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RHODOCOCCUS EQUI: AN APPROACH TO THE METHODOLOGY OF PRESUMPTIVE IDENTIFICATION AND TESTS OF SENSITIVITY TO ANTIMICROBIALS

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Abstract: *Rhodococcus equi* is a Gram-positive bacterial agent and contains mycolic acids in the cell wall, being classified in the group of aerobic actinomycetes. The relevance of *R. equi* is remarkable for veterinary and human medicine, as it is considered a zoonotic agent that causes rhodococcosis in animals and humans, with pulmonary infection being the most common. *R. equi* is considered a pathogenic agent in foals and has emerged as an opportunist in humans, especially associated with human immunodeficiency virus infection. Pulmonary rhodococcosis has clinical and pathological characteristics similar to pulmonary tuberculosis, in immunocompromised and non-immunocompromised patients. In this review, we address a methodology that can be implemented in microbiology laboratories, especially those that carry out diagnosis, in view of the occurrence of bacterial isolates that resemble the phenotypic profiles of *R. equi,* valuing the importance of its isolation and identification, even if presumptively. The study was based on the review of scientific articles, case reports, monographs, dissertations and theses, published in magazines, periodicals, books and materials available online. The presumptive identification of *R. equi* is carried out based on a variety of phenotypic characteristics, initially considering the colonial and cellular morphology, partial resistance to acid alcohol and the production of the *factor equi* demonstrated in the CAMP Test. The treatment of rhodococcosis is still quite incipient and in Brazil there are few works carried out to evaluate the sensitivity profile to the current antimicrobials for this bacterial agent. The standard test recommended by the "Clinical and Laboratory Standards Institute/ National Committee for Clinical Laboratory Standards" for aerobic actinomycetes, including *Rhodococcus* sp, is the determination of the Minimum Inhibitory Concentration (MIC), which can be performed using the broth microdilution method. Müeller Hinton (MH broth) supplemented with cations. There are still no standardized methods for testing the sensitivity of aerobic actinomycetes, using the Disk Diffusion method, however, in recent years, several antimicrobial agents have been tested, such as amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, erythromycin, imipenem, minocycline and sulfamethoxazole- trimethoprim. Several researchers have followed the interpretation criteria of accepted cutoff points for *Staphylococcus* sp. Due to the occurrence of human rhodococcosis with the increase in the number of cases of infections by *R. equi,* there is a need to use more appropriate identification techniques, as well as more clinical studies to delineate a conclusion of diagnosis and treatment schemes. Presumptive identification is often enough to initiate empirical treatment with antimicrobial agents.

Keywords: *Rhodococcus equi,* presumptive identification, "camp *factor*" Cholesterol Oxidase, antimicrobials, Disk Diffusion, CIM.

INTRODUCTION

Bacteria that contain mycolic acid in their cell walls are part of a group of Gram-positive bacillary organisms determined as aerobic actinomycetes, consisting of taxonomically divergent and heterogeneous genera, belonging to the phylum Actinobacteria, in the class Actinomycetes, subclass *Actinobacteridae, order* Actinomycetales *and* suborder *Corynebacterineae* [1.] This group includes the genus *Rhodococcus,* which houses a diversity of species that vary in morphology, growth patterns and biochemical characteristics. They are characterized as Gram positive, strict aerobic, immobile and catalase producing bacteria. They may have branched filaments which, when present, are rudimentary, transient or extensive and fragment into

pleomorphic bacilli and coccoid forms [2].

Rhodococcus species are associated with a variety of environments, being found in soil and animals, particularly in abundance in the excreta of herbivores. Some strains are considered pathogenic for animals and humans. *Rhodococcus equi* is one of the most common species found in soil, along with *R. coprophilus, R. erythropolis, R. globerulus, R. rhodochrous* and *R. ruber.* As for the manure of herbivores, *R. equi* and *R. coprophilus* are the most common species. Other species are related to exclusive means as in the cases of *R. fascians* isolated from plants and intestinal tract of carp; *R. marinonascens* isolated from the uppermost layer of marine sediment and *R. rhodnii* isolated from the intestine of a certain species of bedbug. Certainly, *R. equi* is the main species involved in infections in humans [3,4,5.]

This work aimed to address a methodology that could be implemented in microbiology laboratories, especially those that carry out diagnosis, in the face of the occurrence of Gram-positive bacilli isolates suggestive of *Rhodococcus* sp, valuing the importance of their isolation and identification, even if presumptively. The elaboration was based on the review of bibliographical publications related to rhodococcosis. Scientific articles, case reports, monographs, dissertations and theses, published in magazines, newspapers, periodicals, books and materials available online were consulted. The descriptors used were *Rhodococcus equi,* presumptive identification, "camp *factor"* Cholesterol Oxidase, antimicrobials, Disk Diffusion, MIC.

TAXONOMY AND PATHOGENESIS OF *R. EQUI*

R. equi was originally described by Goodfellow and Alderson in 1977 [6] through a numerical taxonomic classification study with 177 strains representing the "rhodochrous" complex and the genera *Gordona, Mycobacterium* and *Nocardia.* These researchers subjected the strains to 92 unitary traits whose data were analyzed by computer, resulting in three similarly defined clusters at the 75 to 80% level. The first cluster proved to be heterogeneous and corresponded to the "rhodochrous" taxon, while the other two contained strains of *Mycobacterium* and *Nocardia,* respectively. The good correlation between numerical analysis and chemo-taxonomic, serological and genetic data collected in previous studies provided sufficient evidence to elevate the "rhodochrous" taxon to generic status. Thus, the genus *Rhodococcus* was recognized to contain 9 species: *R. rhodochrous, R. bronchialis, R. coprophilus, R. corallinus, R. erythropolis, R. equi, R. rhodnii, R. rubrus, R. rubropertinctus* and *R. terrae* [7.]

Taxonomically, *R. equi* has been the subject of progressive studies in the understanding of its molecular nature. Although it is a recognized zoonotic bacterial pathogen, there has been taxonomic debate since the 1980s regarding the validity of the name *R. equi,* for which changes to *R. hoagii* and *Prescottella equi have been proposed* as official alternative names. Other nomenclatures were also previously used, including *Nocardia restricted, Corynebacterium equi, Bacillus hoagii, Corynebacterium purulentus, Mycobacterium equi, Mycobacterium restricted* and *Proactinomyces restricted* [8,9.]

According to Vázquez-Boland & Meijer (2019) [10] and Vázquez-Boland et al., 2020 [11,] the recent rise of *R. equi* as a new paradigm of multi-host adaptation has been accompanied

by an unusual instability in nomenclature with a confusing succession of names: "*Prescottia equi*", "*Prescottella equi*", *Corynebacterium hoagii* and *Rhodococcus hoagii*. These researchers reviewed current advances in the genomics, biology, and virulence of these actinobacteria, confirming their pathogenesis due to a unique mechanism of plasmid-determined animal host tropism. They also discussed the taxonomic and nomenclatural issues surrounding *R. equi* regarding recent phylogenomic evidence confirming its membership as a bona fide species of the genus *Rhodococcus*. Due to its recognition as a zoonotic pathogen and its bacterial name being solidly established in the veterinary and medical literature, the authors further argued that changing the nomenclature could cause error and confusion. Therefore, they requested the rejection of *R. hoagii* and the conservation of the name *R. equi,* in accordance with the conclusions of their studies.*R. equi* is the agent of rodococcosis, a zoonotic infection generally associated with pyogranulomatous pneumonia in domesticated or wild animals, especially in mammals with a predominant incidence in horses [2,12,13.] Confirmation of *R. equi* as a pathogen that causes pneumonia in foals occurred in 1923 and it was recognized as an agent of infections in humans in 1967 [14.] Since then, it has been described as an opportunistic pathogenic agent, mainly in immunocompromised patients [15,] however, immunocompetent people can acquire the infection[16.]

The occurrence of human rhodococcosis has increased, especially in people with weakened immune systems, including cancer or transplant patients who are using immunosuppressive treatment, as well as those with advanced and untreated HIV/AIDS [17.]

The lung is considered the site of primary infection, which is sometimes characterized by cavitary lung lesions similar to those seen in tuberculosis. Symptoms presented by patients include high fever, productive cough, chest pain and hemoptysis. On chest X-rays, dense pulmonary infiltrate, also described as nodules, is usually seen. These lesions can cavitate and form abscesses with the presence of pleural effusion and have been reported in more than 80% of immunocompromised patients. Thus, rhodococcosis can easily be confused with pulmonary tuberculosis, causing the patient to be subjected to the wrong treatment [18.]

In addition to representative clinical samples from the lung, *R. equi* has been isolated in cultures of representative samples from other sites of infection including the central nervous system, pelvis, subcutaneous tissue and lymphatic system [5,14,19.] Other clinical manifestations of rhodococcosis are represented by abscesses located in various organs such as the kidneys, liver, brain, retroperitoneal region, joints and bones [20.]

Although rhodococcosis is attributed to *R. equi,* other species can be considered as rare causes of infections in humans, including *R. aurantiacus, R. erythropolis, R. globerulus, R. gordoniae, R. corynebacteroides* and *R. rhodochrous* [21,22.]

The cellular morphology of *R. equi* is marked by a morphogenetic cycle that starts with the cocci or short bacilli stage, with different organisms showing a succession of more or less complex morphological stages, by which the complementation of the growth cycle is achieved. Thus, cocci germinate between short bacilli, forming filaments with lateral projections, showing elementary branching or, in most differentiated forms, producing extensively branched hyphae. The next generation of cocci or short bacilli is formed by the fragmentation of these elements. The cell wall of *R. equi* has mycolic acid with a chain of 30 to 54 carbon atoms,

it shows partial resistance to acid alcohol when stained by the Zihel Neelsen method. Due to simple nutritional requirements, this bacterial agent can be recovered in cultures from various environmental sources and clinical samples from humans and animals. All *Rhodococcus* species grow on nutrient agar and other non-selective media. They may have rough, smooth or mucoid colonies, with different colors such as yellow, orange, cream, red, salmon or colorless [2,5, 23.]

In this context, colonial and cellular amorphology, together with partial resistance to acid alcohol are key characteristics for initial characterization [5.] It must be based on the characteristics of the colonies grown in the culture media used and the cellular morphology of the bacteria, observed in the Gram and Kinyoun staining methods, modified for actinomycetes [24.]

PHENOTYPIC CHARACTERISTICS OF *R. EQUI*

MACROSCOPIC CHARACTERISTICS OF THE COLONIES

Colonies develop on non-selective culture media normally used in the microbiology laboratory, including those used for isolating mycobacteria and fungi. In solid culture media they are viscous, mucoid and coalescent, with a size of 2 to 4 mm. They grow best at temperatures ranging from ambient to 37°C. Pigment formation is rarely observed in cultures less than four days old. *R. equi* is strictly aerobic and does not show motility, positive catalase and negative oxidase [2,5.]

Satisfactorily, suspected bacterial isolates of *R. equi* form colonies faster on blood or chocolate agar media (various media can be used as a base, especially Muller Hinton Agar (MHA), Brain Heart Infusion Agar (BHIA) and Triptyc Soy Agar (TSA). Each isolate must be seeded in plates of any of these media, which are incubated aerobically at a temperature of 35° – 37° C for up to 5 days, with a daily reading being taken during this period. The colonies are mucoid in appearance and vary in color from cream, orange, salmon and pink [2.]

Colonial characteristics are more evident after 48 hours of incubation, with entire edges, irregularly round, smooth, semitransparent, shiny, coalescing, mucoid, in the shape of teardrops, varying in size (Figure 1).

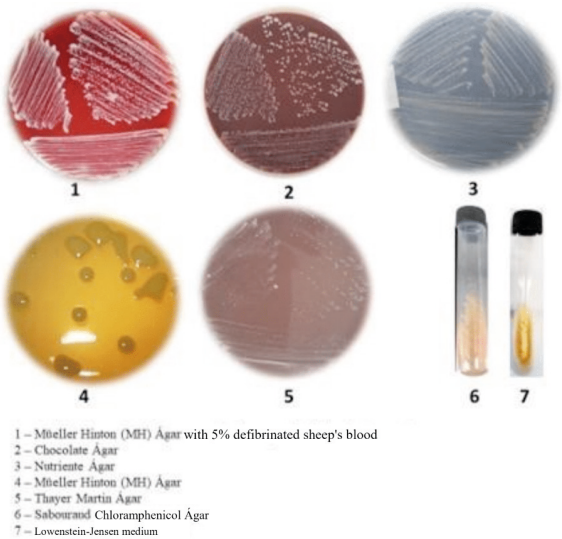


Figure 1. Culture of *R. equi* ATCC 6939 on the related culture media. The photos illustrate the typical (mucoid) appearance and variation in shape of colonies after incubation period in aerobic conditions for up to 5 days at 37°C. Photos: Paulo da Silva.

MICROSCOPIC CHARACTERISTICS OF *R. EQUI*

Each and every bacterial isolate must be submitted to microscopic examination, through smears prepared on slides, from colonies isolated in solid culture media or broth culture media and subjected to modified Gram and Kinyoun stains (described below):

MODIFIED GRAM STAIN FOR ACTINOMYCETES [24.]

Aniline+crystal violet solution (Aniline 4 g in 100 mL of distilled water) + (11.4 mL of Crystal violet, saturated solution in absolute alcohol) *.

*add crystal violet in 100 mL of alcohol, stirring constantly, until complete dissolution.

-Weigh the aniline and place it in a jar with a lid; add the water and shake the sealed bottle to dilute the aniline.

-Filter the mixture through 4 overlapping paper filters moistened with water.

-Add the crystal violet solution to the filtrate.

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

Lugol's solution (1g of resublimated iodine + 2g of potassium iodide in 300 mL of distilled water).

-Weigh and mix the 2 substances in a glass mortar, macerating with a pestle until a homogeneous substance is formed; add water slowly to dissolve.

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

Saturated solution of safranin (2.5g of safranin and dissolved in 100 mL of absolute alcohol).

-Add 10 mL of sol. Saturated with safranin in 90 mL of distilled water.

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

staining procedures

Cover the smear with crystal violet for 2 minutes and rinse; cover the smear with Lugol for 1 minute and rinse; bleach with absolute alcohol for 30 seconds and rinse; cover the smear with safranin for 30 seconds, rinse and allow to dry.

KINYOUN STAINING, MODIFIED FOR ACTINOMYCETES [24.]

Kinyoun's fuchsin solution (4g of basic fuchsin in 20 mL of absolute alcohol + 8g of crystallized phenol; add 100 mL of distilled water.

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

Acidic alcohol (3 mL of concentrated hydrochloric acid in 97 mL of absolute alcohol).

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

Methylene blue solution (2.5g of methylene blue in 100 mL of absolute alcohol).

-Transfer the volume to an amber bottle with a lid. Keep in an open and dry place.

staining procedures

-Make a slide smear. Let it dry and fix it in the flame by flaming it three times.

-Cover the smear with Kinyoun fuchsin for 3 minutes without heating and rinse.

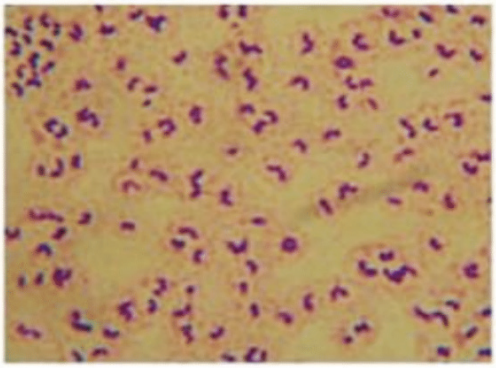
-Bleach with acid alcohol for 5 to 10 seconds and rinse.

-Cover the smear with methylene blue solution for 30 seconds, rinse and allow to dry.

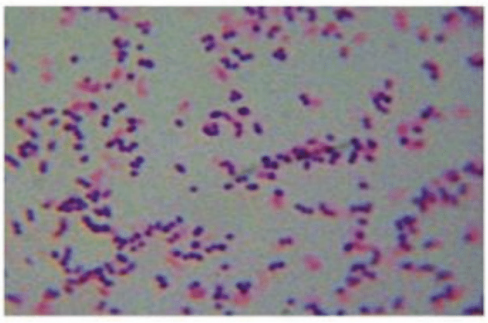
In the smears stained by the modified Gram method, Gram-positive, pleomorphic cocci or coccobacilli are observed and when stained by the Ziehl-Neelsen methods, giving preference to the Kinyoun modification, they are partially alcohol-acid resistant [2,25.]

The microscopic morphology of *Rhodococcus* sp can vary from coccoid to bacillary, depending on the species, the clinical sample investigated, the type and stage of growth in which the bacterium is found, showing a growth cycle in the form of bacilli to the form of cocci [1.] Coccoid forms may be more evident from colonies grown on solid media. In liquid medium (BHI broth), particularly in young cultures, bacilli can be observed

forming long or short filaments, sometimes showing rudimentary ramifications [2]. Figure 2. represents smear microscopy of the culture of *R. equi* ATCC 6939 in MHA medium.



Photograph showing the typical morphological appearance of pleomorphic, Gram-positive coccobacilli



Photograph illustrating the typical morphological appearance of pleomorphic coccobacilli, partially resistant to acid alcohol

1000X magnification in oil immersion

Figure 2. Microscopy of a smear from the culture of *R. equi* ATCC 6939, stained by Gram (Left) and Kinyoun (Right) methods. Photos: Paulo da Silva.

DETERMINING PHENOTYPIC CHARACTERISTICS FOR THE PRESUMPTIVE IDENTIFICATION OF *R. EQUI*

In addition to mycolic acid in the cell wall, which may be directly related to virulence, *R. equi* also presents the "*equi factor* " made up of capsular polysaccharides, which could be involved in inhibiting the phagocytosis of the bacteria, together with some exoenzymes, especially Cholesterol oxidase or *ChoeE* and Phospholipase C. The "*equi factor*" acts on the host's tissues, destroying them, due to its marked membranolytic activity. However, the virulence role of these constituents seems doubtful, as their presence can also be found in non-virulent isolates [26]. The study carried out by Pei et al. (2006) [27] showed the non-importance of the enzyme Cholesterol Oxidase for the virulence of *R. equi,* working with mutant strains showing deletion of the gene that codes for that protein. The strains remained virulent in mice and in experimentally infected foals.

The presumptive identification of *R. equi* must be based on macro and microscopic characteristics [2,28], along with its ability to produce the " *factor equi* " when submitted to the " CAMP Test", which is used to describe the synergistic lysis of red blood cells in the presence of exosubstances diffusible bacteria produced by two different species of bacteria growing adjacent to each other, on the surface of the culture medium with agar added with sheep or ox blood. The method specifies which species to be tested must be grooved in straight lines forming a 90° angle to each other without touching. after incubation adequate, hemolysis can be observed immediately, around the stretch marks, where an enlarged area of lysis of erythrocytes present in the culture medium [29].

Production of the "*equi factor* " – CAMP Test

The CAMP Test is well represented by the method performed according to Bille and Doyle (1991) [30], using the reference strains *R. equi* ATCC 6939, *Listeria ivanovii* ATCC 19119 and *Listeria monocytogenes* ATCC 19112. The phenomenon of synergistic lysis is demonstrated with *L. ivanovii,* which produces the enzyme Sphingomyelinase C and *R. equi* which produces the *ChoeE enzyme.* The test presumptively determines the bacterial isolates

producing the enzyme *ChoeE,* interacting with the enzyme Sphingomyelinase C, produced by *L ivanovii* [31] (Figure 3).

Production of the "equi factor" - CAMP test
Presumptively determines whether the microorganism under investigation can be isolated from Rhodococcus equi, which produces the enzyme Cholesterol Oxidase, which interacts with the enzyme Sphingomyelinase C, produced by Listeria Ivanovii, forming an area of complete hemolysis on MH agar with 5% defibrinated sheep's blood in an arrowhead or shovel configuration.

1- *Listeria ivanovii* – ATCC 19112
2- *Rhodococcus equi* – ATCC 6939
3- *Listeria monocytogenes* – ATCC 19119 (negative control)

Figure 3. Photographic representation of the CAMP Test. Photo: Paulo da Silva.

ADDITIONAL PHENOTYPIC FEATURES USED IN IDENTIFYING *R EQUI.*

The biochemical characteristics represented in Table 1 are complementary evidence for the presumptive identification of *R. equi.*

100% positive reactions: Catalase, Adenine breakdown, Glucose oxidation, Lipase and "Equi *Factor*" production (CAMP test).

100% negative reactions: Amylase, DNase, Beta-galactosidase (ONPG), Caseinase, Thymine decomposition; hypoxanthine and xanthine, Fermentation of glucose, Use of a single source of carbon (acetate, citrate, malonate), Gelatinase, Indole.

Variable Reactions: Oxidase 1-5% (+), H2S 32% (+), Urease 95% (+), Hipurate hydrolysis 1% (+), Esculin hydrolysis 4% (+) and Nitrate reduction 88% (+).

Non-use of the following carbohydrates: Adonitol, Starch, Arabinose, Cellobiose, Dulcitol, Erythritol, Fructose, Galactose, Glycerol, Inositol, Lactose, Maltose, Mannitol, Mannose, Melibiose, Raffinose, Raminose, Sucrose, Salicin, Sorbitol, Trehalose and Xylose.

Table 1. Biochemical characteristics for identification of *R. equi*

Data adapted from Prescott (1991) [2,] Beaman et al. (1995) [4,] Brown et al. (1999) [5,] McNeil and Brown (1994) [25,] Flores et al. (1990) [32;] Fiss and Brooks (1991) [33,] Bizet et al. (1997) [34,] Silva (2009) [35.] Symbols: (+) positive reaction; (–) negative reaction.

CHARACTERIZATION OF *R. EQUI* AS TO SUSCEPTIBILITY TO ANTIMICROBIALS

As with any bacterial agent, it is important to study the antimicrobial susceptibility of *R. equi* for the correct management of patients. Therefore, the antibiotic therapy used in the treatment of rhodococcosis must be based on sensitivity tests for all isolates, avoiding the misuse of antibiotics determined to be resistant. *in vitro* [36.] Case reports of human rhodococcosis have been published with the aim of disclosing the results obtained in the treatment with the evaluation of the sensitivity profile to the current antimicrobials for this bacterial agent [12,37.]

Due to pathogenicity factors, *R. equi* is able to remain inside macrophages, where histopathological studies reveal its presence in pyogranulomatous processes. In the clinical samples investigated, a large number of macrophages, lymphocytes and degenerated neutrophils are observed, in addition to giant cells and a large amount of the microorganism in the cytoplasm of the phagocytes, surrounded by a fibrous capsule [38.] Because of these characteristics, the treatment of rhodococcosis is based on the use of antimicrobials combined with supportive therapy. *R. equi* usually responds to several antimicrobials, especially lipophilic ones, because they reach high concentrations inside the cell and because they act even in the presence of purulent material, which can limit the therapeutic action of conventional antimicrobials. The most indicated antimicrobials have been azithromycin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, imipenem, rifampicin, tobramycin, sulfamethoxazole+trimethoprim, or vancomycin [16,38.]

As a therapeutic scheme for the treatment of rhodococcosis, most investigators agree that combinations of 2 to 3 antimicrobials

with good intracellular penetration must be used. Treatment extends over prolonged periods of 6 weeks or more, with injectable medication, followed by oral treatment for further weeks or even months. One therapeutic suggestion is the combination of vancomycin and erythromycin for at least two months. In addition to antibiotic therapy, surgical resection of necrotic tissue, drainage of suppurative lesions and control of predisposing factors are indicated [16,38,39,40,41].

SUSCEPTIBILITY TESTS OF AEROBIC ACTINOMYCETES, CONTAINING MYCOLIC ACID, INCLUDING *RHODOCOCCUS* SP

The standard test recommended by the "Clinical and Laboratory Standards Institute/ National Committee for Clinical Laboratory Standards" (CLSI/NCCLS, 2003 [42] ; 2011 [43]) for aerobic actinomycetes, including *Rhodococcus* sp, is the determination of the Minimum Inhibitory Concentration (MIC), which can be carried out by the microdilution method in Müeller Hinton broth (MH broth) supplemented with cations. There are still no standardized methods for testing the sensitivity of aerobic actinomycetes, using the Disk Diffusion method, however, in recent years, several antimicrobial agents have been tested, such as: Amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, erythromycin, imipenem, minocycline and sulfamethoxazole -trimethoprim [44,45,46].

BROTH MICRODILUTION METHOD

This method is defined in CLSI (2011) [43], document M24-A2, Susceptibility Testing of *Mycobacteria, Nocardiae,* and Other Aerobic Actinomycetes; Approved Standard replacing document M24-A (CLSI 2003) [42].

The recommended antimicrobial agents are shown in Table 1, which are: **Primary drugs** (first choice) and **Secondary drugs** (second choice).

DISK DIFFUSION METHOD

Although there is no standardization of sensitivity tests for *Rhodococcus* sp, using the Disk Diffusion (DD) method, several authors have followed the accepted interpretation criteria for *Staphylococcus* sp [46,47,48,49].

Tables 2 and 3 represent the suggested antibiotics for testing by the DD method for clinical isolates of *R. equi.*

BETA-LACTAMICS
Penicillin + Beta-Lactamase Inhibitor
Amoxicillin+clavulanic acid (AMC 30µg)
Cephalosporins
3rd generation: Cefotaxime (CTX 30µg); Ceftriaxone (CRO 30µg)
4th generation: Cefepime (CPM 30µg)
Carbapenene
Imipenem (IPM 10µg)
AMINOGLYCOSIDES
Amikacin (AMI 30µg); Tobramycin (10µg)
QUINOLONES:
2nd Generation: Ciprofloxacin (CIP 5µg)
4th Generation: Moxifloxacin (MFX 5µg)
OXAZOLIDINONE
Linezolid (LNZ 30 µg)
M A CROLIDS
Clarithromycin (CLA 15µg)
TETRACYCLINES
Doxycycline (DOX 30µg); Minocycline (MIN 30µg)
SULFONAMIDE AND TRIMETHOPRIM
Sulfamethoxazole+Trimethoprim (SMT 25µg)

Table 2. Antimicrobials with respective classes and concentrations, suggested to be tested with *R. equi* isolates, according to the disk diffusion method*

*Based on CLSI document. Performance Standards for Antimicrobial Susceptibility Testing. Table 2C. Zone Diamenter and MIC Breakpoints for *Staphylococcus* spp. M02 and M07. 30 th ed. CLSI supplement M100. Wayne. PA: Clinical and Laboratory Standards Institute; 2020 [50].

ANTIMICROBIAL AGENT	MIC (µg/mL) BY CATEGORY		
First choice	**Sensitive**	**Intermediary**	**Resistant**
amikacin [c]	≤ 8	-	≥ 16
Amoxicillin+Clavulanic acid	≤ 8/4	8/16	≥ 32/16
Ceftriaxone	≤ 8	16 - 32	≥ 64
Ciprofloxacin [d]	≤ 1	two	≥ 4
Clarithromycin [and]	≤ 2	4	≥ 8
Imipenem	≤ 4	8	≥ 16
Linezolid [f]	≤ 8	-	-
minocycline [c]	≤ 1	2 - 4	≥ 8
Moxifloxacin	≤ 1	two	≥ 4
Sulfamethoxazole+Trimetho-prim	≤ 2/38	-	≥ 4/76
Tobramycin	≤ 4	8	≥ 16
second choice	**Sensitive**	**Intermediary**	**Resistant**
Cefepime	≤ 8	16	≥ 32
Cefotaxime	≤ 8	16 - 32	≥ 64
Doxycycline	≤ 1	2 – 4	≥ 8

Table 1. Interpretation of "Breakpoints" of the broth microdilution method for *Nocardia* sp and other aerobic actinomycetes, including *Rhodococcus* sp [*] [a, b.]

*Based on document providing updated tables for Clinical and Laboratory Standards Institute standards M24-A2-A6 for antimicrobial susceptibility testing. Informative supplement, for global use, developed through the consensus process of the Clinical and Laboratory Standards Institute (CLSI/NCCLS, 2011) [43.]

a- The "Breakpoints" in this table apply to *Nocardia* sp and can be used, tentatively, for other aerobic actinomycetes, based on the distributions of the populations of the microorganism in question, its clinical data and results obtained in the experiences of specialists in this field. Results must be considered suggestive and reported as such, pending further information accumulation.

b- For *R. equi,* the interpretation criteria as indicated in "CLSI document M10048" for *Staphylococcus aureus,* with the inclusion of results for vancomycin and rifampicin. Interpretive categories must be considered and reported tentatively pending additional information.

c- The following antimicrobial "breakpoints" differ from the current "CLSI document M10048" recommendations for microorganisms that grow aerobically: amikacin, minocycline and moxifloxacin.

d- Ciprofloxacin and levofloxacin are interchangeable. Both are less active *in vitro* than the newer generation 8-methoxy fluoroquinolones.

e- Representative class for more recent macrolides.

f- No isolated *Nocardia* sp with MIC value >8 µ g/mL for linezolid has been reported.

Antimicrobial Agent	Disc Content	Inhibition Halo Diameter (mm)		
		R	I	s
Amikacin	30μg _ _	≤ **14**	**15-16**	≥ **17**
Amoxicillin+Ac. clavulanic	20/10 μg _	≤ **19**	-	≥ **20**
Cefepime	30 μg _	≤ **14**	**15-17**	≥ **18**
Cefotaxime	30 μg _	≤ **14**	**15-22**	≥ **23**
Ceftriaxone	30 μg _	≤ **13**	**14-20**	≥ **21**
Ciprofloxacin	5 μg _	≤ **15**	**16-20**	≥ **21**
clarithromycin	15 μg _	≤ **13**	**14-17**	≥ **18**
Doxycycline	30 μg _	≤ **12**	**13-15**	≥ **16**
Imipenem	10μg _ _	≤ **13**	**14-15**	≥ **16**
Linezolid	30 μg _	≤20	-	≥21
Minocycline	30 μg _	≤ **14**	**15-18**	≥ **19**
Moxifloxacin	5 μg _	≤20	**21-23**	≥24
Sulfamethoxazole+Trimethoprim	23.75/ 1.25 μg	≤ **10**	**11-15**	≥ **16**
Tobramycin	10 μg _	≤12	**13-14**	≥15

Table 3. Interpretive Standards of Inhibition Halo Diameters and Equivalent Minimum Inhibitory Concentration (MIC) Cutoff Points for *Staphylococcus* sp*.

S= Sensitive; I= Intermediate; R= Resistant

*Based on CLSI document. Performance Standards for Antimicrobial Susceptibility Testing. Table 2C. Zone Diamenter and MIC Breakpoints for *Staphylococcus* spp. M02 and M07. 30 th ed. CLSI supplement M100. Wayne. PA: Clinical and Laboratory Standards Institute; 2020 [50].

DISCUSSION

In the presumptive identification of bacteria, phenotypic characteristics such as morphology, cultivation, nutrition, biochemistry, metabolism, pathogenesis, serology and ecology must be considered. Thus, the presumptive identification of *R. equi* is no exception to the rule. In this review study, we addressed the morphophysiological characteristics to show a profile compatible with *R. equi,* according to pre-established descriptions [2,4,34].

According to Prescott (1991)[2], variation in colony types in *R. equi cultures* is observed in recent cultures. The classic type of colonies with a viscous and coalescing consistency is normally predominant, however, colonies with a creamy consistency and a diameter of less than 1 mm can also be found. Therefore, pleomorphic colonies may form in the culture of the same isolate. Pigment production is rarely observed in cultures less than 4 days old. Generally, after 4 to 7 days of culture on non-selective medium, colonies may develop a delicate salmon-pink hue, slightly yellow, or may be non-pigmented. Cultures maintained on an inclined medium for prolonged periods without subcultures commonly become rough, dry, and orange-red, but revert to classic colonies on subcultures.

With regard to microscopic morphology and staining properties, *R. equi* presents the Gram-positive bacillus form, varying from distinct coccoid to bacillary forms, depending on the growth conditions. The coccoid forms are usually observed in cultures in solid medium and purulent material from patients. From liquid medium, particularly in young cultures, bacilli are observed forming long or short filaments, which may show rudimentary ramifications [2,4,34].

According to Prescott (1991)[2], the resistance to acid alcohol presented by *R. equi* seems to depend on the staining technique used, the age of the cultures and the culture medium used. Most reports fail to demonstrate this inconsistent feature of the microorganism.

Originally, CAMP Test he was described by Christie et al. (1944) 51 whose initials gave rise to the term CAMP. The test determines an increased hemolytic area, due to the lysis that occurs with ovine or bovine erythrocytes contained in agar. The phenomenon occurs by the synergistic action of sphingomyelinase W (betatoxin) produced by *Staphylococcus aureus* and the CAMP Factor (cohemolysin), a protein of 25.3 kDa secreted by group B streptococci (*Streptococcus agalactiae*). The reaction takes

place with erythrocytes whose Cell membranes contain hair minus 45 mol % sphingomyelin 52 in one process two-step sequential. in the first reaction takes place the hydrolysis of sphingomyelin membrane and phospholipids by action from the sphingomyelinase or phospholipase and in the second reaction, the CAMP factor interacts with the membrane of erythrocytes, causing cell lysis 53, 54.

Many Gram positive and Gram-negative bacteria are known to be CAMP Test positive, including *Listeria* sp [29,] *R. equi* [31,] *Pasteurella* sp [55,] *Aeromonas* sp [56,] *Vibrio* sp [57,] *Streptococcus agalactiae* [58,] *Actinobacillus pleuropneumoniae* [59,] *Bartonella henselae* [52.]

Like *R. equi,* other Gram-positive bacteria that contain mycolic acids, such as *Mycobacterium* sp, *R. erythropolis* and *R. rhodochrous,* show cholesterol oxidase activity [60,] indicating the possibility of being CAMP Test positive. Although the CAMP Test is not a unique characteristic for *R. equi,* it is distinctive for it, and must always be used in its identification, since negative *equi factor isolates have not yet been described* [31.] In this context, this proof must be considered as the most important factor for the presumptive identification of *R. equi.*

Several studies show that, normally, *R. equi* is susceptible to aminoglycosides, erythromycin, fluoroquinolones, glycopeptides, imipenem and rifampicin, while for other antibiotics such as cephalosporins, clindamycin, chloramphenicol, cotrimoxazole and tetracycline, it presents variable susceptibility. In general, the use of Beta-lactams is not recommended, since typically, the isolates are especially resistant to penicillins. Therefore, treatment with these antibiotics must be avoided, even for susceptible isolates, as a rapid acquisition of resistance can occur [16,47,49,61,62.]

Some authors draw attention to the intra-histiocytic survival of *R. equi,* where the importance of the intracellular activity of the antibiotic is not clear. Other authors report the use of antimicrobial combinations containing an agent with intracellular activity, while some claim that bactericidal activity is more important, especially during the initial treatment, when microorganisms present greater numbers, both in extracellular and intracellular conditions [49,63.]

R. equi isolates recovered from sputum cultures, Silva et al. (2010) [46,] obtained results with 100% susceptibility to the antibiotics amikacin, clarithromycin, ciprofloxacin, doxycycline and minocycline. In the study by Torres-Tortosa et al., (2003) [45,] the most effective antimicrobial agents were amikacin (100%), imipenem (97.6%) and ciprofloxacin (94%).

McNeil & Brown (1992) [36] did not find resistance in an isolate of *R. equi* to amoxicillin+clavulanic acid and imipenem.

The study by Silva et al. (2010) [46] showed that the -lactam agents btested did not show good activity, where 90% of the isolates were resistant to Amoxicillin. However, when associated with clavulanic acid, there was a significant increase in sensitivity (88.3%). The detection of resistance-mediating enzymes determined by the production of ß-lactamase was negative, using the nitrocefin disc method, in all *R. equi isolates* recovered from sputum culture, mainly in those that showed resistance to ß-lactams. As for the antimicrobials imipenem and ceftriaxone, the isolates showed sensitivity of 100% and 93.3%, respectively.

Nordmann et al. (1994) [64] did not find in resistant isolates of *R. equi,* a plasmid with a structural gene for b-lactamase. However, they verified the presence of bacteriophages in such isolates which, when tested in mice, showed greater virulence and greater ability to lead to chronic infection. They concluded that, in human b*R. equi isolates, the* β-lactam resistance phenotype may be mediated by phages and be related to virulence.

Linezolid (oxazolidone class) is used in the treatment of Gram-positive bacillus infections, including *Corynebacterium* sp, and little is known about its efficacy in the treatment of rodococcosis due to the lack of information in the literature [65]. In the study by Bowersock et al (2000) [66] linezolid was effective *in vitro* in all 102 strains of *R. equi* (36 isolated from humans and 66 from horses) presenting MIC=2 mg/mL.

Antimicrobials from the macrolide class stand out when compared to other drugs, however, preference must be given to clarithromycin and azithromycin over erythromycin due to their well-documented decrease in sensitivity in recent years and their contribution to gastrointestinal side effects 67,[68,69].

Silva et al. (2010) [46] found 40% sensitivity to sulfamethoxazole+trimethoprim in 60 isolates of *R. equi*. However, when analyzed separately by trimethoprim, only 6.7% were sensitive to this agent. McNeil and Brown (1992) [36] found 5% resistance to sulfamethoxazole+trimethoprim in 98 *R. equi isolates* from human clinical samples. Two other studies of *R. equi* isolated from soil and animals carried out by Barton & Hughes (1984) [70], with 66 isolates and Woolcock and Mutimer (1980) [71], with 100 isolates, demonstrated resistance to sulfonamides of 58 and 50%, respectively.

CONCLUSIONS

Since *R. equi* is a microorganism partially resistant to acid alcohol, which induces a clinical picture similar to that of tuberculosis, the differential diagnosis deserves special importance, especially in countries like Brazil, where the prevalence of tuberculosis is high. This microorganism must always be suspected when cultures, mainly those for the investigation of tuberculosis, present colonies with distinct pigmentation and in the microscopic observations of the same under Gram stain, pleomorphic Gram-positive coccobacilli are found. The suspicion is reinforced by performing microscopy of culture smears stained by the Ziehl-Neelsen method, modified by Kinyoun, and the microorganism shows partial acid resistance. From a laboratory point of view, the identification of these bacteria, even if presumptive, is crucial for these possible etiological agents not to be discarded as simple contaminants.

catalase, oxidase, lipase, DNase, gelatinase and lecithinase tests being complementary.

From a clinical point of view, presumptive identification is often enough to initiate empirical treatment with antimicrobial agents, especially in immunocompromised patients with cavitary infectious lesions, in order to institute specific treatment for rhodococcosis. Sensitivity test is only standardized for the microdilution method in broth for *Nocardia* sp and other aerobic actinomycetes, including *Rhodococcus* sp. In recent years, several researchers have followed the interpretation criteria of accepted cutoff points for *Staphylococcus* sp.

Due to the emergence of *R. equi* as a cause of infections, especially among immunocompromised patients, the need for its routine investigation in microbiology laboratories, using more appropriate identification techniques, as well as more clinical studies to outline a diagnostic conclusion, increases. and treatment regimens.

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