Spread of Multidrug-Resistant Rhodococcus equi, United States

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Spread of emerging multidrug-resistant *Rhodococcus equi* in USA

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Summary line: Antibiotic prophylaxis at equine farms determines the emergence, spread and evolution of multidrug-resistant *Rhodococcus equi* and dissemination of the macrolide resistance plasmid pRErm46.

Running tittle: Spread of emerging MDR *R. equi* in USA

Key Words: Multidrug-resistant *Rhodococcus equi*, MDR *R. equi*, *erm*(46), pRErm46, Tn*RErm46*, macrolide resistance, rifampin resistance.

Abstract

Multidrug-resistance (MDR) has been detected in the animal and zoonotic human pathogen *Rhodococcus equi* following mass macrolide-rifampin (MR) antibioprophylaxis in endemic equine farms in the USA. MDR *R. equi* emerged upon acquisition of pRERm46, a conjugative plasmid conferring resistance to macrolides, lincosamides, streptogramins and, as shown here, also tetracycline. Phylogenomic analyses indicate that its increasing prevalence since first documented in 2002 is due to a single clone, *R. equi* 2287, attributable to co-selection of pRErm46 with a chromosomal *rpoB*^{S531F} mutation driven by the MR combination therapy. pRErm46 spillover to diverse *R. equi* genotypes has been now observed, giving rise to a novel MDR clone (G2016) associated with a distinct *rpoB*^{S531Y} mutation. The findings illustrate how antibiotic overuse for prophylaxis in animals can generate MDR pathogens potentially transmissible to people. Currently spreading in the USA, MDR *R. equi* and pRErm46-mediated resistance is likely to become internationally disseminated via horse movements.

Introduction

Rhodococcus equi is a soil-borne facultative intracellular actinobacterium that causes pyogranulomatous infections in different animal species, including humans. Rhodococcal infections are particularly severe in young foals and immunocompromised people where they typically manifest as a life-threatening purulent bronchopneumonic disease (1-3). Three different host-specific virulence plasmid types, designated pVAPA, pVAPB and pVAPN, enable *R. equi* to colonize equids, pigs and ruminants, respectively (4). Analysis of the virulence plasmids carried by the isolates, and comparison of genomic profiles, indicates that human *R. equi* infections originate from animals (4-6).

R. equi is highly prevalent in horse-breeding farms worldwide (7). For decades, the standard treatment of *R. equi* foal pneumonia has been the administration of the synergistic combination of a macrolide and rifampin (MR therapy) (8). In the absence of effective prevention methods, many horse-breeding farms rely on early ultrasonographic detection of infected foals and initiation of MR prophylaxis prior to development of clinical signs (9). In the

USA where *R. equi* infection is often endemic, implementation of this practice has been linked to the emergence of dual macrolide/rifampin resistance (MR^R) (10-12). First detected in the late 1990's, *R. equi* MR^R isolates are increasingly prevalent (11-14), posing a significant problem because no clinically proven therapeutic alternative is currently available against foal rhodococcosis (8). The MR^R isolates also represent a potential hazard to human health due to the risk of zoonotic transmission.

We determined that the emerging MR^R phenotype among *R. equi* equine isolates was linked to a novel methyltransferase gene, *erm*(46), which confers cross-resistance to macrolides, lincosamides and streptogramins (MLS^R phenotype) (13). *erm*(46) is part of a 6.9-kb transposable element, Tn*RErm46*, carried by the conjugative resistance plasmid pRErm46 (15). Upon pRErm46 acquisition, Tn*RErm46* stabilizes itself in *R. equi* by transposing to the host genome, including the conjugative virulence plasmid pVAPA. Despite its high horizontal spread potential, we found that pRErm46/Tn*RErm46* was restricted to a specific *R. equi* clone, designated 2287, likely due to co-selection with a chromosomal rifampin-resistance $rpoB^{S531F}$ mutation in response to the MR therapy (15).

The identification work of the multidrug-resistant (MDR) *R. equi* 2287 clone was based on the analysis of isolates collected between 2002 and 2011 (15). Here we investigated the spread of the *erm*(46) determinant in a contemporary sample of macrolide-resistant isolates. The data show that *R. equi* 2287 not only continues disseminating in the USA, but also that horizontal spread of pRErm46/Tn*RErm46* is now taking place. This has led to the emergence of a further MDR *R. equi* clone, designated G2016, associated with a novel $rpoB^{S531Y}$ mutation.

Materials and Methods

Bacteria

The genomes of a random selection of 30 macrolide-resistant and 18 macrolide-susceptible *R. equi* equine clinical strains recovered from pneumonic foals in five US states (Florida,

Kentucky, Louisiana, New York, and Texas) during the period 2012 to 2017 were sequenced in this study (Appendix Table 1). Whenever possible, at least one strain from each category were picked per year and US state. The strains from Louisiana were a random collection of 10 convenience-sampled isolates from a single farm. All strains were routinely grown in brain-heart infusion (BHI, BD) medium for 48h at 37°C. Detection of the *erm*(46) gene by PCR was performed as previously described (13, 15).

Antimicrobial susceptibility testing

Susceptibility tests were performed at the Hagyard Equine Medical Institute diagnostic laboratory (Lexington, Kentucky), Texas A&M Veterinary Medical Diagnostic laboratory, and University of Georgia Veterinary Diagnostic Laboratory according to Clinical & Laboratory Standards Institute (CLSI) guidelines (https://clsi.org/). In the absence of specific disk susceptibility interpretive criteria for *R. equi*, CLSI guidelines for *Staphylococcus aureus* were used in accordance to routine practice by veterinary diagnostic laboratories (11, 16). Minimum inhibitory concentrations (MIC) were determined in tryptone soy agar (TSA) medium using Etest strips (bioMérieux) according to the manufacturer's recommendations, as previously described (16). *Staphylococcus aureus* ATCC 29213 was used as a control in all susceptibility tests.

Genome sequencing and phylogenetic analysis

Bacterial genomic DNA was extracted using DNeasy UltraClean Microbial Kit (Qiagen) following the manufacturer's instructions. DNA quality (OD 260/280 ratio of 1.8 to 2) and concentration (>1 µg) of each gDNA sample was verified using a NanoDrop apparatus (ThermoFisher). Single-molecule real-time (SMRT) long-read DNA sequencing was performed at Duke Center for Genomic and Computational Biology (Duke University). SMRTbell Template Prep Kit 2.0 was used for library preparation of 4-6 kb insert for 8 multiplexed

bacterial samples. Samples were run on a PacBio Sequel II system (Pacific Bioscience). Genomes were assembled *de novo* using Canu v 1.9 (17). Whole-genome phylogenetic analysis was performed with ParSNP in the Harvest suite, designed for SNP analysis between closely-related species/strains (≥97% ANI) (18). The program uses FastTree 2 (19) to build approximately Maximum-Likelihood trees from core-genome SNPs. Trees were visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Principal component analysis (PCA) was performed by feeding VCF files extracted from ParSNP alignments to ggfortify package in R software (version 3.6.1; https://cran.r-project.org/web/packages/ ggfortify/index.html)

Statistical analysis

Statistical significance of tetracycline susceptibility data was determined by χ^2 and Student's t-test analysis using Prism version 8 software (GraphPad).

Results

The 30 macrolide-resistant *R. equi* genome sequences determined in this study were subjected to phylogenetic analysis alongside those of a sample of 18 susceptible isolates from the same time period and geographical origins to examine their relationships. The macrolide resistant isolates had previously tested positive to *erm*(46) by PCR and most (*n* = 22, 73 %) were also resistant to rifampin (MR^R phenotype). The fact that eight of the 2012-2017 *R. equi* isolates examined here were macrolide (MLS)-only resistant (M^R) (Appendix Table 1) was interesting because, to date, dual MR^R resistance had been invariably observed (10, 11, 13, 15). To our knowledge, this is the first time that an M^R phenotype is reported in *R. equi* clinical isolates. Illumina whole-genome assemblies from 22 equine isolates characterized in our previous study (*n* = 16 belonging to the 2287 clone, *n* = 6 control susceptible isolates), plus 23 macrolide-susceptible strains representative of the global genomic diversity of *R. equi* (20), were also included in the analysis. Figure 1 shows the core-genome phylogeny of the 93 *R. equi* strains.

109

110 **Clonal spread of MDR *R. equi* 2287**

111 Twenty out of 22 (91%) of the new MR^R isolates clustered together at short genetic distances
112 with the previously characterized MDR 2287 isolates, indicating they correspond to the same
113 clonal population (Figure 1). Accordingly, all the newly sequenced MR^R strains possessed the
114 *rpoB*^{S531F} mutation unique to the 2287 clone. Two of them had lost the pRErm46 plasmid and
115 only carried the Tn*RErm46* transposon (Figure 1), as previously observed in one of the 18
116 isolates from the 2002 to 2011 series (15). Collection times and locations encompassed the
117 entire period from 2012 to 2017 and the five US states for the MDR 2287 clonal population.
118 The lack of spatial-temporal circumscription of MDR 2287 in the analyzed sample is illustrated
119 by a principal components analysis (PCA) where the only grouping factor for the 93 *R. equi*
120 isolates included in the study is the genetic background of the 2287 clone (Figure 2).

121 The phylogenomic analysis was repeated with the 36 *R. equi* 2287 sequences covering
122 the entire 2002 to 2017 to assess the microevolution of the clone. This revealed that MDR 2287
123 had diversified into three major radiations (Figure 3), consistent with the clonal structure of *R.*
124 *equi* evolution (20). Interestingly, one of these subclades gathered 11 of the 16 older isolates
125 from the period 2002 to 2011, all originating from Florida or Kentucky. The remaining five
126 older isolates were distributed in the two other subclades where strains were grouped
127 independently from year of collection or geographical origin. This distribution suggests a
128 pattern of spread defined by the diversification of the MDR 2287 into subclonal lineages and
129 increasing exchange between horse farms of a progressively diverse clonal population

130

131 **Dissemination of pRErm46 and emergence of novel MDR clone**

132 Ten macrolide-resistant isolates also carried pRErm46 but did not belong to the MDR 2287
133 clone and were genetically diverse. Most appeared as singletons interspersed among the
134 different lines of descent in the *R. equi* tree (Figure 1). Eight strains in this group corresponded

to the above-mentioned macrolide-only resistant isolates (i.e. rifampin susceptible, no *rpoB* mutation; MIC <0.125 to 1.25 μg/ml). Interestingly, all but one of these isolates originated from the same farm in Louisiana in which an MDR 2287 isolate (no. 171) was also recovered at the same period. This suggests a scenario where the entry of MDR 2287 into this particular farm resulted in the conjugal spread of pRErm46 to different members of the heterogeneous *R. equi* populations typically found colonizing the horse breeding environments or even individual animals in a farm (21, 22).

Notably, two of the non-2287 macrolide-resistant isolates, nos. 155 and 183 recovered in Kentucky in 2017 and 2016, respectively, were also resistant to rifampin (MIC >32 μg/ml) (Figure 1). These two nearly genomically-identical MR^R strains carried the pRErm46 plasmid and a chromosomal *rpoB* mutation, Ser531Tyr (*Escherichia coli* numbering), distinct from that present in MDR 2287 and novel in *R. equi*. Both MR^R isolates constitute a new emerging MDR *R. equi* clone, first detected in 2016, which we designated G2016.

Collectively, these data indicate that the pRErm46 macrolide resistance plasmid, up to now unique to the 2287 clone, has recently undergone horizontal transfer events to multiple *R. equi* genotypes, giving rise to novel MDR clones when associated with an *rpoB* mutation.

pRErm46 variability and tetracycline resistance

pRErm46 also harbors a class 1 integron (C1I) with a *tetR-tetA* cassette encoding a putative tetracycline efflux pump homologous to TetA(33) from the corynebacterial plasmid pTET3 (15, 23). TetA efflux pumps are often carried by transposons and are one of the most prevalent tetracycline resistance mechanisms (24). Both the C1I and *tetRA* determinant from pRErm46 are virtually identical to those from pTET3, including flanking IS*6100* insertion sequences (15). Blast alignments revealed that the C1I-*tetRA*(33) region was deleted in 17 of the 43 (40%) pRErm46 plasmids (Figures 1, 3), presumably due to recombination between the duplicated IS*6100*s (Figure 4). Similar rearrangements have been reported in other integrons carrying

directly-repeated IS*6100* copies (25, 26). Confirming the predicted functionality of pRErm46's *tetRA*(33) determinant, pRErm46-positive isolates were resistant to tetracycline, in contrast to those carrying the ΔC1I-*tetRA*(33) form of the resistance plasmid (Table 1). However, all *R. equi* isolates were susceptible to the semi-synthetic tetracycline derivative, doxycycline, regardless of pRErm46 plasmid carriage (Table 1). This is consistent with previous data in *Corynebacterium glutamicum* showing that TetA(33) does not confer significant cross-resistance to doxycycline (23).

While a ΔC1I-*tetRA*(33) plasmid deletion was present in only one of the older (2002 to 2011) MDR 2287 isolates, the deletion was found in 10 of the 18 pRErm46-positive clonal isolates recovered in the 2012 to 2017 period (Figure 1). Deleted pRErm46s are observed in each of the clonal radiations of the MDR 2287 population and coexist with complete plasmids in more basal branches (Figure 3), indicating an increasing occurrence due to repeated independent deletion events. Interestingly, the deletion was present in all of the genetically heterogeneous macrolide-only (M^R) resistant *R. equi* isolates and MDR 2287 (isolate no. 171) recovered from the Louisiana farm during the same period, supporting the notion that the latter was the source from which pRErm46 had spread to other locally prevalent *R. equi* genotypes.

Discussion

This study shows that the increasing prevalence of MR^R *R. equi* since its emergence in the late 1990's to early 2000's in equine farms in the USA (11-14) is primarily accounted for by the spread of the recently identified MDR 2287 clone (15). The oldest characterized MDR 2287 isolate dates from 2002 and was recovered in Kentucky (15) (Figure 1) where the clone likely emerged following implementation of mass MR antibiotic prophylaxis in foals (10). Since then, *R. equi* MDR 2287 has been frequently transferred between geographically distant farms, presumably through carrier horses. Active exchange of *R. equi* populations, previously noted in our earlier study (20), is evident in the USA and internationally from our data in Figure 1. For

example, the strains recovered from the Louisiana farm in this study are essentially identical to others found elsewhere in the USA; and terminal branches of the *R. equi* tree contain nearly identical equine isolates from different countries, e.g. USA, France and Netherlands, or in another case Canada, Hungary, Sweden and USA (Figure 1).

Despite the diversity of *R. equi* genotypes that typically circulate in the farms (21, 22), the highly horizontally transferable *erm*(46) (Tn*RErm46*) determinant remains largely confined to MDR 2287. This paradoxical clonal restriction is likely determined by the simultaneous requirement for both *erm*(46) and the *rpoB* mutation under dual MR pressure; more specifically, by the low odds of pRErm46/Tn*RErm46* and a high-resistance *rpoB* mutation (such as Ser531Phe in MDR 2287 or Ser531Tyr in MDR G2016) being concurrently acquired, with the latter effectively linking the mobile *erm*(46) determinant to a specific chromosomal background (15).

The above interpretation implies a few predictions. First, under dual MR pressure, spread of an existing MR^R strain through horse movements is more likely to contribute to the bulk of resistance than the generation of new MR^R strains (15). Second, continued MR therapy may eventually lead to the emergence of new MR^R clones, such as G2016 identified here, detected in 2016 again in Kentucky and characterized by a novel $rpoB^{S531Y}$ mutation. Third, and importantly, if dual MR selection ceases, unrestricted pRErm46/Tn*RErm46* horizontal transfer to other *R. equi* strains may take place. Our data appear to encompass these three possibilities.

Scenarios (i) and (ii) are expected in horse-breeding areas such as Kentucky, Texas or Florida where *R. equi* is endemic and MR antibiotic prophylaxis has been commonly practiced (10, 27, 28). Less intensive and more targeted antibiotic therapy is more likely in areas with smaller horse population such as Louisiana (29), where pRErm46 spillover outside the MDR 2287 clone was detected (scenario iii). We hypothesize that a more relaxed antibiotic pressure, perhaps involving macrolide monotherapy or a macrolide in combination with antibiotics other

than rifampin, disrupted the linkage between *erm*(46) and *rpoB*^{S531F} in the MDR 2287 strain found in the Louisiana farm, enabling the transfer of the plasmid to other locally prevalent *R. equi* strains (Figure 1).

Our analyses show that MDR 2287 has diversified since its first documented isolation into a clonal complex with several radiations (Figure 3). We also detected signs of microevolution in pRErm46, with a substantial rate of deletion of the C1I-*tetRA*(33) region in the 2012 to 2017 macrolide-resistant *R. equi* cohort, resulting in loss of tetracycline resistance. The clinical significance of this finding is unclear because tetracyclines are not used to treat *R. equi* infections in foals. An exception is doxycycline, which owing to its higher oral bioavailability in foals, greater tissue penetration and better activity against Gram-positive bacteria, may be used in case of macrolide intolerance (or resistance) (2, 8, 30). However, our data indicate that the pRErm46-encoded TetA33 pRErm46's does not confer clinically relevant cross-resistance to this semi-synthetic tetracycline derivative. Genetic dispensability due to lack of antibiotic selection/fitness advantage may therefore be the likely reason for the increasing occurrence of ΔC1I-*tetRA*(33) pRErm46 plasmids in the macrolide-resistant *R. equi* population.

MDR *R. equi* shows resistance to several clinically relevant antibiotics including macrolides, lincosamides, streptogramins, and in a significant proportion tetracycline, conferred by the pRErm46 conjugative plasmid, plus rifampin due to a chromosomal *rpoB*$^{S531F/Y}$ mutation. They also show intrinsic resistance to chloramphenicol (Appendix Table 2), often observed in *R. equi*. All these antibiotics are listed as critically or highly important for human medicine by the World Health Organization (31). Around 9% of human *R. equi* infections are caused by equine-derived (pVAPA-positive) strains, and about half of human cases are caused by pVAPB-positive (porcine) isolates (5), which recent *in vitro* data show can also acquire pRErm46 (32). Therefore, in addition to compromising the therapeutic management of equine *R. equi* infection, these isolates represent a potential hazard to human

health due to the risk of zoonotic transmission (or horizontal spread of the pRErm46 resistance plasmid to other pathogens, either directly or via environmental microbiota [32]).

Although not systematic and thereby probably underestimating the extent of MDR *R. equi* spread, our study provides valuable insight into the determinants underlying its emergence and spread. The data suggest a pattern of MDR *R. equi* dissemination and evolution directly determined by the antibiotic pressure in equine farms. The stable therapeutic regimen applied over the years in the case of *R. equi* facilitates a unique understanding of the factors affecting the generation and evolution of MDR clones, and specifically how combination therapy may help in limiting the horizontal transfer of resistance. While MDR *R. equi* is to our knowledge still only circumscribed to the horse population in the USA, our data predict a scenario of international spread with horse movements, calling for interventions to control its dissemination and potential zoonotic transmission.

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Declaration of interest

We declare no competing interests.

Data sharing

New *R. equi* genome assemblies were deposited in GenBank under the accession numbers indicated in Appendix Table 1.

Author Bio

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Table 1. Effect of absence of *tetRA*(33) determinant from pRErm46 plasmid on *R. equi* susceptibility to tetracycline and doxycycline. Determined on macrolide-resistant isolates collected between 2012 and 2017 (see figure 1). Susceptibility data to other relevant antimicrobials are shown in Appendix Table 2.

	pRErm46		pRErm46 ΔC1I-*tetRA*(33)	
	Phenotype [a]	MIC μg/ml [b]	Phenotype	MIC μg/ml
Tetracycline	R (100) [c]	21.33 (8-48) [d]	S (100) [c]	1.97 (0.38-3) [d]
Doxycycline	S (100)	3.35 (0.75-6) [e]	S (100)	1.06 (0.25-3) [e]

[a] Determined by disk diffusion technique. R, resistant; S, susceptible (isolate percentage in parenthesis). Zone diameter susceptibility breakpoints based on CLSI interpretive criteria for *Staphylococcus aureus*, routinely used for *R. equi* susceptibility testing in the absence of specific approved criteria for this species [11,16].

[b] Minimal inhibitory concentration determined using Etest strips. Mean value (range in parenthesis).

[c] $P < 0.001$ χ^2 test.

[d] $P < 0.001$ t-test.

[e] $P < 0.001$ t-test. Presence of TetRA(33) appears to induce a small, statistically significant MIC increase. MIC remains however below the CLSI susceptibility breakpoint for doxycycline (susceptible ≤4 μg/ml, intermediate 8 μg/ml, resistant ≥16 μg/ml)

Figures

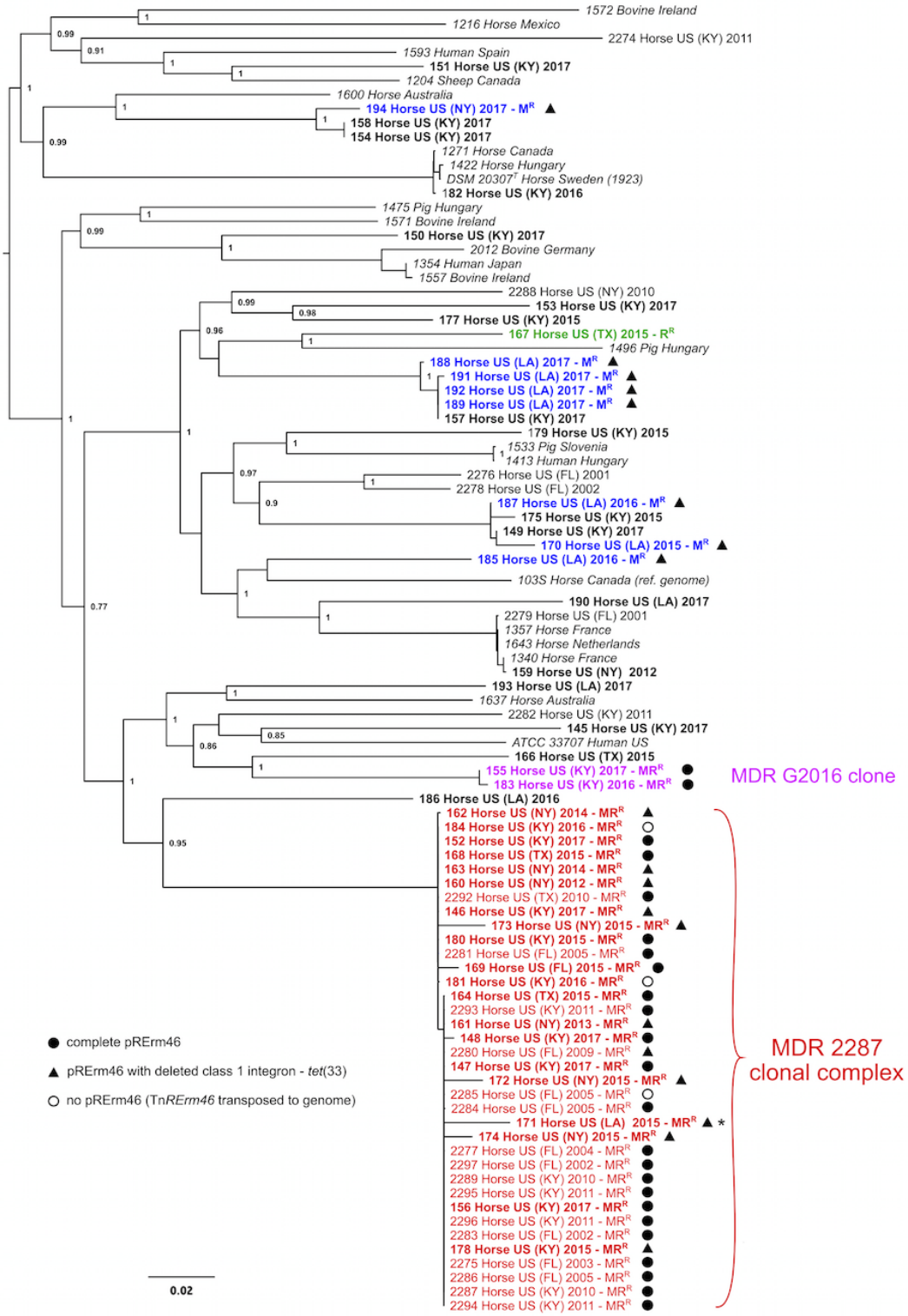

Figure 1. Spread and phylogenetic relationships of MDR *R. equi*. Phylogenetic tree of 93 *R. equi* isolates based on core-genome SNP analysis using ParSNP (18). The genomes analyzed are from 58 *erm*(46)-positive macrolide (MLS)-resistant isolates, 24 control susceptible isolates from same period and geographical origins, plus 23 isolates representative of the genomic

406 diversity of *R. equi* including the reference genome 103S and the type strain DSM 20307^T (see
407 Appendix Table 1). Tip labels show year of collection and resistance phenotype (M^R,
408 macrolides-only; MR^R, macrolide+rifampicin; R^R, rifampicin-only) for the 2001-2017 equine
409 clinical isolates analyzed (in bold the 50 those whose genomes were determined in this study,
410 rest of genomes from previous study in ref 15). Colour codes: red, MDR 2287 clonal complex;
411 violet, novel MDR G2016 clone; blue, genetically diverse M^R isolates recovered from a farm in
412 Louisiana 2015-2017 (MDR 2287 isolate from which they likely acquired the pRErm46
413 plasmid is indicated by an asterisk); green, a R^R isolate (*rpoB* S531K mutation). pRErm46
414 carriage status is indicated by symbols. Tree graph constructed with FigTree
415 (http://tree.bio.ed.ac.uk/ software/figtree/).
416

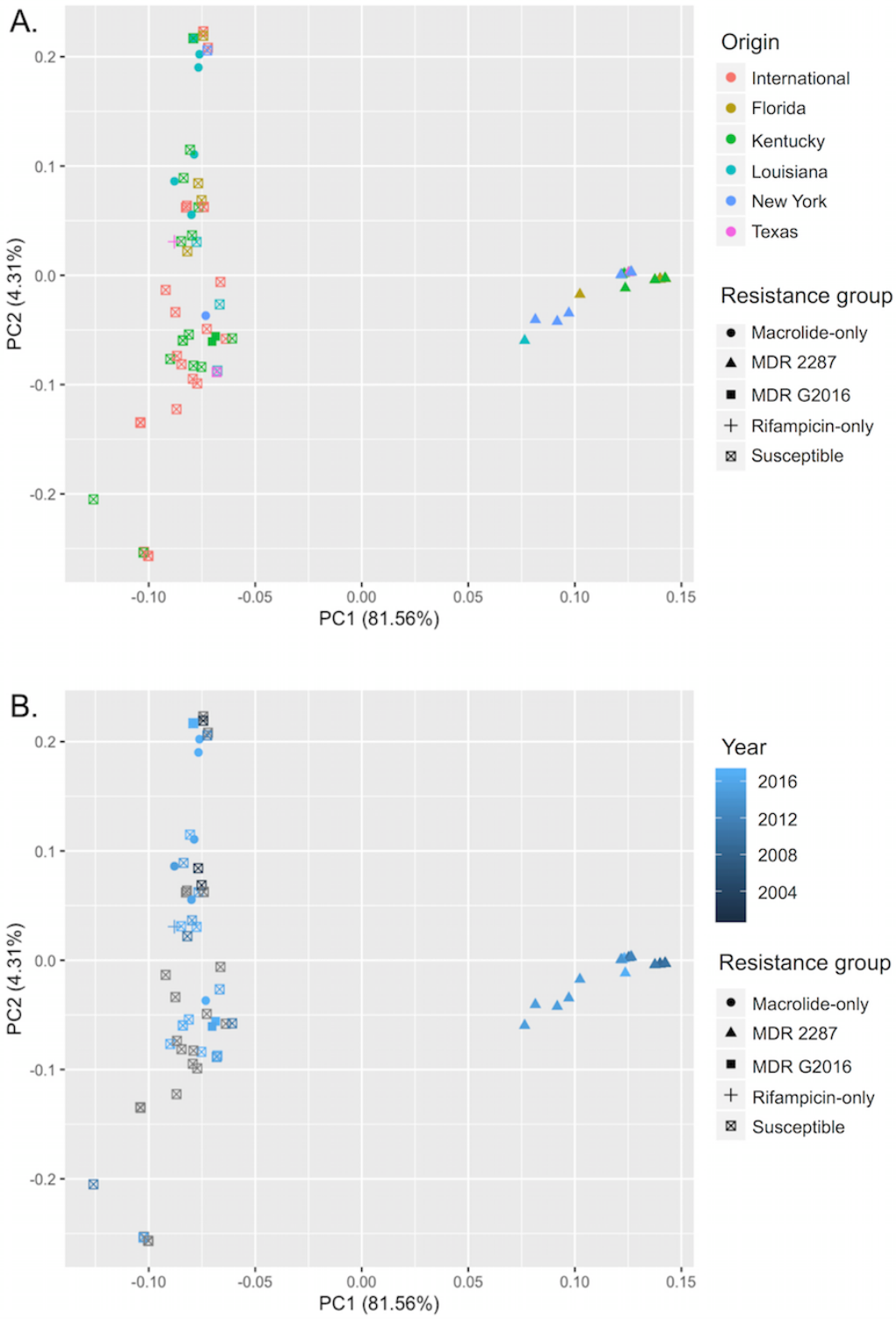

Figure 2. Lack of spatial-temporal circumscription of MDR 2287 clone. Principal component analysis (PCA) plot based on the SNP variant calls obtained in the phylogenetic analysis. Isolates are identified by resistance group and colour-coded by geographical origin (A) or year of isolation (B).

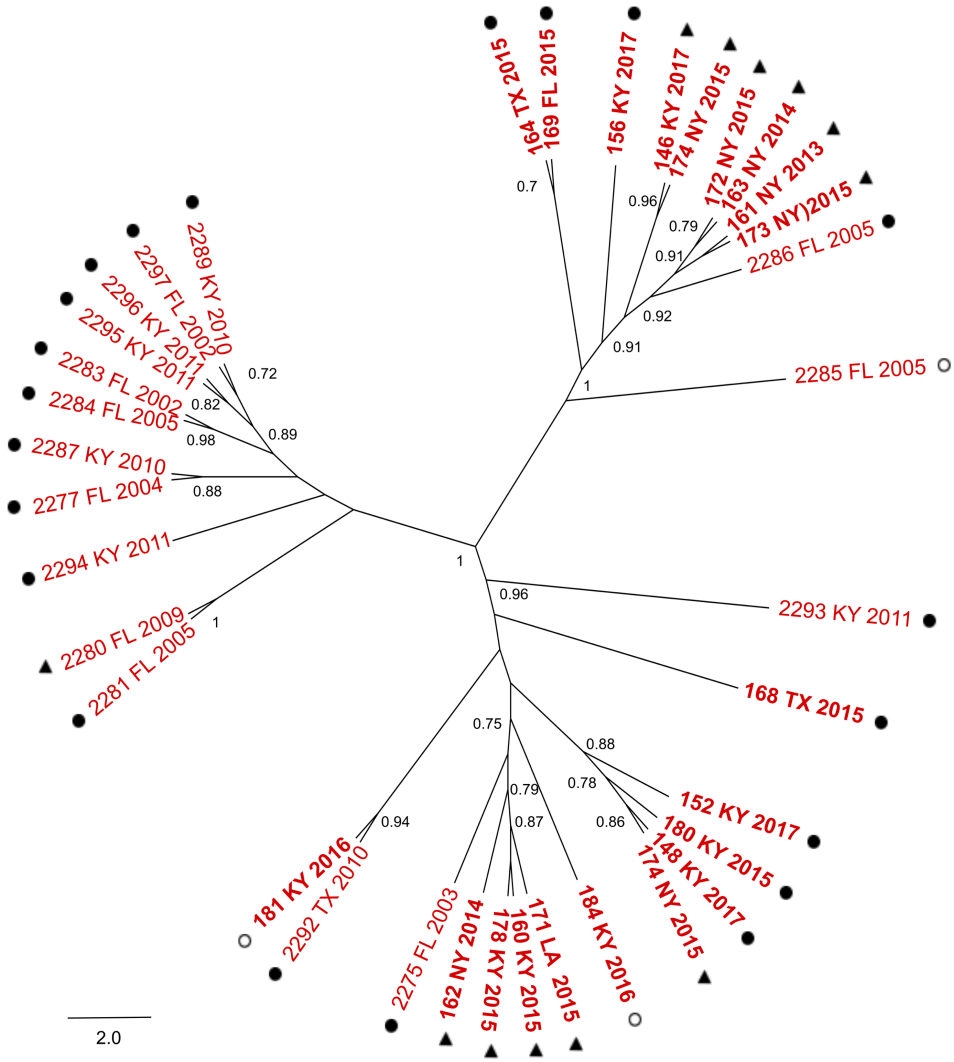

Figure 3. Phylogenetic population structure of MDR 2287 clonal complex. ParSNP core-genome tree of MDR 2287 isolates in Figure 1. Nodes indicate bootstrap support for 1,000 replicates (values >0.7 shown). Tip labels indicate strain name, source (US state) and year of isolation, symbols indicate the type of pRErm46 resistance plasmid carriage (as per Figure 1 inset legend).

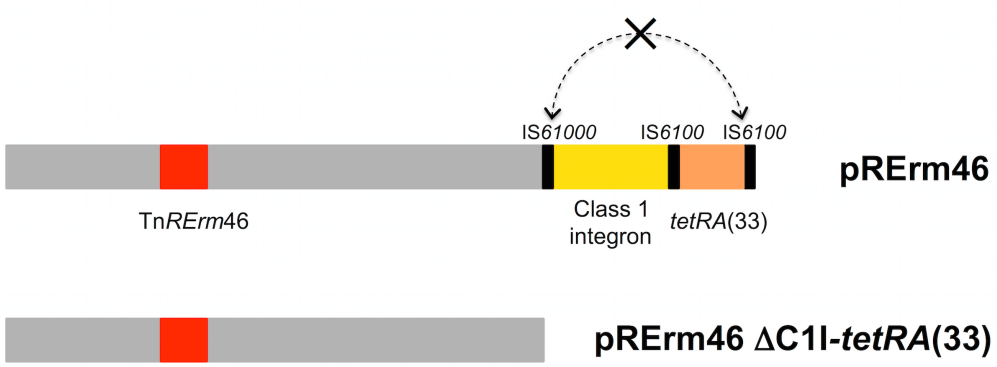

Figure 4. Schematic of ΔC1I-*tetRA*(33) deletion in *R. equi* pRErm46 macrolide resistance plasmid. Top, full size plasmid with the Tn*RErm46* transposon carrying the macrolide resistance *erm*(46) gene (in red, represented at nt position 32,567 [pRErm46 (PAM 2287) coordinates] common to all pRErm46 plasmids, additional Tn*RErm46* copies generated by transposition from original insertion may be present) and class 1 integron (C1I, in yellow) with associated *tetRA*(33) tetracycline resistance cassette (peach). Bottom, pRErm46 plasmid with the ΔC1I-*tetRA*(33) deletion. The deletion likely occurs through double crossover between the directly repeated flanking IS6100 sequences (dotted double arrow). See ref. (15) for detailed descriptions of pRErm46 plasmid and Tn*RErm46* transposon.