Antimicrobial resistance in *Rhodococcus equi*\(^*\)

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*Rhodococcus equi* is an important etiologic agent of respiratory- and non-respiratory tract infections, diseases of animals and humans. Therapy includes the use of various group of chemotherapeutic agents, however resistance acquirement is quite common. To date there is no preferred treatment protocol for infections caused by isolates resistant to macrolides and rifampicin. The resistance acquirement is a result of many molecular mechanisms, some of which include alterations in the cell envelope composition and structure, activity of the efflux pumps, enzymatic destruction or inactivation of antibiotics, and changes in the target site. This paper contains an overview of antimicrobial susceptibility of *R. equi*, and explains the possible molecular mechanisms responsible for antimicrobial resistance in this particular microorganism.

**Key words:** antimicrobial susceptibility, *Rhodococcus equi*

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**INTRODUCTION**

*Rhodococcus equi* is a Gram-positive, pleomorphic rod, commonly found in soil. It is one of the most important pathogens of foals, in which it causes rhodoccosis, a disease manifesting in pyogranulomatous bronchopneumonia, abscesses, lymphadenitis or ulcerative enterocolitis. *R. equi* can be pathogenic to other domestic and wild animals (especially pigs and cattle), and humans as well (Weinstock \& Brown, 2002; Yamshchikov *et al*., 2010; Giguère *et al*., 2011; Witkowski *et al*., 2011; Muscatello, 2012).

Widespread antibiotic usage exerts a selective pressure that acts as a driving force in development of the antibiotic resistance. Prolonged therapy of *R. equi* infections with lipophilic antibiotics leads to a progressive increase in resistance of currently isolated strains. Combination of erythromycin and rifampicin has been the treatment of choice for *R. equi* pneumonia in foals for the past two decades. The existing data indicates that the low-level resistance towards erythromycin and rifampicin may be clinically relevant, and these drugs should be carefully considered for the further treatment of diseased horses. In recent years, newer macrolides, such as azithromycin, clarithromycin and tulathromycin, have become available as promising alternatives for the veterinary use. So has the gallium maltolate, which was also proven to inhibit *R. equi* growth in both rifampicin and macrolide-susceptible and rifampicin and macrolide-resistant strains (Chaffin *et al*., 2009; Coleman *et al*., 2010). Other drugs, such as imipenem, gentamicin, amikacin or vancomycin, have no application in treatment of foals but along with erythromycin and rifampicin are proposed to be used in a human therapy of *R. equi* infections (Gilbert *et al*., 2010).

Our pervious study (Cisek *et al*., 2013) reported differences of susceptibility profiles of *R. equi* strains isolated from wild boars (*Sus scrofa*) in comparison to data obtained from the literature on prevalence over time (Woolcock \& Mutimer, 1980; Nordmann \& Ronco, 1992; Soriano *et al*., 1995; Hsueh *et al*., 1998; Bowersock *et al*., 2000; Tomlin *et al*., 2001; Jacks *et al*., 2003; Niwa *et al*., 2005). Differences regarded five antimicrobials including tetracycline, rifampicin, clindamycin, cephalothin and amoxicillin-clavulanate. For the latter two β-lactam antibiotics, *R. equi* isolated from wild boars was more resistant in comparison to the strains isolated from other sources (literature data). In contrast, tetracycline, rifampicin, and clindamycin were more active against wild-boar isolates. Differences between strains isolated from various sources were also the subject-matter of the study of Girardini *et al.* (2013), in which it was observed that human and environmental isolates possess different susceptibility profiles. This could be due to the antimicrobial pressure, which comes from the fact that humans and farm animals are simply more exposed on antibiotic administration, sometimes inappropriate or unnecessary. This paper contains an overview of *R. equi* susceptibility, and it clarifies the possible molecular mechanisms of resistance acquirement of *R. equi* strains.

**ANTIMICROBIAL SUSCEPTIBILITY AND TREATMENT**

Standard therapy of rhodoccosis consist of a few antibiotics (table 1). Still, the treatment of rhodococcal infection may be difficult, because strains seem to become more resistant, and there is no clear and straightforward protocol that would indicate the preferred antimicrobial combination for the therapy of animals infected by the antimicrobial resistant strains. Another problem comes due to the fact that *R. equi* is an intracellular pathogen, which only shortens the list of active antimicrobials to just a few groups of drugs that are suitable for use.

Among active antimicrobials, macrolides (erythromycin, azithromycin and clarithromycin) demonstrate good inhibitory activity against *R. equi* in comparison to other classes of drugs (Muscatello, 2012). However, in case of...
erthyromycin increasing MIC values are well documented in the 10-year period of time starting from the late 1990s (Buckley et al., 2007). Moreover, usage of erythromycin may contribute to serious side effects. For these two reasons newer and safer macrolides, such as azithromycin and clarithromycin, are proposed as an alternative in the treatment of foals and humans. In fact, study of Villarino & Martin-Jiménez (2013) reports that newer macrolides demonstrate accumulation inside the bronchoalveolar lavage cells at higher extent than erythromycin, which explains why the former are more effective than erythromycin in pneumonia caused by R. equi. Moreover, the use of azithromycin monotherapy provided nearly as high survival rate values in foals as a combination of azithromycin with rifampicin (Venner et al., 2013). Other two macrolides, tularthromycin and tilmicosin, demonstrate low in vitro activity against R. equi, which should be taken under consideration by veterinarians (Giguère et al., 2011). Finally, it should be noted that some study highlights the occurrence of cross-resistance to macrolides, especially among rifampicin-resistant strains (Giguère et al., 2010; Muscatello, 2012).

Erythromycin and other macrolides are preferably coupled with rifampicin that was found very useful in therapy against rhodococcal infections. It has been used so often and eagerly that rifampicin resistance of R. equi became a therapeutic problem. Occurrence of this phenomenon was well documented by Buckley et al. (2007), who demonstrated that increase in prevalence of rifampicin resistance is even faster than that of erythromycin. Because of this ready development of rifampicin resistance, this drug should be combined with other antimicrobials.

Lincosamides (clindamycin and lincomycin), fluoroquinolones (ciprofloxacin, enrofloxacin and norfloxacin), tetracyclines (tetracycline, doxycycline and minocycline) and chloramphenicol are another antibiotics able to penetrate macrophages and kill intracellular pathogens such as R. equi. It is important to know that not all these drugs are entirely effective. Some strains of R. equi demonstrate variable in vitro susceptibility, and the number of multidrug-resistant strains is increasing. For instance, in two studies from the year 1992 and 2010 percentage of drug resistant human strains were ranging between 50% and 90.2% for clindamycin, 9.8% and 16% for norfloxacin, 7.84% and 18% for ciprofloxacin, and 4% and 21.57% for tetracycline (McNeil & Brown, 1992; Silva et al., 2010).

The main advantage of macrolides, rifampicin and other cell-penetrating drugs is the fact that they reach high intracellular concentration in alveolar macrophages and neutrophils (Ribeiro et al., 2006), which is essential for anti-rhodococcal activity. Drugs that inhibited R. equi growth in vitro, (such as combination of penicillin with gentamycin), but were not able to penetrate macrophages, were not effective in the treatment of foals (Giguère et al., 2011). Therefore, antimicrobials that exhibit a poor intracellular activity, such as aminoglycosides, glycopeptides, and β-lactams, should be administered only as a supporting drug in a combination of two or more. In fact, the use of β-lactams should be limited to imipenem, for which the number of susceptible strains accounted for 98.04% (Silva et al., 2010). Therapy based on combination of imipenem with teicoplanin, gentamycin, vancomycin or amikacin, as well as combinations of β-lactam antibiotics with β-lactamase inhibitors (i.e. amoxicillin with clavulanate, or ampicillin with sulbactam) may represent an alternative. For amoxicillin-clavulanate the in vitro susceptibility of R. equi was also 98.04% (Silva et al., 2010). Of course, combination of two drugs that penetrate cells (e.g. rifampicin with a macrolide, rifampicin with a tetracycline, or a macrolide with a tetracycline) is recommended. In such case the use of two antibiotics from different classes decreases the risk of resistance development for either antimicrobial (Venner et al., 2013).

Some antimicrobials demonstrate an antagonistic activity. For instance, use of amikacin alongside with a macrolide/rifampicin, or gentamycin with rifampicin is not recommended (Giguère et al., 2012). Use of β-lactam antibiotics may be problematic since, according to Nor-

Table 1. Antimicrobials used for treatment of Rhodococcus equi infections in horses and humans (Wilson, 2001; Gilbert et al., 2010; Yamshchikov et al., 2010; Giguère et al., 2011; de Bruijn et al., 2013; Venner et al., 2013)

<table>
<thead>
<tr>
<th>Horses</th>
<th>Humans</th>
</tr>
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<tbody>
<tr>
<td><strong>First choice drugs</strong>: erythromycin with rifampicin, clarithromycin with rifampicin, azithromycin with rifampicin</td>
<td><strong>First choice drugs</strong>: erythromycin, rifampicin, imipenem, vancomycin, levofoxacin, aminoglycosides: tobramycin, gentamycin, amikacin</td>
</tr>
<tr>
<td><strong>Alternative choices</strong>: gamithromycin with or without rifampicin, tulathromycin, erythromycin, azithromycin, gentamicin with rifampicin, doxycycline with rifampicin, trimethoprim-sulfamethoxazole with or without rifampicin</td>
<td><strong>Alternative choices</strong>: clindamycin, ciprofloxacin, trimethoprim-sulfamethoxazole, tetracyclines, linezolid, cellulosporins</td>
</tr>
<tr>
<td>Also effective**: azithromycin, clarithromycin, teicoplanin, meropenem, amoxicillin-clavulanate</td>
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*consider 2 agents, **based on clinical studies that ended in patient’s recovery
MOLECULAR MECHANISMS OF ANTIMICROBIAL RESISTANCE

The success of a therapy against \( R. \text{equi} \) relies on two major factors: the \textit{in vitro} susceptibility of the strains to antibiotics, and the effectiveness of the penetration to the body cells where the drug plays its role.

\textit{In vitro} susceptibility of \( R. \text{equi} \) varies among the strains, and this heterogeneity underlies in resistance of the entire population. In a study by McNeil & Brown (1992) less than 5% of human strains isolated from the HIV-infected patients were resistant to erythromycin, rifampicin, tetracycline, and trimethoprim-sulfamethoxazole (McNeil & Brown, 1992). The same study shows that resistance of human isolates were nearly 20% for fluoroquinolones (ciprofloxacin and norfloxacin), and 50% or more for clindamycin and some \( \beta \)-lactam antibiotics, such as ampicillin, cephalothin, oxacillin, and penicillin. Interestingly, human isolates demonstrate higher multidrug resistance than \( R. \text{equi} \) isolated from other sources (McNeil & Brown, 1992). It was also concluded that two out of eight strains of \( R. \text{equi} \) isolated from the HIV-infected patients showed evidence of acquired resistance to \( \beta \)-lactam antibiotics.

Other research made on human isolates presented similar results for rifampicin and erythromycin, which inhibited the \textit{in vitro} growth of 98.04% of \( R. \text{equi} \) strains. Fluoroquinolones, i.e. levofloxacin, ciprofloxacin and norfloxacin, were very effective, inhibiting the growth of approx. 92–96% of strains. About 96% of strains was inhibited by doxycycline, whereas only 78.43% by tetracycline, and 84.31% by chloramphenicol. Much lower antimicrobial activity was observed in case of clindamycin and sulfamethoxazole with trimethoprim, to which only 9.8 and 41.16% of strains were susceptible, respectively. Susceptibility of strains to \( \beta \)-lactams varied, reaching from 1.96% for oxacillin, 5.88% for penicillin, 7.84% for ampicillin, 13.73% for cefazolin, 19.60% for cephalothin, 23.53% for cefotaxime, 45.10% for cefepime, 49.02% for ceftoxitin, and 80.39% for ceftriaxone (Silva \textit{et al.}, 2010). (Silva \textit{et al.}, 2010).

The main mechanisms responsible for acquirement of resistance regard cell wall permeability, efflux pumps, metabolic pathways, and an acquisition of new genes that would result in the increase of tolerance to the antimicrobial drugs (Fig. 1). Resistance genes may be located on the chromosome, or on the mobile genetic elements, i.e. on a plasmid, or on a transposon (de Carvalho, 2010).

Among chromosomal genes, \( rpoB \), a gene encoding RNA polymerase \( \beta \)-subunit, was found to have an influence on antimicrobial resistance profile of \( R. \text{equi} \). In \textit{Mycobacterium tuberculosis} rifampicin resistance is related to
spontaneous mutations in one of three loci in rpoB gene (Lambert et al., 2002). Similar observations were made for R. equi, and it was proven that monotherapy with rifampicin induced mutations in rpoB gene, which resulted in occurrence of rifampicin resistance (Fines et al., 2001; Asoh et al., 2003; Giguère et al., 2011). Spontaneous substitutions of nucleotides located in a 81 bp-long region of rpoB gene, i.e. region between codons 507 and 533 (Escherichia coli numbering), resulted in alterations within the RNA polymerase β-subunit composition in the corresponding positions. This region of polymerase is also known to be a rifampicin target, which means that alterations in the polymerase composition and structure may result in decreased affinity of rifampicin to this enzyme (Fines et al., 2001). The low-level resistant strains of R. equi (MIC 1–8 µg/ml) had the following substitutions: Ser509Pro, Asp516Val, His526Asn and Ser531Leu, whilst high-level resistant strains (MIC ≥128 µg/ml) had Ser531Trp, His526Asp, His526Trp and His526Arg substitutions (Fines et al., 2001; Asoh et al., 2003; Boyen et al., 2011). The His526Arg-mutant was constructed from a rifampicin-susceptible reference strain R. equi ATCC 33701, while the rest of mutations were observed in isolates of an equine and human origin.

Molecular mechanisms of macrolide resistance of R. equi are still unknown. However, bacteria resistant to one macrolide, such as erythromycin, are usually resistant to azithromycin and clarithromycin as well. Moreover, sometimes strains classified as resistant are in fact susceptible and in such case retesting is needed (Giguère et al., 2011).

Quinolone resistance is related to mutations in the gyrA and gyrB genes (encoding DNA gyrase α- and DNA gyrase β-subunit), in particular to the quinolone resistance-determining region (QRDR) of these genes. Studies made on ciprofloxacin-exposed R. equi revealed that there are nine single nucleotide substitutions observed in QRDRs of gyrA and gyrB corresponding to eight amino acid alterations in both gyrase. Mutation in gyrase α-subunit resulted in higher-level resistance (MIC from 8 to >64 µg/ml) of R. equi than mutation in gyrase β-subunit (MIC 4 µg/ml). Moreover, substitutions of serine in position 83 to arginine or isoleucine in the gyrase α-subunit (Ser83Arg or Ser83Ile) led to higher-level resistance than other substitutions of the same enzyme, such as Asp87Asn, Asp87Gly, Asp87His, Asp87Tyr, or Gly81Cys. Such differences may result from the fact that Ser83 is of great importance, and its substitution may be related to reduction of affinity of rifampicin to this enzyme (Fines et al., 2001). Similarly, mutations in gyrB gene, i.e. region between codons 507 and 533 (Bacillus subtilis numbering), resulted in alterations within the DNA gyrase β-subunit in the corresponding positions. This region of gyrase is also known to be a rifampicin target, which means that alterations in the gyrase composition and structure may result in decreased affinity of rifampicin to this enzyme (Fines et al., 2001). The low-level resistant strains of R. equi (MIC 1–8 µg/ml) had the following substitutions: Ser509Pro, Asp516Val, His526Asn and Ser531Leu, whilst high-level resistant strains (MIC ≥128 µg/ml) had Ser531Trp, His526Asp, His526Trp and His526Arg substitutions (Fines et al., 2001; Asoh et al., 2003; Boyen et al., 2011). The His526Arg-mutant was constructed from a rifampicin-susceptible reference strain R. equi ATCC 33701, while the rest of mutations were observed in isolates of an equine and human origin.

Of course, different molecular mechanisms of resistance to β-lactam antibiotics, such as PBP alterations (resulting in a target site changes for the antimicrobials), and efflux pump activity are valid, especially since it has been reported that several strains resistant to β-lactam antibiotics were lacking β-lactamases (McNeil & Brown, 1992; Nordman et al., 1993; Nordman et al., 1994; Linder et al., 1997). Surprisingly, Meroueh et al. (2003) and Martínez (2009) suggested that plasmid-encoded β-lactamases, may have been originally the PBP2s, and their activity against β-lactams might be a side effect of their original function.

The β-lactamases are not the only enzymes produced by R. equi in order to protect it from antimicrobials. Study made on strains exposed to sulfamethoxazole revealed that R. equi had the greatest ability to metabolize sulfamethoxazole amongst other rhodococci. Enzymes proposed for sulfamethoxazole degradation included the alylaminate N-acetyltransferase, an amidase that degraded lysergamide to hygseric acid, an urethanase which hydrolyzes anilides, and the N-acetyl-phenylethylamine hydrolyase which hydrolyzes N-acetylated compounds (Larcher & Yargeau, 2011).

Cell envelope is the main defense barrier of many bacteria. Cell wall of R. equi slightly resembles cell envelope properties of the Gram-negative bacteria. It is highly hydrophobic because of the presence of mycolic acid and glycolipids. These compounds are responsible for increased cellular tolerance to hydrophobic antimicrobials and organic solvents, which cannot diffuse across this hydrophobic layer, and are thought to use the porin channels, which are present in R. equi (de Carvahlo, 2010; Kuyukina & Ivshina, 2010; Sutcliffe et al., 2010).

Resistance to hydrophobic antibiotics, such as rifampicin and quinolones, depends upon the effective efflux pump systems. This mechanism is commonly found in microorganisms, including mycobacteria and Rhodococcus species (de Carvahlo, 2010). Genes encoding exporters and transporters are located both on chromosome and on mobile genetic elements. The chromosome-encoded pump systems are responsible for multidrug resistance, whilst plasmid ones remove only specific groups of antimicrobials, which is usually related to the acquired drug resistance to macrolides, lincosamides, tetacyclines, rifampicin and chloramphenicol. This specific efflux system responsible for chloramphenicol resistance have been well-described for Rhodococcus fascians and Rhodococcus rhodochrous, for which twelve membrane spanning domains were detected as a consequence of cmr and cmrA gene expression, respectively. The cmr gene was found on a conjugative plasmid pRFB2, while cmrA gene is a component of a transposon Tn5561 (Butaye et al., 2003). In R. equi, chloramphenicol-resistance has been observed as well (Vázquez-Boland et al., 2010; Silva et al., 2012), which only leads to an assumption, that this species may possess similar efflux pump systems, which are not described yet.

Apart from the mechanisms responsible for an increase in antimicrobial resistance of R. equi, there is one of an opposite relevance. Surprisingly, antimicrobial susceptibility of R. equi depends on presence of the virulence-associated plasmids (VAPs) as well. These plasmids contain lsr2 gene alongside with the virulence genes sap. The Lsr2 protein is homologous to the one that in Mycobacterium tuberculosis serves as a regulator of the antibiotic-induced responses, phase infections, and plays a role in modifications of the mycolic acid (Arora et al., 2008; Colangelo et al., 2007; Vázquez-Boland et al., 2010). In M. tuberculosis Lsr2 nonspecifically binds to AT-rich sequences,
including those that seem to be antibiotic-induced genes, changes the way that DNA is shaped, and prevents from the antibiotic-induced responses in mycobacteria (Colangeli et al., 2007). Similar mode of action is predicted for R. equi (Vázquez-Boland et al., 2010). This unspecific reaction is responsible for repression of the resistance genes expression, and increases rhodococcal susceptibility to the antimicrobials (Arora et al., 2008).

CONCLUSIONS

Treatment of rhodococcal ususally consists of a combination of at least two antibiotics to which the agent is susceptible. These include macrolides, rifampicin, fluoroquinolones, aminoglycosides, glycopeptides and carbapenems, although the increase of rifampicin and erythromycin resistance is progressing. Other macrolides, such as azithromycin, demonstrate good inhibitory activity, however cross-resistance among macrolides is common. Susceptibility to other antibiotics is variable, and depends upon various resistance mechanisms, characteristic for each group of antimicrobials. The genetic potential for antimicrobial resistance revealed in the R. equi genome, indicates that we may be at a critical junction in effective antimicrobial treatment of rhodococcal infection.

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