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Short Communication

Genotypic and phenotypic detection of efflux pump in *Rhodococcus equi*

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Abstract

The *req_39680* gene, associated to a putative efflux system, was detected in 60% (54/90) of *R. equi* isolates by PCR. The phenotypic expression of efflux mechanism was verified in 20% of the isolates using ethidium bromide. For the first time, the expression of efflux mechanism was demonstrated in *R. equi*.

Key words: ethidium bromide, efflux pump, multidrug resistance, *Rhodococcus equi*, *vapA*.

Efflux systems or efflux pumps are transporter proteins of toxic compounds classified into five families according to their primary structure and energy-coupling mechanism: i. Major Facilitator Superfamily - MFS, ii. Adenosine Triphosphate-Binding Cassette - ABC, iii. Small Multidrug Resistance Family - SMR, iv. Resistance-Nodulation-Cell Division Superfamily - RND, and v. Multidrug and Toxic Compound Extrusion Family - MATE (Kumar and Schweizer, 2005). These proteins were firstly described in *Escherichia coli* associated with tetracycline resistance profile (Mcmurray *et al.*, 1980).

Although several genes encoding efflux pumps can be found on plasmids, there are other efflux pump genes located on the chromosome, which provide for the bacterium an intrinsic mechanism that allows survival in a hostile environment (*e.g.* the presence of antimicrobials) (Webber and Piddock, 2003). Then, the efflux pumps are related to acquired and intrinsic resistance to several antimicrobials in different bacterial species (Li and Nikaido, 2004). Cross-resistance was already described in efflux systems, *i.e.* the exposure to any one agent that belongs to the substrate profile of an efflux pump can allows its overexpression and consequent cross-resistance to different substrates (Webber and Piddock, 2003). These systems are also considered as a major mechanism of resistance among multidrug-resistant pathogenic microorganisms in veterinary medicine (Paulsen, 2003).

Rhodococcus equi is a facultative intracellular pathogen that infects animals and immunocompromised human patients (Takai *et al.*, 1991; Takai *et al.*, 2000). This bacterium is the etiological agent of rhodococcosis, an important disease that typically affects foals less than six months of age, causing severe lesions including pyogranulomatous pneumonia and mesenteric lymphadenitis (Prescott, 1991). In *R. equi*, the most important virulence factor is associated with the presence of virulence proteins (Vap) encode by large plasmids (Takai *et al.*, 1995), which have been used to classify isolates as virulent (VapA); intermediately virulent (VapB) and avirulent (without virulence plasmid) (Ribeiro *et al.*, 2005).

Currently, the treatment of rhodococcosis usually consists of a combined application of macrolides, such as erythromycin, azithromycin or clarithromycin, associated with rifampicin (Muscatello, 2012). However, the increasing resistance in *R. equi* isolates to various antimicrobials, including the macrolides group, has been reported (Takai *et al.*, 1997; Asoh *et al.*, 2003; Buckley and Stanbridge, 2007; Venner *et al.*, 2012). The microorganisms' resistance to

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macrolides has been associated to basically three events: presence of methylases, efflux pump activity and enzymatic inactivation (Sutcliffe *et al.*, 1996).

In *R. equi*, the presence of four putative multidrug efflux proteins was mentioned in the reference strain *R. equi* 103S (Letek *et al.*, 2010). This study reported the sequence of a chromosomal gene known as *req_39680*, which encodes a protein with 308 amino acids, related to putative cation efflux system. Similar efflux mechanism was described in mycobacteria being related to the development of high level drug-resistance (Schmalstieg *et al.*, 2012); however, studies about efflux systems activity have not been described in *R. equi*. Herein, we identified the presence of the *req_39680* gene, as well as verified the phenotypic expression of efflux mechanism in soil, feces of healthy animals and clinical *R. equi* isolates.

R. equi isolates were obtained from ten horse-breeding farms, located in the Brazilian states of Rio Grande do Sul (RS) and Paraná (PR). The isolates were obtained from soil ($n = 30$), feces of healthy animals ($n = 30$) and foals clinical ($n = 30$) samples from 1991 to 2012. The clinical samples were recovered from post-mortem pulmonary lesions subsequent to antimicrobial treatment. The reference strains *R. equi* ATCC 33701P + (*vapA* positive), *R. equi* ATCC 33701P - (*vapA* negative) and *Staphylococcus aureus* ATCC 25923 were used as control in both phenotypic and genotypic assays. *Mycobacterium goodii* SB314/96 and *Nocardia* sp. SB57/2008 were used as negative control in the genotypic assays.

Among 90 bacterial isolates analyzed, 70 were identified phenotypic and genotypically previously by Monego *et al.* (2009), being classified as *vapA* positive *R. equi* ($n = 26$) and *vapA* negative *R. equi* ($n = 44$). These isolates were lyophilized and stored at -20 °C until the tests were performed. Additionally, 20 soil samples (≈ 10 g) were collected, especially for this study, in January of 2012 in three different horse-breeding farms in RS. These samples were collected from mare-foal paddocks ($n = 20$) and stored in sterile plastic bags until the bacteriology culturing. The soil samples were growing in nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) selective medium described by Woolcock *et al.* (1980). Colonies with *R. equi* profile were submitted to phenotypic identification (Quinn *et al.*, 1994) and genotypic analysis for genus, species and *vapA* gene (Takai *et al.*, 1995). All the *R. equi* soil isolates were classified as *vapA* negative and also were lyophilized and stored at -20 °C until testing.

In order to evaluate the characteristics of the protein encoded by *req_39680* gene prior to the genotypic tests, it was performed a bioinformatics analysis. The sequence of the protein was recovered from *R. equi* 103S (GenBank access YP_004008630.1) and submitted to analysis by the Phyre V 2.0 (Protein Homology/Analogy Recognition Engine) program (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

Following, the polymerase chain reaction (PCR) method was used to detect the *req_39680* gene in the *R. equi* isolates. Primarily, each strain was grown on Müeller Hinton agar (Himedia® Laboratories) and incubated at 37 °C for 24 h. Three to five colonies were suspended in 500 µL of Milli-Q water and submitted to DNA extraction by cethyltrimethylammonium bromide (CTAB) protocol (Sambrook and Russell, 2001). The extracted DNA was measured using the Picopet 01 DNA Calculator (Cambridge, England). The PCR primers employed were: forward - RE1 (5'-CCGCGATCCCTCGACACACG-3') and reverse - RE2 (5'-CCCACCCGCATCCGCAAGAT-3'). Both primers were designed using Primer-BLAST program (http://www.ncbi. nlm.nih.gov/tools/primer-blast/) based on DNA sequence of the reference strain *R. equi* 103S (GenBank access: NC_014659.1) (http://www.ncbi.nlm.nih.gov) in order to amplify the complete gene. The PCR was carried out in a total volume of 25 µL containing: 10 µmoles of each primer, 200 µM of deoxynucleotides (dNTPs, Invitrogen®), 1 U of DNA polymerase (GoTaq, Invitrogen®), 1X of the 5X enzyme buffer, and 60 ng of DNA sample. The amplifications were performed using PTC-100 Programmable Thermal Controller (MJ Research) with the following cycling profile: initial denaturation at 94°C/1min, 35 cycles of 94 °C/30 s, 63 °C/30 s and 72 °C/1 min 30 s, and a final extension at 72 °C/5 min. PCR products (10 µL) were run in 1% agarose gel, stained with ethidium bromide, visualized under UV light and photo documented by L-PIX ST (Loccus® Brazil). All PCR products were purified, in quadruplicate, with the PureLink PCR Purification Kit (Invitrogen®) and sent for DNA sequencing with the same primers for *req_39680* gene. DNA sequencing was performed in an automated sequencer ABI-PRISM 3100 Genetic Analyzer (ACT Gene Molecular Analysis Ltd., Biotechnology Center/UFRGS, Porto Alegre, RS). DNA consensus sequences were generated by Gap program of the Staden package 4 software (Staden *et al.*, 2000) and analyzed by Basic Local Alignment Search Tool (BLAST) (NCBI/DNASIS software version 2.5, Software Engineering Co. Ltd., SanBruno, California, US).

The phenotypic expression of efflux mechanism was performed in agar containing ethidium bromide (EtBr). This assay was based on the use of a fluorescent cationic dye (EtBr) to visualize the efflux mechanism in bacterial cells (adapted from Martins *et al.*, 2006). Six *R. equi* isolates from clinical ($n = 2$), feces ($n = 2$) and soil ($n = 2$) samples were tested with different concentrations of EtBr (0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 µg/mL) in order to determine the toxicity level of this dye for cells. Each group had an isolate presenting *req_39680* gene. The reference strain *S. aureus* ATCC 25923 was used as positive control according Couto *et al.* (2008). All microorganisms were

cultivated in tryptic soy agar (TSA) medium (Himedia® Laboratories) for 24 h at 37 °C. A single colony of each bacterium was transferred to tryptic soy broth (TSB) medium (Himedia® Laboratories) and maintained at 37 °C overnight. For each microorganism, an inoculum suspension was prepared in 0.9% saline, adjusted to the turbidly of 0.5 on the McFarland' scale, and absorbance readings were performed in a spectrophotometer at 600 nm-wavelength. Subsequently, dilutions in TSB were carried out to obtain an inoculum containing approximately 1,000 CFU/mL. This inoculum was applied with swab on plates containing EtBr incorporated into the TSA and incubated at 37 °C for 48 h under aerobic conditions. Afterward, these plates were visualized on UV light. Both viability of cells and capacity of detection of efflux systems at the different concentrations were observed. The 0.2 μg/mL EtBr concentration was selected to be used in all isolates (90), since provided the adequate physiological conditions to development of the *R. equi*. All tests were performed in duplicate. After the visualization of the results on UV light the plates were photo documented by PIX-L ST (Loccus® Brazil). Bacteria were considered positive to efflux system expression when the fluorescent colonies were not observed under UV light and negative when the colonies showed fluorescence.

The data were analyzed by SAS statistical software (SAS, 2001). The nonparametric Chi-square test was used to calculate the difference in the *req_39680* gene frequency among *R. equi* isolates from different sources. Likewise, it was used the Spearman correlation analysis in order to evaluate the correlation between the presence of this gene with the phenotypic expression of efflux mechanism, as well as the occurrence of *vapA* gene. The minimum significance level considered was $p < 0.05$.

The protein encoded by *req_39680* gene demonstrated 100% confidence and 96% coverage with MATE family transporter protein; it was classified as multi antimicrobial extrusion protein and characterized as cation-bound multidrug and toxin compound extrusion protein when analyzed by Phyre V 2.0. This finding is very relevant since MATE family transporter proteins are categorized among the multidrug efflux transporter families present in various pathogenic microorganisms (Kuroda and Tsuchiya, 2009). Based on these results we selected the *req_39680* gene to perform this study using *R. equi* isolates.

The *req_39680* gene was detected in 60% (54/90) of *R. equi* isolates tested (Table 1), as well as in *R. equi* ATCC 33701. This result corroborates with the described by Rahman *et al.* (2003), which demonstrated that *R. equi* ATCC 33701 has 12 putative genes encoding efflux proteins, as well as 25 proteins of the ABC family, some of which are related to drug resistance. All PCR- products amplified a specific DNA fragment of approximately 840 bp (Figure 1). The identity of *R. equi* PCR products was confirmed by DNA sequence consensus analyses, which showed 100% of identity with the reference strain *R. equi* 103S (GenBank access NC_014659.1). A DNA sequence from a Brazilian *R. equi* isolate (SB 54/97) was deposited in GenBank (access JX_512957).

The *req_39680* gene was detected in *R. equi* isolates from clinical (66.7%), soil (66.7%) and feces (46.7%) samples. No difference was observed in the frequency of the gene among *R. equi* isolates from different sources, thus it

Table 1 - Molecular detection of the gene *req_39680* and phenotypic expression of efflux mechanism in agar containing EtBr in clinical, feces and soil *R. equi* isolates ($n = 90$).

Source	Positive/total of *R. equi* isolates	
	req_39680 gene[1]	Phenotypic expression of efflux mechanism
Clinical	20/30*	11/30*
Feces	14/30	03/30
Soil	20/30	04/30
Total	54/90	18/90

[1]Presence of *req_39680* gene detected by PCR assay.
*A high correlation index (0.66; $p < 0.0001$) was verified between the presence of the *req_39680* gene and phenotypic expression of efflux mechanism by Spearman correlation analysis.

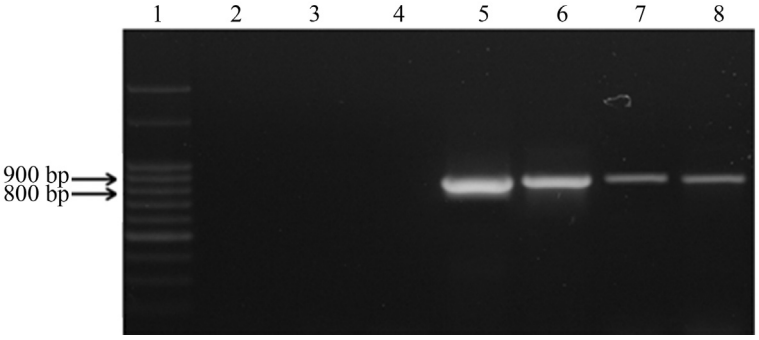

Figure 1 - PCR amplification patterns of *req_39680* gene in *R. equi* isolates. Amplicons were generated from a single PCR assay with *R. equi req_39680* gene-specific primers (approx. 840 bp). Line 1: Molecular weight marker, 100 bp-DNA ladder (Ludwig Biotech®, Brazil); Lines 2-4: Negative controls (Ultra-pure water, *Mycobacterium goodii* SB314/962, *Nocardia* sp., respectively); Line 5: reference strain *R. equi* ATCC 33701 (positive control); Lines 6-8: Samples from clinical (SB54/97), feces (SB490/95) and soil (SB20/12) isolates *R. equi*.

is present in a comparable proportion of isolates recovered from all three sources. We suppose that the presence of the *req_39680* gene is not an adaptation to the host but may be just as relevant to its saprophytic status, given that aerosols from soil or fecal derived strains are the most likely source of rhodoccocal pneumonia (Muscatello *et al.*, 2006). Likewise, according Letek *et al.* (2010) some antimicrobial resistance genes may be associated to efflux systems in clinical isolates as well as other environmental rhodococci. Other members of the genus *Rhodococcus*, such as *Rhodococcus erythropolis* and *Rhodococcus fascians*, assure their survival in the environment through the efflux pumps (Desomer *et al*., 1992; Nagy *et al.*, 1997). According Martinez (2009) drug resistance determinants present in soil organisms typically may have an important impact on the clinical management of microbial infections

The assessment of phenotypic expression of efflux mechanism by methodologies using EtBr as substrate is widely employed in phenotypic studies to verify efflux pumps (Li and Nikaido, 2004); however, it is based on qualitative results and have also show limitation due to physiological characteristics of each microorganism (Martins *et al.*, 2006). In this sense, we firstly standardized the phenotypic expression of efflux mechanism assay with different EtBr concentrations, in order to know the concentration that did not affect the best growth of the bacterial cells. We suggest that this methodological approach could be a useful to further screening assays of efflux systems in *R. equi*, furthermore, this is an instrument-free method for the demonstration of efflux pump activity of bacteria (Martins *et al.*, 2006).

The efflux mechanism by extruding of EtBr was detected in 20% (18/90) of *R. equi* isolates tested, including: 61.1% (11/18) clinical, 22.2% (4/18) soil and 16.7% (3/18) feces isolates (Table 1). Although the *req_39680* gene has been detected in all positive samples for efflux mechanism by extruding of EtBr, we cannot establish a relationship between efflux and presence or absence of *req_39680* gene. On the other hand, 66.6% (36/54) of *req_39680* gene positive *R. equi* isolates did not show phenotypic expression of efflux mechanism. We suppose that this mechanism may be expressed by these isolates in other circumstances to be evaluated. In this respect, Viveiros *et al.* (2005) demonstrated that an *E. coli* strain sensitive to tetracycline became resistant due to the efflux mechanism induced after slow and gradual exposure to the antibiotic and this expression was reverted by serial transfer to drug-free medium or by exposure to inhibitors of efflux pumps.

A high correlation index (0.66; $p < 0.0001$) was verified between the presence of the *req_39680* gene and phenotypic expression of efflux mechanism in clinical isolates. However, no correlation was observed in *R. equi* isolates from feces and soil. We believe that the fact of these clinical samples have been recovered from foals postmortem pulmonary lesions, subsequent to antimicrobial treatment against *R. equi*, may be driving an expression of the efflux mechanism in the host. The treatment failures observed in infections by *R. equi* may be associated to the extrusion of chemical compounds (*e.g.* antimicrobials) once resistance mechanisms to macrolides also include two families of efflux pumps (Roberts *et al.*, 1999), increasing the chances of failure in infection control.

According to Letek *et al.* (2010) the correlation between the presence of virulence and antimicrobial resistance genes in *R. equi* is not well documented. As stated by these same authors, it is possible the occurrence of a direct regulatory interaction or only a random effect between these genes. In this study, no correlation was observed between the presence of both *vapA* and *req_39680* genes in *R. equi* isolates. This finding may be better explained by the location of *req_39680* gene, since it has a chromosomal *locus* and the *vapA* is a plasmidial gene.

In conclusion, the *req_39680* gene is equally distributed among *R. equi* isolates from different sources and a high correlation index between phenotypic expression of efflux mechanism and presence of *req_39680* gene was observed in clinical samples. Thus, there is a potential possibility of efflux systems to be an emerging form of adaptation to pathogenic and saprophytic life by *R. equi* isolates; however, additional studies are required to confirm this hypothesis.

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