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Antimicrobial resistance spectrum conferred by pRErm46 of emerging macrolide (multidrug)-resistant *Rhodococcus equi*

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Running Title: Resistance profile of pRErm46 from MDR *R. equi*

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ABSTRACT

Clonal multidrug resistance recently emerged in *Rhodococcus equi*, complicating the therapeutic management of this difficult-to-treat animal and human pathogenic actinomycete. The currently spreading multidrug-resistant (MDR) “2287” clone arose in equine farms upon acquisition, and co-selection by mass macrolide-rifampin therapy, of the pREm46 plasmid carrying the *erm*(46) macrolides-lincosamides-streptogramins resistance determinant, and an *rpoB*^{S531F} mutation. Here, we screened a collection of susceptible and macrolide-rifampin-resistant *R. equi* from equine clinical cases using a panel of 15 antimicrobials against rapidly growing mycobacteria (RGM), nocardiae and other aerobic actinomycetes (NAA). *R. equi* –including MDR isolates– was generally susceptible to linezolid, minocycline, tigecycline, amikacin and tobramycin according to *Staphylococcus aureus* interpretive criteria, plus imipenem, cefoxitin and ceftriaxone based on Clinical & Laboratory Standards Institute (CLSI) guidelines for RGM/NAA. Ciprofloxacin and moxifloxacin were in the borderline category according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria. Molecular analyses linked pREm46 to significantly increased MICs for trimethoprim-sulfamethoxazole and doxycycline in addition to clarithromycin within the RGM/NAA panel, and to streptomycin, spectinomycin and tetracycline resistance. pREm46 variants with spontaneous deletions in the class 1 integron (C1I) region, observed in ≈30% of *erm*(46)-positive isolates, indicated that the newly identified resistances were attributable to C1I’s sulfonamide (*sul1*) and aminoglycoside (*aaA9*) resistance cassettes and adjacent *tetRA*(33) determinant. Most MDR isolates carried the *rpoB*^{S531F} mutation of the 2287 clone, while different *rpoB* mutations (S531L, S531Y) detected in two cases suggest the emergence of novel MDR *R. equi* strains.

Rhodococcus equi is a soil-borne facultative intracellular actinomycete that causes chronic pyogranulomatous infections in animals and immunocompromised people (1-3). Young foals are particularly susceptible to *R. equi* carrying the equine-specific virulence plasmid pVAPA (4-7) and develop a life-threatening purulent bronchopneumonia with frequent extrapulmonary involvement (1, 8). No effective vaccine is currently available and control of the disease largely relies on prolonged courses of antimicrobial therapy (9, 10).

Many antimicrobials are active against *R. equi in vitro* but their clinical efficacy or applicability is compromised for a variety of reasons, such as poor intracellular penetration, reduced oral bioavailability, inadequate pulmonary pharmacokinetics, undesirable effects in foals, or lack of randomized efficacy studies (11). Intrinsic and mutational resistance, compounded by the permeability barrier afforded by a mycolic acid-containing cell envelope similar to that of *Mycobacterium* and other actinomycetes, may also result in inconsistent drug susceptibility, as reported for chloramphenicol, β-lactams or quinolones (12-22). Consequently, co-administration of a macrolide (erythromycin, later clarithromycin or azithromycin) and rifampin has remained the mainstay therapy against foal rhodococcosis since clinical experience in the 1980's (23, 24), further supported by *in vitro* (15, 25, 26) and *in vivo* (27) experimental data, demonstrated the efficacy of this drug combination (11).

While rifampin resistance caused by *rpoB* mutations has been regularly reported in *R. equi* (15, 20, 21, 28), resistance to macrolides only recently emerged, interestingly, always associated with rifampin resistance (29, 30). Dual resistance to macrolides and rifampin was first detected in the late 1990's in equine farms where mass macrolide-rifampin antibioprophylaxis was systematically practiced (31, 32), and is more frequent among foals exposed to the macrolide-rifampin combination (33, 34). Macrolide resistance was found to be mediated by *erm*(46), a novel self-transmissible rRNA methylase determinant conferring cross-resistance to lincosamides and streptogramins B (MLSB) (30). *erm*(46) is carried on a conjugative plasmid, pRErm46 (35), as part of a 6.9-kb transposon, Tn*RErm46*. The latter is

highly mobile and becomes stabilized in *R. equi* by actively transposing onto the host genome and the pVAPA virulence plasmid (35). Despite pRErm46's high conjugal transferability, *erm*(46) remains largely restricted to a clonal *R. equi* subpopulation characterized by a unique *rpoB*^{S531F} mutation, presumably as a result of strong co-selection driven by the combination therapy (35, 36). pRErm46 has recently been shown to also confer tetracycline resistance via a *tetRA*(33) determinant associated to a class 1 integron (C1I) (35) virtually identical to those found in the corynebacterial plasmid pTET3 (37).

The *erm*(46)-carrying multidrug-resistant (MDR) *R. equi* clone, designated 2287 (35, 36), is increasingly prevalent across equine farms in the USA, is likely to spread internationally (36), and poses a substantial threat because of the lack of clinically-proven alternative antimicrobials to treat affected foals. The aim of this study was (i) to determine the genetic basis of macrolide resistance in *R. equi* equine isolates from Kentucky, USA, where foal rhodococcosis is endemic and MDR 2287 was first identified (31); and (ii) to assess the activity against MDR *R. equi* of a panel of antimicrobials used for susceptibility testing of closely related rapidly growing mycobacteria (RGM) and nocardiae/other aerobic actinomycetes (NAA).

MATERIALS AND METHODS

Bacteria. The *R. equi* isolates analyzed in this study were recovered from 70 necropsied foals with severe rhodococcal infection diagnosed between 1989 to 2019 at the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL). Necropsy specimens typically included lung, liver, small intestine, colon and any other organ/tissues with *R. equi*-compatible lesions. Isolation was performed on blood agar, Columbia (colistin/nalidix acid) agar and eosin methylene blue agar plates incubated at 37 ºC microaerophilically for 24 h followed by a minimum additional 24 h aerobically. *R. equi* identification was based on standard criteria including colony morphology, gram staining, biochemical tests, CAMP-like

co-operative hemolysis with sphingomyelinase C-producing indicator bacteria (*S. aureus* or *Listeria ivanovii*) (38, 39), and PCR detection of the *R. equi*-specific *choE* and *vapA* gene markers (40, 41). *R. equi* isolates were stored at -80 ºC until used.

***In vitro* susceptibility testing.** Inocula containing ≈1×10^5 CFU as verified by plate counting were prepared by the direct colony suspension method according to CLSI guidelines. Minimal inhibitory concentrations (MICs) for erythromycin and rifampin (and confirmatory determinations for trimethoprim-sulfamethazole [TMP-SMX] and tetracycline) were performed using gradient concentration Etest® strips as per the manufacturer's instructions (BioMérieux, Durham, NC and Basingstoke, Hampshire, UK) using *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 as controls. Since there are currently no approved breakpoint criteria for susceptibility testing of *R. equi* in horses, interpretation was extrapolated from MIC-based human interpretative criteria (erythromycin: S ≤0.5 µg/ml, I = 1-4 µg/ml, R ≥8 µg/ml; rifampin: S ≤1 µg/ml, I = 2 µg/ml, R ≥4 µg/ml) (42). Susceptibility testing for antimicrobials against RGM and NAA was performed by the broth microdilution method using Sensititre™ RapMyco AST Plates (Trek diagnostics, Thermo Fisher Scientific, Grand Island, NY), comprising the following 15 antimicrobials: amikacin, amoxicillin/clavulanic acid, cefepime, cefoxitin, ceftriaxone, ciprofloxacin, clarithromycin, doxycycline, imipenem, linezolid, minocycline, moxifloxacin, tigecycline, tobramycin, and TMP-SMX. *S. aureus* ATCC 29213 and *Mycobacterium peregrinum* ATCC 700686 were used as controls as per CLSI guidelines (43). Susceptibility to different aminoglycosides was determined by the diffusion method using the following disks (Oxoid, Basingstoke, Hampshire, UK): streptomycin (S, 25µg), spectinomycin (SH, 25 µg), gentamicin (CN, 50 µg), kanamycin (K, 30 µg) and apramycin (APR, 15 µg).

Molecular characterization of MDR *R. equi* and pRErm46. Total bacterial DNA was prepared by heating isolated colonies at 100 °C in 100 µl of ultrapure water and centrifugation for 90 s at 16,000 × *g*. PCR reactions were carried out using Quick-load 2× Taq

master mix (New England Biolabs) as previously described (30). Oligonucleotide primers used are listed in Table S1. RpoB substitutions were determined by sequencing an 827-bp *rpoB* region amplified by PCR from *R. equi* genomic DNA using previously reported oligonucleotides primers (20) and Kapa HiFi HotStart ReadyMix (Roche). PCR amplicons were purified from agarose gels using QIAquick kit (Qiagen, Manchester, UK) and sequenced by the Sanger method at Source BioScience (Nottingham, UK). The deduced amino acid sequence was aligned to the RpoB sequence from the *R. equi* 103S reference genome (accession no. FN563149) (13) using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis. MIC_{50}/MIC_{90} values were determined as previously reported (44). Data were also analyzed by descriptive statistics including modal MICs (the most common MIC), average and range when appropriate. The statistical significance of the differences in the MICs was calculated using Mann-Whitney, Kruskal-Wallis or Fisher´s exact test. Data were analyzed using GraphPad Prism version 9.1.0 software for Mac, GraphPad Software, San Diego, California USA (www.graphpad.com).

RESULTS

Resistance to macrolides and rifampin. We analyzed a selection of 70 *R. equi* clinical strains recovered post-mortem from severe cases of foal rhodococcosis, including 15 macrolide-resistant isolates identified through erythromycin susceptibility screening (modal MIC 24/32 µg/ml, range 8-96 µg/ml) (Table 1). All erythromycin-resistant (Erm^R) isolates also showed high rifampin MICs (≥32 µg/ml), consistent with the previously reported dual Erm^R/rifampin-resistant (Rif^R) phenotype of the *R. equi* 2287 clone (35, 36). The remaining strains were erythromycin susceptible (Erm^S, MIC_{90} 0.75/1 µg/ml, range 0.016-6 µg/ml) and included 21 Rif^R isolates (MIC_{90} ≥32 µg/ml) (Table 1).

Molecular characterization. Using a previously described PCR test (30), *erm*(46) was detected in 14 of the 15 Erm^R/Rif^R isolates (Table 1). Sequencing of the *rpoB* gene determined that 13 of the 14 *erm*(46)-positive Erm^R/Rif^R isolates carried the Ser531Phe substitution (TCG→TTC transversion) characteristic (and so far unique) (35, 36) to the MDR 2287 clone (35). The other *erm*(46)-positive isolate carried a distinct RpoB substitution also at position 531 within the rifampin resistance determining region (RRDR-1), Ser531Leu, previously described in *R. equi* several times (20, 21, 45).

The only *erm*(46)-negative Erm^R/Rif^R isolate also carried a distinct *rpoB* mutation, again at position 531, resulting in a Ser→Tyr substitution not described before in clinical isolates of *R. equi*. An $rpoB^{S531Y}$ substitution was recently reported by Huber et al. in an Erm^R clonal *R. equi* population apparently restricted to the environment and which carried a variant *erm*(46) gene, designated *erm*(51) (46). Attempts to detect *erm*(51) in our *erm*(46)-negative Erm^R isolate (and all other Erm^R strains plus a selection of Erm^S isolates) using different primer sets (Table S1) were unsuccessful. Moreover, the *rpoB* Tyr531 codon in the *erm*(46)/*erm*(51)-negative isolate (TAC) is different from that found in the *erm*(51)-positive clonal isolates (TAT) (46), indicating that both correspond to genetically distinct Erm^R/Rif^R *R. equi* subpopulations.

erm(46) was not detected in any of the 21 Erm^S/Rif^R isolates nor the 34 susceptible *R. equi* clinical isolates (Table 1). Analysis of the *rpoB* sequences from the Erm^S/Rif^R isolates identified different RpoB substitutions (to be described elsewhere). As expected, no *rpoB* mutations were found in a random selection of the Rif^S isolates.

Screening against antimicrobial panel for related pathogenic actinomycetes. Most of the 15 RapMyco antimicrobials for RGM/NAA susceptibility testing were active against *R. equi* irrespective of the Erm/Rif phenotype. MIC_{90}'s were ≤1 µg/ml for linezolid, minocycline and tigecycline, 1 µg/ml for ciprofloxacin and moxifloxacin, 1 to 2 µg/ml for doxycycline and tobramycin, 2 µg/ml for amikacin, ≤2 µg/ml for imipenem, and ≤4 µg/ml for ceftriaxone.

Higher MIC_{90}'s were observed for cefoxitin, cefepime and amoxicillin-clavulanic (16 µg/ml) (Table 2).

Significant MIC differences between Erm^R and Erm^S isolates were observed, as expected, for the macrolide clarithromycin (8 to ≥16 µg/ml vs 0.06-0.5 µg/ml, respectively; *P* <0.0001), but interestingly also TMP-SMX (>8 and 2 µg/ml, respectively; *P* = 0.0025) (Table 2). The differential TMP-SMX susceptibility was confirmed using gradient MIC Etest strips (≥32 µg/ml for Erm^R, 0.5-1 µg/ml for Erm^S; Table 3). Diffusion essays with sulfamethoxazole disks showed it was linked to sulfonamide resistance (no halo for most Erm^R isolates, 27.3±2.4 mm mean diameter for Erm^s ones).

pRErm46-mediated sulfonamide resistance. pRErm46's C1I carries a *sul1* gene (35) (Fig. 1) that could explain the association between macrolide and TMP-SMX resistances in MDR *R. equi* (47). Using specific PCR primers (Table S1), we confirmed that all isolates displaying sulfamethoxazole resistance (Smx^R) possessed *sul1* and associated C1I genes, whereas all Smx^S strains were negative (Table 3).

A notable exception was the prototype strain of the MDR 2287 clone, PAM 2287 (35), which we tested as a control. Despite carrying the *sul1* gene (35), PAM 2287 was susceptible to TMP-SMX (MIC 1 µg/ml) unlike most members of the 2287 clonal population (MIC >32 µg/ml, determined by Etest). This is likely because, in PAM 2287's pRErm46, a copy of the Tn*RErm46* transposon is inserted within the C1I's *aadA9* gene (35), preventing read-through transcription of the downstream *sul1* cassette from the integron's promoter (Fig 1).

While there was 100% correlation between the presence of a functional *sul1* and sulfonamide resistance, not all *erm*(46)-positive Erm^R isolates exhibited an Smx^R phenotype. Specifically, six (43%) of the 14 *erm*(46)-positive isolates showed low TMP-SMX MICs similar to those of the Erm^S group (1 - 2 µg/ml vs >8 µg/ml for Erm^R strains, *P* = 0.69) (Table 3**)**. This profile would be expected in case of pRErm46 plasmid loss with retention of the

erm(46) (Tn*RErm46*) element by transposition onto the host genome, observed in a proportion of MDR *R. equi* isolates (35, 36).

To examine the above possibility, we assessed the presence of pRErm46 indirectly by using the C1I-associated *tetRA*(33) tetracycline resistance determinant (36) as a phenotypic marker using Etest strips, and directly by PCR mapping with primers targeting the plasmid backbone and the C1I-*tetRA*(33) region (Table S1). This analysis showed that three of the six *erm*(46)-positive/Smx^S isolates were indeed negative to all pRErm46 markers. However, the three others retained the pRErm46 backbone but were negative to C1I and *tetRA*(33), consistent with the deletion of this region, previously observed in a subset of MDR 2287 isolates (36). The data also revealed a perfect correlation between the sulfonamide and tetracycline susceptibility phenotypes (Table 3), except in one case. This single Smx^R and tetracycline susceptible isolate was positive to all pRErm46 markers except *tetRA*(33) (Table 3), suggesting a specific deletion of the latter. The pRErm46 C1I/*tetRA*(33) deletions were confirmed by PCR mapping using external primers (Fig. 1, Table S1).

Collectively, our results indicate that pRErm46 also confers resistance to sulfonamides in addition to macrolides and tetracycline in *R. equi*, and that complete or partial deletions of the C1I-*tetRA*(33) region (Fig 1) take place in a proportion of the plasmid population (4 of 14 plasmids analyzed), resulting in corresponding loss of sulfonamide and/or tetracycline resistance.

Other pRErm46-associated antimicrobial resistances. pERrm46's C1I also codes for an aminoglycoside-modifying enzyme identical to the ANT(3")-Ia family adenylyltranferase encoded by the *aadA9* cassette from the homologous C1I of the pTET3 plasmid from *Corynebacterium glutamicum* LP-6 (37). Consistent with the substrate range of the corynebacterial AadA9 enzyme (37), disk diffusion assays showed that all *R. equi* isolates carrying a pRErm46 plasmid with intact C1I (n = 8) were resistant to streptomycin and spectinomycin but susceptible to a range of other aminoglycosides (gentamicin, kanamycin

and apramycin in addition to amikacin and tobramycin), whereas those carrying C1I-deleted plasmids (and pRErm46-negative isolates) were susceptible (Table 4). Of note, the prototype MDR 2287 strain, PAM 2287, which carries a Tn*RErm46*-disrupted *aadA9* cassette (see above and Fig. 1), was also susceptible to streptomycin and spectinomycin.

Finally, while there were no significant differences in doxycycline susceptibility between *erm*(46)-positive (Erm^R) and -negative (Erm^S) isolates in the global analysis (Table 2), consistent with previous reports (34, 35), there was a small but significant increase in the MIC for the *erm*(46)-positive Erm^R isolates carrying *tetRA*(33) compared to those lacking the tetracycline resistance determinant (modal MIC 2 μg/ml, range 1-2 μg/ml vs 1 μg/ml, range 0.12-1 μg/ml; $P < 0.0001$). These data indicate that the TetA(33) efflux pump system encoded in pRErm46 confers some degree of cross-resistance to the semisynthetic tetracycline, doxycycline.

DISCUSSION

The emergence of MDR *R. equi* renders ineffective the macrolide-rifampin combination used as mainstay therapy against foal rhodococcosis (11) and, often, in the treatment of human rhodococcal infections (2, 19). To aid in the identification of alternative drugs, we used a panel of antimicrobials for susceptibility testing of RGM and NAA, to which *R. equi* is phylogenetically, pathogenically and physiologically closely related. We found that the pRErm46 MLSB resistance plasmid of MDR *R. equi* (35) also confers resistance to sulfonamides and, at low level, doxycycline (see below), both recognized as potential therapeutic options against *R. equi* (3, 9, 11, 23, 48, 49).

Sulfonamide resistance is linked to the *sul1* cassette of pRErm46's C1I. Horizontally acquired *sul1* genes encode alternative dihydropteroate synthase (DHTS) enzymes that functionally complement the core bacterial DHTS, allowing to bypass the inhibitory effect of sulfonamides (47). Doxycycline resistance is conferred by the *tetRA*(33) element adjacent to

the C1I (Fig 1), previously linked to pRErm46-specified tetracycline resistance (35), which we confirm here. We also report that pRErm46 additionally encodes streptomycin and spectinomycin resistance via pRErm46's C1I *aadA9* cassette (37) (Fig 1).

Our data show that all the newly identified non-MLSB resistances can be lost by either (i) spontaneous deletion of the plasmid's C1I-*tetRA*(33) region (36), presumably by homologous recombination between the flanking directly repeated IS*6100* copies (50, 51) (Fig 1); or (ii) pRErm46 curing after transposition of the Tn*RErm46* element to the host genome (35). Although based on the analysis of a limited number of $Erm^R/Smx^S/Tet^S$ isolates, both events appear to occur at similar frequency in the MDR *R. equi* population. pRErm46 ΔC1I-*tetRA*(33) variants are increasingly observed among clonal MDR 2287 isolates (36) (29% in this study), possibly reflecting genetic dispensability due to lack of antibiotic selection, because neither sulfonamides nor streptomycin, spectinomycin or tetracycline (and doxycycline) are used in the mass *R. equi* antibioprophylaxis at equine farms. We also show that deletion of the *tetRA*(33) locus alone, causing loss of tetracycline –but not the C1I-specified sulfonamide (and streptomycin-spectinomycin)– resistance (Fig 1, Tables 3 and 4), can also occur, as detected in one of the Erm^R isolates.

Consistent with the known substrate range of tetracycline resistance mechanisms (52), pRErm46's TetA(33) efflux pump seems inactive against minocycline and tigecycline. For doxycycline, a word of caution is in order because the *tetRA*(33) determinant was associated with a statistically significant increase in the MIC from 0.5-1 µg/ml to 2 µg/ml (Table 3). While perhaps not clinically relevant in humans, the poor oral bioavailability of doxycycline in adult horses (53, 54) makes MDR *R. equi* isolates carrying pRErm46 plasmids with an intact *tetRA*(33) locus to be classified as doxycycline resistant according to CLSI's criteria for this animal species (PK/PD breakpoint ≥0.5 µg/ml) (42). Although doxycycline's pharmacokinetic variables are more favorable in foals, maximum serum activity values (C_{max} 2.54 and 2.89 µg/ml after intragrastric administration of 10 and 20 mg/kg) (48) would remain

close to the MIC in MDR 2287 (2 µg/ml), meaning it might be difficult to achieve the two- to four-times over-MIC concentrations required for time-dependent antibacterial activity (55). Moreover, doxycycline may also contribute to pRErm46 selection, either in *R. equi* or in other members of the environmental microbiota in which it can be potentially maintained (56)

In the absence of specific interpretive guidelines, *S. aureus* breakpoints are tentatively applied to *R. equi* (43, 57). This may in certain cases be inapplicable because of lack of breakpoint criteria for some antimicrobials, and even be questionable given the significant drug susceptibility-relevant physiological differences between *S. aureus* and *R. equi*. For example, β-lactam susceptibility testing in *S. aureus* relies on the cephalosporin, cefoxitin, as a marker of *mecA/mecC*-mediated methicillin-resistance (MRSA) and predictor of resistance to all antibiotics within this group, including cephems and carbapenems (58). With these criteria, *R. equi* would be resistant to cefoxitin and, by inference, generally to all β-lactams. This is at odds with an interpretation based on RGM and/or NAA criteria (43, 59), with which *R. equi* would be susceptible to imipenem (MIC_{90} ≤2 µg/ml), ceftriaxone (MIC_{90} ≤4 µg/ml) and cefoxitin (MIC_{90} 16 µg/ml), and intermediate to cefepime and amoxicillin/clavulanate (MIC_{90} 16 µg/ml). The RGM/NAA guidelines take into account that MICs tend to be higher in this bacterial group owing to their less permeable cell envelope or typical abundance of intrinsic resistance mechanisms (e.g. the *R. equi* 103S genome encodes 10 putative β-lactamase homologs and an array of 11 penicillin-binding proteins [13]).

To circumvent these problems, we interpreted the *R. equi* susceptibility data by integrating CLSI's criteria for *S. aureus* (57) and RGM/NAA (43, 59), and the EUCAST criteria for both *S. aureus* and corynebacteria (60). Based on MIC_{90} values, *R. equi* clinical isolates, including Erm^R (MDR) strains, can be considered to be generally susceptible to linezolid, minocycline, tigecycline, amikacin and tobramycin. Linezolid and tigecycline reach satisfactory plasma and pulmonary concentrations and would be adequate candidates, eventually in combination, to treat *R. equi* infections (61). However, both are listed as

critically important antibiotics in human medicine (62) and their use in animals is restricted. Minocycline can be administered orally with good bioavailability and offers potentially favorable pharmacokinetic characteristics to treat equine rhodococcosis (63, 64), although a caveat is that it was found to be inactive against *R. equi* in a nude mouse infection model (65). Amikacin was also found to be weakly effective against *R. equi* in nude mice, possibly related to a high frequency of resistant mutants and limited intracellular uptake (65), while the effectiveness window for tobramycin according to pharmacokinetic studies in horses (MIC of 1 to 2 µg/ml) (66) may be too close to the *R. equi* MIC. However, both aminoglycosides may be useful in combination to other antimicrobials. Although assumed to be largely resistant to β-lactams based on the relatively high MICs (15, 19, 67-73), the application of the RGM/NAA breakpoints may enable a wider the use of these antibiotics against MDR *R. equi*, eventually in combination with β-lactamase inhibitors and other antimicrobials, as exemplified with the highly drug-resistant *Mycobacterium abscessus* (74). While β-lactams do not concentrate intracellularly, they permeate into mammalian cells and display intracellular activity (75, 76), as observed with ceftiofur and imipenem in equine monocyte-derived macrophages infected with *R. equi* (77). With a MIC_{90} of 1 µg/ml (Table 2), ciprofloxacin and moxifloxacin would be also largely active against *R. equi*, consistent with previously reported data (2, 11, 17, 78). However, susceptibility to ciprofloxacin would be borderline while moxifloxacin would fall in the resistance category according to EUCAST criteria, more stringent based on PK/PD analyses and the potential impact of low-level resistance on clinical outcomes (resistance breakpoints for *S. aureus*/corynebacteria >1 µg/ml for ciprofloxacin, 0.25/0.5 µg/ml for moxifloxacin) (60). Of note, a poor response to fluoroquinolones has been invoked in human *R. equi* infections (14, 17, 69, 71, 79, 80). Quinolones are also associated with risks of arthropathy in foals (11).

Finally, our data further confirm the predominance of the MDR 2287 clonal population, characterized by the *rpoB* Ser531Phe mutation (35, 36), among Erm^R equine

clinical isolates (90%). One pRErm46-harboring isolate carried a distinct *rpoB* substitution, Ser531Leu, indicative of spillover of pRErm46 to other *R. equi* genotypes and potential emergence of novel MDR clones carrying different *rpoB* mutations (36). We also identified an Erm^R/Rif^R isolate with a novel *rpoB* Ser531Tyr mutation (recently also found in an emerging MDR clone in Kentucky) (36) in which neither *erm*(46)/pRErm46 markers nor the *erm*(51) variant recently discovered in environmental isolates of *R. equi* (46), were detected. This strain warrants further investigation and indicates that diverse resistance mechanisms are being actively selected in *R. equi* in response to the antibiotic pressure imposed by the macrolide-rifampin combination therapy commonly used at equine farms.

In summary, this study adds to the known resistance spectrum of pRErm46 (macrolides, lincosamides, streptogramins and tetracycline) four additional antimicrobials (sulfonamides/trimethoprim-sulfamethoxazol, doxycycline, streptomycin and spectinomycin), and identifies alternative drugs for potential consideration in the treatment of infections by MDR *R. equi*.

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AUTHORS CONTRIBUTIONS

EE: study design, research, data analysis and interpretation, manuscript drafting, critical revisions. MS: experimental design, molecular and susceptibility studies, data analysis and interpretation, manuscript writing, critical revisions. JF, MP: susceptibility determinations. JV-B: study design, conceptualization, data analysis and interpretation, writing of final manuscript.

REFERENCES

1. Prescott JF. 1991. *Rhodococcus equi*: an animal and human pathogen. Clin Microbiol Rev 4:20-34.
2. Weinstock DM, Brown AE. 2002. *Rhodococcus equi*: an emerging pathogen. Clin Infect Dis 34:1379-1385.
3. Vazquez-Boland JA, Giguere S, Hapeshi A, MacArthur I, Anastasi E, Valero-Rello A. 2013. *Rhodococcus equi*: the many facets of a pathogenic actinomycete. Vet Microbiol 167:9-33.
4. Takai S, Hines SA, Sekizaki T, Nicholson VM, Alperin DA, Osaki M, Takamatsu D, Nakamura M, Suzuki K, Ogino N, Kakuda T, Dan H, Prescott JF. 2000. DNA sequence and comparison of virulence plasmids from *Rhodococcus equi* ATCC 33701 and 103. Infect Immun 68:6840-7.
5. MacArthur I, Anastasi E, Alvarez S, Scortti M, Vazquez-Boland JA. 2017. Comparative Genomics of *Rhodococcus equi* virulence plasmids indicates host-driven evolution of the *vap* pathogenicity island. Genome Biol Evol 9:1241-1247.
6. Jain S, Bloom BR, Hondalus MK. 2003. Deletion of *vapA* encoding Virulence Associated Protein A attenuates the intracellular actinomycete *Rhodococcus equi*. Mol Microbiol 50:115-128.
7. Muscatello G, Gerbaud S, Kennedy C, Gilkerson JR, Buckley T, Klay M, Leadon DP, Browning GF. 2006. Comparison of concentrations of *Rhodococcus equi* and virulent *R. equi* in air of stables and paddocks on horse breeding farms in a temperate climate. Equine Vet J 38:263-265.
8. Giguere S, Cohen ND, Chaffin MK, Hines SA, Hondalus MK, Prescott JF, Slovis NM. 2011. *Rhodococcus equi*: clinical manifestations, virulence, and immunity. J Vet Intern Med 25:1221-1230.
9. Giguere S, Cohen ND, Chaffin MK, Slovis NM, Hondalus MK, Hines SA, Prescott JF. 2011. Diagnosis, treatment, control, and prevention of infections caused by *Rhodococcus equi* in foals. J Vet Intern Med 25:1209-1220.
10. Venner M, Astheimer K, Lammer M, Giguere S. 2013. Efficacy of mass antimicrobial treatment of foals with subclinical pulmonary abscesses associated with *Rhodococcus equi*. J Vet Intern Med 27:171-176.
11. Giguere S. 2017. Treatment of infections caused by *Rhodococcus equi*. Vet Clin North Am Equine Pract 33:67-85.
12. Jacks SS, Giguere S, Nguyen A. 2003. *In vitro* susceptibilities of *Rhodococcus equi* and other common equine pathogens to azithromycin, clarithromycin, and 20 other antimicrobials. Antimicrob Agents Chemother 47:1742-1745.
13. Letek M, Gonzalez P, Macarthur I, Rodriguez H, Freeman TC, Valero-Rello A, Blanco M, Buckley T, Cherevach I, Fahey R, Hapeshi A, Holdstock J, Leadon D, Navas J, Ocampo A, Quail MA, Sanders M, Scortti MM, Prescott JF, Fogarty U, Meijer WG, Parkhill J, Bentley SD, Vazquez-Boland JA. 2010. The genome of a pathogenic *Rhodococcus*: cooptive virulence underpinned by key gene acquisitions. PLoS Genet 6: e1001145
14. Mascellino MT, Iona E, Ponzo R, Mastroianni CM, Delia S. 1994. Infections due to *Rhodococcus equi* in three HIV-infected patients: microbiological findings and antibiotic susceptibility. Int J Clin Pharmacol Res 14:157-163.
15. Nordmann P, Ronco E. 1992. *In-vitro* antimicrobial susceptibility of *Rhodococcus equi*. J Antimicrob Chemother 29:383-393.
16. Makrai L, Fodor L, Csivincsik A, Varga J, Senoner Z, Szabo B. 2000. Characterisation of *Rhodococcus equi* strains isolated from foals and from immunocompromised human patients. Acta Vet Hung 48:253-259.
17. Nordmann P, Rouveix E, Guenounou M, Nicolas MH. 1992. Pulmonary abscess due to a rifampin and fluoroquinolone resistant *Rhodococcus equi* strain in a HIV infected patient. Eur J Clin Microbiol Infect Dis 11:557-558.

18. Nordmann P, Keller M, Espinasse F, Ronco E. 1994. Correlation between antibiotic resistance, phage-like particle presence, and virulence in *Rhodococcus equi* human isolates. J Clin Microbiol 32:377-383.

19. Yamshchikov AV, Schuetz A, Lyon GM. 2010. *Rhodococcus equi* infection. Lancet Infect Dis 10:350-359.

20. Riesenberg A, Fessler AT, Erol E, Prenger-Berninghoff E, Stamm I, Bose R, Heusinger A, Klarmann D, Werckenthin C, Schwarz S. 2014. MICs of 32 antimicrobial agents for *Rhodococcus equi* isolates of animal origin. J Antimicrob Chemother 69:1045-1049.

21. Petry S, Sevin C, Kozak S, Foucher N, Laugier C, Linster M, Breuil MF, Dupuis MC, Hans A, Duquesne F, Tapprest J. 2020. Relationship between rifampicin resistance and RpoB substitutions of *Rhodococcus equi* strains isolated in France. J Glob Antimicrob Resist 23:137-144.

22. Niwa H, Hobo S, Anzai T. 2006. A nucleotide mutation associated with fluoroquinolone resistance observed in *gyrA* of *in vitro* obtained *Rhodococcus equi* mutants. Vet Microbiol 115:264-268.

23. Sweeney CR, Sweeney RW, Divers TJ. 1987. *Rhodococcus equi* pneumonia in 48 foals: response to antimicrobial therapy. Vet Microbiol 14:329-36.

24. Hillidge CJ. 1987. Use of erythromycin-rifampin combination in treatment of *Rhodococcus equi* pneumonia. Vet Microbiol 14:337-342.

25. Giguere S, Lee EA, Guldbech KM, Berghaus LJ. 2012. In vitro synergy, pharmacodynamics, and postantibiotic effect of 11 antimicrobial agents against *Rhodococcus equi*. Vet Microbiol 160:207-213.

26. Berghaus LJ, Giguere S, Guldbech K. 2013. Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against *Rhodococcus equi*. Vet Microbiol 166:670-675.

27. Burton AJ, Giguere S, Berghaus LJ, Hondalus MK. 2015. Activity of clarithromycin or rifampin alone or in combination against experimental *Rhodococcus equi* infection in mice. Antimicrob Agents Chemother 59:3633-3636.

28. Asoh N, Watanabe H, Fines-Guyon M, Watanabe K, Oishi K, Kositsakulchai W, Sanchai T, Kunsuikmengrai K, Kahintapong S, Khantawa B, Tharavichitkul P, Sirisanthana T, Nagatake T. 2003. Emergence of rifampin-resistant *Rhodococcus equi* with several types of mutations in the *rpoB* gene among AIDS patients in northern Thailand. J Clin Microbiol 41:2337-2340.

29. Giguere S, Lee E, Williams E, Cohen ND, Chaffin MK, Halbert N, Martens RJ, Franklin RP, Clark CC, Slovis NM. 2010. Determination of the prevalence of antimicrobial resistance to macrolide antimicrobials or rifampin in *Rhodococcus equi* isolates and treatment outcome in foals infected with antimicrobial-resistant isolates of *R equi*. J Am Vet Med Assoc 237:74-81.

30. Anastasi E, Giguere S, Berghaus LJ, Hondalus MK, Willingham-Lane JM, MacArthur I, Cohen ND, Roberts MC, Vazquez-Boland JA. 2015. Novel transferable *erm*(46) determinant responsible for emerging macrolide resistance in *Rhodococcus equi*. J Antimicrob Chemother 70:3184-3190.

31. Burton AJ, Giguere S, Sturgill TL, Berghaus LJ, Slovis NM, Whitman JL, Levering C, Kuskie KR, Cohen ND. 2013. Macrolide-and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. Emerg Infect Dis 19:282-285.

32. Huber L, Giguere S, Slovis NM, Carter CN, Barr BS, Cohen ND, Elam J, Erol E, Locke SJ, Phillips ED, Smith JL. 2019. Emergence of resistance to macrolides and rifampin in clinical isolates of *Rhodococcus equi* from foals in central Kentucky, 1995 to 2017. Antimicrob Agents Chemother 63:e01714-18

33. Huber L, Giguere S, Cohen ND, Slovis NM, Hanafi A, Schuckert A, Berghaus L, Greiter M, Hart KA. 2019. Prevalence and risk factors associated with emergence of *Rhodococcus equi* resistance to macrolides and rifampicin in horse-breeding farms in Kentucky, USA. Vet Microbiol 235:243-247.

34. Erol E, Locke S, Saied A, Cruz Penn MJ, Smith J, Fortner J, Carter C. 2020. Antimicrobial susceptibility patterns of *Rhodococcus equi* from necropsied foals with rhodococcosis. Vet Microbiol 242:108568.

35. Alvarez-Narvaez S, Giguere S, Anastasi E, Hearn J, Scortti M, Vazquez-Boland JA. 2019. Clonal confinement of a highly mobile resistance element driven by combination therapy in *Rhodococcus equi*. mBio 10: e02260-19.

36. Alvarez-Narvaez S, Giguere S, Cohen N, Slovis N, Vazquez-Boland JA. 2021. Spread of multidrug resistant *Rhodococcus equi*, United States. Emerg Infect Dis 27:529-537.

37. Tauch A, Gotker S, Puhler A, Kalinowski J, Thierbach G. 2002. The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenyltransferase gene cassette *aadA9* and the regulated tetracycline efflux system Tet33 flanked by active copies of the widespread insertion sequence *IS6100*. Plasmid 48:117-129.

38. Ripio MT, Geoffroy C, Dominguez G, Alouf JE, Vazquez-Boland JA. 1995. The sulphydryl-activated cytolysin and a sphingomyelinase C are the major membrane-damaging factors involved in cooperative (CAMP-like) haemolysis of *Listeria* spp. Res Microbiol 146:303-313.

39. Navas J, Gonzalez-Zorn B, Ladron N, Garrido P, Vazquez-Boland JA. 2001. Identification and mutagenesis by allelic exchange of *choE*, encoding a cholesterol oxidase from the intracellular pathogen *Rhodococcus equi*. J Bacteriol 183:4796-4805.

40. Ladron N, Fernandez M, Aguero J, Gonzalez Zorn B, Vazquez-Boland JA, Navas J. 2003. Rapid identification of Rhodococcus equi by a PCR assay targeting the *choE* gene. J Clin Microbiol 41:3241-3245.

41. Rodriguez-Lazaro D, Lewis DA, Ocampo-Sosa AA, Fogarty U, Makrai L, Navas J, Scortti M, Hernandez M, Vazquez-Boland JA. 2006. Internally controlled real-time PCR method for quantitative species-specific detection and *vapA* genotyping of *Rhodococcus equi*. Appl Environ Microbiol 72:4256-4263.

42. Clinical and Laboratory Standards Institute. 2020. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals. 5th edit, CLSI supplement VET01S. CLSI.

43. Clinical and Laboratory Standards Institute. 2011. Susceptibility testing of Mycobacteria, *Nocardia* spp and other aerobic actinomycetes. 2nd edit. CLSI document M24-A2. CLSI.

44. Schwarz S, Silley P, Simjee S, Woodford N, van Duijkeren E, Johnson AP, Gaastra W. 2010. Assessing the antimicrobial susceptibility of bacteria obtained from animals. J Antimicrob Chemoth 65:601-604. (Editorial.)

45. Fines M, Pronost S, Maillard K, Taouji S, Leclercq R. 2001. Characterization of mutations in the *rpoB* gene associated with rifampin resistance in *Rhodococcus equi* isolated from foals. J Clin Microbiol 39:2784-2787.

46. Huber L, Giguere S, Slovis NM, Alvarez-Narvaez S, Hart KA, Greiter M, Morris ERA, Cohen ND. 2020. The novel and transferable *erm*(51) gene confers macrolides, lincosamides and streptogramins B (MLSB) resistance to clonal *Rhodococcus equi* in the environment. Environ Microbiol 22:2858-2869.

47. Capasso C, Supuran CT. 2020. Dihydropteroate synthase (sulfonamides) and dihydrofolate reductase inhibitors, p 163-172. *In* Bonev BB, Brown, N.M. (ed), Bacterial Resistance to Antibiotics -from Molecules to Man. John Wiley & Sons Ltd.

48. Womble A, Giguere S, Lee EA. 2007. Pharmacokinetics of oral doxycycline and concentrations in body fluids and bronchoalveolar cells of foals. J Vet Pharmacol Ther 30:187-193.

49. Wetzig M, Venner M, Giguere S. 2020. Efficacy of the combination of doxycycline and azithromycin for the treatment of foals with mild to moderate bronchopneumonia. Equine Vet J 52:613-619.

50. Targant H, Doublet B, Aarestrup FM, Cloeckaert A, Madec JY. 2010. IS6100-mediated genetic rearrangement within the complex class 1 integron In104 of the *Salmonella* genomic island 1. J Antimicrob Chemother 65:1543-1545.

51. Partridge SR, Recchia GD, Stokes HW, Hall RM. 2001. Family of class 1 integrons related to In4 from Tn*1696*. Antimicrob Agents Chemother 45:3014-3020.

52. Grossman TH. 2016. Tetracycline antibiotics and resistance. Cold Spring Harb Perspect Med 6:a025387

53. Davis JL, Salmon JH, Papich MG. 2006. Pharmacokinetics and tissue distribution of doxycycline after oral administration of single and multiple doses in horses. Am J Vet Res 67:310-316.

54. Bryant JE, Brown MP, Gronwall RR, Merritt KA. 2000. Study of intragastric administration of doxycycline: pharmacokinetics including body fluid, endometrial and minimum inhibitory concentrations. Equine Vet J 32:233-238.

55. Cunha BA, Domenico P, Cunha CB. 2000. Pharmacodynamics of doxycycline. Clin Microbiol Infect 6:270-273.

56. Alvarez-Narvaez S, Giguere S, Berghaus LJ, Dailey C, Vazquez-Boland JA. 2020. Horizontal spread of *Rhodococcus equi* macrolide resistance plasmid pRErm46 across environmental *Actinobacteria*. Appl Environ Microbiol 86: e00108-20.

57. Clinical and Laboratory Standards Institute. 2021. Performance standards for antimicrobial susceptibility testing, 31st edit, CLSI supplement M100. CLSI.

58. Bard JD, Hindler JA, Gold HS, Limbago B. 2014. Rationale for eliminating *Staphylococcus* breakpoints for β-lactam agents other than penicillin, oxacillin or cefoxitin, and ceftaroline. Clin Infect Dis 58:1287-1296.

59. Brown-Elliott BA, Woods GL. 2019. Antimycobacterial susceptibility testing of nontuberculous mycobacteria. J Clin Microbiol 57:e00834-19

60. European Committee on Antimicrobial Susceptibility Testing. 2021. Breakpoint tables for interpretation of MICs and zone diameters Version 11.0. http://www.eucast.org.

61. Russo G, Lichtner M, Carnevalini M, Mascellino MT, Mengoni F, Oliva A, Miccoli GA, Iannetta M, Trinchieri V, Massetti AP, Mastroianni CM, Vullo V. 2010. Primary retroperitoneal abscesses due to *Rhodococcus equi* in a patient with severe nephrotic syndrome: successful antibiotic treatment with linezolid and tigecycline. Int J Infect Dis 14:E533-E535.

62. Collignon PJ, Conly JM, Andremont A, McEwen SA, Aidara-Kane A, World Health Organization Advisory Group BMoISoAR, Agerso Y, Andremont A, Collignon P, Conly J, Dang Ninh T, Donado-Godoy P, Fedorka-Cray P, Fernandez H, Galas M, Irwin R, Karp B, Matar G, McDermott P, McEwen S, Mitema E, Reid-Smith R, Scott HM, Singh R, DeWaal CS, Stelling J, Toleman M, Watanabe H, Woo GJ. 2016. World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies to control antimicrobial resistance from food animal production. Clin Infect Dis 63:1087-1093.

63. Schnabel LV, Papich MG, Divers TJ, Altier C, Aprea MS, McCarrel TM, Fortier LA. 2012. Pharmacokinetics and distribution of minocycline in mature horses after oral administration of multiple doses and comparison with minimum inhibitory concentrations. Equine Vet J 44:453-458.

64. Giguere S, Burton AJ, Berghaus LJ, Haspel AD. 2017. Comparative pharmacokinetics of minocycline in foals and adult horses. J Vet Pharmacol Ther 40:335-341.

65. Nordmann P, Kerestedjian JJ, Ronco E. 1992. Therapy of *Rhodococcus equi* disseminated infections in nude mice. Antimicrob Agents Chemother 36:1244-1248.

66. Newman JC, Prange T, Jennings S, Barlow BM, Davis JL. 2013. Pharmacokinetics of tobramycin following intravenous, intramuscular, and intra-articular administration in healthy horses. J Vet Pharmacol Ther 36:532-541.

67. Harvey RL, Sunstrum JC. 1991. *Rhodococcus equi* infection in patients with and without human immunodeficiency virus infection. Rev Infect Dis 13:139-145.

68. Nordmann P, Nicolas MH, Gutmann L. 1993. Penicillin-binding proteins of *Rhodococcus equi*: potential role in resistance to imipenem. Antimicrob Agents Chemother 37:1406-1409.

69. McNeil MM, Brown JM. 1992. Distribution and antimicrobial susceptibility of *Rhodococcus equi* from clinical specimens. Eur J Epidemiol 8:437-443.

70. Macken E, de Jonge H, Van Caesbroeck D, Verhaegen J, Van Kerkhoven D, Van Wijngaerden E, Kuypers D. 2015. *Rhodococcus equi* sepsis in a renal transplant recipient: a case study. Transplantat Direct 1:e11.

71. Hsueh PR, Hung CC, Teng LJ, Yu MC, Chen YC, Wang HK, Luh KT. 1998. Report of invasive *Rhodococcus equi* infections in Taiwan, with an emphasis on the emergence of multidrug-resistant strains. Clin Infect Dis 27:370-375.

72. Soriano F, Fernandez-Roblas R, Calvo R, Garcia-Calvo G. 1998. *In vitro* susceptibilities of aerobic and facultative non-spore-forming gram-positive bacilli to HMR 3647 (RU 66647) and 14 other antimicrobials. Antimicrob Agents Chemother 42:1028-1033.

73. Kedlaya I, Ing MB, Wong SS. 2001. *Rhodococcus equi* infections in immunocompetent hosts: case report and review. Clin Infect Dis 32:E39-46.

74. Van Bambeke F, Barcia-Macay M, Lemaire S, Tulkens PM. 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: Current views and perspectives. Curr Opin Drug Discov Devel 9:218-230.

75. Lemaire S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. 2005. Activity of three beta-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*. J Antimicrob Chemoth 55:897-904.

76. Giguere S, Berghaus LJ, Lee EA. 2015. Activity of 10 antimicrobial agents against intracellular *Rhodococcus equi*. Vet Microbiol 178:275-278.

77. Story-Roller E, Maggioncalda EC, Cohen KA, Lamichhane G. 2018. *Mycobacterium abscessus* and β-Lactams: Emerging Insights and Potential Opportunities. Front Microbiol 9:2273.

78. Niwa HH, S; Anzai, T; Higuchi, T. 2005. Antimicrobial susceptibility of 616 *Rhodococcus equi* strains isolated from tracheobronchial aspirates of foals suffering from respiratory disease in Japan. J Equine Sci 16:99-104.

79. Niwa H, Lasker BA. 2010. Mutant selection window and characterization of allelic diversity for ciprofloxacin-resistant mutants of *Rhodococcus equi*. Antimicrob Agents Chemother 54:3520-3523.

80. Kwa AL, Tam VH, Rybak MJ. 2001. *Rhodococcus equi* pneumonia in a patient with human immunodeficiency virus: case report and review. Pharmacotherapy 21:998-1002.

FIGURE LEGEND

Fig. 1. Schematic representation of the antimicrobial resistance elements of pRErm46 from *R. equi* MDR 2287. The Tn*RErm46* mobile element is represented in pale red with the IS*Re46* transposase highlighted in deep red and the macrolides-lincosamides-streptogramins (MLS) resistance determinant *erm*(46) in black. Tn*RErm46* is highly mobile and can be present in several copies in the pRErm46 plasmid and the host genome, including the pVAPA virulence plasmid (35). The class 1 integron (C1I) with integrase gene (*intI1*) and aminoglycoside (*aadA9*) and sulfonamide (*sul1*) resistance cassettes are represented in yellow, the adjacent *tetRA*(33) tetracycline resistance element in green, and the flanking IS*6100* copies in magenta. The C1I and *tetRA*(33) elements are found in a 100% identical (but reverse) arrangement in the pTET3 plasmid from *C. glutamicum* (35,37), suggesting that they can be mobilized en bloc via the IS*6100*s. Deletion of both elements, or only *tetRA*(33), presumably via homologous recombination between the directly repeated IS*6100* copies (35, 50, 51), are observed in a proportion of the MDR 2287 isolates and result in loss of the corresponding resistances (see text). The pRErm46 plasmid of the prototype strain of the MDR 2287 clone, PAM 2287, is unique in that it carries one of its three Tn*RErm46* copies inserted within the C1I's *aadA9* (streptomycin/spectinoycin resistance) cassette (35). This insertion does not affect tetracycline resistance despite abolishing *sul1*-mediated sulfonamide resistance, indicating that the *IS6100*-flanked *tetRA*(33) determinant is expressed independently of the integron's promoter that drives the expression of C1I's *aadA9* and *sul1* cassettes (Pc). The gene markers for PCR detection of pRErm46's backbone (Table 3), and the position of the oligonucleotide primers for confirmation of the pRErm46 deletions (Table S1), are indicated.

Table 1. Erythromycin and rifampicin MICs and presence/absence of *erm*(46) in *R. equi* isolates.

Phenotype		*n*	*erm*(46)	MIC_{50}/MIC_{90} (range) µg/ml	
				Erm	**Rif**
Erm^R / Rif^R		14	+	24 / 64 (8-96) [a]	≥32 / ≥32 (≥32) [b]
		1	–	24	≥32
Erm^S /	Rif^S	34	–	0.5 / 0.75 (0.016-1) [a]	0.12 / 0.5 (0.032-0.75) [b]
	Rif^R	21	–	0.5 / 1 (0.125-6) [a]	≥32 / ≥32 (8-≥32) [b]

[a] $P < 0.0001$, Mann-Whitney test; comparison of Erm^R *vs* Erm^S.
[b] $P < 0.0001$, Kruskal-Wallis test; comparison of Erm^S/Rif^S *vs* Erm^R/Rif^R or Erm^S/Rif^R.

Table 2. *In vitro* activity against *R. equi* of 15 antimicrobials used in susceptibility testing of rapidly growing mycobacteria, nocardiae and aerobic actinomycetes (RapMyco panel). Asterisks indicate statistically significant differences between Erm^R (MDR) and Erm^S isolates.

	MIC_{50} / MIC_{90} (range) µg/ml				
	Global	**Erm^R** [a]	**Erm^S**		
			All	**Rif^S**	**Rif^R**
Amikacin	≤1/2 (≤1-4)	≤1/2 (≤1-2) [b]	≤1/2 (≤1-4)	≤1/2 (≤1-2)	≤1/2 (≤1-4)
Amox/clavul. [c]	8/16 (4-32)	8/16 (8-16)	8/16 (4-32)	8/16 (4-32)	8/16 (8-16)
Cefepime	8/16 (2 to-32) [d]	4/8 (2-8)	8/16 (2->32)	8/8 (2-16)	8/16 (4->32)
Cefoxitin	16/16 (≤4-32) [d]	8/16 (8-16)	16/16 (≤4-32)	16/16 (≤4-16)	8/16 (8-32)
Ceftriaxone	≤4/≤4 (≤4) [d]	≤4/≤4 (≤4)	≤4/≤4 (≤4)	≤4/≤4 (≤4)	≤4/≤4 (≤4)
Ciprofloxacin	1/1 (0.5-2)	1/1 (0.5-1)	1/1 (0.25-2)	1/1 (0.25-1)	1/1 (0.5 - 2)
Clarithromycin	≤0.06/>16 (≤0.06->16)	>16/>16 (8->16) * [e]	≤0.06/≤0.06 (≤0.06-0.5) * [e]	≤0.06/≤0.06 (≤0.06-0.12)	≤0.06/≤0.06 (≤0.06-0.5)
Doxycycline [f]	1/1 (≤0.12-2)	1/2 (0.5-2) [f]	1/1 (≤0.12-1)	1/1 (≤0.12-1)	1/1 (0.5-1)
Imipenem	≤2/≤2 (≤2-16) [d]	≤2/≤2 (≤2-4)	≤2/≤2 (≤2-16)	≤2/≤2 (≤2-16)	≤2/≤2 (≤2-4)
Linezolid	≤1/≤1 (≤1)	≤1/≤1 (≤1)	≤1/≤1 (≤1)	≤1/≤1 (≤1)	≤1/≤1 (≤1)
Minocycline	≤1/≤ (≤1) [g]	≤1/≤1 (≤1)	≤1/≤1 (≤1)	≤1/≤1 (≤1)	≤1/≤1 (≤1)
Moxifloxacin	0.5/1 (≤0.25-1)	0.5/1 (0.5-1)	0.5/1 (≤0.25-1)	0.5/1 (≤0.25-1)	0.5/1 (≤0.25-1)
Tigecycline	0.12/0.25 (≤0.015-0.25)	0.12/0.12 (0.06-0.12)	0.12/0.25 (≤0.015-0.25)	0.12/0.25 (≤0.015-0.25)	0.12/0.25 (0.06-0.25)
Tobramycin	≤1/2 (≤1-2)	≤1/≤1 (≤1) [b]	≤1/2 (≤1-2) [h]	≤1/≤1 (≤1-2)	≤1/2 (≤1-2)
TMP/SMX [i]	2/2 (1->8)	2/>8 (1->8) * [j]	2/2 (1-2) * [j]	2/2 (1-2)	2/2 (1-2)

[a] Erm^R strains were also Rif^R.
[b] See also Table 4.
[c] Amoxicillin/clavulanate (2:1) expressed as MIC values for amoxicillin.
[d] The most active ß-lactams were imipenem (MIC_{90} ≤2 µg/ml, RGM/NAA resistance breakpoints ≥32/≥16 µg/ml), ceftriaxone (MIC_{90} ≤4 µg/ml, NAA resistance breakpoint ≥64 µg/ml) and cefoxitin (MIC_{90} = 16 µg/ml, RGM resistance breakpoint ≥128 µg/ml); cefepime and amoxicillin/clavulanate (MIC_{90} = 16 µg/ml) would be in the intermediate range for NAA.
[e] $*P < 0.0001$, Mann-Whitney test; comparison of Erm^R *vs* Erm^S.
[f] See also Table 3.
[g] Minocycline MICs were all ≤1 µg/ml, i.e. the lower detection limit of RapMyco, which is above the susceptibility/resistance breakpoints for horses (≤0.12/≥0.5 µg/ml) but within the susceptible category for human use (≤4/≥16 µg/ml).
[h] 14% of *R. equi* isolates (all in the Erm^S category) had tobramycin MICs of 2 µg/ml, which while within the CLSI susceptibility range for RGM and NAA (susceptibility/resistance breakpoints ≤2/≥8 µg/ml and ≤4/≥16 µg/ml, respectively), is above the EUCAST resistance breakpoint for *S. aureus* (>1 µg/ml).
[i] TMP/SMX: Trimethoprim/sulfamethoxazole (1:19), expressed as the MIC values of trimethoprim.
[j] $*P < 0.0025$, Mann-Whitney test; comparison of Erm^R *vs* Erm^S.

Table 3. Sulfonamide (TMP/SMX), tetracycline (Tet) and doxycycline (Dox) susceptibility and relationship with pRErm46 components.

Phenotype	*n*	pRErm46 markers				MIC (range) µg/ml [d]		
		Tn*RErm46* [a]	*sul1* [b]	*tetRA*(33)	plasmid backbone [c]	TMP-SMX	Tet	Dox
Erm^R	5	+	+	+	+	≥32 (≥32) * [e]	16 (12-32) * [f]	2 (1-2) * [g]
	1	+	+	–	+	≥32	1	0.5
	3	+	–	–	+	0.5 (0.5-1)	0.25 (0.25-0.5)	0.5 (0.5)
	3	+	–	–	–	0.5 (0.5-1)	0.25 (0.25-0.38)	1 (0.5-1)
	1	–	–	–	–	0.5	0.25	0.5
Erm^S [h]	6	–	–	–	–	0.5 (0.5-1.5)	0.5 (0.25-0.75)	1 (0.5-1)

[a] Determined by PCR detection of *erm*(46) and IS*Re46* transposase (35) (see Fig. 1).
[b] *sul1* gene was always detected together with other C1I gene markers (*IS6100, intI1, addA9* and *qacE*) (see Fig. 1).
[c] Determined using gene markers *mobP, mobC, pRERM_0200* and *pRERM_0740* (35) (see Fig. 1).
[d] Determined by eTest for TMP/SMX and Tet, RapMyco microdilution plate for Dox. Data expressed as modal MIC.
[e] $*P < 0.0001$, Mann-Whitney test; comparison of *sul1*-positive Erm^R *vs* all *sul1* negative.
[f] $*P < 0.0001$, Mann-Whitney test; comparison of *tetRA*(33)-positive Erm^R *vs* all *tetRA*(33) negative.
[g] $*P = 0.0004$, Mann-Whitney test; comparison of *tetRA*(33)-positive Erm^R *vs* all *tetRA*(33) negative.
[h] Erm^S *R. equi* isolates (n =6 randomly selected) were included as controls.

Table 4. Susceptibility to aminoglycosides and presence of pRErm46's C1I in Erm^R (MDR) *R. equi* isolates

Phenotype	C1I [a]	*n*	Mean diameter in mm (range) [b]					MIC µg/ml (range) [c]	
			Str	Spt	Gen	Kan	Apr	Amk	Tob
Erm^R	+	8	1.5 * (0-12)	0 * (0)	28.2 (25-31.5)	18.1 (16-21.5)	27.9 (24.5-31)	≤1 (≤1-2)	≤1 (≤1)
	–	7	24.8 (21.5-28)	17.9 (15-20.5)	27.7 (24-31)	18 (16-22)	25.3 (19-30)	≤1 (≤1)	≤1 (≤1)
Erm^S [d]	–	6	19.3 (22-26)	18.7 (15-24)	24.9 (26-30)	18.3 (16-21)	28.1 (28-30)	≤1 (≤1-2)	≤1 (≤1)

[a] Class 1 integron, +/– means positive/negative to PCR markers for *addA9*, *intI1, qacE*, *sul1* and IS*6100* (35) (see Fig. 1).
[b] Determined by disk diffusion. Spt, spectinomycin; Stp, streptomycin; Gen, gentamicin; Kan, kanamycin; Apr, apramycin.
[c] Determined by RapMyco microdilution plates. Amk, amikacin; Tob, tobramycin. Data expressed as modal MIC.
[d] Erm^S isolates (n = 6 randomly selected) included as a control.
*P < 0.0001, Kruskal-Wallis test; comparison of Erm^R/C1I (+) *vs* Erm^R/C1I (–) or Erm^S.