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BIOLOGICAL CHARACTERISTICS OF RHODOCOCCUS EQUI ISOLATED IN THE REPUBLIC OF KAZAKHSTAN

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Abstract

Rhodococcus equi is characterized by pyogranulomatous bronchopneumonia, septic arthritis, osteomyelitis, ulcerative enterocolitis, mesenteric lymphadenopathy, neonatal diarrhea, and sudden death of young animals. The development of a clinical disease is associated with the immunocompetence of the body of foals. As a worldwide soil infection, it is responsible for approximately 3% of all foal deaths, with a mortality rate of approximately 50%.

The aim of our research was to study the morphological and biochemical properties of the putative *R. equi* strain isolated by us on the territories of our republic.

At about 3-4 months of age, foals are capable of mounting an immune response against *R. equi*, which is why vaccination is given at an early age. The horse industry worldwide urgently needs an effective vaccine to prevent *R. equi* disease in foals, and current scientific research is focused on developing a vaccine using a local strain.

We collected 27 pools manure and 27 pools soil samples from the Almaty, Zhambyl, Turkestan, and East Kazakhstan regions for bacteriological research. The samples were taken from exercise pens, areas within 100 meters of stables, passages, ditches, roads, and flower beds. We isolated a characteristic strain of *R. equi* and studied its cultural, morphological, tinctorial, and biochemical properties. Out of all

the isolates, only the one from the Almaty region was a typical representative of the species *Rhodococcus equi*.

Key words: biochemical characteristic; blood agar; isolate; tryptone soy agar; ram erythrocyte; *Rhodococcus equi*.

Basic position and introduction

Rhodococcus equi is a soil aerobic actinomycete bacterium that infects animals and humans. Human infections are thought to be opportunistic and zoonotic in origin, and may be related to environmental exposure on farms [1-3]. Although clinical cases of *R. equi* are relatively rare in most animal species, foals are often sick, and the incidence is often high on horse farms in countries where horses are bred [4].

For more than 80 years, *R. equi* has been recognized as a lung pathogen in horses. Infection can spread from the lungs to other organs and joints when granulomatous lesions in the lungs rupture and infection of the intestinal mucosa causes diarrhea with ulcerative enteritis and *R. equi* mucosal invasion, which is often seen in chronic disease. Immune complex deposition can cause polysynovitis, which contributes to the development of uveitis, anemia, or thrombocytopenia in infected foals. Sometimes osteomyelitis and arthritis are also observed [5].

R. equi is a Gram-positive, aerobic, non-motile, non-spore-forming and metabolically diverse bacterium. Representatives of the genus Rhodococcus (red pigmented cocci) belong to the phylogenetic group described as actinomycetes of the nocardia form. The infection causes subacute or chronic abscess or purulent bronchopneumonia, ulcerative lymphangitis, enteritis and is the cause of zoonotic infection in foals aged 1-4 months [6, 7].

Although infections can occur in healthy adult horses, they are more common and severe in foals due to their weakened immune systems. It has been found that only a small fraction of all *R. equi* in soil can cause infection, and only *R. equi* carrying virulence plasmids can cause disease in foals [8].

It is known that in some strains of R. equi, the presence of a plasmid encoding a 15–17 kilodaltons protein, called protein A (Vap A), associated with virulence, is responsible for virulence [9]. In 85% of cases, the presence of the Vap A virulence plasmid has been associated with *R. equi* infection in foals over the past few decades [10]. Experiments have shown that the presence of the Vap A expression plasmid in *R. equi* can increase the percentage of macrophages killed in a standard trypan blue assay by 20-70% compared to an equivalent strain without the plasmid.

The present study was undertaken to study the biological characteristics of R. equi, isolated by us in the horse breeding farm of the Almaty region.

Materials and Methods

Collection and bacteriological examination of material from horse breeding farms (feces, soil) to isolate the culture of *R. equi*. From each farm, material was collected from three points:

1 - manure;

2 - "pen for exercise", within 100 meters from the stable;

3 - soil within 15 meters of the stable (passages, ditches, roads, flower beds, etc.).

Manure was collected from randomly selected horses. All samples were scraped from the ground with a small spoon and placed in individual sterile containers designed for collection of biomaterials with a screw cap in an individual package. They were stored at 4°C until further processing.

Samples collected from each of the three points in a given farm were combined to create a single pool. The number of samples are given in tables 1-3.

Table 1 - List of samples obtained from the territory of the Almaty region

No.	District name	Name of the village, farms	Number of samples		
			1	2	3
1	Karasai	v. Kairat	4	3	3
		v. Turar, IE "Dias"	3	3	3
2	Ili	v. Akshi	2	2	2
3	Zhambyl	v. Uzynagash	3	3	3
4	Talgar	v. Panfilovo, Bayserke-Agro LLP	6	4	4
		Nur p/a	6	5	3
5	Enbekshikazakh	Karazhota r/d., “Seisenbaev Zh.” p/a	8	4	4
		Karazhota r/d, “Maukenov N.” p/a	6	3	3
		Akshi r/d, “Kasenov Rahman” p/a	6	5	3
6	Kaskelen	“Aitumar” p/a	7	5	3
		"Gaziz" p/a	5	5	3
7	Kegen	Karkara r/d, “Kumteke” p/a	6	5	4
		Zhylysai r/d, “Bagasharov” p/a	8	5	3
8	Almaty city	Almaty Hippodrome	5	5	4
	Number of pools		14	14	14
Note: v - village; IE - individual entrepreneur; r/d - rural district; p/a - peasant agriculture					

Thus, materials were collected for bacteriological examination from all points in 14 pools from the horse breeding farms of the Almaty region.

The ranking of samples in the Zhambyl region is shown in Table 2.

Table 2 - List of samples received from the territory of the Zhambyl region

No.	District name	Name of the village, farms	Number of samples		
			1	2	3
1	Kordai	Kenen r/d, IE "Tlepbergenov"	7	5	3
		Kakpat r/d, IE "Kumbasheva"	8	5	3

2	Shu	Baluan Sholak r/d, "Kalka" p/a	8	5	4
		Zhanakogam r/d, "Ospanov" p/a	7	5	3
3	Merke	Aktogan r/d, "Tuzelbay" p/a	6	5	3
		Aktogan r/d, “Yesen" p/a	8	5	3
		Zhambyl r/d, "Myrzakhan" p/a	7	5	3
	Number of pools		7	7	7
Note: v - village; IE - individual entrepreneur; r/d - rural district; p/a - peasant agriculture					

Thus, from the horse breeding farms of the Zhambyl region, materials were collected for bacteriological examination from all points in 7 pools.

Thus, materials were collected for bacteriological examination from all points in 6 pools from the horse breeding farms of the Turkestan region (table 3).

Table 3 - List of samples obtained from the territory of the Turkestan region

No.	District name	Name of the village, farms	Number of samples		
			1	2	3
1	Baidibek	v. Zhanatalap, IE "Turdykulov"	7	5	4
		Almaly r/d, IE "Akhataev"	5	5	3
2	Kazykurt	Sharbulak r/d, "Sapa" p/a	8	5	4
		Sharbulak r/d, LLP "Kayyp ata"	6	5	3
3	Tulkubas	Keltemashat r/d, "Uzyn Bulak" p/a	7	5	3
		Ryskulov r/d, "Ak bastau" p/a	8	5	4
	Number of pools		6	6	6
Note: v - village; IE - individual entrepreneur; r/d - rural district; p/a - peasant agriculture					

The total number of samples collected in all three areas was as follows:

1) manure - 27 pools;

2) "Pen for exercise," within 100 meters from the stable - 27 pools;

3) soil within 15 meters of the stable (passages, ditches, roads, flower beds, etc.) - 27 pools.

Additionally, 40 fecal samples from horse breeding farms belonging to the East Kazakhstan region were also subjected to bacteriological research.

Bacteriological research. For selective isolation of *R. equi*, trypton-soy agar medium with ram erythrocyte was used at concentrations of 5-10%.

To prepare the nutrient medium, tryptic soy agar (dry) was mixed with 39.5 g of nutrient medium per 1 liter of distilled water and boiled for 2 minutes until the agar was completely dissolved. The medium was then poured into test tubes of 7-8 cm3 and 500 ml flasks, which were sterilized at 121°C for 15 minutes in an autoclave. After cooling the medium (45-50)ºC,

sheep erythrocytes were added and thoroughly mixed.

Sheep erythrocytes were prepared by taking sheep blood into a sterile mounted flask with beads, defibrinating for 10-15 minutes, and washing with sterile saline until a clear supernatant was obtained at 2500-3000 rpm for 15 minutes.

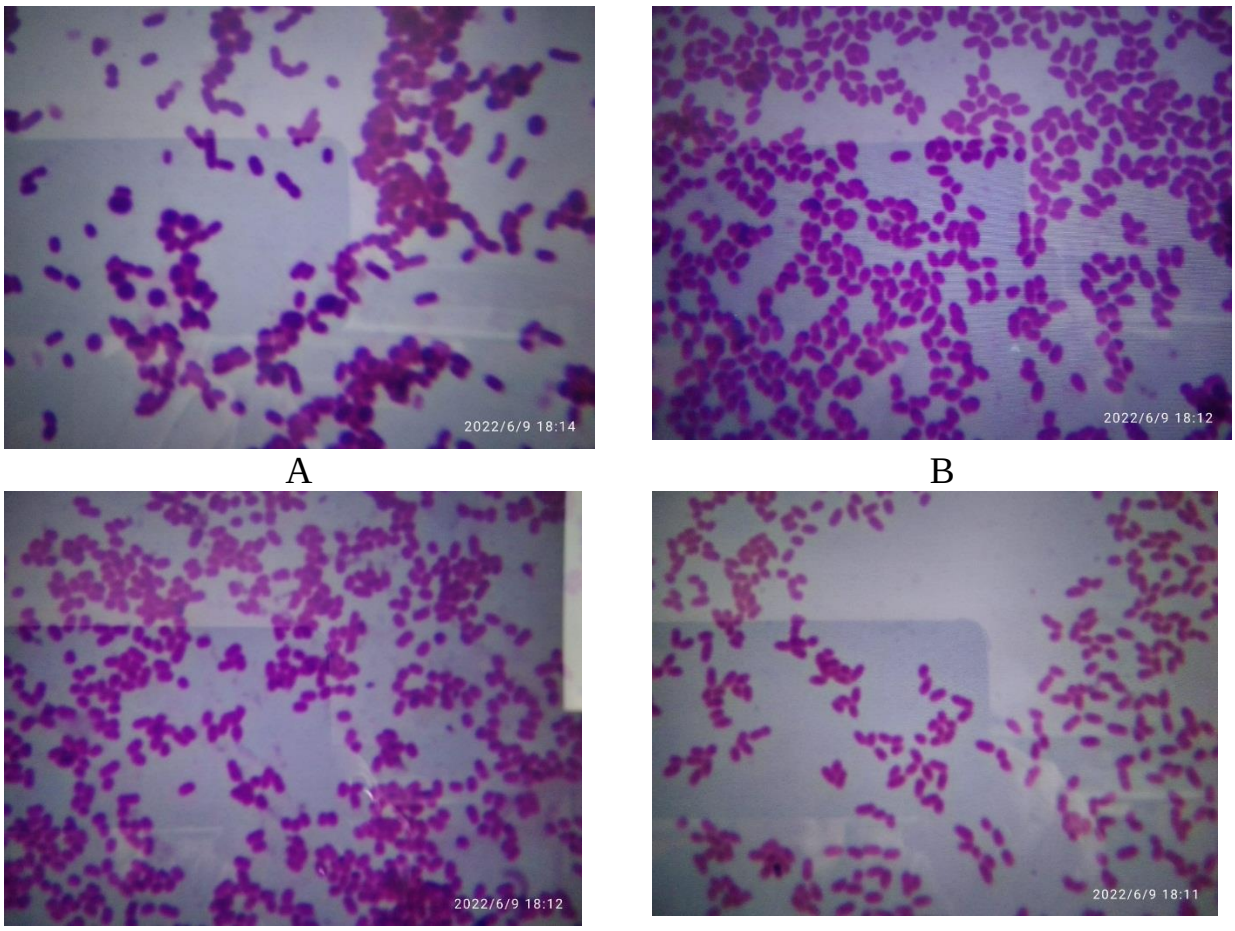
The nutrient medium was poured into Petri dishes and tested for sterility in a thermostat at 37°C for 48 hours. A sterile saline solution was added to the biological material before inoculation until the sample was completely immersed. Inoculations were made on a nutrient medium by applying the seed material with a loop on the surface of the nutrient agar and placing it in a thermostat at 37°C. A visual inspection for culture growth was performed daily, and when characteristic colonies appeared, they were marked with a marker, subjected to microscopy, and subcultured into test tubes with slant agar.

Biochemical research was conducted using the following materials: a Gram stain kit, Hiss medium, Himedia, India, 0.7% meat-peptone agar, 1.2% meat-peptone agar (MPA), meat-peptone broth (MPB), 3% hydrogen peroxide solution, and 1% tetramethyl paraphenylenediamine dihydrochloride solution. The culture was sown in test tubes with MPA and Hiss medium, cultivated at 37ºC for one day, and the results were taken into account.

Prepared MPA in 4 test tubes. Three of them were cultured and one tube served as a control. Then, the daily culture was inoculated into 24 tubes with Hiss medium (with glucose, sucrose, lactose, maltose, mannitol, and sorbitol) with semi-liquid agar (Himedia, India). The crops were cultivated at 37 ºC for one day, then the results were taken into account.

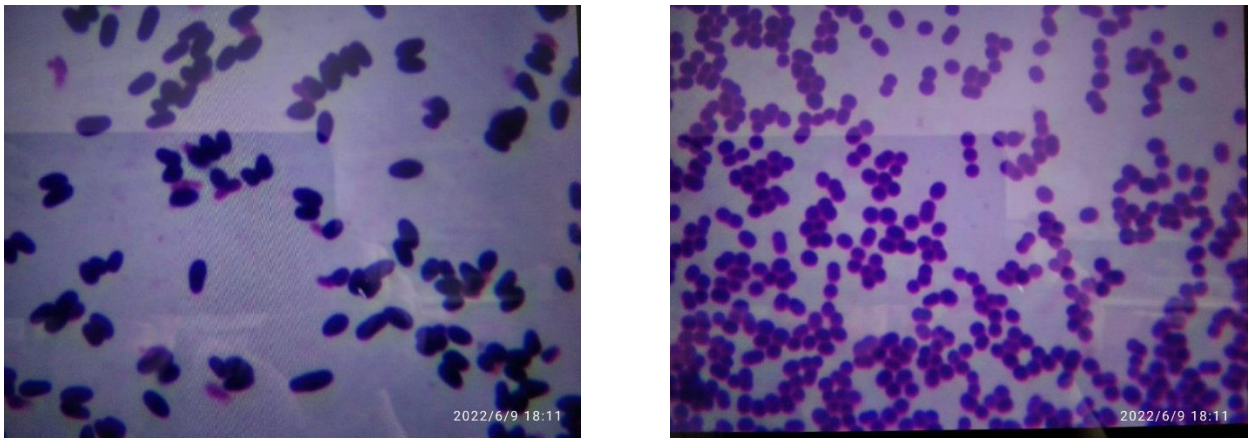
Results

The results of bacteriological studies of pools from the exercise pen and soil showed that smears were stained according to Gram. Gram-negative bacteria were stained in purple or blue, and Gram-positive bacteria were stained in red. *E. coli, Staphylococcus, Streptococcus, Diplococcus, Tetracoccus, rod-shaped* microorganisms, and fungi were found. In the study of manure in 27 pools, micrococcus, short, non-motile, Gram-positive rods were found in 12 (Figures 1, 2), and they were subject to genetic analysis. The ranking showed that 5 of them are from the Almaty region, 4 from Zhambyl, and 3 from Turkestan.

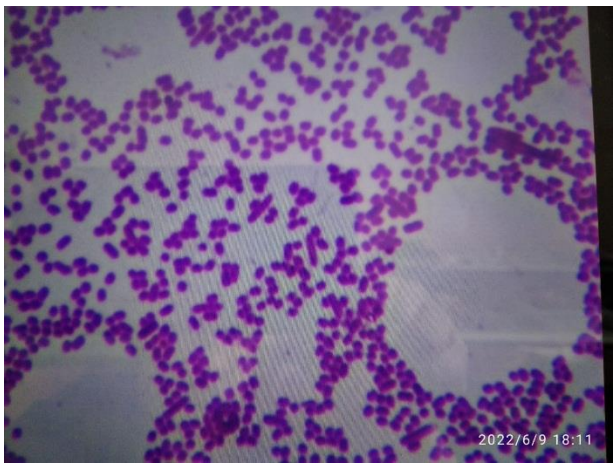


A B C D

Figure 1 - Swabs prepared from the feces of horses belonging to the farms of the Almaty region (A, B, C, D - smears)



A B



C

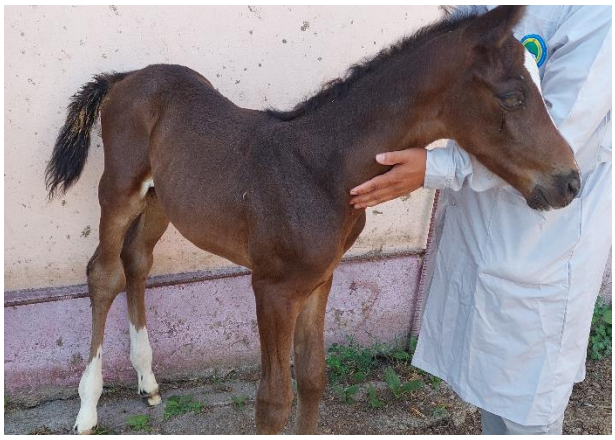
D

Figure 2 - Swabs prepared from the feces of horses belonging to the farms of the Zhambyl region (A, B, C, smears; D - growth of cultures in blood agar)

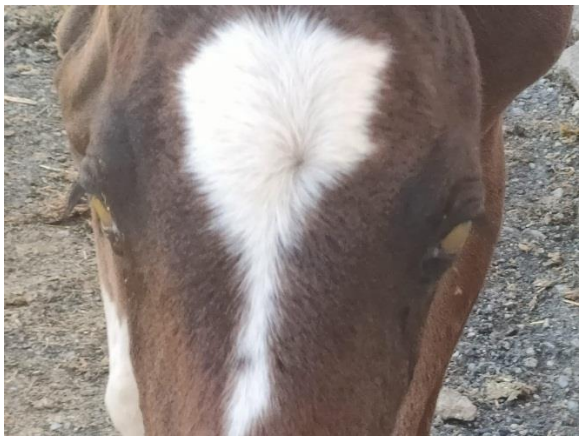
Of note, blood agar showed growth of small, smooth, shiny white colonies after 24 hours of incubation.

Bacteriological studies of 40 fecal samples from farms belonging to the East Kazakhstan region also showed a negative result for *R. equi*.

We conducted clinical studies of horses and foals in a horse breeding farm located in the village of Arkabay, Talgar district, Almaty region, where there was a sporadic case among foals. During the examination, we found one foal exhibiting the following clinical signs: diarrhea, emaciation, pollution of the rear part of the body, swelling of the joints of the fore and hind limbs. Additionally, opacities were also observed in both eyes (Fig. 3).



A



B

Figure 3 - Foal with swelling of the joints (A), blurred eyes (B)

Feces were taken from this animal and from the mother mare for bacteriological examination. Blood agar cultures of both showed growth of small, smooth, shiny and non-hemolytic colonies after 24 hours of incubation, but became larger, slimier, and salmon pink in color with age (Fig. 4). The smears were Gram-stained positively.

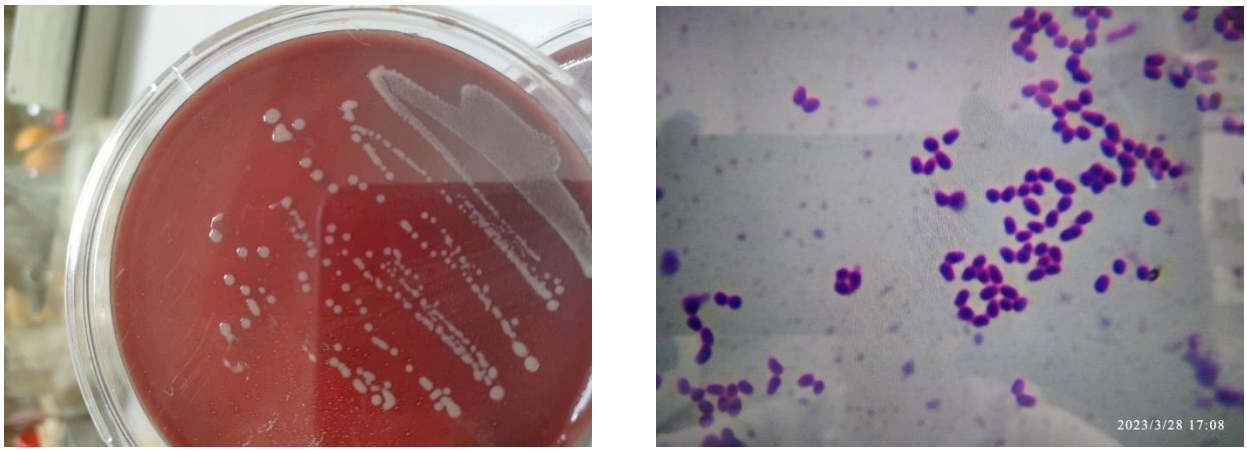


Figure 4 – Colony and smear from foal feces

To differentiate the isolated bacterial culture, its biochemical properties were studied on Hiss nutrient media. The *Rhodococcus equi* culture was found to be catalase-positive and oxidase-negative. It tested negative for glucose, lactose, sucrose, maltose, and mannitol.

Discussion

The isolated bacterial culture from the sick foal was identified as *Rhodococcus equi*. This bacterium is well-known for causing pyogranulomatous pneumonia in foals and as an opportunistic pathogen in other animals and humans. However, there is limited information available on the biochemical properties of animal isolates of this species.

The culture was found to be catalase-positive, oxidase-negative, and negative for glucose, lactose, sucrose, maltose, and mannitol in biochemical studies. The colonies of the culture grew irregularly, were smooth and slimy, and turned salmon-pink to yellow after a week of growth. The smears were Gram-stained positively. The morphology of *Rhodococcus equi* varies from bacillary to coccoid, depending on the growth conditions. Some strains have pili or appendages, but they do not have flagella.

The sick foal showed various clinical signs, including diarrhea, emaciation, hindquarters soiling, joint swelling, and eye clouding.

According to other researchers, the morphology of *Rhodococcus equi* varies from bacillary to coccoid, depending on the growth conditions. If the bacteria are rod-shaped after 4 hours of growth in a culture broth, then after a day of growth in a liquid medium or on blood agar, they become coccoid [11]. *Rhodococcus equi* do not have flagella [12], but some strains have pili or appendages [13]. *Rhodococcus equi* grow in irregular, smooth and slimy colonies that turn salmon pink to yellow after a week of growth [12]. The positive result for acid resistance found in some studies probably depends on the growth conditions and the technique used. *Rhodococcus equi* is a Gram-positive obligate aerobic bacterium. They are catalase-positive, mostly urease-

positive and oxidase-negative, and their optimum growth temperature ranges from 30 to 37ºC. *Rhodococcus equi* produce soluble "equi factor(s)" associated with phospholipase and cholesterol oxidase activity [14]. This factor interacts with phospholipase D from Corynebacterium pseudotuberculosis, b -toxin from Staphylococcus aureus or hemolysin from Listeria monocytogenes, causing complete hemolysis of erythrocytes in sheep and cattle [12]. Nutrient requirements are simple, and carbon can be used from many different sources, including simple organic acids such as propionate or acetate [15], which are found in abundance in herbivore manure [16, 17]. Predilection *R. equi* to lipids as a carbon source is also supported by analysis of the *R. equi* chromosome, which encodes - like in mycobacteria - many genes involved in lipid metabolism, and no gene for sugar transport [18].

The *R. equi* isolate is immobile, and growth in semi-liquid agar is localized strictly in the upper part of the surface of the medium, indicating that aerobic conditions are optimal for this culture.

The most common clinical signs in foals with *R. equi* reflect lower respiratory tract infections and include cough, fever, increased respiratory rate and effort (including flaring of the nostrils), increased heart rate, and abnormal breath sounds in the trachea (often referred to as tracheal rales) and in the lungs (coughs, rales, or both may be heard) [1, 19]. Foals may also show extrapulmonary signs (EPS) of *R. equi* infection [20]. The most common EPS are diarrhea, ulcerative enterocolitis, suspected immune-mediated synovitis, intra-abdominal lymphadenitis or abscess, and uveitis.

Polysynovitis can occur in 40% or more of affected foals. The most commonly affected joints are the tarsocral, carpal, and pastern joints, but other synovial structures may also be involved. These swellings usually cause little more than mild pain and reduced range of motion, whereas foals with septic arthritis usually show severe lameness.

Conclusion

During bacteriological studies of feces in a horse breeding farm, where a foal with clinical signs characteristic of *R.equi* (diarrhea, emaciation, swelling of the joints of the fore and hind limbs, clouding of both eyes) was found, an isolate was isolated. It showed the growth of small, smooth, shiny and non-hemolytic colonies on blood agar and was Gram-positive.

In terms of cultural, morphological, tinctorial and biochemical properties, in general, the isolate was a typical representative of the *Rhodococcus equi* species.

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