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Molecular basis of rifampicin resistance in methicillin-resistant *Staphylococcus pseudintermedius* isolates from dogs

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Background: Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) often display resistance to almost all classes of antimicrobial agents used in veterinary medicine. In the present study, we investigated the emergence of rifampicin resistance in MRSP, the persistence of these isolates and identified the corresponding mutations in the *rpoB* gene.

Methods: In addition to two rifampicin-resistant MRSP isolates from a multicentre study, consecutive MRSP isolates collected prior to and after rifampicin therapy from nine dogs at five Dutch veterinary hospitals were included in this study. The isolates were tested for resistance to rifampicin and other antimicrobial agents. The rifampicin resistance-determining region (RRDR) within the *rpoB* gene of the rifampicin-resistant and -susceptible isolates was amplified by PCR and sequenced. PFGE served to determine the genetic relationships of the MRSP isolates.

Results: Two MRSP isolates of the multicentre study showed mutations at position 513 or 522 in the RRDR of the *rpoB* gene. In contrast to the rifampicin-susceptible isolates, all rifampicin-resistant MRSP isolates showed mutations at one or two of the amino acid positions 508, 509, 513, 516, 522, 526 and 531. In most strains, a single amino acid exchange was observed. PFGE analysis confirmed that the rifampicin-resistant MRSP isolates were indistinguishable from or closely related to the rifampicin-susceptible isolate obtained from the same dog prior to rifampicin application.

Conclusions: Therapy of MRSP infections with rifampicin results in the rapid emergence of rifampicin resistance and these isolates can persist for months. As a consequence, single therapy with rifampicin is not recommended.

Keywords: MRSP, antimicrobial agents, *rpoB* gene, mutation, multiresistance

Introduction

Staphylococcus pseudintermedius is the most frequent causative agent of canine pyoderma and is also associated with surgical and non-surgical wound infections, urinary tract infections, otitis externa and various other infections in dogs. In the past, *S. pseudintermedius* isolates were generally susceptible to β-lactam antibiotics, but recently methicillin-resistant *S. pseudintermedius* (MRSP) has emerged.[1] Two major clonal lineages of MRSP have disseminated in Europe/Hong Kong and in North America, respectively, and both of them display resistance to almost all classes of antimicrobial agents used in veterinary medicine.[2,3] During a recent multicentre study, 2 rifampicin-resistant MRSP isolates were identified among 103 isolates from dogs while 0 of the 12 MRSP isolates from cats showed this resistance property.[4] To date, rifampicin resistance in staphylococci of animal origin and also in MRSP has been detected very rarely.[2] Rifampicin acts by binding to the β-subunit of the DNA-dependent RNA polymerase. In most bacteria, rifampicin resistance is mediated by mutations in the *rpoB* gene encoding the β-subunit of RNA polymerase.[5] Such mutations have been described in *Staphylococcus aureus*.[6] The mechanism of rifampicin resistance in MRSP is currently unknown.

Different names for this antimicrobial agent are used in different parts of the world and may be a source of confusion. Rifampicin is the International Nonproprietary Name (INN) and is also the designation listed as British Approved Name (BAN) and Japanese Accepted Name (JAN). However, in North America, the official US Pharmacopeia (USP) name and the US Adopted Name (USAN) are rifampin.

The objectives of the present study were to investigate (i) the molecular basis of rifampicin resistance in the two canine isolates from the multicentre study and (ii) the emergence of rifampicin resistance in canine MRSP isolates after rifampicin

treatment of the corresponding dogs, the persistence of these isolates over time and to further characterize these isolates.

Materials and methods

Bacterial strains and species identification

Both canine MRSP isolates from the multicentre study originated from wound infections, one isolate was from the Netherlands and the other from Italy.[2] The database of the Veterinary Microbiological Diagnostic Centre (VMDC) of Utrecht University was screened for more patients with rifampicin-resistant MRSP. Another nine dogs were identified that had wound infections ($n=2$), dermatitis ($n=5$), paronychia ($n=1$) or otitis externa ($n=1$). From all dogs, at least one rifampicin-susceptible isolate had previously been isolated from the same infection site. Per patient, two to five isolates, including one or two rifampicin-susceptible isolates, taken at different timepoints and stored as part of VMDC's strain collection, were included in this study. This resulted in a total of 33 MRSP isolates from these nine dogs. All dogs had been treated with rifampicin.

All isolates were classified as methicillin resistant by oxacillin MICs of at least 0.5 mg/L. The isolates had been identified as members of the *Staphylococcus intermedius* group using standard techniques (colony morphology, Gram's stain, catalase and coagulase tests) and the ID 32 STAPH system (bioMérieux, Marcy l'Étoile, France). Species confirmation as *S. pseudintermedius* was conducted by MboI digestion of a PCR-amplified internal fragment of the *pta* gene.[2,7]

Antimicrobial susceptibility testing and PFGE analysis

Antimicrobial susceptibility testing was performed by broth microdilution using custom-made microtitre panels (MCS Diagnostics, Swalmen, The Netherlands). In total, 10–12 concentrations in 2-fold dilution series were tested for 25 antimicrobial agents and two combinations of antimicrobial agents. These comprised penicillins (penicillin G, ampicillin, amoxicillin/clavulanic acid 2:1 and oxacillin), cephalosporins (cefalotin, cefotaxime, cefoperazone, cefquinome and ceftiofur), tetracyclines (tetracycline and doxycycline), macrolides (erythromycin, tilmicosin, tylosin, tulathromycin and spiramycin), lincosamides (clindamycin and pirlimycin), folate pathway inhibitors (trimethoprim and trimethoprim/sulfamethoxazole 1:19), an aminoglycoside (gentamicin), an aminocyclitol (spectinomycin), phenicols (chloramphenicol and florfenicol), a glycopeptide (vancomycin), a fluoroquinolone (enrofloxacin) and a pleuromutilin (tiamulin). Rifampicin MICs were determined by broth macrodilution using an initial 2-fold dilution series of 2–1024 mg/L. Isolates that showed no growth at the lowest concentration were re-tested using a 2-fold dilution series of 0.002–2 mg/L. Performance of the tests and evaluation of the MIC values followed the recommendations given in the documents M31-A3 and M100-S20 of the CLSI.[8,9] The reference strains *S. aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 served as quality control strains.

To determine the genetic relatedness of isolates obtained from the same dog, PFGE using either SmaI or ApaI was applied. DNA preparation and digestion of the DNA followed previous specifications.[2,10] The SmaI fragment patterns were separated over 24 h at 5.6 V/cm and with pulse time ramping from 2 to 5 s.[2] The pulse times for ApaI digests were increased from 2 to 5 s for 20 h.[10]

Analysis of the rifampicin resistance-determining region (RRDR)

In other bacteria such as *E. coli*, *Rhodococcus equi* and *Mycobacterium tuberculosis*, the region between codons 503 and 533 (*E. coli* numbering) of the *rpoB* gene has been identified as the RRDR. Structural comparisons with the partial sequence of the *rpoB* gene of *S. intermedius* (database entry AF325869)[11] led to the detection of the RRDR. Based on this sequence, the primers rpoB-fw (5′-GCCGTCTACGTTCAGTTGGT-3′) and rpoB-rev (5′-CGCCATCGTTGTGTTGTTAC-3′) were selected and used to amplify a 593 bp segment of the *rpoB* gene, which comprised the complete RRDR. A standard PCR program, which includes 2 min of initial denaturation at 94°C, followed by 30 cycles each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 57°C and 30 s of primer extension at 72°C as well as a terminal elongation step of 5 min at 72°C, was applied. The amplicons obtained were sequenced (MWG Eurofins, Ebersberg, Germany) on both strands using the same primers. The *rpoB* sequences were checked for mutations that resulted in amino acid alterations within the RRDR.

Results

Rifampicin-resistant MRSP from the multicentre study

The two MRSP isolates from the multicentre study revealed high rifampicin MICs of ≥2048 mg/L and showed two different mutations. The MRSP isolate from the Netherlands had CGA instead of the wild-type CAA at position 513, which resulted in the amino acid change Gln513→Arg513. The MRSP isolate from Italy had the single nucleotide exchange GCT→GAT in the RRDR of *rpoB*, which led to the amino acid alteration Ala→Asp at codon 522.

Rifampicin-resistant MRSP from the VMDC of Utrecht University

The 11 rifampicin-susceptible MRSP isolates showed low rifampicin MICs ranging between ≤0.002 and 0.015 mg/L, whereas the 21 rifampicin-resistant isolates had MICs of 128 to ≥2048 mg/L. A single isolate was classified as intermediate by its rifampicin MIC of 2 mg/L (Table 1). The sampling dates of all isolates as well as the start of rifampicin therapy are given in Table 1. The nine dogs from which these isolates originated had been treated for various diseases in at least one of the five veterinary hospitals A—E. All isolates were classified as multiresistant (Table 1) since they exhibited resistance to members of more than three different classes of antimicrobial agents.[12] All rifampicin-resistant isolates exhibited at least one mutation within the RRDR of the *rpoB* gene, while no mutations were detectable in any of the susceptible isolates and the intermediate isolate. A summary of the mutations detected in the resistant isolates is given in Table 1 while the PFGE patterns are provided in Figure 1.

Patient 1 from hospital A in the province of Utrecht suffered from a non-surgical wound infection and was treated with amoxicillin/clavulanic acid and thereafter with rifampicin. Among the five isolates collected, the single rifampicin-susceptible isolate 1-1 differed in its SmaI pattern by one fragment from the resistant isolates 1-2, 1-3 and 1-4 and by two fragments from the resistant isolate 1-5. All isolates showed a similar multiresistance phenotype. The four resistant isolates had the same *rpoB* mutation at codon 526: CAC (His)→CGC (Arg).

Patient 2 from hospital B in the province of Utrecht had a surgical wound infection after a car accident. The dog received rifampicin for 7 days and tetracycline thereafter. A rifampicin-susceptible MRSP was cultured from a sample taken during

Table 1. Characteristics of the rifampicin-susceptible and the rifampicin-resistant MRSP isolates obtained from nine dogs admitted to veterinary hospitals in the Netherlands

Patient	MRSP isolate no.	Sampling date	Start of rifampicin therapy	Resistance phenotype[a]	Rifampicin MIC (mg/L)	*rpoB* mutation	PFGE pattern
1	1-1	10-12-2007		BLA-TET-ML-SXT-GEN-ENR	0.004	—	SmaI – 1
	1-2	04-02-2008	01-02-2008	BLA-TET-ML-SXT-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 1[c]
	1-3	11-06-2008		BLA-TET-ML-SXT-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 1[c]
	1-4	11-08-2008		BLA-TET-ML-SXT-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 1[c]
	1-5	02-09-2008		BLA-TET-ML-SXT-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 1[d]
2	2-1	12-02-2009		BLA-TET-ML-SXT-CHL-GEN-ENR	≤0.002	—	SmaI – 2
	2-2	13-03-2009	20-02-2009	BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	Ser531→Leu531	SmaI – 2
	2-3	10-04-2009		BLA-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 2
	2-4	21-12-2009		BLA-ML-SXT-GEN-ENR-RIF	≥2048	Ser531→Leu531	SmaI – 2
3	3-1	13-05-2009		BLA-TET-ML-SXT-CHL-GEN-ENR	0.015	—	SmaI – 3
	3-2	14-08-2009		BLA-TET-ML-SXT-CHL-GEN-ENR	0.004	—	SmaI – 3
	3-3	01-07-2009		BLA-TET-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Arg526	SmaI – 3
	3-4	20-10-2009 ear	06-10-2009	BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Tyr526	SmaI – 3
	3-5	20-10-2009 skin		BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Tyr526	SmaI – 3
4	4-1	25-11-2008		BLA-TET-ML-SXT	0.008	—	SmaI – 4
	4-2	22-01-2009		BLA-TET-ML-SXT	0.004	—	SmaI – 4
	4-3	03-03-2009	01-02-2009	BLA-TET-ML-SXT-RIF	≥2048	His526→Arg526	SmaI – 4
	4-4	02-04-2009		BLA-TET-ML-SXT-RIF	≥2048	His526→Arg526	SmaI – 4
	4-5	12-05-2009		BLA-TET-ML-SXT-RIF	≥2048	His526→Arg526	SmaI – 4
5	5-1	05-06-2009		BLA-ML-SXT-GEN-ENR	0.004	—	SmaI – 5
	5-2	06-07-2009	11-06-2009	BLA-ML-SXT-GEN-ENR-RIF	1024	Ala522→Asp522	SmaI – 5
6	6-1	06-01-2009		BLA-TET-ML-SXT-CHL-GEN-ENR	0.004	—	ApaI – 1
	6-2	24-02-2009	15-01-2009	BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Pro526	ApaI – 1
	6-3	09-03-2009		BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Pro526	ApaI – 1
7	7-1	28-05-2009		BLA-TET-ML-SXT-CHL-GEN-ENR	0.008	—	ApaI – 1
	7-2	24-09-2009	30-05-2009	BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	His526→Arg526	ApaI – 1
8	8-1	02-07-2009		BLA-TET-ML-SXT	≤0.002	—	SmaI – 6
	8-2	11-08-2009 skin	11-07-2009	BLA-ML-SXT-GEN-ENR-RIF	≥2048	His526→Tyr526	SmaI – 6
	8-3	11-08-2009 nose		BLA-TET-ML-SXT-GEN-TIA[b]-SPT-RIF	≥2048	Gln513→Leu513	SmaI – 7
	8-4	11-02-2010		BLA-ML-SXT-GEN-ENR	2	—	SmaI – 6[d]
9	9-1	02-07-2009		BLA-TET-ML-SXT-GEN-TIA[b]-SPT	≤0.002	—	SmaI – 7
	9-2	03-09-2009	12-07-2009	BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	128	Ser508→Asn508 + Ser509→Pro509	SmaI – 8
	9-3	06-10-2009		BLA-TET-ML-SXT-CHL-GEN-ENR-RIF	≥2048	Ser509→Pro509 + Asp516→Asn516	SmaI – 8

[a]BLA, β-lactams including oxacillin; TET, tetracyclines; ML, macrolides and lincosamides; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; GEN, gentamicin; ENR, enrofloxacin; TIA, tiamulin; SPT, spectinomycin; RIF, rifampicin.

[b]Although there are no CLSI-approved breakpoints to classify staphylococci as resistant to tiamulin or spectinomycin, MRSP isolates with tiamulin MICs of ≥128 mg/L and spectinomycin MICs of ≥512 mg/L were considered tiamulin- and spectinomycin-resistant, respectively.

[c]One band difference in PFGE pattern compared with the pattern of the rifampicin-susceptible isolate of the same patient.

[d]Two bands difference in PFGE pattern compared with the pattern of the rifampicin-susceptible isolate of the same patient.

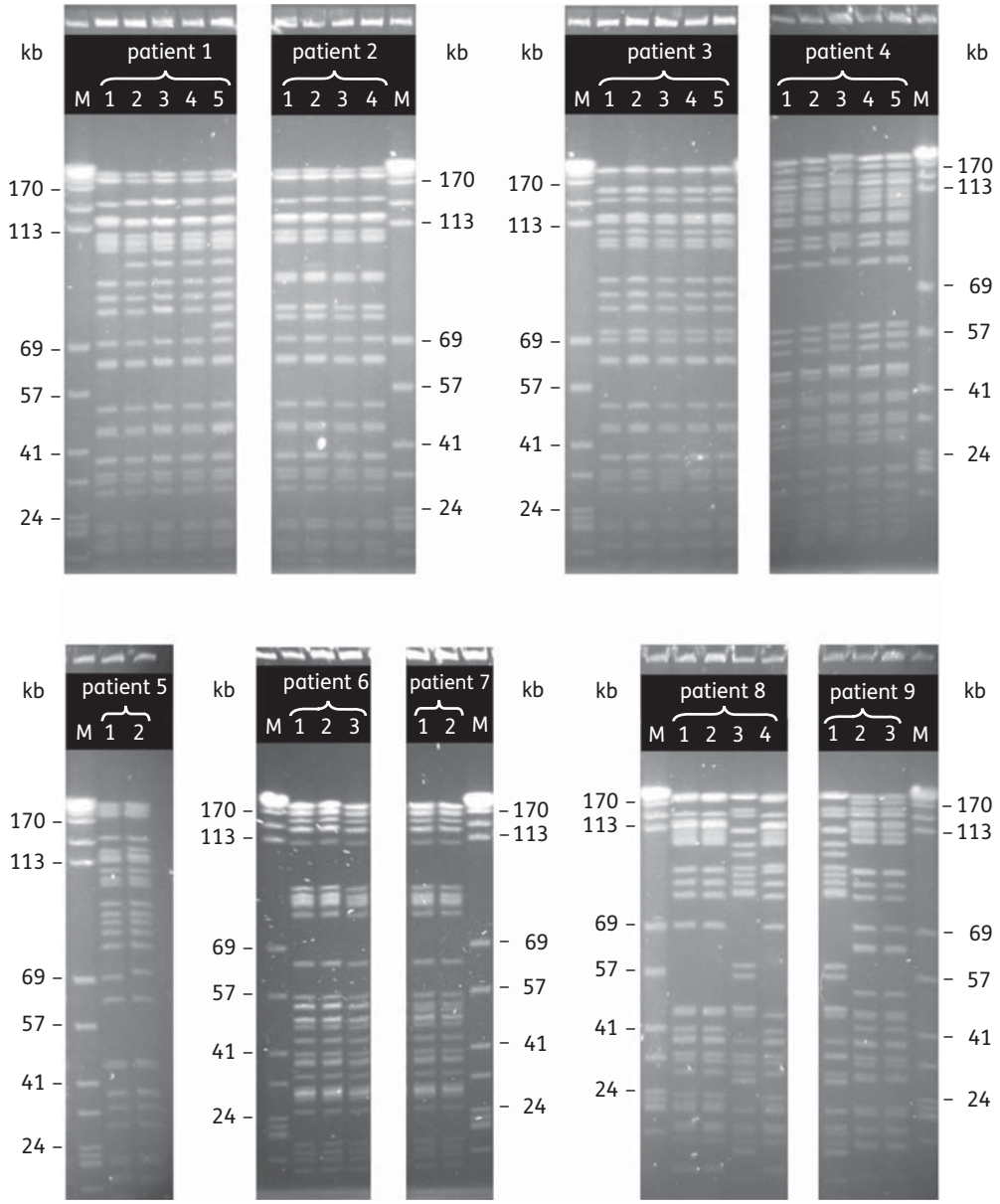

Figure 1. PFGE patterns of the MRSP isolates obtained from the nine dogs listed in Table 1. Lanes marked with an M contain the SmaI digest of *S. aureus* 8325. The fragment patterns of the isolates of patients 1–5, 8 and 9 were obtained with SmaI and those of patients 6 and 7 were obtained with ApaI.

surgery to repair a fracture and showed the same SmaI fragment pattern as the three rifampicin-resistant isolates obtained after rifampicin application. The rifampicin-resistant isolates 2-3 and 2-4 were tetracycline susceptible and isolate 2-4 was also chloramphenicol susceptible. The resistant isolates 2-2 and 2-4 showed the mutation TCA (Ser)→TTA (Leu) at codon 531, whereas the resistant isolate 2-3 exhibited the mutation CAC (His)→CGC (Arg) at codon 526.

Patient 3 had been treated in hospital C in the province of Noord-Brabant for dermatitis and in hospital D in the province of Noord-Holland for otitis externa. This dog received treatment with rifampicin + fusidic acid for 2 weeks. Five isolates, two rifampicin susceptible and three rifampicin resistant, were available. They showed indistinguishable SmaI fragment patterns. It should be noted that the first rifampicin-resistant MRSP isolate 3-3 was cultured 3 months prior to rifampicin therapy. This isolate had the mutation CAC (His)→CGC (Arg) at codon 526, whereas isolates 3-4 and 3-5 shared the same *rpoB* mutation CAC (His)→TAC (Tyr) at codon 526. In contrast to all other isolates from patient 3, isolate 3-3 proved to be susceptible to macrolides and lincosamides.

Patient 4 from hospital D had dermatitis and was first treated with enrofloxacin and then with rifampicin + trimethoprim/sulphonamide for 1 month and with enrofloxacin and fusidic acid thereafter. The two rifampicin-susceptible isolates obtained prior to rifampicin therapy and the three rifampicin-resistant

isolates obtained after rifampicin therapy showed indistinguishable SmaI fragment patterns and showed, besides rifampicin resistance, the same multiresistance pattern. The three resistant isolates 4-3, 4-4 and 4-5 exhibited the same mutation CAC (His)→CGC (Arg) at codon 526.

Patient 5 had paronychia and had been treated at clinic E in the province of Zuid-Holland with marbofloxacin followed by rifampicin+tetracycline. A rifampicin-susceptible and a rifampicin-resistant isolate were available. Both isolates showed the same multiresistance phenotype for non-rifampicin antimicrobial agents and also did not differ in their SmaI fragment patterns. The rifampicin-resistant isolate revealed the presence of the mutation GCT (Ala)→GAT (Asp) at codon 522.

Patients 6 and 7 both had pyoderma and had been treated at hospital D. Patient 6 received rifampicin+trimethoprim/sulphonamide for 14 days and fusidic acid+amikacin thereafter. Patient 7 was treated with a cephalosporin and then with rifampicin+fusidic acid for 4 months. The three isolates from patient 6 and the two isolates from patient 7 exhibited the same multiresistance phenotype for antimicrobial agents other than rifampicin. All five isolates were non-typeable with SmaI, but showed indistinguishable ApaI fragment patterns. The two resistant isolates 6-2 and 6-3 from patient 6 exhibited the mutation CAC (His)→CCC (Pro) at codon 526, whereas the single resistant isolate 7-2 from patient 7 had the mutation CAC (His)→CGC (Arg) at codon 526.

Patient 8 had also been treated for pyoderma at hospital D with a cephalosporin followed by rifampicin+fusidic acid for 1 month. The rifampicin-susceptible isolate 8-1 and the resistant isolate 8-2 shared indistinguishable SmaI fragment patterns from which the SmaI fragment pattern of isolate 8-4 differed by two fragments. The resistant isolate 8-3 differed distinctly in its SmaI fragment pattern from the other isolates obtained from this dog. Isolates 8-1 and 8-3 were enrofloxacin susceptible and tetracycline resistant, whereas isolates 8-2 and 8-4 were enrofloxacin resistant and tetracycline susceptible. Isolate 8-3 also showed a high MIC of tiamulin of ≥128 mg/L. Isolate 8-2 had the mutation CAC (His)→TAC (Tyr) at codon 526 while isolate 8-3 had the mutation CAA (Gln)→CTA (Leu) at codon 513. Isolate 8-4 had a rifampicin MIC of 2 mg/L and did not reveal a mutation in the RRDR of the *rpoB* gene.

Patient 9 had been treated for otitis externa at hospital D and had received fusidic acid+neomycin and thereafter rifampicin+fusidic acid for 1 month. The rifampicin-susceptible isolate 9-1 differed in its SmaI fragment pattern as well as in its additional resistances from isolates 9-2 and 9-3, but was indistinguishable from the rifampicin-resistant isolate 8-3 of patient 8. The two resistant isolates 9-2 and 9-3 shared the same SmaI fragment pattern. Both isolates had two *rpoB* mutations. Besides the mutation TCT (Ser)→CCT (Pro) at codon 509, which was present in both isolates, isolate 9-2 had the additional mutation AGC (Ser)→AAC (Asn) at codon 508 and isolate 9-3 the additional mutation GAC (Asp)→AAC (Asn) at codon 516.

Discussion

Rifampicin was introduced >40 years ago and is used mainly in the treatment of *M. tuberculosis* infections in humans and occasionally for *R. equi* infections in horses and immunocompromised humans. In addition, it is used for the eradication of *S. aureus* carriage in humans.[13] Rifampicin resistance develops quickly during treatment and rifampicin monotherapy is generally not recommended.[13] Therefore, combinations of rifampicin with other antimicrobial drugs such as tetracycline or fusidic acid are used to prevent the emergence of rifampicin resistance during therapy. In those cases, the drug used in combination with rifampicin should also display *in vitro* activity against the strain causing the infection. Based on the observation that MRSP strains involved in canine pyoderma often exhibit antimicrobial multiresistance, empirical therapy of this disease is not recommended. It should also be noted that systemic antibiotic therapy of canine pyoderma is often carried out in combination with antiseptic shampoos. Clinical studies in which dogs are treated using this combination are required to assess the efficacy of rifampicin for combination therapy of MRSP-associated canine pyoderma.

The data from the present study showed that rifampicin resistance emerged rapidly during rifampicin therapy even if rifampicin was used in combination with other antimicrobial drugs. However, patients 4 and 6 had been infected with MRSP isolates that were already resistant to trimethoprim/sulfamethoxazole and as such the combination rifampicin+trimethoprim/sulphonamide should not have been used for therapy. It should be noted that one rifampicin-resistant isolate from patient 3 was obtained 3 months before the dog was treated with rifampicin. This observation may either point to a previous non-recorded rifampicin treatment or the transfer of a rifampicin-resistant isolate from a previously treated dog, or indicate that the observed *rpoB* mutation occurred spontaneously. Rifampicin-resistant isolates with indistinguishable PGFE patterns and identical or similar resistance patterns were cultured for weeks or even months after rifampicin therapy had stopped, indicating that the resistant organisms can persist for months without selective pressure. It is generally thought that isolates with resistance-mediating mutations are less fit than their susceptible counterparts in the absence of selective pressure. The *rpoB* gene is an essential housekeeping gene and mutations in this gene could potentially compromise transcription efficiency resulting in a loss of fitness of the bacteria. Wichelhaus *et al.*[14] demonstrated that *in vitro*-selected mutations within the *rpoB* gene in *S. aureus* resulted in a reduced level of fitness of the bacteria. Clinical rifampicin-resistant *S. aureus* isolates, however, were not associated with a reduced level of fitness. This is in accordance with the findings of Enne *et al.*[15] who showed that the fitness cost of rifampicin-resistant *Enterococcus faecium* is variable and sometimes even absent and mutants with substitutions His526Gln were most fit *in vitro* and *in vivo*. This could be explained by the fact that the loss of biological fitness of the resistant bacteria can be overcome by the acquisition of compensatory mutations, thereby stabilizing the resistant bacteria within a population.[14] Previous studies on *R. equi* have also shown that different types of mutation within the RRDR of the *rpoB* gene can result in different levels of rifampicin resistance.[16,17] In contrast to these observations, all canine MRSP isolates investigated in this study displayed high-level rifampicin resistance (MICs ≥128 mg/L) regardless of the numbers of mutations present, the codons affected or the amino acids exchanged.

Our retrospective analysis of rifampicin-resistant MRSP isolates from dogs revealed a considerable number of mutations in the RRDR of the *rpoB* gene. In total, 10 different mutations involving seven codons were found. Similar observations have also been made with other bacteria, such as *R. equi* and *M. tuberculosis*.[16–19] In our test population, the mutation CAC (His)→CGC (Arg) at codon 526 was most frequently seen. It was detected in isolates from five of the nine dogs. Other mutations at codon 526, e.g. CAC (His)→TAC (Tyr) and CAC (His)→CCC (Pro), or mutations at codons 508, 509, 513, 516, 522 and 531 were seen more rarely (Table 1). Previous studies on field isolates of *R. equi* revealed that the positions Asp516, His526 and Ser531 play a key role in rifampicin resistance of *R. equi*. Mutations that resulted in a His526Arg exchange have also been detected in highly resistant *in vitro* mutants of *R. equi*.[16] A His526Tyr mutation has been identified in a rifampicin-resistant *R. equi* strain of an AIDS patient.[17] In rifampicin-resistant *M. tuberculosis*, the exchanges Ser531Leu, Asp516Val, His526Asp and His526Arg were detected most frequently.[19–21] In contrast to mutations at other positions in the RRDR, mutations at position 513 seem to occur rarely in *M. tuberculosis*.[21] In the present study two MRSP isolates, one from a Dutch dog (isolate 8-3) and one from another Dutch dog from the multicentre study, were found to have mutations at this position (Gln513Leu and Gln513Arg, respectively). Mutations at positions 508 and 509 also seem to be rare in *M. tuberculosis*,[5] but were found in the present study in two isolates from patient 9, in one isolate as a double mutation (Ser508Asn + Ser509Pro) and in the other isolate in combination with a mutation at codon 516 (Ser509Pro + Asp516Asn).

An interesting finding of the present study was that different mutations were found in MRSP isolates with similar or indistinguishable PFGE and resistance patterns isolated at different timepoints from the same infection site of the same patient; in two cases, two mutations even occurred in the same isolate. This finding underlines that mutations in the *rpoB* gene occur frequently in MRSP. Most studies on MRSP are one-point prevalence studies. However, studies investigating MRSP isolates from the same patients over time are an important tool in gaining a better understanding of the epidemiology of MRSP. Although six of the patients had visited the same veterinary hospital, the isolates they harboured had different mutations and/or different resistance patterns and, apart from a few exceptions, distinct SmaI PFGE patterns. Although the isolates from patients 6 and 7 shared indistinguishable ApaI fragment patterns, they exhibited different mutations at codon 526. Thus, it can be assumed that no resistant clone was present and transferred to dogs admitted to this hospital, but that mutations occurred in genetically diverse MRSP isolates.

Even though clinical reports on the use of rifampicin are limited, there is at least one report of clinical efficacy using rifampicin monotherapy for the control of canine pyoderma.[22] Nevertheless, the use of rifampicin as monotherapy or combination therapy might rapidly result in high-level resistance to this drug by single or double nucleotide changes within the RRDR of the *rpoB* gene as observed in the present study. Therefore, monotherapy with this drug cannot be recommended for the treatment of infections due to multidrug-resistant MRSP. When combined with other antimicrobial agents, it is indispensable to determine their *in vitro* efficacy against the causative MRSP strain prior to the start of therapy. However, the results of this study showed that the use of rifampicin in combination with other antimicrobial agents does not necessarily prevent MRSP from developing resistance-mediating *rpoB* mutations. Rifampicin-resistant isolates with indistinguishable or similar PFGE patterns, but sometimes with different mutations, were found in isolates from the same patient obtained at different timepoints, indicating that rifampicin resistance can persist for longer periods and that diverse mutations occur in isolates with the same genetic background.

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Transparency declarations

None to declare.

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