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Research Article

Characterization of *Rhodococcus equi* Isolates from Domestic Farm Animals

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Abstract

Rhodococcus equi (R. equi) is a Gram-positive, coco-bacillus bacterium found in the soil. It is an intracellular pathogen which causes rhodococcosis, a condition that can be confused with tuberculosis. The symptoms of *R. equi* infections are often attributed to *Mycobacterium tuberculosis* or different bacteria with similar characteristics in the actinomycete group since those bacteria also cause infections similar to infections caused by *R. equi.* This renders antibiotics ineffective when administered to infected organisms by physicians. This study aimed to characterize bacterial isolates from lymph nodes of domestic farm animals suggestive of *R. equi.* The phenotypic identification includes microbiology methods such as Culturing, where a Salmon-Pink colony on a Brain Heart Infusion Agar is recorded positive for the presence of *R. equi.* Further studies using biochemical methods of Gram-staining and Acid fast staining were also carried out to stain mycolic acids present in the cell envelope of *R. equi* bacteria. Among the 19 samples which were cultured on Brain Heart Infusion agar, 11 showed a distinctive growth of Salmon-Pink color as a phenotypic feature of *R. equi* and 8 showed morphological characteristics of *Mycobacterium marisnus*. Only 10 of them were known to be Gram-positive bacteria. Out of the 10 Gram-positive bacteria, 8 were cocci shaped, 1 was rod shaped and only 1 had both rod and cocci (rodococci) shape among suspected domestic animals at the Kumasi Abattoir Company Limited. All characterizations were based on literature data. All biochemical methods were done using the stock and none of the bacteria were Acid-fast bacilli. This means *R. equi* can be characterized among various bacteria that cause similar infections from the lymph nodes of domestic farm animals by culturing and the use of staining techniques.

Keywords: *Rhodococcus equi*; *Mycobacterium* Species; Gram Staining; Acid Fast Staining; Domestic Farm Animals

Introduction

In the past years, *Rhodococcus equi* (*R. equi*) has been one of the major interesting research subjects due to diseases it causes to farm animals and human beings. The bacterium has been investigated by many researchers using various methods to identify its phenotypic features. *R. equi* is an intracellular opportunistic pathogen causing pneumonia in foals up to 3 - 5 months of age. The bacterium forms smooth and irregular mucoid colonies and acquires Salmon- Pink color on BHI agar in 4 to 5 days [1]. It is classified among *Corynebacterium* genus and infects different domesticated animal host such as cattle, sheep, pigs and goats [2]. Recently, researchers have shown an increased interest in the organism and clinical studies on cats and dogs have given an evidence of pyogranulomatous and pneumonia associated with the bacterium *R. equi* [3]. It can also be found in the submaxillary lymph nodes of some healthy domestic farm animals. The bacterium causes hepatic abscesses, disseminated pulmonary, lymphadenitis and pneumonia in goats. It is also recognized as an opportunistic pathogen that causes pulmonary abscesses in immunocompromised humans [4]. *R. equi* is a saprophytic inhabitant of soil. The clinical disease of the bacteria is enzootic and is widespread on some farms of

domestic animals, sporadic on others, and not recognized in most farms [5]. Diseases mostly affect young foals and rarely affect adult horses. The bacteria cause severe complications to the animals such as bone and joint infection, diarrhea, abscesses in the abdomen and lymphadenopathy. *R. equi* has recently been reclassified due to more findings and put into the suprageneric taxon nocardioform actinomycetes group. A study found that the bacterium contains mycolic acids in its cell wall [6]. However, the detection of *R. equi* with clinical signs of respiratory disease and pulmonary lesions probably mislead many scientists for different organism such as *Mycobacterium*.

Previous study reveals that both *M. tuberculosis* and *R. equi* are characterized by the presence of a special cell envelope consisting of mycolic acids. This makes them distinct and are phylogenetically placed in a group called Mycolata [7]. This unique envelope forms a distinct barrier which is permeable to hydrophilic compounds and enhances granuloma formation and this enables the organism to multiply and destroy macrophages [8]. Apart from their phenotypic similiarities and mode of action, the common symptoms of *R. equi* in the host are also similar to symptoms of *Mycobacteria species* and these factors make it difficult for scientists to achieve an effective diagnoses and treatment during infection.

To our knowledge, research shows no records on *R. equi* infections among domestic farm animals in Ghana and according to a study [9], differentiating between infections caused by *R. equi* and infections caused by other pathogens is problematic especially in surroundings of domestic farm animals without any previous records of infections caused by *R. equi*. Moreover, the reliability for the identification of the bacteria amongst other pathogens in the actinomycete group is limited, resulting in misidentification of the bacteria to other bacteria in the same order [10]. Therefore, this experiment was carried out to characterize the presence of *Rhodococcus equi* in domestic farm animals using culture media for their morphological studies. It addresses a unique procedure to characterize the presence of mycolic acid in *R. equi* using acid fast staining. The findings of this study will help characterize *R. equi* among the various bacteria such as *Mycobacterium bovis* that cause similar infection in domestic farm animals. This research method will also enhance scientist to detect *R. equi* infection and introduce farmers to new pragmatic ways to prevent the bacteria. It will also increase the efficacy of antibiotics against *R. equi* symptoms that mimics the infections of *Mycobacterium* species to establish a simple procedure to identifying the pathogen and ensure an effective treatment after infection.

Materials and Methods

Materials used

Brain Heart Infusion (BHI), Phosphate Buffer Saline (PBS), Crystal violet, Iodine, Safranin, Carbol fuchsin, 20% sulphuric acid, 90% alcohol, Methylene Blue, Distilled water, Falcon tubes, Glass microscope slides, inoculating loop, tongs, Pasteur pipette, Glass bottle and Sterile knife.

Study Area

The lymph nodes of the domestic animals were taken from the Kumasi Abattoir Company Limited (KACL) in the Ashanti region of Ghana.

Methods

Data Collection

Questionnaire administration

Data was collected from animals with the help of a veterinary officer using a structured questionnaire which seeks information on the type of animal (goat, sheep, cattle, pig), origin (local or foreign), sex (male or female) and breed.

Isolation and culturing

Lymph nodes were taken from animals with a sterile knife into coded Falcon tubes containing 10ml of Phosphate Buffer Saline. The falcon tubes containing the lymph nodes and PBS were stored on ice and were brought to the microbiology laboratory of the department of Biochemistry and Biotechnology, KNUST. The lymph nodes were taken from the falcon tubes and cut into smaller pieces on a flat plate under sterile conditions. It was then transferred back into the falcon tubes containing the PBS and shaken vigorously.

A sterilized inoculating needle was used to collect the sample and dragged across the surface of the set up agar BHI (23.5g in 500 ml of distilled water). The cover of the dish was then replaced and incubated at 30˚C in aerobic conditions for 4 days. The media was examined after 24 hours to 4 days.

Gram staining

To ensure the presence of the Gram positive bacteria, an inoculation loop was used to spread the specimen from the stock (cut lymph nodes in PBS) on the slide. The slide was passed through

a flame. Drops of crystal violet stain were added to the fix culture for 1 minute and afterwards the stain was rinsed with excess tap water. Few drops of iodine solution were added to the smear and left for 1 minute. The smear was then rinsed with 95% alcohol. The smear was rinsed with tap water and was flooded with safranin for 30 seconds. It was then rinsed with distilled water, left to dry for 20 minutes and observed under light microscopy using 100× (oil immersion).

Acid fast stain

To detect the presence of mycolic acid in the bacteria, the inoculation loop was used to spread the stock on the slide. It was then air dried to fix the slide. The slide was flooded with carbol fuchsin and was heated until vapor came off the slide. Afterwards the carbol fuchsin was left to remain on the smear for 5 minutes. It was then rinsed off with excess tap water and afterwards 20% sulphuric acid was then added to the smear for 1 minute. The sulphuric acid was rinsed with distilled water and the smear was flooded with methylene blue for 2 minutes. It was rinsed with distilled water and then observed under oil immersion.

Results and Discussion

The lymph nodes of nineteen (19) domestic animals comprising of sheep, goats, cattle and pigs were used for the study. The number of foreign breeds were seven (7) representing 36.84% and the local breeds were twelve (12) representing 63.16%. The sex and the name of breed used are shown in table 1.

Rhodococcus equi found in foals and domestic animals can survive inside macrophages, a characteristic considered as the basis for its pathogenicity. *R. equi* infection on domestic animals as described by Khurana [9] led to the selection of domestic farm animals used in this study. The study found *R. equi* in goats and cattle and it is consistent to literature [11]. The lymph nodes are known to harbor some microorganisms and this research reflects on the findings [12,13] that used lymph nodes of wild boars to characterize some bacteria from it, including *Rhodococcus equi*. Domestic farm animals that show signs like pneumonia, osteomyelitis, lymphadenitis and abscess are likely to have either one of the genus group such as *Mycobacterium* or *Rhodococcus* in their lymph node. Culturing and other biochemical tests help to distinguish the different types of microorganism in the lymph node of these farm animals. The lymph nodes collected in Falcon tubes containing 10ml PBS were stored on ice to keep the bacteria alive

Animal	Number designated	Sex	Origin	Breed
Cattle	CO1	M	Local	West African Short horn
	CO2	M	Foreign	Muturu
	CO3	F	Local	West African short horn
	CO4	M	Foreign	Muturu
	CO5	M	Foreign	Budali
	CO6	M	Foreign	Budali
Goat	GO1	F	Local	Ashanti dwarf
	GO2	F	Local	Ashanti dwarf
	GO3	F	Local	Ashanti dwarf
	GO4	M	Foreign	Swahalian
	GO5	M	Foreign	Swahalian
	GO6	F	Local	Ashanti dwarf
	GO7	F	Local	Ashanti dwarf
Pig	PO1	M	Local	Large white
	PO2	M	Local	Large white
	PO3	M	Local	Large white
Sheep	SO1	M	Local	Ashanti dwarf
	SO2	M	Local	West African dwarf
	SO3	M	Foreign	Swahalian

Table 1: Types of animal used and their respective breeds.

M: Male; F: Female.

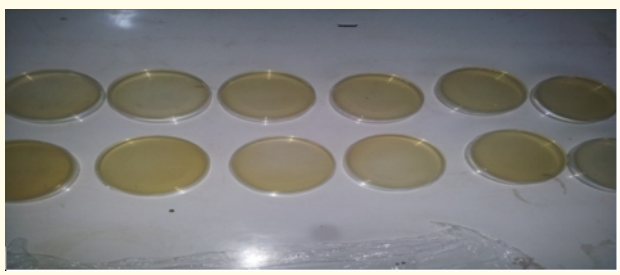

Figure 1: BHI in Petri dishes after 24hours.

There was no growth of any organism on the Agar after sterility-check was done after 24 hours. This shows that there was no contamination before culturing. It indicates that the agar used was free from contaminants and that the nutrients to support the

growth of certain bacteria were in correct proportions as produced by the manufacturer. This was also consistent with the findings of [13] when BHI was used in their experiment. The study found out that there was no growth on the BHI during the sterility check [13]. The stock spread on the agar was incubated at 30°C to enable the growth of *R. equi*. This is supported by another study which reveals that 30°C is a favorable temperature for the growth of the bacteria [14]. The bacteria *R. equi* are facultative organisms and keeping them under aerobic conditions wouldn't affect them. This was in agreement to previous studies and that was the reason why the incubation was done in aerobic conditions [15,16].

On observing that there was no growth on the agar plates, the stock that was spread on all the agar plates and incubated at 30°C after day one all showed growth of yellow colonies as shown below.

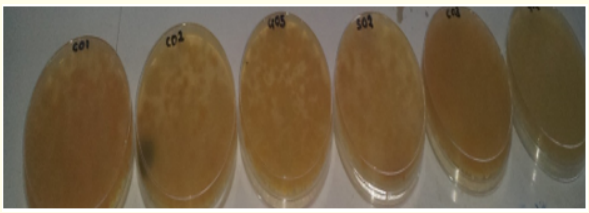

Figure 2: Growth of bacteria colonies on agar after 24hours.

On day 4, it was observed that there was growth of Salmon-Pink colonies which were smooth, irregular and mucoid on some agars and some also contained yellowish colonies.

After 24 hours, the growth of yellow colonies observed on the agar in figure 2 shows that the BHI is rich in nutrient that supports a faster growth of bacteria. As some of the agar formed yellow colonies on them, even more distinctive on day 4 (Figure 3), others formed Salmon-Pink colonies. The formation of yellow and pink colonies on some of the media agrees to findings which concluded that the BHI media is non-selective and non-differential because it supports growth of different bacteria. According to the findings these individual colonies must be sub cultured onto additional media to ensure bacterial purity and then further characterized by standard microbiological test to obtain a confirmatory diagnosis [17].

The characteristics described by previous studies on *R. equi* were investigated in the confirmed isolates [18].

The growth of a smooth, irregular, mucoid and Salmon-Pink colony on BHI seen in figure 4 agrees well with previous studies [18]. This can be stated in confirmation to literature that the Salmon-Pink color on some of the agar signifies the presence of *R. equi*. Pigment production was not observed in cultures less than 2 days old, but up to 4 days colonies developed a delicate salmon-pink color. The present finding also supports the study which also concluded that *R. equi* bacteria develops its morphology as a delicate salmon-pink color from day 4 to day 7 during incubation [14]. The yellowish mucoid colour on other agars in figure 4 denotes the presence of different bacteria. Phenotypically, *Mycobacterium marinum* from the same phylum Actinobacteria with *R. equi* forms yellowish colonies [19] hence the yellowish colonies seen on some of the agar. The bacterium lives in water and can contaminate the water given to domestic animals. The infected organ in domestic farm animals is unknown and because BHI is non-differential, it enhanced the growth morphology of the organism.

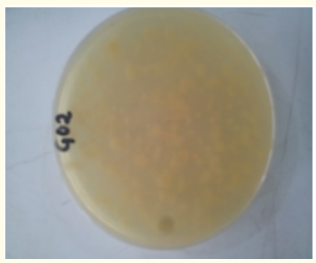

Figure 3: Growth of yellowish colony on agar (GO2).

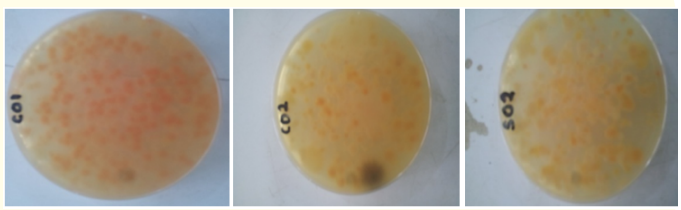

Figure 4: Growth of Salmon-Pink, smooth, irregular and mucoid colonies on some agar.

After day four (4), the growth of yellow colony was observed on the agar labeled GO2 as indicated in figure 3 above. Yellow colonies were also observed on eight (8) Agar plates in all.

On day four (4), Salmon-Pink and Yellow colonies were formed on the Agars and table 2 gives a summary of the colored colonies formed on various samples.

Colored colonies formed	Sample code
Salmon-Pink	CO1, CO2, CO3, CO6, PO2, PO3, SO2, GO1, and GO3
Salmon-Pink and Yellow	SO3 and GO6
Yellow	CO4, CO5, PO1, SO1, GO2, GO4, GO5, GO7

Table 2: Colored Colonies formed on each sample on day four (4).

Escherichia coli also forms yellowish colonies on BHI and because the bacterium is not found in the lymph nodes of domestic farm animals, it can be said that the colonies formed which denotes the presence of *E. coli* on the BHI could be as a result of contamination. Both *M. marinum* and *E. coli* have similar morphology as *Rhodococcus equi.* It is therefore possible that *M. marinum* and *E. coli* were among the bacteria that formed the yellowish colonies on BHI agar as shown in table 2. It is important to note that BHI is non-selective and non-differential medium, which supports the growth of aerobes, anaerobes and other fastidious microorganisms.

Identification of *R. equi* has been carried out severally by many researchers using microscopic and molecular techniques. The use of Gram staining and acid fast staining was used to identify the bacteria present in this research work, which is suitable for *R. equi* identification and differentiation [20]. Regarding the Gram staining properties, identification of a Gram positive bacterium in each of the samples gave a confirmation to the morphology of the bacteria suspected to be *R. equi.* Since *R. equi* is a Gram positive bacterium, the results for Gram staining confirms the result by earlier literature [12,18] that found *R. equi* to be a Gram positive bacterium.

Out of the nineteen (19) samples, 10 contained Gram positive bacteria within the lymph nodes and 9 had only Gram negative bacteria. The calculations of this work suggest that 47% are Gram negative and 53% had Gram positive bacteria. Figure 5 shows the percentage samples that have Gram positive and Gram negative bacteria.

Sample code	Gram staining and shape of bacteria		Acid fast staining	
	Positive	Negative	Acid fast	Non-Acid fast
CO1	Cocci	Cocci	-	Yes
CO2	Cocci	-	-	Yes
CO3	Cocci	Cocci	-	Yes
CO4	-	Rods, cocci	-	Yes
CO5	-	Rods	-	Yes
CO6	Cocci	-	-	Yes
GO1	-	Cocci	-	Yes
GO2	-	Cocci	-	Yes
GO3	Rods	Cocci	-	Yes
GO4	-	Rods	-	Yes
GO5	-	Cocci	-	Yes
GO6	Cocci	Rods	-	Yes
GO7	-	Rods, cocci	-	Yes
PO1	-	Rods, cocci	-	Yes
PO2	Rods, cocci	Rods	-	Yes
PO3	Cocci	Rods	-	Yes
SO1	-	Rods, cocci	-	Yes
SO2	Cocci	Rods, cocci	-	Yes
SO3	Cocci	Rods, cocci	-	Yes

Table 3: Results for Gram staining and acid fast staining of the stock.

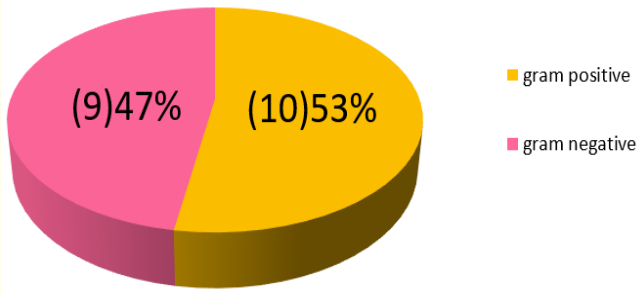

Figure 5: The percentage of gram positive bacteria.

Out of the ten (10) Gram positive bacteria, eight (8) were cocci-shaped, 1 was rod-shaped and 1 contained both rod and cocci shapes.

After examining under a light microscope, it was observed that 80% of all samples that had Gram positive bacteria in them were cocci-shaped. 10% were rod-shaped and 10% were rod and cocci shaped having a rod-like to cocci-shapes taking the shape of *R. equi.*

The figure below denotes the number of rods, cocci, and rhodococcal shapes of the different Gram positive bacteria.

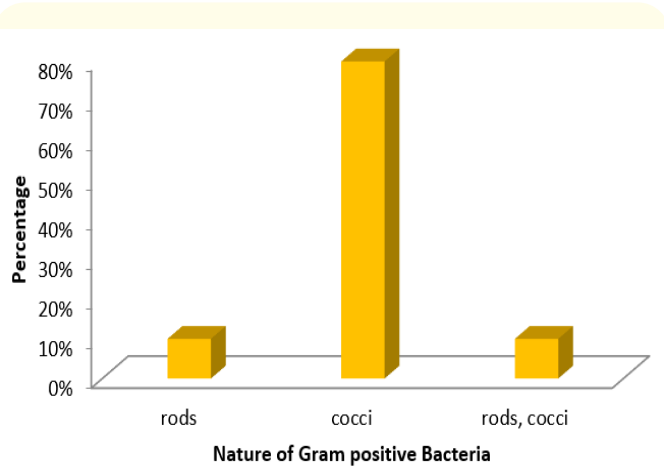

Figure 6: Percentage of rods and cocci shapes in all sample.

Previous studies characterized *R. equi* as a Gram positive pleomorphic coccobacillus varying from distinctively coccoid to bacillary. The sample PO2 gave a distinctive morphology of *R. equi* when a mucoid, smooth and irregular Salmon-Pink colonies was observed. Further studies using Gram staining proved the presence of *R. equi* in the stock when a rod to coccus (*Rhodococcus*) shape was examined under the microscope. The present features observed as characteristics of *R. equi* agrees well with literature [2,12,21]. All the characteristics observed in both culturing and Gram staining proved positively to the physiological and chemical characteristics of *R. equi.*

In comparison to literature, it can be said that Gram positive rod in sample GO3 could be the bacterium *Bacillus anthracis*. Since this bacterium is a Gram positive rod shape that lives in the lymph nodes of domestic farm animals and causes pulmonary necrosis in domestic farm animals. However, the Gram negative bacterium could possibly be a *Diplococcus* species. Species of *Diplococcus* also affect the lymphatic system when it gets into domestic animals. *Diplococcus* does not live in the lymph node of domestic farm animals but could be as a result of contamination from the sample collection. The Gram positive cocci bacterium present in samples CO1, CO2, CO3, CO6, GO3, PO3, SO2 and SO3 could also be *Streptococcus pneumoniae*. This bacterium also resides in the lymph nodes of domestic farm animals and causes pneumonia to farm animals. *Salmonella* which is a facultative anaerobe is a Gram negative rod-shape that lives in the lymph nodes of domestic farm animals. It is zoonotic and causes gastroenteritis and osteomyelitis to animals. It can therefore be postulated that the Gram negative rod-shaped bacteria in the lymph nodes seen in the three samples from sheep, CO4, CO5, GO4, GO6, GO7, PO1, PO2 and PO3 contained *salmonella species* or *Diplococcus species* that lives in the lymph nodes of domestic animals. Adding to the fact that the bacteria *Diplococcus* present in the sample could be as a result of contamination from the sample collection. Polymerase chain reaction which is specific, and a sensitive method use to characterize the bacteria genotypically will help determine the specific organism present since it will target the correct genetic sequence of the specific bacteria. The findings show that the number of cocci bacteria in lymph nodes of the domestic animals used outnumbers the number of rods and it means that the prevalence rate of Diplococcus species among domestic farm animals is very high. This is in consistent with earlier research [22] that pointed out clearly that the prevalence rate of *Diplococcus* is epidemic among healthy or unhealthy domestic farm animals.

Although growth of yellow colonies on the media for samples CO4, CO5, GO2, GO4, GO5, GO7, PO1 and SO1, the *Mycobacterium marinum* suspected to be present could not react positively to the Gram staining. This is not a characteristic of the organism as seen in the results. The Gram staining was carried on the stock and the peptidoglycan wall of the bacterium was not fully differentiated to retain the crystal violet. It would have been different if the Gram staining was carried out on the colony seen.

Moreover, the acid fast gave a negative result for all samples used. Some bacteria are able to resist the decolorisation of acid alcohol and give a light pink distinct colour in a blue background when examined under oil immersion using 100× objective lens. The results indicate that none of the Gram positive bacteria was

acid fast bacteria. The result is in agreement with the study which indicates that the presence of cell envelope with Mycolic acid in a bacterium makes it possible for that bacterium to be termed as an acid fast bacterium [14]. Examples are *Rhodococcus equi, Mycobacterium tuberculosis* and *Nocardia*. Since the suspected rods and cocci Gram positive bacteria found in the samples do not fall under this group, they are thus termed as non-acid fast bacteria. Although the phenotypic characterization of *R. equi* was observed in some agars, it wasn't clearly examined under the microscope to get correct shape of the organism since the stock was used. A Gram-stained on a sub cultured selective agar would give a positive result of the pleomorphic coccobacillus shape of the bacteria as used in previous studies [5]. On the contrary, a Gram staining on the sub cultured colonies would prove better than the use of the stock since the use of the stock had a limited positive reaction with the reagents to all suspected colonies seen in the Agars. A light microscope with a high power objective lens might help reveal the shape of the bacteria in cases where the stock is Gram stained. The Gram stain on the sub cultured colony would give a positive result as carried out by a different study [18] in the characterization of *R. equi.* The bacterium has an inconsistent feature and according to previous studies [14], *R. equi* can be acid fast positive or non-acid fast depending on the growth age of the culture. Molecular techniques would help detect the presence of the bacterium. However, it can be clearly seen that the two methods used produced similar results in the characterization of *R. equi* in domestic farm animals.

Conclusion

The purpose of this study was to characterize phenotypically the presence of *R. equi* in domestic farm animals. The study has shown that *R. equi* indeed forms Salmon-Pink colonies, which are smooth, mucoid and irregular shape on BHI agar.

The second major finding was that the conformation of *R. equi* can be determined using microbiology techniques such as Gram staining and acid fast staining to detect the rod and cocci forms of the bacteria. The findings from this study make contributions to the current literature. First, *R. equi* is present in domestic animals and microbiology techniques can help to characterize it. Farmers should ensure regular and proper cleaning of the environment of domestic animals to help reduce the levels of bacteria effectively to prevent the incidence of *R. equi* infection.

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