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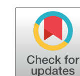
Relationship between rifampicin resistance and RpoB substitutions of *Rhodococcus equi* strains isolated in France



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ABSTRACT

Objectives: Study of the rifampicin resistance of *Rhodococcus equi* strains isolated from French horses over a 20-year period.
Methods: Rifampicin susceptibility was tested by disk diffusion (DD) and broth macrodilution methods, and *rpoB* gene sequencing and MLST were performed on 40 *R. equi* strains, 50.0% of which were non-susceptible to rifampicin.
Results: Consistency of results was observed between rifampicin susceptibility testing and *rpoB* sequencing. Strains non-susceptible to rifampicin by DD had a substitution at one of the sites (Asp516, His526 and Ser531) frequently encountered and conferring rifampicin resistance. High-level resistance was correlated with His526Asp or Ser531Leu substitutions; low-level resistance was correlated with Asp516Tyr substitution, a novel substitution for *R. equi*. Strains susceptible to rifampicin by DD showed no substitution in the three sites, except for two strains carrying, respectively, the His526Asn and Asp516Val substitutions (previously correlated with low-level rifampicin resistance). Both strains were isolated from an animal from which ten other strains were also isolated and found to be rifampicin-non-susceptible by DD. MLST showed the presence of 10 STs (including the novel ST43), but no association was observed with rifampicin resistance.
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 non-clonal dissemination of rifampicin-resistant strains in France. Standard DD 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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1. Introduction

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

Following its introduction in 1967, rifampicin has become a mainstay of therapy in the treatment of various diseases, including tuberculosis, methicillin-resistant *Staphylococcus aureus* (MRSA) infections and *Neisseria meningitidis* infections [10]. It is also recommended as an alternative treatment for infections caused by the tick-borne pathogens *Borrelia burgdorferi* and *Anaplasma phagocytophilum* [10] and is part of the multidrug therapy used to treat 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 recommended treatment for foals infected with *R. equi* [12–14]. The

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rifampicin–macrolide combination has reduced foal mortality but has also led to the emergence of resistance in *R. equi*. The first observations of rifampicin resistance [15–17] and progressive development of resistance to both rifampicin and erythromycin [16] were reported in the 1990s. An alarming situation has recently been observed in Kentucky, USA [18–22] where rifampicin and erythromycin resistance have increased by 13.8% and 12.9%, respectively, between the periods 1995–2006 and 2007–2017 [22] owing to a specific *R. equi* clone that carries a chromosomal mutation and a mobile element conferring this dual resistance to, respectively, rifampicin and macrolides [23].

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

Apart from two French reports on the rifampicin resistance of equine *R. equi* strains [15,30], very few data are available in France, a major European horse-breeding country. Nevertheless, according to the study by Duchesne et al. [30], which found that only 1.7% of the *R. equi* strains isolated from 2006 to 2016 were rifampicin-resistant, the French situation could be 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 in France over a 20-year period (1998–2018) in order to determine the relationship between rifampicin susceptibility determined using the disk diffusion (DD) and broth macrodilution (BMD) methods, *rpoB* gene sequencing and multilocus sequence typing (MLST).

2. Materials and methods

2.1. Bacterial strains, culture media and growth conditions

From 1995 to 2018, a total of 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 were selected to reflect the diversity of the collection (Table 1). The 40 strains studied were maintained using cryobeads (AES or bioMérieux) at −80 °C. They were confirmed as *R. equi* by colony morphology on Columbia CNA agar with 5% sheep blood (bioMérieux) and by PCR to detect the presence of the plasmid *vapA* gene [31] and the chromosomal *choE* gene [32,33]. If the *vapA* PCR result proves negative, plasmid-positive and plasmid-negative strains are distinguished following PCR targeting the *traA* gene encoding a protein of the conserved conjugal transfer machinery of *Rhodococcus* spp. plasmids [6]. *Rhodococcus equi* ATCC 33701 was used as a rifampicin-susceptible reference strain.

2.2. In vitro antibiotic susceptibility testing

Antibiotic susceptibility was determined using the standard DD method on Mueller–Hinton agar supplemented with 5% lysed horse blood (Oxoid) and 20 μg/mL β-nicotinamide adenine dinucleotide (β-NAD) (Thermo Fisher Scientific) according to the 2019 Antibiogram Committee of the French Society for Microbiology (CA-SFM) guidelines [34]. Antibiotic discs (Oxoid) containing oxacillin (5 μg), gentamicin (500 μg), streptomycin (500 μg), erythromycin (15 μg), spiramycin (100 μg), lincomycin (15 μg), enrofloxacin (5 μg), trimethoprim/sulfamethoxazole (SXT; 1.25/23.75 μg), azithromycin (15 μg), clarithromycin (15 μg) and rifampicin (30 μg) were used. In view of the absence of clinical breakpoints for *R. equi*, inhibition zone diameters were interpreted applying rifampicin breakpoints approved by the 2013 CA-SFM guideline for *Staphylococcus* spp. and *Streptococcus* spp. [35] and breakpoints for other antibiotics approved by the veterinary part of the 2019 CA-SFM guideline for *Streptococcus* spp. [36]; in the absence of recommendations, azithromycin and clarithromycin breakpoints are the same 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 to indicate that they were no longer wild-type strains.

Rifampicin minimum inhibitory concentrations (MICs) were determined by the BMD method using Mueller–Hinton broth supplemented with 5% lysed horse blood (Oxoid) and 20 μg/mL β-NAD (Thermo Fisher Scientific) according to the 2019 CA-SFM guideline for slow-growing bacteria including *Streptococcus* spp. and *Corynebacterium* spp. [34]. The protocol was performed as previously described [37]. Results were recorded as the lowest concentrations of rifampicin that inhibited visible growth of *R. equi*.

2.3. DNA extraction

A 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.

2.4. rpoB sequencing

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

2.5. MLST and phylogenetic tree

Seven housekeeping genes (*gapdh*, *tpi*, *mdh*, *icl*, *rpoB*, *recA* and *adk*) were amplified and sequenced as previously described [33]. The *Rhodococcus* MLST website (https://pubmlst.org/rhodococcus/) sited at the University of Oxford [38] was used to assign the allele numbers and sequence types (STs) after having previously trimmed to equivalent lengths all the sequences of each individual MLST locus like on the *Rhodococcus* MLST website. For each unique ST, sequences of the seven MLST loci were concatenated, giving a 4017-bp in-frame sequence. Concatenated sequences were aligned and a phylogenetic tree was drawn up using MEGA v.7.0 software [39] via the maximum-likelihood method based on the Tamura–Nei model. Support for internal nodes was estimated using the non-parametric bootstrap method with 1000 replicates. The tree was rooted with the ST06-concatenated sequences of the

Table 1
Origin, chromosomal and virulence plasmid PCR detection, rifampicin resistance, and RpoB amino acid substitutions for the 40 *Rhodococcus equi* strains studied.

Strain (animal) ID in the study	Strain alias	Year	Sample source	Equine breed	Sex	Age	Country	PCR detection			MLST	Rifampicin[a]		RpoB (AAs 477–705; *Escherichia coli* numbering) profile
								choE	*vapA*	*traA*		ZOI (mm) [S/I/R]	MIC (μg/mL)	
#01 (01)	MBE 755	2018	Abdominal abscess	Thoroughbred	Male	5 months	Normandy (FR)	+	+	+	ST1	20 [R]	64	X
#02 (02)	MBE 756	2018	Lung abscess	Thoroughbred	Male	5 months	Normandy (FR)	+	+	+	ST43	13 [R]	128	XIV
#03 (03)	MBE 578	2012	Muscle abscess	Pony[b]	Male	12 years	Mayotte (FR)	+[c]	+[c]	+[c]	ST7[c]	24 [I]	16	X
#04 (04)	MBE 597	2012	Perianal abscess	Pony[b]	Female	13 years	Mayotte (FR)	+[c]	+[c]	+[c]	ST10[c]	34 [S]	1	I (wild-type)
#05 (05)	MBE 363	2009	Lung abscess	Thoroughbred	Male	2 months	Normandy (FR)	+[c]	+[c]	+[c]	ST13[c]	34 [S]	1	I (wild-type)
#06 (06)	MBE 355	2009	Lung abscess	Thoroughbred	Male	3.75 months	Normandy (FR)	+[c]	+[c]	+[c]	ST16[c]	34 [S]	1	I (wild-type)
#07 (07)	MBE 757	2008	Lung	Thoroughbred	Male	3.25 months	Normandy (FR)	+	+	+	ST24	19 [R]	128	X
#08 (08)	MBE 758	2008	Lung abscess	French Trotter	Female	2.5 months	Normandy (FR)	+	+	+	ST24	20 [R]	64	X
#09 (09)	MBE 759	2008	Lung	Thoroughbred	Male	2 months	Normandy (FR)	+	+	+	ST2	34 [S]	1	I (wild-type)
#10 (10)	MBE 760	2007	Bladder	Thoroughbred	Male	4.5 months	Normandy (FR)	+	+	+	ST24	20 [R]	64	XI
#11 (11)	MBE 226	2006	Bone	French Trotter	Female	5.5 months	Normandy (FR)	+[c]	+[c]	+[c]	ST24[c]	34 [S]	1	III
#12 (12)	MBE 761	2006	Lung	Thoroughbred	Female	6 months	Normandy (FR)	+	+	+	ST13	35 [S]	2	VII
#13 (13)	MBE 192	2006	Placenta	French Trotter	Female	Foetus	Normandy (FR)	+[c]	+[c]	+[c]	ST24[c]	20 [R]	256	XII
#14 (14)	MBE 190	2006	Placenta	Thoroughbred	Male	Foetus	Normandy (FR)	+[c]	+[c]	+[c]	ST19[c]	36 [S]	0.5	II
#15 (15)	MBE 762	2005	Lung	French Trotter	Female	2 months	Normandy (FR)	+	+	+	ST3	34 [S]	1	I (wild-type)
#16 (16)	MBE 763	2005	Lung	French Trotter	Female	3.5 months	Normandy (FR)	+	+	+	ST24	36 [S]	1	I (wild-type)
#17 (17)	MBE 174	2005	Eye	French Trotter	Male	1.75 months	Normandy (FR)	+[c]	+[c]	+[c]	ST24[c]	38 [S]	2	VII
#18 (18)	MBE 173	2005	Bone	French Trotter	Male	2.75 months	Normandy (FR)	+[c]	+[c]	+[c]	ST16[c]	35 [S]	2	VI
#19 (19)[d]	MBE 764	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	ST19	22 [R]	64	X
#20 (19)[d]	MBE 765	2004	Lung abscess	Unknown	Unknown	Unknown	France	+	+	+	ST19	7 [R]	1024	XIV
#21 (19)[d]	MBE 171	2004	Tracheobronchial lymph node	Unknown	Unknown	Unknown	France	+[c]	+[c]	+[c]	ST19[c]	22 [R]	64	X
#22 (20)	MBE 167	2004	Bone	French Trotter	Female	3.5 months	Normandy (FR)	+[c]	+[c]	+[c]	ST16[c]	35 [S]	1	I (wild-type)
#23 (21)	MBE 163	2004	Liver	French Trotter	Female	2 h	Normandy (FR)	+[c]	+[c]	+[c]	ST1[c]	33 [S]	1	V
#24 (22)	MBE 158	2002	Lung	French Trotter	Male	2 months	Normandy (FR)	+[c]	+[c]	+[c]	ST1[c]	34 [S]	1	VI
#25 (23)	MBE 766	2001	Lung	French Trotter	Male	2 months	Normandy (FR)	+	+	+	ST2	36 [S]	1	VI
#26 (24)	MBE 767	1999	Faeces	Unknown	Unknown	Foal	Normandy (FR)	+	+	+	ST19	36 [S]	0.5	I (wild-type)
#27 (25)[e]	MBE 768	1998	Lung	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	1024	XIV
#28 (25)[e]	MBE 769	1998	Lung abscess	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	1024	XIV
#29 (25)[e]	MBE 770	1998	Uterus	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	22 [R]	32	X
#30 (25)[e]	MBE 771	1998	Spleen	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	15 [R]	1024	XIV
#31 (25)[e]	MBE 772	1998	Tracheobronchial lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	1024	XIV
#32 (25)[e]	MBE 773	1998	Colic lymph node	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	512	XIV
#33 (25)[e]	MBE 774	1998	Digestive abscess n°1	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	27 [I]	8	IX
#34 (25)[e]	MBE 775	1998	Digestive abscess n°2	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	31 [S]	4	VIII
#35 (25)[e]	MBE 776	1998	Joint	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	30 [S]	1	IV
#36 (25)[e]	MBE 777	1998	Small colon	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	512	XIII
#37 (25)[e]	MBE 778	1998	Tracheobronchial fluid	Thoroughbred	Female	2.8 years	Normandy (FR)	+	+	+	ST24	7 [R]	1024	XIV
#38 (26)	MBE 123	1998	Lung	Thoroughbred	Female	2 months	Normandy (FR)	+[c]	+[c]	+[c]	ST1[c]	36 [S]	0.5	I (wild-type)
#39 (27)	MBE 122	1998	Lung	Thoroughbred	Female	1.5 months	Normandy (FR)	+	–[f]	–[c]	ST16[c]	36 [S]	0.5	I (wild-type)

Table 1 (*Continued*)

Strain (animal) ID in the study	Strain alias	Year	Sample source	Equine breed	Sex	Age	Country	PCR detection			MLST	Rifampicin[a]		RpoB (AAs 477–705; *Escherichia coli* numbering) profile
								choE	*vapA*	*traA*		ZOI (mm) [S/I/R]	MIC (μg/mL)	
#40 (25)[e]	MBE 179	1998	Faeces	Thoroughbred	Female	2.8 years	Normandy (FR)	+	–[f]	–	ST24	20 [R]	128	X
ATCC 33701		Unknown	Lung	Unknown	Unknown	Foal	Ontario (CA)	+[c]	+[c]	+[c]	ST1[c]	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)

MLST, multilocus sequence typing; ZOI, zone of inhibition; S, susceptible; I, intermediate; R, resistant; MIC, minimum inhibitory concentration; FR, France; CA, Canada.
[a] 30 μg rifampicin inhibition zone diameters (mm) and rifampicin MICs (μg/mL) were determined, respectively, by disk diffusion and broth macrodilution.
[b] From the atypical outbreak of *R. equi* infections in adult ponies on Mayotte in 2012 [45].
[c] PCR and MLST results extracted from Duquesne et al. [33]: +, detected; –, not detected; ND, not determined.
[d] Same animal (no. 19) but different sample sources.
[e] Same animal (no. 25) but different sample sources; the strain from faeces was isolated 2 days before necropsy.
[f] No virulence plasmid was detected.
[g] Rifampicin susceptibility of the reference ATCC 33701 and 103S strains was taken from the literature [15,40].

Rhodococcus sp. MBE 538 strain as the outgroup, extracted from the *Rhodococcus* MLST website.

3. Results

The 40 *R. equi* strains studied were from 27 horses, with 1 strain per animal except for horses no. 11 (3 strains) and no. 25 (12 strains) (Table 1). They were confirmed as *R. equi* by PCR (positive *choE* PCR result), and the equine pVAPA plasmid was detected by PCR in 38 of them (positive *traA* and *vapA* PCR results). The two plasmid-less strains (negative *traA* and *vapA* PCR results) were both isolated in 1998, including strain #39 from the lung of animal no. 27 and strain #40 from the faeces of animal no. 25 collected 2 days before its death and necropsy; interestingly, 11 pVAPA strains (#27 to #37) (Table 1) were isolated from different organs of this same animal (no. 25).

3.1. General susceptibility study

The susceptibility of the 40 strains to 11 antibiotics was determined by DD (Table 2). All 40 were susceptible to gentamicin, streptomycin, erythromycin, azithromycin, clarithromycin and enrofloxacin but were non-susceptible to lincomycin and oxacillin, except one strain susceptible to lincomycin. Approximately one-half of the strains were also non-susceptible to spiramycin (45.0%), rifampicin (50.0%) and SXT (57.5%). A focus on rifampicin susceptibility determined by DD and BMD is presented in Table 1. The 20 strains classified by DD as susceptible to rifampicin (inhibition zone diameters of 30–36 mm) had MICs of ≤4 μg/mL, whilst the 20 strains classified by DD as non-susceptible to rifampicin (inhibition zone diameters of 7–24 mm) had MICs of ≥8 μg/mL. The strains that were not susceptible to rifampicin were isolated between 1998 and 2018 from nine equids of widely varying breed, sex, age and sample source.

3.2. RpoB sequence analysis

RpoB amino acid sequences encompassing positions 477–705 (*E. coli* numbering) were obtained for the 40 strains and *R. equi* ATCC 33701. Fourteen different RpoB amino acid sequences, designated RpoB profiles I–XIV, were observed, including the wild-type RpoB (GenBank accession no. CBH49657) [40] of the rifampicin-susceptible *R. equi* 103S (Table 1 and Fig. 1). RpoB profile I corresponded to the wild-type sequence. It was found in ten strains with MICs of 0.5–1 μg/mL and characterised by DD as susceptible to rifampicin as well as in the rifampicin-susceptible *R. equi* ATCC 33701. RpoB profiles II–XIV differed from the wild-type sequence by the presence of one to five substitutions per profile, for a total of nine different substitutions at four sites in cluster I (Thr508Ala, Asp516Val, Asp516Tyr, His526Asn, His526Asp and Ser531Leu) and three sites outside clusters I, II and III (Thr491Ser, Tyr653Asn and Asn678His) (Fig. 1). RpoB profiles II–VIII were found in ten strains with MICs of 0.5–4 μg/mL and characterised by DD as susceptible to rifampicin, whilst RpoB profiles IX–XIV were found in 20 strains with MICs of 8–1024 μg/mL and characterised by DD as non-susceptible to rifampicin (Table 1 and Fig. 1).

Comparative analysis of in vitro rifampicin susceptibility testing and RpoB substitutions shows that: (i) substitutions outside clusters I–III and the Thr508Ala substitution in cluster I do not appear to confer rifampicin resistance because they were found both in strains that are susceptible and strains that are not susceptible to rifampicin by DD and with MICs of 0.5–512 μg/mL. They were observed in a single substitution for six strains found by DD to be susceptible to rifampicin with MICs ≤2 μg/mL or in RpoB profiles with multiple substitutions; (ii) the Asp516Tyr (MIC, 8 μg/mL), Ser531Leu (MIC, 16–256 μg/mL) and His526Asp (MIC, 512–

Table 2
Antimicrobial resistance in the 40 *Rhodococcus equi* strains studied as determined by disk diffusion.

Antimicrobial class	Antibiotic	Breakpoints (mm) S≥/R<	No. of non-susceptible strains	Proportion (%) of non-susceptible strains (95% CI)[a]
β-Lactams	Oxacillin	21/21[b]	40	100
Aminoglycosides	Gentamicin	17/11[b]	0	0
	Streptomycin	14/12[b]	0	0
Macrolides	Erythromycin	22/17[b]	0	0
	Azithromycin	22/17[c]	0	0
	Clarithromycin	22/17[c]	0	0
	Spiramycin	18/14[b]	18	45.0 (29.6–60.4)
Lincosamides	Lincomycin	21/17[b]	39	97.7 (92.7–100)
Fluoroquinolones	Enrofloxacin	22/17[b]	0	0
Folate pathway inhibitors	SXT	16/10[b]	23	57.5 (42.2–72.8)
Rifamycins	Rifampicin	29/24[d]	20	50.0 (34.5–65.5)

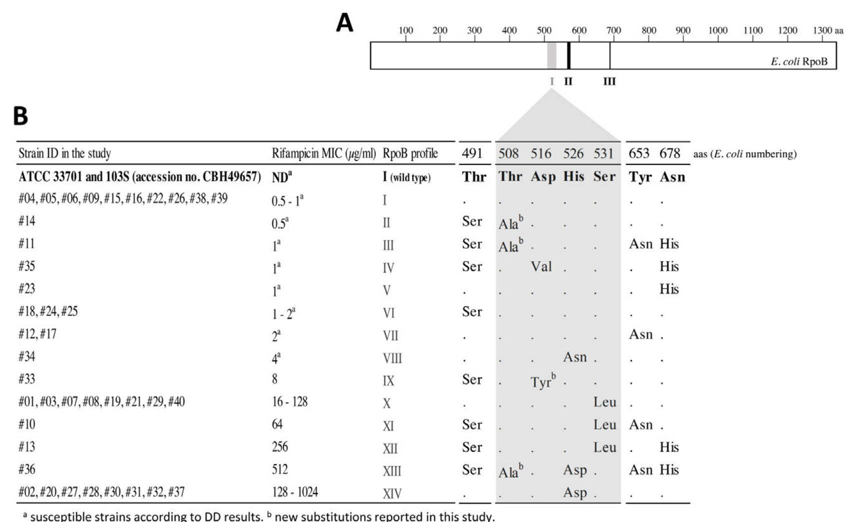
S, susceptible; R, resistant; SXT, trimethoprim/sulfamethoxazole.
[a] The indicator of resistance was defined as the ratio between the number of non-susceptible strains and the total number of strains ($n = 40$). Their confidence intervals (CIs) were calculated using the exact binomial method.
[b] Approved by the veterinary part of the CA-SFM [36] for *Streptococcus* spp.
[c] Breakpoints for azithromycin and clarithromycin are the same as for erythromycin.
[d] Approved by the CA-SFM [35] for *Staphylococcus* spp. and *Streptococcus* spp.

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 μg/mL, as a single substitution or in RpoB profiles with multiple substitutions not appearing to confer rifampicin resistance; (iii) the His526Asn substitution was observed in a single substitution (RpoB profile IV) in strain #34, which was found by DD to be susceptible to rifampicin but with an MIC of 4 μg/mL, suggesting it might actually be considered as having some degree of resistance; (iv) the Asp516Val substitution was observed together with multiple other substitutions apparently unrelated to rifampicin resistance (RpoB profile IV) in strain #35, found by DD to be susceptible to rifampicin with a MIC of 1 μg/mL (Figs. 1 and 2).

3.3. MLST analysis

MLST analysis was used to investigate a possible association between RpoB profiles and STs. The 40 strains and *R. equi* ATCC 33701 were divided into nine existing STs as well as 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 all cases only one ST was found per animal (Table 1). The ST distribution in the phylogenetic tree showed two distinct phylogenetic clusters: one (ST7 and ST10) with both strains from Mayotte (a French overseas island in the Indian Ocean), and one with *R. equi* ATCC 33701 from Ontario (Canada) and the 38 strains from metropolitan France (Fig. 3). No association was observed between RpoB profiles and dominant ST1, ST19 and ST24, or even the Mayotte and Normandy clusters. It is noteworthy that ST16 was found only in strains 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.

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. 1).



Strain ID in the study	Rifampicin MIC (μg/ml)	RpoB profile	491	508	516	526	531	653	678
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]	I	.	.	.	.	.	.	.
#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	X	.	.	.	.	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	.	.	.

aas (*E. coli* numbering)

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

Fig. 1. Distribution of rifampicin inhibition zone diameters and minimum inhibitory concentrations (MICs) as determined by, respectively, disk 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 *Rhodococcus 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.

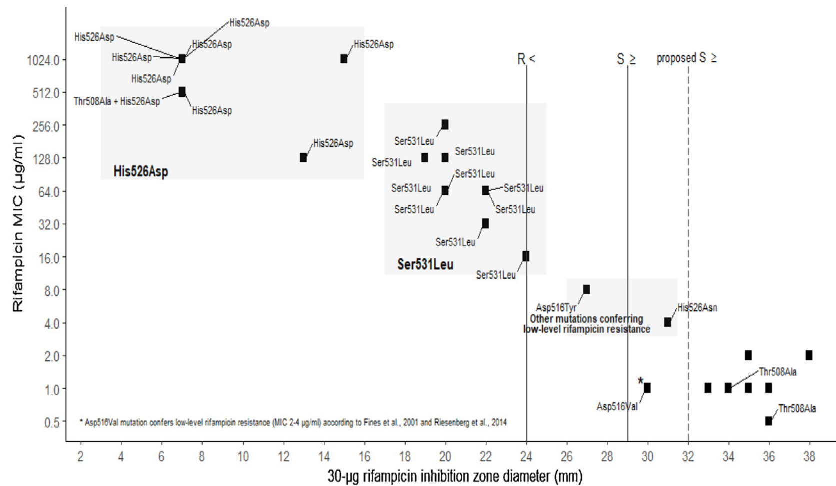


Fig. 2. Phylogenetic tree of *Rhodococcus equi* species based on multilocus sequence typing (MLST) sequences. The maximum-likelihood phylogenetic tree was based on the alignment of 4017-bp concatenated DNA sequences of the seven MLST loci for 37 *R. equi* STs recorded in the *Rhodococcus* MLST database (https://pubmlst.org/rhodococcus/) and the new ST (ST43) obtained in this study. The tree was rooted with the ST06-concatenated sequences of the *Rhodococcus* sp. MBE 538 strain as the outgroup, extracted from the *Rhodococcus* MLST database. The bootstrap values were calculated from 1000 replicates. Black arrows represent the STs of the 40 strains studied and *R. equi* ATCC 33701. RpoB profiles I–XIV are indicated after the black arrows with the number of horses concerned in parentheses; the year of collection was indicated when animals were concerned with the isolation of rifampicin-non-susceptible strains. [a]His526Asp or Ser531Leu correlated with high-level rifampicin resistance; [b]Asp516Tyr correlated with low-level rifampicin resistance; [c]His526Asn or Asp516Val previously reported as correlated with low-level rifampicin resistance, and observed, respectively, in strain #34 (susceptible to rifampicin by DD with an MIC of 4 μg/mL) and in strain #35 (susceptible to rifampicin by DD with a MIC of 1 μg/mL). Both strains were isolated from animal no. 25 from which ten other strains were also isolated, all being non-susceptible to rifampicin by DD with MICs of 8–1024 μg/mL. DD, disk diffusion; MIC, minimum inhibitory concentration.

4. Discussion

This study reports the presence of nine French cases of rifampicin resistance in horses infected by *R. equi* between 1998 and 2018. The 20 *R. equi* strains recovered from these cases and found to exhibit resistance to rifampicin correspond to 1% of the *R. equi* ANSES collection built up between 1995 and 2018 from equine samples collected during necropsies or environmental samples from stud farms. These strains were all susceptible to macrolides. 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% were erythromycin-resistant according to the data presented. Thus, the French situation appears to differ from that in Kentucky, USA, where rifampicin and macrolide resistance has considerably increased over the past two decades [18–22] 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.

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. and *Streptococcus* spp. breakpoints approved by the 2013 CA-SFM guideline [35], and rifampicin MICs were compared with DD and *rpoB* sequencing results. The interpretative results of inhibition zone diameters and MICs are consistent with each other, with the 20 strains found by DD to be non-susceptible to rifampicin all having MICs with higher values than the 20 strains found by DD to be susceptible to rifampicin. The consistency of results was also observed between rifampicin susceptibility testing and *rpoB* sequencing. All of the strains found by DD to be non-susceptible to rifampicin systematically had a substitution at one of the three sites (Asp516, His526 and Ser531) in cluster I that are frequently encountered and confer rifampicin resistance [10,24]. However, 20% of these strains also showed one to four additional substitutions (outside clusters I–III and Thr508Ala in cluster I) apparently not involved in rifampicin resistance. The clinical significance of this finding needs to be further explored.

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, high-level resistance was correlated with the presence of His526Asp (MIC, 512–1024 μg/mL; nine strains) or Ser531Leu (MIC, 16–256 μg/mL; ten strains), whilst low-level resistance was correlated with the presence of Asp516Tyr (MIC, 8 μg/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 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 rifampicin resistance of 8 μg/mL [15,28]. Substitutions in other regions of RpoB than clusters I–III or other mechanisms of rifampicin resistance may explain this finding and even the presence of rare rifampicin-resistant *R. equi* strains with no substitution in clusters I–III of RpoB [28]. To our knowledge, Asp516Tyr has never been described before in *R. equi* but was previously described in rifampicin-resistant *Mycobacterium tuberculosis* strains [41,42].

Regarding the 20 strains found by DD to be susceptible to rifampicin, 50% showed no substitution in RpoB amino acid sequences 477–705 and 40% showed only substitutions not appearing to confer rifampicin resistance. Regarding the remaining 10%, strains #34 and #35 had one substitution in cluster I (with additional substitutions outside clusters I–III in the case of strain #35), being His526Asn and Asp516Val, respectively. These have previously been described in *R. equi* strains as leading to low-level rifampicin resistance of 1–8 μg/mL [15,26,28]. Both strains were isolated from animal no. 25 from which ten other strains were also isolated, all being found by DD to be non-susceptible to rifampicin and with MICs of 8–1024 μg/mL. In this context, strains #34 and #35 should be considered non-susceptible to rifampicin and we propose that ≥32 mm should be used as a breakpoint for susceptibility instead of ≥29 mm for a 30 μg rifampicin disk (Fig. 2). However, our findings do not allow us to propose

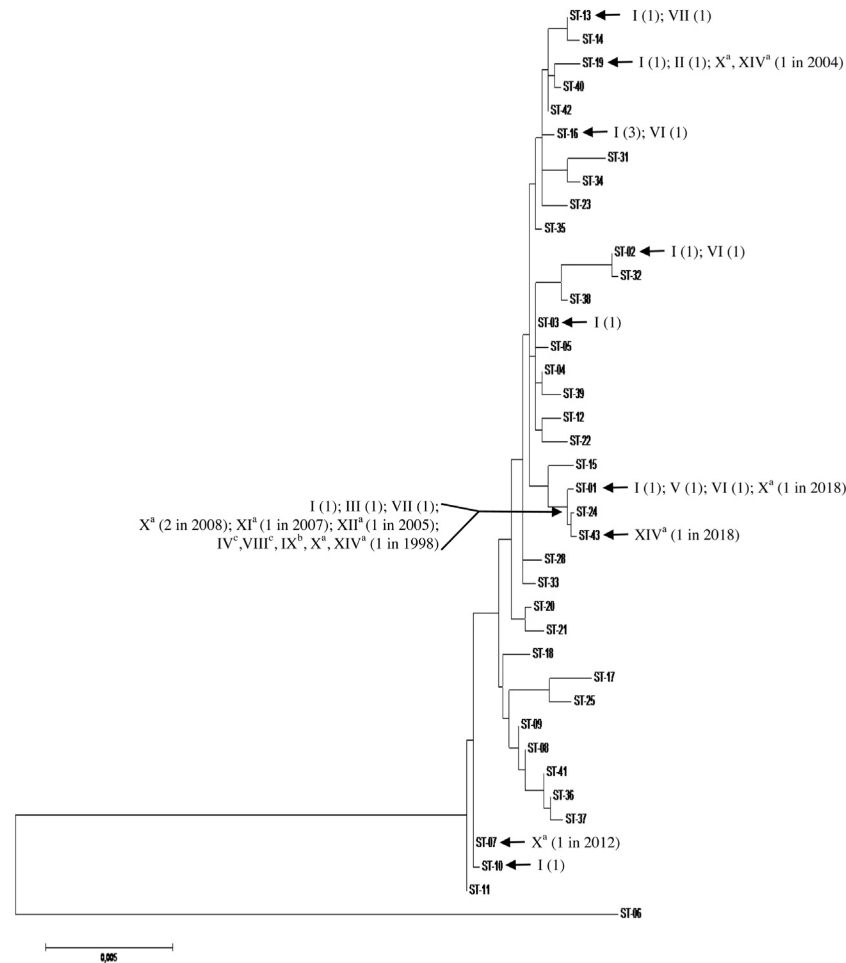


Fig. 3. Schematic positions of RpoB substitutions in the 40 *Rhodococcus equi* strains studied. (A) Map of *Escherichia coli* RpoB with the location of rifampicin clusters I (507–533), II (563–572) and III (687). (B) Distribution of substitutions observed in RpoB amino acid sequences 477–705 (*E. coli* numbering). Fourteen RpoB amino acid sequences, artificially named RpoB profiles I–XIV, were observed, including the wild-type RpoB (GenBank accession no. CBH49657). The rifampicin minimum inhibitory concentrations (MICs) determined by broth macrodilution are indicated. aa, amino acid; DD, disk diffusion.

breakpoints for rifampicin MICs obtained by the BMD method. Breakpoints of ≤1 μg/mL for susceptible and ≥4 μg/mL for resistant have already been reported in the literature for *R. equi* and correspond to Clinical and Laboratory Standards Institute (CLSI) interpretative criteria for *S. aureus* [43], which is probably the most consistent with our DD and *rpoB* sequencing results.

In this study, the nine horses concerned with the isolation of rifampicin non-susceptible strains were distributed throughout the phylogenetic tree of *R. equi* species based on MLST sequences (Fig. 3) and no association was observed between rifampicin resistance and dominant ST1, ST19 and ST24 (or any other information on horses, such as the year of collection) (Fig. 3). These findings suggest a non-clonal dissemination of rifampicin-resistant strains in France, contrary to what has been observed from equine *R. equi* strains isolated in the USA [44]. It should, however, be noted that MLST results generated with next-generation sequencing (NGS) result in a better detection of this clonality than Sanger sequencing [44]. In our context, it is unlikely that NGS-MLST would have allowed us to conclude differently.

In conclusion, this study reports nine French cases of rifampicin resistance in horses infected by *R. equi* between 1995 and 2018. The relationship between rifampicin susceptibility tested using both DD and BMD methods and the substitutions observed in RpoB amino acid sequences 477–705 (*E. coli* numbering) revealed the presence of substitutions that were correlated with high-level (His526Asp, Ser531Leu), low-level (Asp516Tyr), and low- or borderline-level (His526Asn and Asp516Val) rifampicin resistance, with the Asp516Tyr substitution having never been previously described in *R. equi*. We observed that the standard 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. Further studies are needed to define specific *R. equi* clinical breakpoints for rifampicin and macrolides used in combination to treat *R. equi* infections.

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Conflict of interest

None declared.

Ethical approval

Not required.

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