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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-03493193v1>**

Submitted on 17 Oct 2022

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

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20 **Abstract**

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

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

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

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

43

44 **Keywords**

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

46

## 47 **1. Introduction**

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

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

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

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

89

## 90 **2. Materials and methods**

### 91 *2.1. Bacterial strains, culture media and growth conditions*

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

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

106

## 107 2.2. *In vitro* antibiotic susceptibility testing

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

121 resistant strains were grouped together in the non-susceptible strain population so as to  
122 indicate 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 µg/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

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

133

### 134 2.4. rpoB sequencing

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

144

### 145 2.5. MLST and phylogenetic tree

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

158

### 159 **3. Results**

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

168

#### 169 *3.1. General susceptibility study*

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

181

### 182 3.2. *RpoB* sequence analysis

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

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

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

214

### 215 3.3. MLST analysis

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

220 artificially enlarged by the presence of several strains isolated from a single animal, since in  
221 all cases, only one ST was found per animal (Table 1). The ST distribution in the  
222 phylogenetic tree showed two distinct phylogenetic clusters: one (ST7 and ST10) with both  
223 strains from Mayotte (a French overseas island in the Indian Ocean), and one with the *R. equi*  
224 ATCC 33701 from Ontario (Canada) and the 38 strains from metropolitan France (Fig. 1). No  
225 association was observed between RpoB profiles and dominant ST1, ST19, and ST24, or even  
226 the Mayotte and Normandy clusters. It is noteworthy that ST16 was found only in strains  
227 found by DD to be susceptible to rifampicin with wild-type RpoB profile I or RpoB profile VI  
228 in which case the Thr491Ser substitution did not appear to confer rifampicin resistance.  
229 Several strains isolated from a single animal could be considered to represent a single clone  
230 when pVAPA, MLST, rifampicin susceptibility and RpoB sequencing results were identical.  
231 Thus, two clones were characterised among the three strains from animal no. 11, strains #19  
232 and #21 giving the same results; likewise, six clones were characterised among the 12 strains  
233 from animal no. 25, strains #27, #28, #30, #31, #32, and #37 giving the same results (Fig. 2).

234

#### 235 **4. Discussion**

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

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

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

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

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

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

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

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

315

### 316 **Acknowledgements**

317 The authors are grateful to Maud Bernez-Romand, Clarisse Brocquevieuille, Benoit Ecolivet,  
318 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.

326

327 **Funding:** None

328 **Competing interests:** None declared

329 **Ethical approval:** Not required

330

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

487

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

494

495 **Fig. 3.** Distribution of rifampicin inhibition zone diameters and MICs as determined by  
496 respectively disc diffusion and broth macrodilution methods. Vertical solid lines represent the  
497 interpretative zone diameter breakpoints for *Staphylococcus* spp. and *Streptococcus* spp. (35)  
498 and the vertical dotted line represents a proposed interpretative susceptible zone diameter  
499 breakpoint for *R. equi*. The strain frequency per square is not represented; one square may  
500 contain the results from one to seven strains. The amino acid substitutions observed in RpoB  
501 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.

Strain (Animal) ID in the study	Strain alias	Year	Sample source	Equine breed	Sex	Age	Country	PCR detection			MLST (ST)	Rifampicin <sup>f</sup>		RpoB (aas 473 to 705; <i>E. coli</i> numbering) profile
								<i>choE</i>	<i>vapA</i>	<i>traA</i>		mm; S/I/R	MIC (µg/ml)	
#01 (01)	MBE 755	2018	Abdominal abscess	Thoroughbred	Male	5 months	Normandy (FR)	+	+	+	1	20; R	64	X
#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 <sup>c</sup>	Male	12 years	Mayotte (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	7 <sup>d</sup>	24; I	16	X
#04 (04)	MBE 597	2012	Perianal abscess	Pony <sup>c</sup>	Female	13 years	Mayotte (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	10 <sup>d</sup>	34; S	1	I (wild type)
#05 (05)	MBE 363	2009	Lung abscess	Thoroughbred	Male	2 months	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	13 <sup>d</sup>	34; S	1	I (wild type)
#06 (06)	MBE 355	2009	Lung abscess	Thoroughbred	Male	3.75 months	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	16 <sup>d</sup>	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	X
#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)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	24 <sup>d</sup>	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	Foetus	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	24 <sup>d</sup>	20; R	256	XII
#14 (14)	MBE 190	2006	Placenta	Thoroughbred	Male	Foetus	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	19 <sup>d</sup>	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)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	24 <sup>d</sup>	38; S	2	VII
#18 (18)	MBE 173	2005	Bone	French Trotter	Male	2.75 months	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	16 <sup>d</sup>	35; S	2	VI
#19 (19) <sup>a</sup>	MBE 764	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	19	22; R	64	X
#20 (19) <sup>a</sup>	MBE 765	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	19	7; R	1024	XIV
#21 (19) <sup>a</sup>	MBE 171	2004	Tracheobronchial lymph node	Unknown	Unknown	Unknown	France	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	19 <sup>d</sup>	22; R	64	X
#22 (20)	MBE 167	2004	Bone	French Trotter	Female	3.5 months	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	16 <sup>d</sup>	35; S	1	I (wild type)
#23 (21)	MBE 163	2004	Liver	French Trotter	Female	2 hours	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	1 <sup>d</sup>	33; S	1	V
#24 (22)	MBE 158	2002	Lung	French Trotter	Male	2 months	Normandy (FR)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	1 <sup>d</sup>	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) <sup>b</sup>	MBE 768	1998	Lung	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#28 (25) <sup>b</sup>	MBE 769	1998	Lung abscess	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#29 (25) <sup>b</sup>	MBE 770	1998	Uterus	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	22; R	32	X
#30 (25) <sup>b</sup>	MBE 771	1998	Spleen	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	15; R	1024	XIV
#31 (25) <sup>b</sup>	MBE 772	1998	Tracheobronchial lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	1024	XIV
#32 (25) <sup>b</sup>	MBE 773	1998	Colic lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	512	XIV
#33 (25) <sup>b</sup>	MBE 774	1998	Digestive abscess n°1	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	27; I	8	IX
#34 (25) <sup>b</sup>	MBE 775	1998	Digestive abscess n°2	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	31; S	4	VIII
#35 (25) <sup>b</sup>	MBE 776	1998	Joint	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	30; S	1	IV
#36 (25) <sup>b</sup>	MBE 777	1998	Small colon	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	24	7; R	512	XIII
#37 (25) <sup>b</sup>	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)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	1 <sup>d</sup>	36; S	0,5	I (wild type)
#39 (27)	MBE 122	1998	Lung	Thoroughbred	Female	1.5 months	Normandy (FR)	+ <sup>c</sup>	- <sup>c, e</sup>	- <sup>d</sup>	16 <sup>d</sup>	36; S	0,5	I (wild type)
#40 (25) <sup>b</sup>	MBE 179	1998	Faeces	Thoroughbred	Female	2.8 years	Normandy (FR)	+	- <sup>e</sup>	-	24	20; R	128	X
ATCC 33701		Unknown	Lung	Unknown	Unknown	Foal	Ontario (CA)	+ <sup>d</sup>	+ <sup>d</sup>	+ <sup>d</sup>	1 <sup>d</sup>	ND; S <sup>§</sup>	ND	I (wild type)
103S (accession no. CBH49657)		1979	Lung	Unknown	Unknown	Foal	Ontario (CA)	ND	ND	ND	ND	ND; S <sup>§</sup>	ND	I (wild type)

504 <sup>a</sup> Same animal (no. 19) but different sample sources.

505 <sup>b</sup> Same animal (no. 25) but different sample sources; the strain from the faeces was isolated two days before necropsy.

506 <sup>c</sup> From the atypical outbreak of *R. equi* infections in adult ponies on Mayotte in 2012 (46).

507 <sup>d</sup> PCR and MLST results extracted from Duquesne et al. (33). +, detected; -, not detected; ND, not determined.

508 <sup>e</sup> No virulence plasmid was detected.

509 <sup>f</sup> 30- $\mu$ g rifampicin inhibition zone diameters (mm) and rifampicin MICs ( $\mu$ g/ml) were determined respectively by DD and BMD.

510 <sup>g</sup> The rifampicin susceptibility of the reference ATCC 33701 and 103S strains was taken from the literature (15,47).

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

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

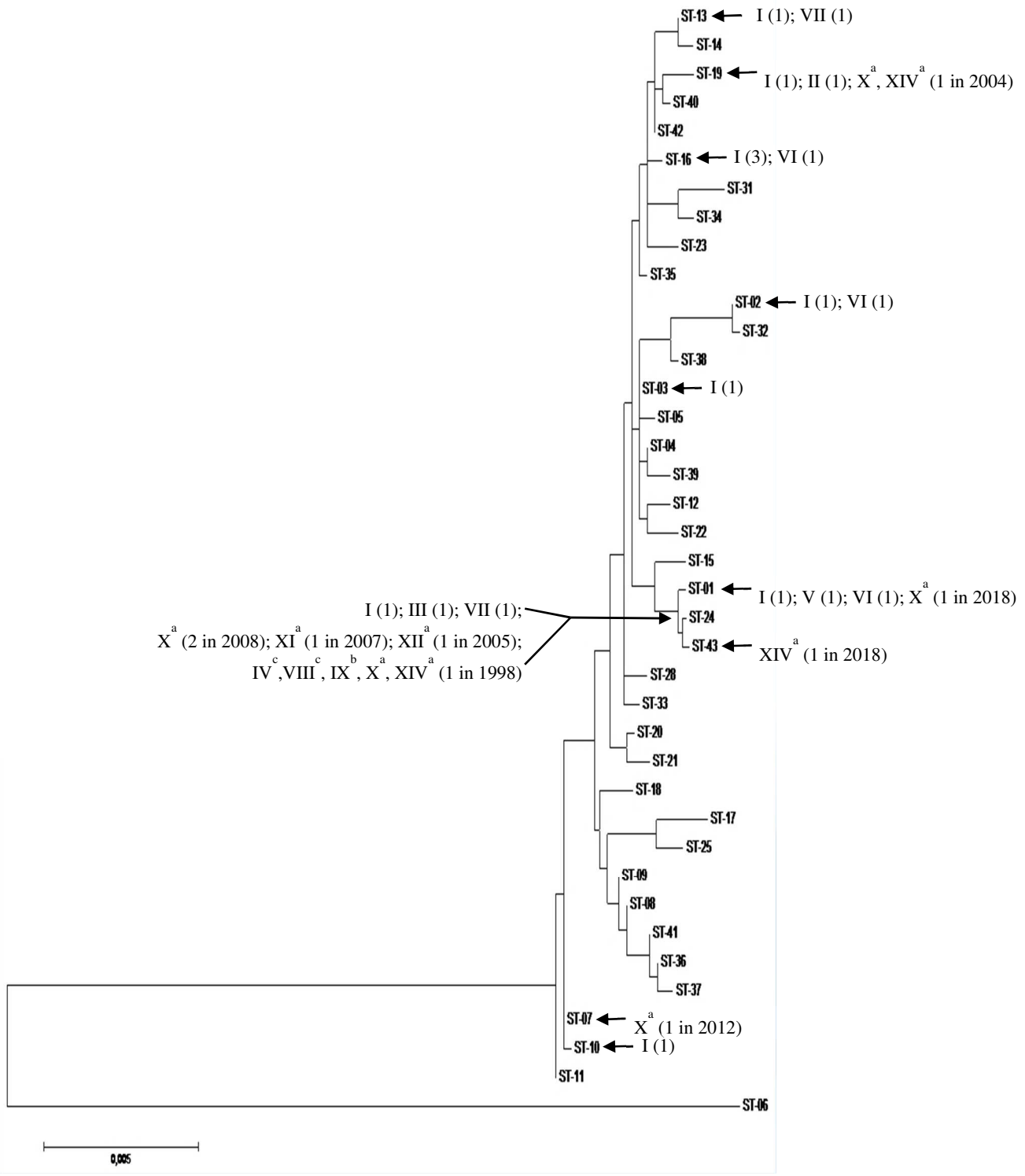
512 S, susceptible; R, resistant.

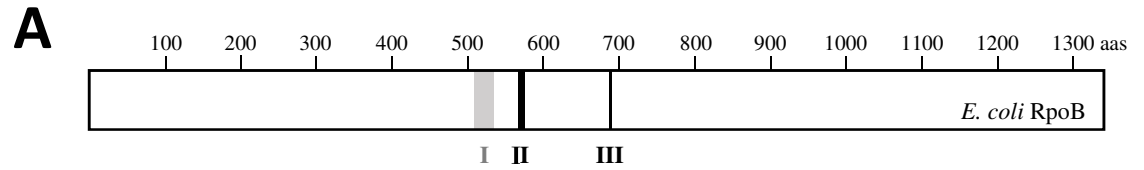
513 <sup>a</sup> Approved by the veterinary part of the CASFM (36) for *Streptococcus* spp.

514 <sup>b</sup> Breakpoints for azithromycin and clarithromycin are the same as for erythromycin.

515 <sup>c</sup> Approved by the CASFM (35) for *Staphylococcus* spp. and *Streptococcus* spp.

516 <sup>d</sup> 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.





**B**

Strain ID in the study	Rifampicin MIC ( $\mu\text{g/ml}$ )	RpoB profile	491	508	516	526	531	653	678	aas ( <i>E. coli</i> numbering)
<b>ATCC 33701 and 103S (accession no. CBH49657)</b>	<b>ND<sup>a</sup></b>	<b>I (wild type)</b>	<b>Thr</b>	<b>Thr</b>	<b>Asp</b>	<b>His</b>	<b>Ser</b>	<b>Tyr</b>	<b>Asn</b>	
#04, #05, #06, #09, #15, #16, #22, #26, #38, #39	0.5 - 1 <sup>a</sup>	I	.	.	.	.	.	.	.	
#14	0.5 <sup>a</sup>	II	Ser	Ala <sup>b</sup>	.	.	.	.	.	
#11	1 <sup>a</sup>	III	Ser	Ala <sup>b</sup>	.	.	.	Asn	His	
#35	1 <sup>a</sup>	IV	Ser	.	Val	.	.	.	His	
#23	1 <sup>a</sup>	V	.	.	.	.	.	.	His	
#18, #24, #25	1 - 2 <sup>a</sup>	VI	Ser	.	.	.	.	.	.	
#12, #17	2 <sup>a</sup>	VII	.	.	.	.	.	Asn	.	
#34	4 <sup>a</sup>	VIII	.	.	.	Asn	.	.	.	
#33	8	IX	Ser	.	Tyr <sup>b</sup>	.	.	.	.	
#01, #03, #07, #08, #19, #21, #29, #40	16 - 128	X	.	.	.	.	Leu	.	.	
#10	64	XI	Ser	.	.	.	Leu	Asn	.	
#13	256	XII	Ser	.	.	.	Leu	.	His	
#36	512	XIII	Ser	Ala <sup>b</sup>	.	Asp	.	Asn	His	
#02, #20, #27, #28, #30, #31, #32, #37	128 - 1024	XIV	.	.	.	Asp	.	.	.	

<sup>a</sup> susceptible strains according to DD results. <sup>b</sup> new substitutions reported in this study.

