

Susceptibility profile of Brazilian *Rhodococcus equi* isolates against azithromycin, clarithromycin and erythromycin

Perfil de susceptibilidade de isolados brasileiros de *Rhodococcus equi* frente à azitromicina, claritromicina e eritromicina

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- NOTE -

ABSTRACT

Rhodococcus equi infection treatment is usually a macrolide (azithromycin - AZM, clarithromycin - CLR and erythromycin - ERY) and rifampicin combination. However, resistance cases have been reported, especially for ERY. In view of the need of a study about Brazilian isolates susceptibility profile, this study aimed to characterize the minimum inhibitory concentration (MIC) of the macrolides - AZM, CLR and ERY - against 44 *R. equi* isolates. It was found all isolates CLR and AZM sensitive; however, for ERY, 27% (12/44) were classified as intermediate sensitivity. *R. equi* Brazilian isolates used here showed a large susceptibility profile, except against ERY, for which it was observed some resistance evidence. In order to avoid failures in the equine rhodococcosis therapy it was highlighted the importance of microbiological culture and antimicrobial susceptibility testing in vitro before beginning treatment.

Key words: macrolide, minimum inhibitory concentration, antimicrobial susceptibility, rhodococcosis.

RESUMO

No tratamento de infecções por *R. equi*, usualmente emprega-se uma combinação de macrolídeos (azitromicina – AZI, claritromicina – CLARI e eritromicina – ERI) e rifampicina. Todavia, casos de resistência frente a esses antimicrobianos vêm sendo reportados, especialmente à ERI. Tendo em vista a necessidade de um estudo sobre o perfil de susceptibilidade de isolados brasileiros, buscou-se verificar a concentração inibitória mínima (CIM) de AZI, CLARI e ERI frente a 44 isolados de *R. equi* de diferentes origens. Todos os isolados analisados demonstraram perfil de sensibilidade à AZI e CLARI; em contraste, frente à ERI, 27% (12/44) apresentaram sensibilidade intermediária. A fim de evitar falhas no tratamento da rodococose equina, destaca-se a importância da cultura microbiológica e a realização de testes in vitro de susceptibilidade antimicrobiana de *R. equi* antes do início do tratamento.

Palavras-chave: concentração inibitória mínima, macrolídeos, susceptibilidade antimicrobiana, rodococose.

Rhodococcus equi is a gram-positive facultative and intracellular pathogen and etiologic agent of equine rhodococcosis (QUINN et al., 1994). The main clinical manifestation of rhodococcosis is pyogranulomatous bronchopneumonia in foals under six months of age (QUINN et al., 1994). This disease is responsible for large economic losses due to mortality, since pneumonia is a major cause of death in foals (GIGUÈRE et al., 2011). *In vitro*, *R. equi* has to be sensitive to a wide variety of antibiotics, such as aminoglycosides, beta-lactams and second-generation of cephalosporins (GIGUÈRE et al., 2010). However, their ability to replicate in macrophages and neutrophils limited the therapeutic options to drugs able to penetrate in lipophilic environment (BUCKLEY et al., 2007).

In 1980 the combination of erithromycin (ERY) and rifampicin become the treatment of equine rhodococcosis highly efficient and dramatically reduced the mortality in foals (GIGUERE et al., 2010). Since then, *R. equi* resistance to these drugs has been observed (BUCKLEY et al., 2007). In this respect, azithromycin (AZM) and clarithromycin (CLR) have been proposed as alternatives to ERY for the treatment of *R. equi* infections in foals (JACKS et al., 2003). Furthermore, when compared with ERY these drugs are chemically more stable, possess greater bioavailability and achieve

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higher concentrations in macrophages and neutrophils (JACKS et al., 2003). However, some studies have shown the emergence of strains resistant also to AZM and CLR. On the basis of the lack of data about Brazilian *R. equi* isolates, this study aimed to characterize the minimum inhibitory concentration (MIC) of AZM, CLR and ERY against 44 *R. equi* isolates from different sources.

R. equi were isolated from clinical (n=15), soil (n=15), feces (n=9) and installations (n=5) samples. All those were collected at equine breeding farms, located in Rio Grande do Sul state, Brazil, between 1991 and 2013 year. Clinical samples were isolated from postmortem lung lesions in foals, feces samples were collected from healthy mares, soil samples were taken from paddocks and installations samples were collected from wall stables, drinkers and feeders. All isolates were characterized as *R. equi* by phenotypic features (QUINN et al., 1994), and then confirmed genotypically according to MONEGO et al. (2009). All isolates remained lyophilized and stored at -20°C until time of testing. The MIC tests were performed in Mueller-Hinton broth (MHB) medium (Himedia® Laboratories) using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). All microorganisms were cultured in MHB for 24h aerobically at 37°C. For each microorganism, an inoculum suspension was prepared in 0.9% saline, adjusted to the turbidity of 0.5 on the McFarland scale, and absorbance readings were performed in a spectrophotometer at 600 nm-wavelength. These suspensions were diluted in MHB and approximately 1×10^5 CFU ml⁻¹ were inoculated into the microtiter plates. The antimicrobials (AZM, CLR and ERY) were tested at 0.03 up 128 µg ml⁻¹. The standard strain, *R. equi* ATCC 33701, known to be sensitive to AZM, CLR and ERY, was used as quality control tests (GIGUÈRE et al., 2010). The MIC was defined as the lowest concentration of antimicrobials that inhibited the visible growth of *R. equi* isolates after overnight incubation evidenced by the addition of 20 µL of 2,3,5-triphenyltetrazolium chloride (Vetec®) 1%. *R. equi* isolates were classified as sensitive (≤ 2 µg ml⁻¹ - AZM and CLR, and ≤ 0.5 µg ml⁻¹ - ERY) or resistant (≥ 8 µg ml⁻¹ - AZM, CLR and ERY) (CLSI, 2009). In addition, isolates with MIC values between the concentrations mentioned above were classified as intermediate sensitivity. All of the susceptibility tests were performed using at least three biological replicates and two technical replicates. The Kruskal-Wallis test was employed to calculate the association among antimicrobials MIC and the source of the isolates and the year of sample collection. The Bonferroni test was used to calculate the media when it was observed differences. The minimum significance level considered

was $P < 0.05$. The data were analyzed by SAS statistical software.

Among the 44 *R. equi* isolates evaluated, none was resistant for antimicrobials tested. All isolates were sensitive against AZM and CLR, with media MIC value of 0.49 µg ml⁻¹ and 0.11 µg ml⁻¹, respectively. However, 27% (12/44) of *R. equi* isolates were classified as intermediate sensitivity for ERY, with media MIC value of 0.54 µg ml⁻¹. This result for ERY can be explained by its use in combination with rifampin as treatment of choice since the 1980s (GIGUÈRE et al., 2011). Probably, this profile could be worse if rifampin was not used, since this combination reduces the likelihood of *R. equi* resistance to either drug (PRESCOTT & NICHOLSON, 1984). It is noteworthy that ERY resistance can drive to AZM and CLR resistance. The molecular mechanisms of macrolide resistance of *R. equi* isolates have not been determined, but it is known that cross-resistance among macrolides is common (GIGUÈRE et al., 2011; MUSCATELLO, 2012); thus if an isolate is resistant to ERY probably will be also resistant to AZM and CLR.

BUCKLEY et al. (2007) evaluated 94 *R. equi* isolates from Ireland between 2000 and 2006 and demonstrated an increase from 0.2 to 0.5 µg ml⁻¹ in the MIC values for ERY. In this regard, analysis of the distribution of resistant *R. equi* isolates collected between 1997 and 2008 from different American states suggests that the prevalence of antimicrobial resistance to macrolide antimicrobials is increasing (GIGUÈRE et al., 2010). Differently, in this study it was not observed significant increase in the MIC values for isolates collected from 1991 to 2013 ($P > 0.05$). This finding can be associated with the mass macrolide treatment of foals with subclinical pulmonary lesions and, especially, the antimicrobial chemoprophylaxis against *R. equi* foal pneumonia, common in other countries (GIGUÈRE et al., 2011; MUSCATELLO, 2011). However, for CLR, the clinical samples showed the major MIC values when compare with *R. equi* isolates from feces, soil and installations samples ($P < 0.05$). This result is according to selection pressure hypothesis, since rhodococcosis is a chronic disease and repeated treatment can easily target to macrolide resistance (GIGUÈRE et al., 2010).

RIBEIRO et al. (2006) demonstrated strong efficacy of AZM against Brazilian *R. equi* isolates ($MIC_{90} < 1.5$ µg ml⁻¹). Similarly, in the present study, it was observed MIC_{90} values between 0.5 and 1 µg ml⁻¹, reinforcing the effectiveness of AZM in Brazilian isolates. Our results corroborate with MIC values observed by others studies, despite the timing among the publications to be more than 10 years (JACKS et al., 2003; CARLSON et al., 2010; RIESENBERG et al., 2013) (Table 1).

Table 1 - Minimum inhibitory concentration (MIC) of three antimicrobial agents against *R. equi* Brazilian isolates from clinical (n=15), feces (n=9), installations (n=5) and soil (n=15) samples.

ATM	Source	MIC ($\mu\text{g/ml}$) values/number of isolates								MIC's		SP (%)		
		<0.03	0.03	0.06	0.12	0.25	0.5	1	2	MIC ₅₀ *	MIC ₉₀ *	S	IS	R
ERY	Clinical	0	0	1	2	7	2	3	0	0.25	1	80	20	0
	Feces	0	0	0	0	2	3	4	0	0.5	1	73	27	0
	Installations	0	0	0	1	3	0	0	1	0.25	2	93	7	0
	Soil	0	0	0	1	2	8	4	0	0.5	1	73	27	0
AZM	Clinical	0	0	1	3	1	8	1	1	0.5	1	100	0	0
	Feces	0	0	0	0	4	5	0	0	0.5	0.5	100	0	0
	Installations	0	0	0	1	3	1	0	0	0.25	0.5	100	0	0
	Soil	0	0	0	0	4	6	5	0	0.5	1	100	0	0
CLR	Clinical	3	2	4	4	2	0	0	0	0.06	0.25	100	0	0
	Feces	5	0	2	2	0	0	0	0	<0.03	0.12	100	0	0
	Installations	1	3	0	0	1	0	0	0	0.03	0.25	100	0	0
	Soil	10	1	3	1	0	0	0	0	<0.03	0.06	100	0	0

* MIC₅₀ and MIC₉₀ were defined as the lowest concentrations of antimicrobials capable of inhibiting the growth of 50% and 90% of isolates, respectively.

ATM=antimicrobial, SP=susceptibility profile, S=sensitivity, IS=intermediate sensitivity and R=resistance.

S: =2 $\mu\text{g ml}^{-1}$ (AZM and CLR) and =0.5 $\mu\text{g/ml}$ (ERY); R: =8 $\mu\text{g ml}^{-1}$ (AZM, CLR and ERY), IS: MIC values between the concentrations mentioned (breakpoints according to CLSI, 2009).

Although in other countries *R. equi* macrolide resistance appears to be in a more advanced step, *R. equi* Brazilian isolates used here showed a large susceptibility profile, except against ERY, for which it was observed some resistance evidence due the intermediate sensitivity profile verified. In order to avoid this process, since the antimicrobials evaluated herein are the best rhodococcosis treatment options, it was highlighted the importance of microbiological culture and *R. equi* antimicrobial susceptibility testing *in vitro* before beginning treatment.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and National Council for Scientific and Technological Development (CNPq) for the scholarship of Marcelo Luis Schwab and Bibiana Petri da Silveira.

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