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▶ To cite this version:

Sandrine Petry, Corinne Sévin, Sofia Kozak, Nathalie Foucher, Claire Laugier, et al.. Relationship between rifampicin resistance and RpoB substitutions of Rhodococcus equi strains isolated in France. Journal of Global Antimicrobial Resistance, 2020, 23, pp.137 - 144. 10.1016/j.jgar.2020.08.006 . hal-03493193

HAL Id: hal-03493193 https://hal.science/hal-03493193

Submitted on 17 Oct 2022

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Version of Record: https://www.sciencedirect.com/science/article/pii/S2213716520302113 Manuscript_067533de9c9b40beeab471f82b0d83d8

1	Relationship between rifampicin resistance and RpoB substitutions of Rhodococcus equi
2	strains isolated in France
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20 Abstract

Objectives: Study the rifampicin resistance of *Rhodococcus equi* strains isolated from French
horses over a 20-year period.

Methods: Rifampicin susceptibility tested by disc diffusion and broth macrodilution methods,
 rpoB gene sequencing and MLST were performed on 40 *R. equi* strains, half of which were
 non-susceptible to rifampicin.

26 Results: Consistency of results was observed between rifampicin susceptibility testing and rpoB sequencing. The non-susceptible strains to rifampicin by disc diffusion had a 27 substitution at one of the sites, Asp516, His526, and Ser531, frequently encountered and 28 29 conferring rifampicin resistance. High-level resistance was correlated with His526Asp or Ser531Leu substitutions; low-level resistance was correlated with Asp516Tyr substitution, a 30 novel substitution for R. equi. The susceptible strains to rifampicin by disc diffusion showed 31 32 no substitution in the three sites, except for two strains carrying respectively the His526Asn and Asp516Val substitutions (previously correlated with low-level rifampicin resistance). 33 34 Both strains were isolated from an animal where ten other strains were also isolated and found to be non-susceptible to rifampicin by disc diffusion. MLST showed the presence of 10 STs 35 (including the novel ST43) but no association was observed with rifampicin resistance. 36

Conclusions: This study confirms that certain substitutions in RpoB are more likely to confer high- or low-level rifampicin resistance, describes a new substitution conferring rifampicin resistance in *R. equi* and suggests a non-clonal dissemination of rifampicin resistant strains in France. The standard disc diffusion method may miss strains with a low-level rifampicin resistant substitution; further studies are needed to remedy the absence of *R. equi*-specific clinical breakpoints.

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44 Keywords

Rhodococcus equi, horse, pneumonia, rifampicin resistance, *rpoB*, MLST

47 **1. Introduction**

48 Rhodococcus equi is a soil-dwelling and facultative intracellular bacterium that causes severe to fatal pulmonary and extrapulmonary pyrogranulomatous infections in animals and humans 49 (1,2). While affecting a variety of animal species, R. equi is a major pathogen in the horse-50 breeding industry, R. equi infections being endemic on many stud farms worldwide (3). The 51 virulence of equine R. equi strains is related to the presence of host-associated plasmid 52 pVAPA (4-7). Together with porcine pVAPB and ruminant pVAPN, equine pVAPA plasmids 53 can be found in R. equi strains isolated from humans (4-6,8), consistent therefore with a 54 zoonotic source of infection (9). 55

56 Following its introduction in 1967, rifampicin has become a mainstay therapy in the treatment of various diseases, including tuberculosis, methicillin-resistant Staphylococcus aureus 57 infections and Neisseria meningitidis infections (10). It is furthermore recommended as an 58 59 alternative treatment for infections caused by tick-borne pathogens Borrelia burgdorferi and Anaplasma phagocytophilum (10), and is also part of the multi-drug therapy used to treat 60 61 leprosy (11). Since the 1980s, rifampicin has been used in combination with a macrolide such as erythromycin or, more recently, azithromycin or clarithromycin, as the standard 62 recommended treatment for foals infected with R. equi (12-14). The rifampicin-macrolide 63 combination has reduced foal mortality but has also led to the emergence of R. equi 64 resistance. The first observations of rifampicin resistance (15-17) and a progressive 65 development of resistance to both rifampicin and erythromycin (16) were reported in the 66 1990s. An alarming situation has recently been observed in Kentucky, USA (18-22), where 67 rifampicin and erythromycin resistance has increased by 13.8% and 12.9% respectively 68 between the 1995-2006 and 2007-2017 periods (22) due to a specific R. equi clone that carries 69 70 a chromosomal mutation and a mobile element conferring this dual resistance to respectively, rifampicin and macrolides (23). 71

The primary mechanism of resistance to rifampicin consists from substitutions that alter 72 73 residues of the rifampicin-binding pocket on the β -subunit of the RNA polymerase (RpoB), resulting in a decreased affinity for rifampicin. Substitutions that confer rifampicin resistance 74 75 are typically located in three distinct clusters, I to III, of RpoB and in particular at three sites located in cluster I: Asp516, His526, and Ser531 (Escherichia coli numbering) (10,24). For R. 76 equi, at least 15 different substitutions at five sites in cluster I — Ser509, Gln513, Asp516, 77 His526, and Ser531 — have been reported (15,23,25-29). Substitutions at His526 and Ser531 78 confer the highest levels of rifampicin resistance (20). 79

Apart from two French reports on the rifampicin resistance of equine R. equi strains (15,30), 80 very few data are available in France, a major European horse-breeding country. 81 Nevertheless, according to Duchesne's study (30), which found that only 1.7% of the R. equi 82 strains isolated from 2006 to 2016 were rifampicin-resistant, the French situation could be 83 84 much less alarming than in Kentucky, USA. In this context, our objective was to study the rifampicin resistance of R. equi strains isolated from infected horses over a 20-year period 85 (1998 to 2018) in order to determine the relationship between the rifampicin susceptibility 86 tested using the disc diffusion (DD) and broth macrodilution (BMD) methods, rpoB gene 87 sequencing and multilocus sequence typing (MLST). 88

89

90 2. Materials and methods

91 2.1. Bacterial strains, culture media and growth conditions

From 1995 to 2018, 1949 *R. equi* strains were isolated during bacteriological analyses performed at the ANSES Laboratory for Animal Health in Normandy on samples collected during equine necropsies or epidemiological surveys on stud farms. Among these strains, 20 were non-susceptible to rifampicin and none to erythromycin. Forty *R. equi* strains from the ANSES collection were used in this study, including the 20 strains non-susceptible to

rifampicin. The strains susceptible to rifampicin were not randomly selected but to reflect the 97 diversity of the collection (Table 1). The 40 strains studied were maintained using cryobeads 98 (AES or BioMérieux) at -80°C. They were confirmed as *R. equi* by their colony morphology 99 100 on Columbia CNA agar with 5% sheep blood (BioMérieux) and by PCRs to detect the presence of the plasmid vapA gene (31) and the chromosomal choE gene (32-33). If the vapA 101 PCR result proves negative, plasmid-positive and plasmid-negative strains are distinguished 102 after a PCR targeting the *traA* gene coding a protein of the conserved conjugal transfer 103 104 machinery of Rhodococcus species plasmids (6). R. equi ATCC 33701 was used as a rifampicin-susceptible reference strain. 105

106

107 2.2. In vitro antibiotic susceptibility testing

Antibiotic susceptibility was determined using the standard DD method on Mueller-Hinton 108 109 agar supplemented with 5% lysed horse blood (Oxoid) and 20 μg/ml β-nicotinamide adenine dinucleotide (\beta-NAD) (ThermoFisher Scientific) according to the 2019 Antibiogram 110 111 Committee of the French Society for Microbiology (CASFM) guideline (34). Discs (Oxoid) 112 containing 5 µg oxacillin, 500 µg gentamicin, 500 µg streptomycin, 15 µg erythromycin, 100 µg spiramycin, 15 µg lincomycin, 5 µg enrofloxacin, 1.25/23.75 µg trimethoprim-113 sulfamethoxazole, 15 µg azithromycin, 15 µg clarithromycin and 30 µg rifampicin were used. 114 In view of the absence of clinical breakpoints for R equi, inhibition zone diameters were 115 interpreted applying rifampicin breakpoints approved by the 2013 CASFM guideline for 116 Staphylococcus spp. and Streptococcus spp. (35) and breakpoints for other antibiotics 117 approved by the veterinary part of the 2019 CASFM guideline for Streptococcus spp. (36); in 118 the absence of recommendations, azithromycin and clarithromycin breakpoints are the same 119 120 as for erythromycin. The breakpoints are presented in Table 1. In this study, intermediate and

resistant strains were grouped together in the non-susceptible strain population so as toindicate that they were no longer wild-type strains.

123 Rifampicin MICs were determined using the BMD method with a Mueller-Hinton broth 124 supplemented with 5% lysed horse blood (Oxoid) and 20 μ g/ml β -NAD (ThermoFisher 125 Scientific) according to the 2019 CASFM guideline for slow-growing bacteria including 126 *Streptococcus* spp. and *Corynebacterium* spp. (34). The protocol was performed as previously 127 described (37). Results were recorded as the lowest concentrations of rifampicin that inhibited 128 visible growth of *R. equi*.

129

130 2.3. DNA extraction

The NucleoSpin[®] Tissue kit (Macherey-Nagel) was used for DNA extraction according to the
manufacturer's instructions for hard-to-lyse bacteria in the support protocol for bacteria.

133

134 2.4. rpoB sequencing

The 827-bp *rpoB* partial gene was amplified and sequenced as previously described (28). The 135 rpoB gene fragment studied comprises the three distinct clusters I, II, and III identified by Jin 136 and Gross (24) in E. coli where amino acid substitutions leading to rifampicin resistance were 137 typically located; this was different from the fragment studied by MLST. The RpoB amino 138 acid sequences from amino acids 477 to 705 (E. coli numbering) were deduced and compared 139 to the RpoB amino acid sequence of the rifampicin-susceptible R. equi 103S (GenBank 140 accession no. CBH49657) used as a wild-type reference. The RpoB amino acid sequences of 141 the 40 R. equi strains studied can be found under GenBank accession nos. MT363641 to 142 MT363680. 143

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145 2.5. MLST and phylogenetic tree

The seven housekeeping genes (gapdh, tpi, mdh, icl, rpoB, recA, and adk) were amplified and 146 147 sequenced as previously described (33). The Rhodococcus MLST website (https://pubmlst.org/rhodococcus/) sited at the University of Oxford (38) was used to assign 148 the allele numbers and the sequence types (STs) after having previously trimmed to 149 equivalent lengths all the sequences of each individual MLST locus like on the Rhodococcus 150 MLST website. For each unique ST, sequences of the seven MLST loci were concatenated, 151 giving a 4017-bp in-frame sequence. Concatenated sequences were aligned and a 152 phylogenetic tree drawn up using the MEGA software version 7.0 (39) via the maximum 153 likelihood method based on the Tamura-Nei model. Support for internal nodes was estimated 154 155 using the nonparametric bootstrap method with 1000 replications. The tree was rooted with the ST06-concatenated sequences of the Rhodococcus sp. MBE 538 strain as the outgroup, 156 extracted from the Rhodococcus MLST website. 157

158

159 **3. Results**

The 40 R. equi strains studied were from 27 horses, with one strain per animal except for 160 horses no. 11 (three strains) and no. 25 (12 strains) (Table 1). They were confirmed as R. equi 161 by PCR (positive *choE* PCR results) and the equine pVAPA plasmid was detected by PCR in 162 38 of them (positive traA and vapA PCR results). The two plasmidless strains (negative traA 163 and vapA PCR results) were both isolated in 1998, strain #39 from the lung of animal no. 25, 164 and strain #40 from the faeces of the same animal two days before its death and necropsy; 165 interestingly, 11 pVAPA strains (#27 to #37) (Table 1) were isolated from different organs of 166 this same animal (no. 25). 167

168

169 *3.1. General susceptibility study*

The susceptibility of the 40 strains to 11 antibiotics was determined by DD (Table 2). All 40 170 were susceptible to gentamicin, streptomycin, erythromycin, azithromycin, clarithromycin, 171 and enrofloxacin but non-susceptible to lincomycin and oxacillin, except one strain 172 173 susceptible to lincomycin. About half of the strains were also non-susceptible to spiramycin (45.0%), rifampicin (50.0%) and trimethoprim-sulfamethoxazole (57.5%). A focus on 174 rifampicin susceptibility determined by DD and BMD is presented in Table 1. The 20 strains 175 classified by DD as susceptible to rifampicin (inhibition zone diameters of 30-36 mm) had 176 177 MICs $\leq 4 \mu g/ml$, while the 20 strains classified by DD as non-susceptible to rifampicin (inhibition zone diameters of 7-24 mm) had MICs $\geq 8 \mu g/ml$. The strains that were not 178 179 susceptible to rifampicin were isolated between 1998 and 2018 from nine equids of widely varying breed, sex, age, and sample source. 180

181

182 *3.2. RpoB sequence analysis*

RpoB amino acid sequences encompassing positions 477 to 705 (E. coli numbering) were 183 obtained for the 40 strains and the R. equi ATCC 33701. Fourteen different RpoB amino acid 184 sequences, designated RpoB profiles I to XIV, were observed, including the wild-type RpoB 185 (GenBank accession no. CBH49657) (39) of the rifampicin-susceptible R. equi 103S (Table 1 186 and Fig. 2). RpoB profile I corresponded to the wild-type sequence. It was found in ten strains 187 with MICs of 0.5 to 1 µg/ml and characterised by DD as susceptible to rifampicin, and in the 188 rifampicin-susceptible R. equi ATCC 33701. RpoB profiles II to XIV differed from the wild-189 type sequence by the presence of one to five substitutions per profile, for a total of nine 190 different substitutions at five sites in cluster I (Thr508Ala, Asp516Val, Asp516Tyr, 191 His526Asn, His526Asp, and Ser531Leu) and three sites outside clusters I, II, and III 192 (Thr491Ser, Tyr653Asn, and Asn678His) (Fig. 2). RpoB profiles II to VIII were found in ten 193 strains with MICs of 0.5 to 4 µg/ml and characterised by DD as susceptible to rifampicin, 194

while RpoB profiles IX to XIV were found in 20 strains with MICs of 8 to 1024 μ g/ml and characterised by DD as non-susceptible to rifampicin (Table 1 and Fig. 2).

The comparative analysis of in vitro rifampicin susceptibility testing and RpoB substitutions 197 shows that: (i) substitutions outside clusters I to III and the Thr508Ala substitution in cluster I 198 do not appear to confer rifampicin resistance because they were found in both strains that are 199 susceptible and strains that are not susceptible to rifampicin by DD and with MICs of 0.5 to 200 512 µg/ml. They were observed in a single substitution for six strains found by DD to be 201 202 susceptible to rifampicin with MICs $\leq 2 \mu g/ml$ or in RpoB profiles with multiple substitutions; (ii) the Asp516Tyr (MIC, 8 µg/ml), Ser531Leu (MIC, 16-256 µg/ml), and 203 204 His526Asp (MIC, 512-1024 µg/ml) substitutions appear to confer rifampicin resistance because they were found by DD only in strains non-susceptible to rifampicin with MICs ≥ 8 205 206 μ g/ml, as a single substitution or in RpoB profiles with multiple substitutions not appearing to 207 confer rifampicin resistance; (iii) the His526Asn substitution was observed in a single substitution (RpoB profile VIII) in strain #34, which was found by DD to be susceptible to 208 209 rifampicin but with a MIC of 4 μ g/ml suggesting it might actually be considered as having 210 some degree of resistance; (iv) the Asp516Val substitution was observed together with multiple other substitutions apparently unrelated to rifampicin resistance (RpoB profile VIII) 211 212 in strain #35, found by DD to be susceptible to rifampicin with a MIC of 1 μ g/ml (Fig. 2 and Fig. 3). 213

214

215 *3.3. MLST analysis*

MLST analysis was used to investigate a possible association between RpoB profiles and STs. The 40 strains and the *R. equi* ATCC 33701 were divided into nine existing STs and ST43 representing a novel ST in the *Rhodococcus* MLST website. ST24 (n = 19), ST1 (n = 5), ST19 (n = 5), and ST16 (n = 4) were the most prevalent STs; ST19 and ST24 may nevertheless be

artificially enlarged by the presence of several strains isolated from a single animal, since in 220 all cases, only one ST was found per animal (Table 1). The ST distribution in the 221 phylogenetic tree showed two distinct phylogenetic clusters: one (ST7 and ST10) with both 222 223 strains from Mayotte (a French overseas island in the Indian Ocean), and one with the R. equi ATCC 33701 from Ontario (Canada) and the 38 strains from metropolitan France (Fig. 1). No 224 association was observed between RpoB profiles and dominant ST1, ST19, and ST24, or even 225 the Mayotte and Normandy clusters. It is noteworthy that ST16 was found only in strains 226 227 found by DD to be susceptible to rifampicin with wild-type RpoB profile I or RpoB profile VI in which case the Thr491Ser substitution did not appear to confer rifampicin resistance. 228

Several strains isolated from a single animal could be considered to represent a single clone
when pVAPA, MLST, rifampicin susceptibility and RpoB sequencing results were identical.
Thus, two clones were characterised among the three strains from animal no. 11, strains #19
and #21 giving the same results; likewise, six clones were characterised among the 12 strains
from animal no. 25, strains #27, #28, #30, #31, #32, and #37 giving the same results (Fig. 2).

234

235 4. Discussion

This study reports the presence of nine French cases of rifampicin resistance in horses 236 infected by R. equi between 1998 and 2018. The 20 R. equi strains recovered from these cases 237 and found to exhibit resistance to rifampicin correspond to 1% of the R. equi ANSES 238 collection built up between 1995 and 2018 from equine samples collected during necropsies 239 or environmental samples from stud farms. These strains were all susceptible to macrolides. 240 241 This report is in accordance with a recent French study (30) which revealed that 1.7% of the R. equi strains isolated between 2006 and 2016 were rifampicin-resistant, and 1.7% 242 erythromycin-resistant according to the data presented. Thus, the French situation appears to 243 differ from that in Kentucky, USA, where rifampicin and macrolide resistance has 244

considerably increased over the past two decades (19-22,41), and is associated with both the
density of animals and the number of animals treated with antimicrobials (21). Whatever the
case, as rifampicin is a mainstay of treatment for various human diseases (10), the isolation of
an equine rifampicin-resistant *R. equi* strain must be managed in order to avoid zoonotic
transmission.

250 As no clinical breakpoints are available for application of DD and BMD methods to R. equi, rifampicin inhibition zone diameters were interpreted in keeping with *Staphylococcus* spp. 251 252 and Streptococcus spp. breakpoints approved by the 2013 CASFM guideline (35) and rifampicin MICs were compared to DD and rpoB sequencing results. The interpretative 253 results of inhibition zone diameters and MICs are consistent with each other, the 20 strains 254 found by DD to be non-susceptible to rifampicin all having MICs with higher values than the 255 20 strains found by DD to be susceptible to rifampicin. The consistency of results was also 256 257 observed between rifampicin susceptibility testing and rpoB sequencing. All the strains found by DD to be non-susceptible to rifampicin systematically had a substitution at one of the three 258 259 sites — Asp516, His526, and Ser531 — in cluster I that are frequently encountered and confer 260 rifampicin resistance (10,24). However, 20% of these strains also showed one to four additional substitutions (outside clusters I to III and Thr508Ala in cluster I) apparently not 261 262 involved in rifampicin resistance. The clinical significance of this finding needs to be further explored. 263

Our results support previous reports highlighting the fact that certain substitutions in RpoB are more likely to confer high-level resistance (15,20,23,28-29). Indeed, in this study, highlevel resistance was correlated with the presence of His526Asp (MIC, 512-1024 μ g/ml; nine strains) or Ser531Leu (MIC, 16-256 μ g/ml; ten strains), while low-level resistance was correlated with the presence of Asp516Tyr (MIC, 8 μ g/ml; one strain). His526Asp and Ser531Leu had already been described in rifampicin-resistant *R. equi* strains (26-28), and

when comparative data were present in the same study, the MICs were higher for His526Asp 270 271 than for Ser531Leu (15,28). However, the Ser531Leu substitution was previously observed in R. equi strains with high-level rifampicin resistance of 128 µg/ml (28) or with low-level 272 273 rifampicin resistance of 8 µg/ml (15,28). Substitutions in other regions of RpoB than the clusters I to III or other mechanisms of rifampicin resistance may explain this finding and 274 even the presence of rare rifampicin resistant R. equi strains with no substitution in the 275 clusters I to III of RpoB (28). To our knowledge, Asp516Tyr has never been described before 276 277 in R. equi but was previously described in rifampicin-resistant Mycobacterium tuberculosis strains (42-43). 278

279 Regarding the 20 strains found by DD to be susceptible to rifampicin, 50% showed no substitution in RpoB amino acid sequences 477 to 705, and 40% showed only substitutions 280 not appearing to confer rifampicin resistance. As concerns the remaining 10%, strains #34 and 281 282 #35 had one substitution in cluster I (with additional substitutions outside clusters I to III in the case of strain #35) — His526Asn and Asp516Val respectively. These have previously 283 been described in R. equi strains as leading to low-level rifampicin resistance of 1-8 µg/ml 284 (15,26,28). Both strains were isolated respectively from animal no. 25 during which ten other 285 strains were also isolated, all being found by DD to be non-susceptible to rifampicin and with 286 MICs of 8 to 1024 µg/ml. In this context, strains #34 and #35 should be considered non-287 susceptible to rifampicin and we propose that ≥ 32 mm should be used as a breakpoint for 288 susceptibility instead of ≥ 29 mm for a 30-µg rifampicin disc (Fig. 3). However, our findings 289 do not allow us to propose breakpoints for rifampicin MICs obtained by the BMD method. S 290 $\leq 1 \,\mu$ g/ml and R $\geq 4 \,\mu$ g/ml have already been reported in the literature for R. equi and 291 correspond to CLSI interpretative criteria for *Staphylococcus aureus* (44), which is probably 292 293 the most consistent with our DD and *rpoB* sequencing results.

In this study, the nine horses concerned with the isolation of rifampicin non-susceptible 294 295 strains were distributed throughout the phylogenetic tree of R. equi species based on MLST sequences (Fig. 1) and no association was observed between the rifampicin resistance and 296 297 dominant ST1, ST19 and ST24 (or any other information on horses, like the year of collection) (Fig. 1). These findings suggest a non-clonal dissemination of rifampicin resistant 298 strains in France contrary to what has been observed from equine R. equi strains isolated in 299 the USA (45). It should however be noted that MLST results generated with next generation 300 sequencing (NGS) had resulted in a better detection of this clonality than Sanger sequencing 301 (45). In our context, it is unlikely that NGS-MLST would have allowed us to conclude 302 differently. 303

In conclusion, this study reports nine French cases of rifampicin resistance in horses infected 304 by R. equi between 1995 and 2018. The relationship between rifampicin susceptibility tested 305 306 using both DD and BMD methods, and the substitutions observed in RpoB amino acid sequences 477 to 705 (E. coli numbering) revealed the presence of substitutions that were 307 308 correlated with high-level (His526Asp, Ser531Leu), low-level (Asp516Tyr), and low or 309 borderline-level (His526Asn and Asp516Val) rifampicin resistance, the Asp516Tyr substitution having never been previously described in R. equi. We observed that the standard 310 311 DD method widely used in clinical laboratories may miss isolates with RpoB substitutions, such as strains #34 and #35 with substitutions His526Asn and Asp516Val respectively. 312 Further studies are needed to define specific R. equi clinical breakpoints for rifampicin and 313 macrolides used in combination to treat R. equi infections. 314

315

316 Acknowledgements

The authors are grateful to Maud Bernez-Romand, Clarisse Brocquevieille, Benoit Ecolivet,and Armand Pontin for technical assistance, Aurélie Merlin for discussions on the R

319	programming language (ANSES, Laboratory for Animal Health in Normandy), and Marisa
320	Haenni for discussions on antibiotic resistance (ANSES, Lyon Laboratory). We also wish to
321	thank Delphine Libby-Claybrough, professional translator and native English speaker, for her
322	editorial assistance. The ANSES Laboratory for Animal Health, Normandy site, is a member
323	of the equine research scientific interest group "GIS Centaure" and the authors are grateful to
324	the Regional Council of Normandy for providing an excellent scientific and work
325	environment.

- **Funding:** None
- **Competing interests:** None declared
- 329 Ethical approval: Not required

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Fig. 1. Phylogenetic tree of R. equi species based on MLST sequences. The maximum 470 likelihood phylogenetic tree was based on the alignment of 4017-bp concatenated DNA 471 sequences of the seven MLST loci for 37 R. equi STs recorded in the Rhodococcus MLST 472 473 database (https://pubmlst.org/rhodococcus/) and the new one, ST43, obtained in this study. The tree was rooted with the ST06-concatenated sequences of the Rhodococcus sp. MBE 538 474 strain as the outgroup, extracted from the *Rhodococcus* MLST database. The bootstrap values 475 were calculated from 1000 replications. The black arrows represent the STs of the 40 strains 476 477 studied and *R. equi* ATCC 33701. The RpoB profiles I to XIV are indicated after the black arrows with in parentheses the number of horses concerned; the year of collection was 478 indicated when animals were concerned with the isolation of rifampicin non-susceptible 479 strains. ^a, His526Asp or Ser531Leu correlated with high-level rifampicin resistance; ^b, 480 Asp516Tyr correlated with low-level rifampicin resistance; ^c, His526Asn or Asp516Val 481 482 previously reported as correlated with low-level rifampicin resistance, and observed respectively in the strain #34 (susceptible to rifampicin by DD with a MIC of 4 µg/ml) and in 483 484 the strain #35 (susceptible to rifampicin by DD with a MIC of 1 μ g/ml). Both strains were 485 isolated from the animal no. 25 during which ten other strains were also isolated, all being non-susceptible to rifampicin by DD with MICs of 8 to $1024 \mu g/ml$. 486

487

Fig. 2. Schematic positions of RpoB substitutions in the 40 *R. equi* strains studied. A: Map of
the *E. coli* RpoB with the location of rifampicin cluster I (507 to 533), II (563 to 572), and III
(687). B: Distribution of substitutions observed in RpoB amino acid sequences 477 to 705 (*E. coli* numbering). Fourteen RpoB amino acid sequences, artificially named RpoB profiles I to
XIV, were observed, including the wild-type RpoB (GenBank accession no. CBH49657). The
rifampicin MICs determined by BMD are indicated.

Fig. 3. Distribution of rifampicin inhibition zone diameters and MICs as determined by respectively disc diffusion and broth macrodilution methods. Vertical solid lines represent the interpretative zone diameter breakpoints for *Staphylococcus* spp. and *Streptococcus* spp. (35) and the vertical dotted line represents a proposed interpretative susceptible zone diameter breakpoint for *R. equi*. The strain frequency per square is not represented; one square may contain the results from one to seven strains. The amino acid substitutions observed in RpoB cluster I are indicated. S, susceptible; R, resistant. 502 **Table 1.** Origin, chromosomal and virulence plasmid PCR detection, rifampicin resistance, and RpoB amino acid substitutions for the 40 *R. equi* strains

503 studied.

								PCR detection				Rifampicin ^f		
Strain (Animal) ID											MLST	mm;	MIC	RpoB (aas 473 to 705; E.
in the study	Strain alias	Year	Sample source	Equine breed	Sex	Age	Country	choE	vapA	traA	(ST)	S/I/R	(µg/ml)	coli numbering) profile
#01 (01)	MBE 755	2018	Abdominal abscess	Thoroughbred	Male	5 months	Normandy (FR)	+ + +		+	1	20; R	64	Х
#02 (02)	MBE 756	2018	Lung abscess	Thoroughbred	Male	5 months	Normandy (FR)	+	+	+	43	13; R	128	XIV
#03 (03)	MBE 578	2012	Muscle abscess	Pony ^c	Male	12 years	Mayotte (FR)	+ ^d	+ ^d	+ ^d	7 ^d	24; I	16	Х
#04 (04)	MBE 597	2012	Perianal abscess	Pony ^c	Female	13 years	Mayotte (FR)	+ ^d	+ ^d	+ ^d	10 ^d	34; S	1	I (wild type)
#05 (05)	MBE 363	2009	Lung abscess	Thoroughbred	Male	2 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	13 ^d	34; S	1	I (wild type)
#06 (06)	MBE 355	2009	Lung abscess	Thoroughbred	Male	3.75 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	16 ^d	34; S	1	I (wild type)
#07 (07)	MBE 757	2008	Lung	Thoroughbred	Male	3.25 months	Normandy (FR)	+	+	+	24	19; R	128	X
#08 (08)	MBE 758	2008	Lung abscess	French Trotter	Female	2.5 months	Normandy (FR)	+	+	+	24	20; R	64	Х
#09 (09)	MBE 759	2008	Lung	Thoroughbred	Male	2 months	Normandy (FR)	+	+	+	2	34; S	1	I (wild type)
#10 (10)	MBE 760	2007	Bladder	Thoroughbred	Male	4.5 months	Normandy (FR)	+	+	+	24	20; R	64	XI
#11 (11)	MBE 226	2006	Bone	French Trotter	Female	5.5 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	24 ^d	34; S	1	III
#12 (12)	MBE 761	2006	Lung	Thoroughbred	Female	6 months	Normandy (FR)	+	+	+	13	35; S	2	VII
#13 (13)	MBE 192	2006	Placenta	French Trotter	Female	Fœtus	Normandy (FR)	+ ^d	+ ^d	+ ^d	24 ^d	20; R	256	XII
#14 (14)	MBE 190	2006	Placenta	Thoroughbred	Male	Fœtus	Normandy (FR)	+ ^d	+ ^d	+ ^d	19 ^d	36; S	0,5	II
#15 (15)	MBE 762	2005	Lung	French Trotter	Female	2 months	Normandy (FR)	+	+	+	3	34; S	1	I (wild type)
#16 (16)	MBE 763	2005	Lung	French Trotter	Female	3.5 months	Normandy (FR)	+	+	+	24	36; S	1	I (wild type)
#17 (17)	MBE 174	2005	Eye	French Trotter	Male	1.75 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	24 ^d	38; S	2	VII
#18 (18)	MBE 173	2005	Bone	French Trotter	Male	2.75 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	16 ^d	35; S	2	VI
#19(19) ^a	MBE 764	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	19	22; R	64	Х
$#20(19)^{a}$	MBE 765	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	19	7; R	1024	XIV
#21 (19) ^a	MBE 171	2004	Tracheobronchial lymph node	Unknown	Unknown	Unknown	France	+ ^d	+ ^d	+ ^d	19 ^d	22; R	64	Х
#22 (20)	MBE 167	2004	Bone	French Trotter	Female	3.5 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	16 ^d	35; S	1	I (wild type)
#23 (21)	MBE 163	2004	Liver	French Trotter	Female	2 hours	Normandy (FR)	+ ^d	+ ^d	+ ^d	1 ^d	33; S	1	V
#24 (22)	MBE 158	2002	Lung	French Trotter	Male	2 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	1 ^d	34; S	1	VI
#25 (23)	MBE 766	2001	Lung	French Trotter	Male	2 months	Normandy (FR)	+	+	+	2	36; S	1	VI
#26 (24)	MBE 767	1999	Faeces	Unknown	Unknown	Foal	Normandy (FR)	+	+	+	19	36; S	0,5	I (wild type)
#27 (25) ^b	MBE 768	1998	Lung	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#28 (25) ^b	MBE 769	1998	Lung abscess	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#29 (25) ^b	MBE 770	1998	Uterus	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	22; R	32	Х
#30(25) ^b	MBE 771	1998	Spleen	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	15; R	1024	XIV
#31 (25) ^b	MBE 772	1998	Tracheobronchial lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#32 (25) ^b	MBE 773	1998	Colic lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	512	XIV
#33 (25) ^b	MBE 774	1998	Digestive abscess n°1	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	27; I	8	IX
#34 (25) ^b	MBE 775	1998	Digestive abscess n°2	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	31; S	4	VIII
#35 (25) ^b	MBE 776	1998	Joint	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	30; S	1	IV
#36(25) ^b	MBE 777	1998	Small colon	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	512	XIII
#37 (25) ^b	MBE 778	1998	Tracheobronchial fluid	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#38 (26)	MBE 123	1998	Lung	Thoroughbred	Female	2 months	Normandy (FR)	+ ^d	+ ^d	+ ^d	1 ^d	36; S	0,5	I (wild type)
#39 (27)	MBE 122	1998	Lung	Thoroughbred	Female	1.5 months	Normandy (FR)	+ ^c	_ c, e	_ d	16 ^d	36; S	0,5	I (wild type)
#40(25) ^b	MBE 179	1998	Faeces	Thoroughbred	Female	2.8 years	Normandy (FR)	+	- ^e	-	24	20; R	128	X
ATCC 33701		Unknown	Lung	Unknown	Unknown	Foal	Ontario (CA)	+ ^d	+ ^d	+ ^d	1 ^d	ND; S ^g	ND	I (wild type)
103S (accession no.	CBH49657)	1979	Lung	Unknown	Unknown	Foal	Ontario (CA)	ND	ND	ND	ND	ND; S ^g	ND	I (wild type)

^a Same animal (no. 19) but different sample sources.

- ^b Same animal (no. 25) but different sample sources; the strain from the faeces was isolated two days before necropsy.
- ^c From the atypical outbreak of *R. equi* infections in adult ponies on Mayotte in 2012 (46).
- ^d PCR and MLST results extracted from Duquesne et al. (33). +, detected; -, not detected; ND, not determined.
- ^e No virulence plasmid was detected.
- ^f 30- μ g rifampicin inhibition zone diameters (mm) and rifampicin MICs (μ g/ml) were determined respectively by DD and BMD.
- ^g The rifampicin susceptibility of the reference ATCC 33701 and 103S strains was taken from the literature (15,47).

Antimicrobial class	Antibiotic	Breakpoints (mm) $S \ge / R \le$	Number of non- susceptible strains	Proportion of non- susceptible strains (%; 95% CI) ^d
β-lactams	Oxacillin	21/21 ^a	40	100
Aminoglycosides	Gentamicin	17/11 ^a	0	0
	Streptomycin	14/12 ^a	0	0
Macrolides	Erythromycin	22/17 ^a	0	0
	Azithromycin	22/17 ^b	0	0
	Clarithromycin	22/17 ^b	0	0
	Spiramycin	18/14 ^a	18	45.0 (29.6, 60.4)
Lincosamides	Lincomycin	21/17 ^a	39	97.7 (92.7, 100)
Fluoroquinolones	Enrofloxacin	22/17 ^a	0	0
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	16/10 ^a	23	57.5 (42.2, 72.8)
Rifamycins	Rifampicin	29/24 °	20	50.0 (34.5, 65.5)

511 **Table 2.** Antimicrobial resistance in the 40 *R. equi* strains studied as determined by DD.

512 S, susceptible; R, resistant.

^a Approved by the veterinary part of the CASFM (36) for *Streptococcus* spp.

^b Breakpoints for azithromycin and clarithromycin are the same as for erythromycin.

^c Approved by the CASFM (35) for *Staphylococcus* spp. and *Streptococcus* spp.

^d The indicator of resistance was defined as the ratio between the number of non-susceptible

517 strains and the total number of strains (n=40). Their confidence intervals (CI) were calculated

518 using the exact binomial method.



	Α	100 200	300	400	500	600	700	800	900	0 1000	1100 1200) 1300 aas
	Γ										E. col	i RpoB
	_				Ι	П	III					
В												
Strain ID in the study	Rifampicin MIC (µg/ml)	RpoB profile	491	508	516	526	531	653	678	aas (E. co	<i>i</i> numbering)	1
ATCC 33701 and 103S (accession no. CBH49657)	ND ^a	I (wild type)	Thr	Thr	Asp	His	Ser	Tyr	Asn	-		
#04, #05, #06, #09, #15, #16, #22, #26, #38, #39	0.5 - 1 ^a	Ι	•		•	•		•				
#14	0.5 ^a	II	Ser	Ala ^b	•	•		•				
#11	1 ^a	III	Ser	Ala ^b				Asn	His			
#35	1 ^a	IV	Ser		Val			•	His			
#23	1 ^a	V		•	•	•		•	His			
#18, #24, #25	$1 - 2^{a}$	VI	Ser					•				
#12, #17	2^{a}	VII			•			Asn				
#34	4 ^a	VIII				Asn	-	•				
#33	8	IX	Ser		Tyr ^b			•				
#01, #03, #07, #08, #19, #21, #29, #40	16 - 128	Х					Leu	•				
#10	64	XI	Ser				Leu	Asn				
#13	256	XII	Ser			•	Leu		His			
#36	512	XIII	Ser	Ala ^b		Asp		Asn	His			
#02, #20, #27, #28, #30, #31, #32, #37	128 - 1024	XIV		•		Asp		<u>. </u>	•			

^a susceptible strains according to DD results. ^b new substitutions reported in this study.

