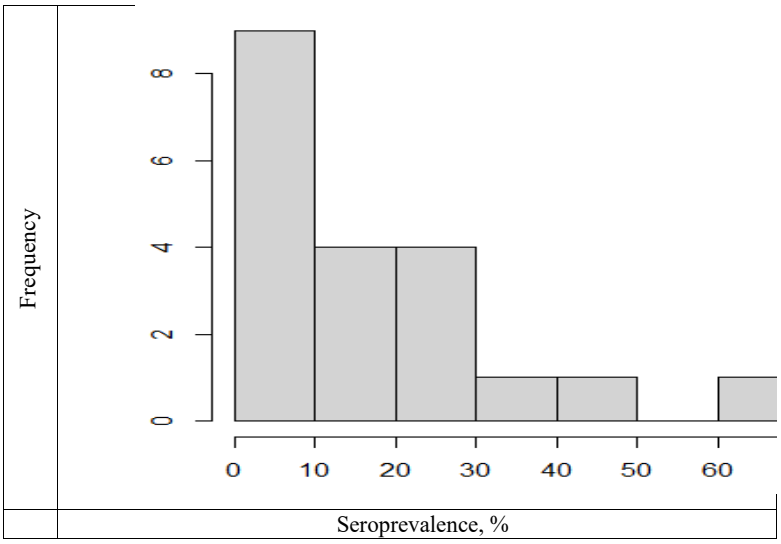


Figure 1. Distribution of R. equi antibody titers among positive samples (n = 51)

Within the structure of positive reactions, the 1:10 titer predominated (56.9%), whereas high titers (1:20-1:40) accounted for 25.5%, indicating pathogen circulation and active epizootic foci.

R. equi infection in horses occurs endemically on some breeding farms and sporadically on others, and it remains unrecognized on most farms [26]. Despite these epidemiological differences, immunological evidence of infection develops in most horses [27, 28], although higher antibody levels are most commonly observed on farms where the disease is endemic. Extrapolating from these epidemiological characteristics, as well as from in vitro studies of macrophage – *R. equi* interactions [29], it appears that young foals may overcome infection when exposed to low numbers of *R. equi*; however, intense or continuous exposure predisposes them to clinical disease, particularly in the absence of antibodies or fully competent cell-mediated immune mechanisms. A progressive increase in environmental contamination with *R. equi* has been observed on horse-breeding farms [30], which is associated with the duration of farm use for housing horses (presumably reflecting horse density and manure disposal practices, summer temperatures, soil type, and whether the farm is used for raising foals).

A statistical summary of the seroprevalence distribution across farms demonstrates pronounced right-skewness: the minimum value and the first quartile are both 0%, indicating a substantial proportion of unaffected farms; the median is 15%, reflecting a moderate typical level; and the arithmetic mean (17.97%) exceeds the median, suggesting the influence of a limited number of high values. This result is further supported by a third quartile of 30% and a maximum of 66.67%. This distribution highlights the heterogeneous nature of the epizootic process, with the formation of localized high-intensity foci against an overall favorable situation in most surveyed sites (Figure 2).



Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
0.00	0.00	15.00	17.97	30.00	66.67

Figure 2. Overall distribution of seroprevalence values in the districts under study

Epizootiological assessment. The predominance of an antibody titer of 1:10 suggests previous exposure or latent infection. High titers (≥1:20) indicate active circulation of the pathogen on multiple farms.

The presence of seropositive animals in all districts indicates a wide distribution of R. equi. The predominance of low and moderate titers is characteristic of an endemic focus with continuous pathogen circulation. The detection of a 1:40 titer necessitates epizootiological control and monitoring of young stock.

Conclusions

Based on the serological survey, infection caused by R. equi is widespread in the studied region but statistically homogeneous at the administrative district level, as evidenced by the absence of significant inter-district differences ($p = 0.231$). The markedly heterogeneous, clustered distribution of the pathogen at the individual farm level is the key epizootiological feature. This is manifested by the formation of local foci with high seroprevalence (up to 66.67%) against a background of predominantly unaffected or minimally affected populations (median 15%, first quartile 0%). This pattern, together with the predominance of the 1:10 titer among positive results and a substantial proportion of high titers (≥1:20), is typical of the pathogen's sustained endemic circulation. Under such conditions, the primary risks and control measures should be focused on specific affected farms rather than based on a territorial approach.

Authors' Contribution

GI and MZ: conceived and designed the study, performed a comprehensive literature search, analyzed the collected data, and prepared the manuscript. MZ, KO, and BV: participated in sample collection, serum separation, and storage. GI, BO, and BV: prepared reaction components and participated in performing the assays, recording the data, and interpreting the results. ED: performed the biostatistical analysis of the obtained results. All authors have read, reviewed, and approved the final version of the manuscript.

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