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Genomic and *in vitro* pharmacodynamic analysis of rifampicin resistance in multidrug-resistant canine *Staphylococcus pseudintermedius* isolates

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Background – Antimicrobial resistance is a growing concern in canine *Staphylococcus pseudintermedius* dermatitis. Treatment with rifampicin (RFP) is considered only in meticillin-resistant and multidrug-resistant *S. pseudintermedius* (MDR-MRSP).

Hypothesis/Objectives – To determine an optimal RFP dosing for MDR-MRSP treatment without induction of RFP resistance and identify causal mutations for antimicrobial resistance.

Methods and materials – Time–kill assays were performed in a control isolate and three MDR-MRSP isolates at six clinically relevant concentrations [32 to 1,024 × MIC (the minimum inhibitory concentration)]. Whole-genome resequencing and bioinformatic analysis were performed in the resistant strains developed in this assay.

Results – The genomic analysis identified nine antimicrobial resistance genes (ARGs) in MDR-MRSP isolates, which are responsible for resistance to seven classes of antibiotics. RFP activity against all four isolates was consistent with a time-dependent and bacteriostatic response. RFP resistance was observed in six of the 28 time–kill assays, including concentrations 64 × MIC in MDR-MRSP1 isolates at 24 h, 32 × MIC in MDR-MRSP2 at 48 h, 32 × MIC in MDR-MRSP3 at 48 h and 256 × MIC in MDR-MRSP3 at 24 h. Genome-wide mutation analyses in these RFP-resistant strains discovered the causal mutations in the coding region of the *rpoB* gene.

Conclusions and clinical relevance – A study has shown that 6 mg/kg per os results in plasma concentrations of 600–1,000 × MIC of *S. pseudintermedius*. Based on our data, this dose should achieve the minimum MIC (×512) to prevent RFP resistance development; therefore, we recommend a minimum daily dose of 6 mg/kg for MDR-MRSP pyoderma treatment when limited antibiotic options are available.

Introduction

The number of infections caused by meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) in veterinary medicine has been on the rise in the last decade.[1] The resistance of *S. pseudintermedius* to meticillin, and inherently to all beta-lactam antimicrobials, is mediated by the carriage of the *mecA* gene.[2,3] This gene is carried on a transmissible mobile DNA element, staphylococcal cassette chromosome *mec* (SCC*mec*), which can be

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transferred between *Staphylococcus* isolates of different species resulting in a potential zoonotic spread of antimicrobial resistance (AMR).[2,4]

Some MRSP isolates also are multidrug-resistant (MDR),[5] which is defined as resistance to at least three antimicrobial classes.[6,7] This could be due to the presence of other AMR genes on SCC*mec*,[3] or the repeated use of antimicrobials and resultant selection for resistance.[8] This poses a serious challenge for veterinarians when faced with treating these MDR infections in clinical practice.[3,8,9]

As a consequence of the increased prevalence of MDR-MR infections, the high-tier antibiotic rifampicin (RFP; also known as rifampin in the United States) is gaining popularity for the treatment of canine MDR-MRSP pyoderma.[10] RFP is a highly lipophilic, semisynthetic derivative of rifamycin and is utilized in people for the treatment of *Mycobacterium tuberculosis* and Gram-positive organisms, notably, MR *S. aureus*.[11] RFP exerts its antimicrobial effect by binding specifically to the β-subunit of bacterial DNA-dependent RNA polymerase, which is encoded by the *rpoB* gene.[12,13] A "rifampicin resistance-determining region" (RRDR) has been identified on *rpoB,* which harbours most RFP resistance mutations, occurring at a frequency of 10^{-10} ~ 10^{-7}. Because most bacterial infection loads are $>10^{10}$, RFP monotherapy often is discouraged, and a second antibiotic to which the isolate is susceptible is recommended to be prescribed concurrently.[14–16] However, these recommendations were made initially regarding the long-term use of RFP for tuberculosis, and finding a suitable second antibiotic can be challenging.[17] Current guidelines suggest limiting RFP usage to when no better antibiotic alternative exists. Despite this, veterinarians are using this drug as monotherapy for the treatment of canine MRSP pyoderma with success,[18,19] although RFP resistance developed in six of 11 dogs in one study.[18] Dogs that received RFP in combination therapy also were reported to develop RFP resistance.[17] Systematic studies of RFP pharmacokinetics are in urgent need to determine the optimal dose to prevent AMR development.

Recent studies suggest that RFP's killing properties against *S. pseudintermedius* and the pharmacokinetics in dogs may be different from what is known in people.[20] Exposure of canine meticillin-susceptible *S. pseudintermedius* and MRSP isolates to RFP concentrations ranging from 0 to 32 × MIC_{90} (minimum inhibitory concentration inhibiting growth of 90% of organisms) demonstrated that RFP acts in a time-dependent fashion with both bacteriostatic and bactericidal properties. Pharmacokinetic data in dogs revealed that following multiple oral dosing (mean dose 5.9 ± 1.1 mg/kg), plasma RFP concentrations ranged from 600 (C_{min}) to 1,000 (C_{max}) × MIC_{90} of *S. pseudintermedius* (MIC_{90} 0.008 μg/mL),[10] suggesting that the *in vitro* killing behaviours described previously for RFP may not be representative of the *in vivo* characteristics. The canine isolates tested were not considered MDR.[20] The objectives of this study were to characterize the *in vitro* killing properties of clinically relevant RFP concentrations for canine MDR-MRSP isolates, to investigate whether RFP resistance occurs following exposure to RFP at higher concentrations, and to identify the causal mutations in these isolates responsible for antibiotic resistance. The information learned from this research will help guide the use of RFP treatment for MDR-MRSP canine pyoderma.

Methods and materials

MDR-MRSP isolate selection

Three MDR-MRSP canine isolates were selected from the Auburn University College of Veterinary Medicine diagnostic microbiology laboratory archive (2018–2019). The isolates were obtained from skin biopsies from dogs with superficial pyoderma. They were identified as *S. pseudintermedius* using traditional biochemical testing, including coagulase, catalase, acetoin, and acid production from mannitol, D-maltose and D-trehalose, as well as whole-genome sequencing.[21] Antimicrobial susceptibility testing was performed by broth microdilution using the Vitek II (bioMérieux; Durham, NC, USA). Testing parameters and interpretive guidelines were obtained from documents M100 and VET08 of the Clinical Laboratory Standards Institute (CLSI).[22,23] All three isolates were classified as MR by expressing oxacillin MICs of ≥0.5 ug/mL, and exhibited resistance to three or more antimicrobial drug classes comprising aminoglycosides, macrolides, lincosamides, fluoroquinolones, potentiated sulfonamides and tetracyclines. *Staphylococcus aureus* subsp. *aureus* ATCC 25923 was included as a control.

Determination of MIC

The RFP MIC for each isolate was determined using ETEST (bioMérieux). Briefly, saline suspensions from 18 to 24-h-old cultures of each isolate were prepared to a density comparable to 0.5 McFarland standard. A bacterial lawn was applied to Mueller–Hinton agar with a cotton swab and rifampin test strip placed on the agar surface. Following overnight incubation at ambient conditions, the MIC was determined from the inhibition ellipse that intersects the scale on the strip. CLSI breakpoints for *S. aureus* were utilized for RFP susceptibility as these have not yet been established for *S. pseudintermedius*. Isolates were considered susceptible when MIC ≤ 1 μg/mL and resistant when MIC ≤ 4 μg/mL.[23] ETEST was used in lieu of standard MIC determination methodology (broth microdilution).

Rifampicin time–kill studies

All four isolates were subjected to time–kill studies according to CLSI standards.[22,24] Rifampicin sterile powder (MP Biomedicals; Rockville, MI, USA) was solubilized in methanol to create a stock solution. A series of dilutions were performed to create final concentrations at 32, 64, 128, 256, 512 and 1,024 × MIC of the isolate tested. Before testing, isolates were subcultured three times. For each isolate, a suspension from an overnight culture was prepared using physiological saline with a density comparable to a 0.5 McFarland Standard. A 250 μL aliquot of the isolate was added to each tube in the RFP dilution series resulting in a final bacterial concentration of 7.5×10^6 colony forming units (CFU)/mL. The tubes were incubated at 37°C, and viable cell counts were measured in triplicate at time points 0, 2, 4, 12, 24 and 48 h. At each time point, 100 μL aliquots were transferred to a 96 well flat-bottom plate (Corning, Tewksbury, MA, USA) and luciferase assay (BacTiter-Glo Microbial Cell Viability Assay, Promega; Madison, WI, USA) reagent was added. Plates were incubated for 5 min and luminescence was measured using the Appliskan filter-based multimode microplate reader (Thermo Fisher Scientific; Waltham, MA, USA).[10] This methodology has been validated for viable cell counts against a gold standard of quantitative plate counts previously.[10] A positive control was included at each time point to confirm accuracy. Negative controls of RFP alone and Mueller–Hinton broth alone were used to detect and measure nonspecific luminescence. Viable CFU/mL measurements were compared to that of a standard curve, and the natural log of CFU/mL was plotted for each isolate. The standard curve was performed in triplicate, and known bacterial concentrations were determined using quantitative plate counts. The lower limit of detection was 1×10^1 CFU/mL, and an R^2 value of 0.99 was obtained, suggesting a suitable fit.

Determination of post-RFP exposure MIC

Two 100 μL aliquots were removed at each time point for plating onto both control agar (Mueller–Hinton agar with no antimicrobial) and Mueller–Hinton agar containing 4 μg/mL RFP. Aliquots from the positive control (bacteria without antibiotic) and negative control (RFP alone) were plated to ensure that contamination did not occur. Isolates that showed growth on RFP-containing agar were considered either RFP-tolerant or RFP-resistant, and the post-exposure MIC was determined using ETEST as described previously. Tolerance was defined as bacterial growth in the face of exposure to the antibiotic at concentrations that should be lethal without a change in the MIC. Resistant organisms exhibited a shift in MIC.[25]

DNA extraction and whole-genome sequencing

Bacterial genomic DNA samples were extracted from cell pellets of three selected RFP-resistant strains (Table 1) using Allprep PowerFecal DNA/RNA Kit (Qiagen; Germantown, MD, USA). DNA concentration was measured using a Qubit fluorometer 3.0 (Invitrogen) using a dsDNA high-sensitive assay kit. One microgram of input DNA was fragmented by an M220 Focused-ultrasonicator (Covaris; Woburn, MA, USA). DNA sequencing libraries were prepared using a NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs; Ipswich, MA, USA). Library quality control was performed using a Perkin-Elmer HT LabChip GX Touch nucleic acid analyzer (Perkin-Elmer; Bilerica, MA, USA). The libraries were sequenced on an Illumina NovaSeq6000 machine. Raw sequencing data are available at the NCBI Short Read Archive (accession no. PRJNA662578).

Mutational analysis

Genomic sequencing reads were quality-checked using FastQC.[26] NEBNext adapter sequences and low-quality bases were trimmed by Trimmomatic v0.39.[27] High-quality filtered reads were mapped using BWA aligner v0.7.17[28] to RFP-susceptible *S. pseudintermedius* genomes PRJNA623239 and PRJNA623240, which were assembled in previous research.[21] Indel realignment and *de novo* SNP calling were performed using GATK v3.8.[29]

Prediction of antimicrobial-resistant genes (ARGs) and bioinformatic analysis of mutational consequences

Assembled MDR-MRSP genomes were screened to predict ARGs using Resistance Gene Identifier (RGI, v4.0)[30] and ResFinder (v5.1.1).[31] To understand the potential effect of the mutations, a homology model of *S. pseudintermedius RNA polymerase B-subunit* was constructed by the Modeller9 v11 program[32] with a crystal structure of the *Escherichia coli* RNA polymerase and RFP complex (PDB ID: 5UAC) as a template. The identity between the template and target sequences was approximately 60%. Detailed structural visualization, comparison and analysis were conducted using the Pymol program (https://pymol.org/2/).

Results

Antimicrobial-resistant gene analysis in MDR-MR *S. pseudintermedius* isolates

Nine ARGs were identified in each MDR-MRSP isolate responsible for resistance to six classes of the antibiotics, including aminoglycoside, beta-lactam, macrolide, nucleoside, tetracycline and trimethoprim (Table 2). In *S. pseudintermedius*, fluoroquinolone resistance was reported to be conferred by mutations in the *gyrA* gene (Ser84Leu and Glu88Gly) or *grlA* gene (Ser80Ile and Asp84Leu).[5] We identified the Ser84Leu mutation in the gyrA gene, and the ARGs responsible for all tested antibiotic resistance were annotated. Through the analysis of gene neighbourhoods of ARGs, we discovered that they are located in close proximity to each other, such as *erm (B)* and *dfrG* on scaffold01, and *aph(3')-IIIa*, *sat4* and *ant (6')-Ia* on scaffold 32 of the M1R strain (Figure 1). Furthermore, ARGs are associated with other genes, including functionally important genes to regulate ARG expression and several transposases that produce transposons (Figure 1). Our results indicate that the above genes constitute the antibiotic-resistant cassettes that have undergone horizontal gene transfers (HGT) across bacteria.

Four RFP resistant isolates were identified from RFP time–kill kinetics assays

In the RFP time–kill experiments, the rate of killing did not increase with higher concentrations in *S. aureus* (ATCC 25923) and three MDR-MRSP strains (Figure 2). Therefore, the RFP activity against all four isolates was consistent with a time-dependent response.[23] All four isolates demonstrated a bacteriostatic response at all concentrations tested because the reduction in CFU/mL at 24 h was less than three logarithmic$_{10}$ reductions compared to the starting inoculum. They exhibited low RFP MICs before exposure, ranging from 0.004 to 0.016 μg/mL (Table 1). Bacterial growth was observed in the presence of RFP at either one (MDR-MRSP1, MDR-MRSP2) or two (*S. aureus*, MDR-MRSP3) concentrations at either 24 or 48 h post-exposure (Figure 2 and Table 1).

Three post-exposure isolates (M1R, M2R and M3R) were RFP-resistant strains based on CLSI standards (MIC ≥ 4 μg/mL). M2R belongs to the low-level resistance group (MIC 1–4 μg/mL), whereas M1R and M2R have high-level resistance (>8 μg/mL)[33,34] with a MIC of

Table 1. A list of multidrug-resistant and meticillin-resistant *Staphylococcus pseudintermedius* (MDR-MRSP) and control *S. aureus* isolates used in this study

Strain	Concentration (× MIC, times the minimum inhibitory concentration)	Time (h)	Pre-exposure MIC (μg/mL)	Postexposure MIC (μg/mL)	Resistant strain
S. aureus ATCC 25923	32	24	0.008	≥32	N/A
S. aureus ATCC 25923	128	24	0.008	≥32	N/A
MDR-MRSP1	64	24	0.004	≥32 (high level)	M1R
MDR-MRSP2	32	48	0.016	4 (low level)	M2R
MDR-MRSP3	256	24	0.008	≥32 (high level)	M3R
MDR-MRSP3	32	48	0.008	1	N/A

MIC minimal inhibitory concentration.

Table 2. A list of antibiotic-resistant genes in multidrug-resistant and meticillin-resistant *Staphylococcus pseudintermedius* (MDR-MRSP) isolates

Gene name	M1R ID*	M2/3R ID†	Class	Product
erm(B)	HFP11_00015	HFP12_13170	Macrolide	23S rRNA (adenine(2058)-N(6))-methyltransferase
dfrG	HFP11_00035	HFP12_13190	Trimethoprim	trimethoprim-resistant dihydrofolate reductase
tet(M)	HFP11_01820	HFP12_05255	Tetracycline	tetracycline resistance ribosomal protection
mecA	HFP11_04990	HFP12_02135	Beta-lactam	PBP2a family beta-lactam-resistant peptidoglycan
aph(2'')-Ia	HFP11_09360	HFP12_13445	Aminoglycoside	aminoglycoside O-phosphotransferase APH(2'')-Ia
blaZ	HFP11_12125	HFP12_08255	Beta-lactam	BlaZ family penicillin-hydrolyzing class A
aph(3')-IIIa / aphA-3	HFP11_13665	HFP12_13305	Aminoglycoside	aminoglycoside O-phosphotransferase
sat4	HFP11_13670	HFP12_13310	Nucleoside	streptothricin N-acetyltransferase Sat4
ant(6')-Ia / aadE	HFP11_13675	HFP12_13315	Aminoglycoside	aminoglycoside nucleotidyltransferase ANT(6)-Ia

*M1R gene IDs from genome assembly PRJNA623239.
†M2R and M3R gene IDs from genome assembly PRJNA623240.

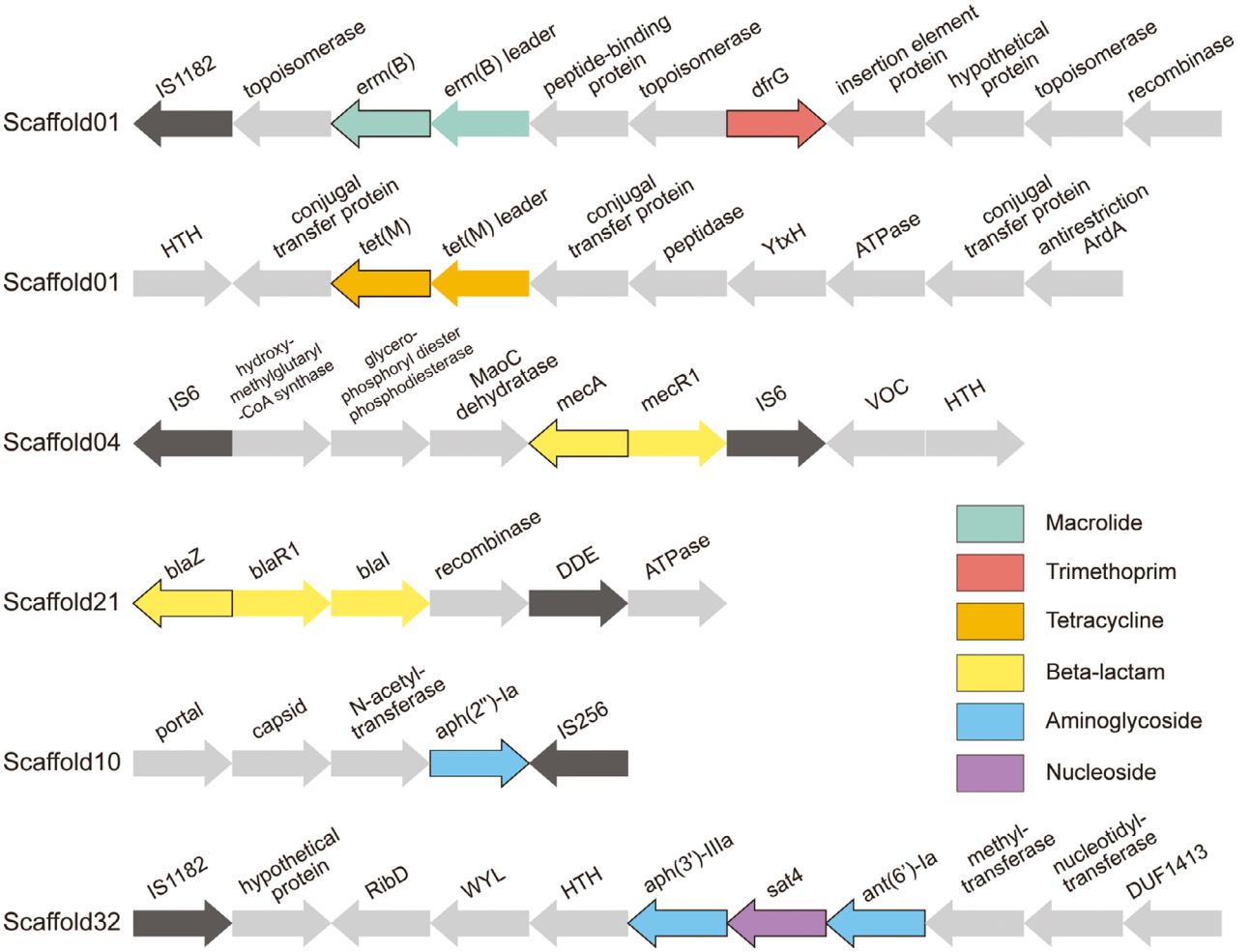

Figure 1. Genomic context of the antibiotic-resistant genes in the multidrug-resistant and meticillin-resistant *Staphylococcus pseudintermedius* (MDR-MRSP) isolate.
Open reading frames (ORFs) are symbolized by arrowed boxes with their gene names shown above. The highlighted colours of the arrowed boxes indicate the class of the antibiotics that the antibiotic-resistant genes target (colour legends are shown in the right panel). The dark grey boxes indicate the transposases, while light grey boxes include other potential resistant genes and transposon components.

>32 μg/mL. The upper limit of ETEST measurements is 32 μg/mL, so the exact MIC cannot be quantified. To confirm the RFP resistance in an independent experiment, 384 aliquots of these isolates at all time points were plated on RFP-free control plates and RFP-containing agar plates (4 μg/mL). Bacterial growth was observed on all control plates, and growth on RFP-containing agar corresponded with those concentrations showing exponential regrowth in the luminescence assay (Figure 2).

Whole-genome resequencing of three RFP-resistant strains identified causal mutations

Totals of 24,303,346, 59,910,834 and 66,673,156 150 bp reads were obtained from M1R, M2R, and M3R RFP-resistant strains, respectively, corresponding to 1,293×,

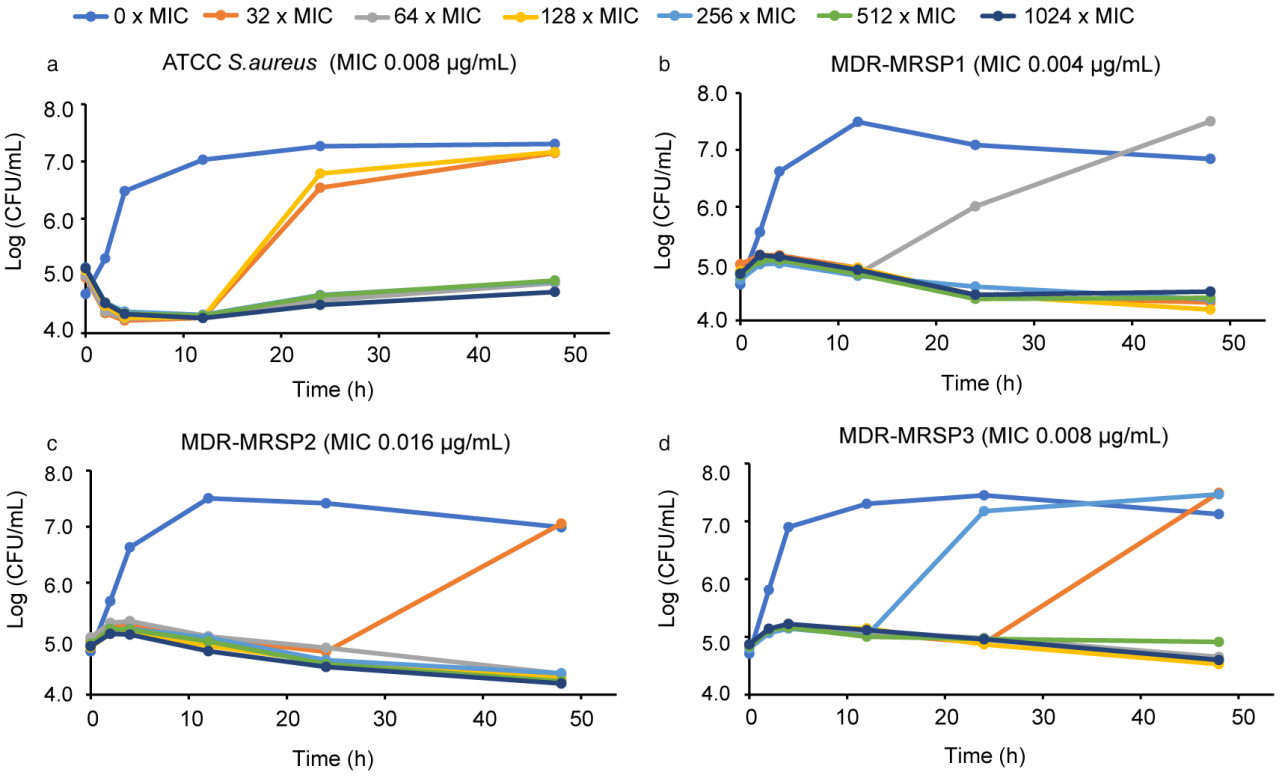

Figure 2. Rifampicin time–kill curves for three multidrug-resistant and meticillin-resistant *Staphylococcus pseudintermedius* (MDR-MRSP) isolates and the control *S. aureus* isolate.
Rifampicin concentration at 32, 64, 128, 256, 512 and 1,024 times the minimum inhibitory concentration (x MIC) in the logarithmic phase of growth for (a) *S. aureus* control isolate, (b) MDR-MRSP1 isolate, (c) MDR-MRSP2 isolate and (d) MDR-MRSP3 isolate. The *x*-axis represents time (h) and the *y*-axis represents the concentration of viable cells [colony forming units (CFU)/mL] measured by luminescence assay.

3,283 × and 3,654 × sequencing depths. On average, 2.84% of sequencing reads with adapter contamination and low-quality bases were trimmed, and 99.86% of the remaining high-quality reads were aligned to the RFP-susceptible genome assemblies. Three point mutations were identified in the M1R genome in response to RFP exposure. *rpoB* has a G-to-A change causing a serine-to-leucine mis-sense mutation (S486L) in the coding region (Table 3). A T-to-A point mutation results in a mis-sense mutation (F172L) in the *HFP11_03010* gene, which encodes a 189 amino acid residue hypothetical protein (Table 3). This gene is highly conserved in different *S. pseudintermedius* strains and with 82% sequence similarity to aquatic *S. delphini*. An A-to-G change causing an asparagine-to-serine mis-sense mutation (N486S) was found in the *HFP11_08430* gene, which encodes an aminopeptidase P family protein metallopeptidase M24. The MDR-MRSP1 isolate has two plasmids in its genome (pAUM1_1 and pAUM1_2). pAUM1_1 is 2,743 bp in length, and it is present in the M1R genome with 1,798 × depth. pAUM1_2 has a 16,531 bp circular genome with 20 protein-coding genes (Table S1), and it is absent in the M1R genome (0.057 × depth).

Both M2R and M3R only had a single mutation in the RFP target gene *rpoB*. A G-to-A mutation in M3R is the same as the one in M1R, which results in a serine-to-leucine change (S486L). M2R has an independent G-to-A mutation causing a mis-sense mutation (A477V) 17 bp away (Table 3).

Structural modelling reveals the mechanisms of *rpoB* mutations in three RFP-resistant strains

The two causal mutations in the *rpoB* gene, A477V and S486L, were found in three resistant strains. RFP is known to target RpoB at the DNA:RNA binding groove (Figure 3a), thereby blocking the RNA extension. By utilizing the crystal structure of the *E. coli* RNA polymerase and RFP complex as a template, we generated a homology model for the *S. pseudintermedius* RpoB protein. The model shows that the Ser486 situated at a deep portion of the RFP-binding pocket, interacts directly with the RFP naphthalene ring through a hydrogen bond (Figure 3a). Therefore, the S486L mutation identified in both M1R or M3R is predicted to reduce the RFP affinity significantly, which is consistent with our experimental observation that both M1R and M3R have high-level resistance to RFP.

The mutated residue in M2R, A477, is located behind the D471 residue and constitutes the major residue forming the back wall of the RFP-binding pocket together with H481 (Figure 3a). Therefore, A477 does not interact with RFP directly. Interestingly, D471 and H481 are the two other most common mutation sites for RFP resistance, in addition to the previously discussed S486. We propose that the A477V mutation will affect the structural confirmation of the D471, further disrupting the RFP-binding pocket to prevent RFP binding. Indeed, our experiments showed that this mutation has less detrimental effects on RFP-binding than the S486L mutation, and that the M2R strain is in the low-level resistant category.

Table 3. Mutations identified in resistant *Staphylococcus pseudintermedius* isolates after rifampicin (RFP) exposure

RFP resistant strain	Concentration (× MIC, times the minimum inhibitory concentration)	Time (h)	Reference genome	Position	Locus	Consequence
M1R	64	24	PRJNA623239	SCAFFOLD03:178473	*rpoB* (G→A)	Ser486Leu
M1R	64	24	PRJNA623239	SCAFFOLD02:137042	HFP11_03010 (T→A)	Phe172Leu
M1R	64	24	PRJNA623239	SCAFFOLD08:81927	HFP11_08430 (A→G)	Asn209Ser
M2R	32	48	PRJNA623240	SCAFFOLD09:49447	*rpoB* (G→A)	Ala477Val
M3R	256	24	PRJNA623240	SCAFFOLD09:49430	*rpoB* (G→A)	Ser486Leu

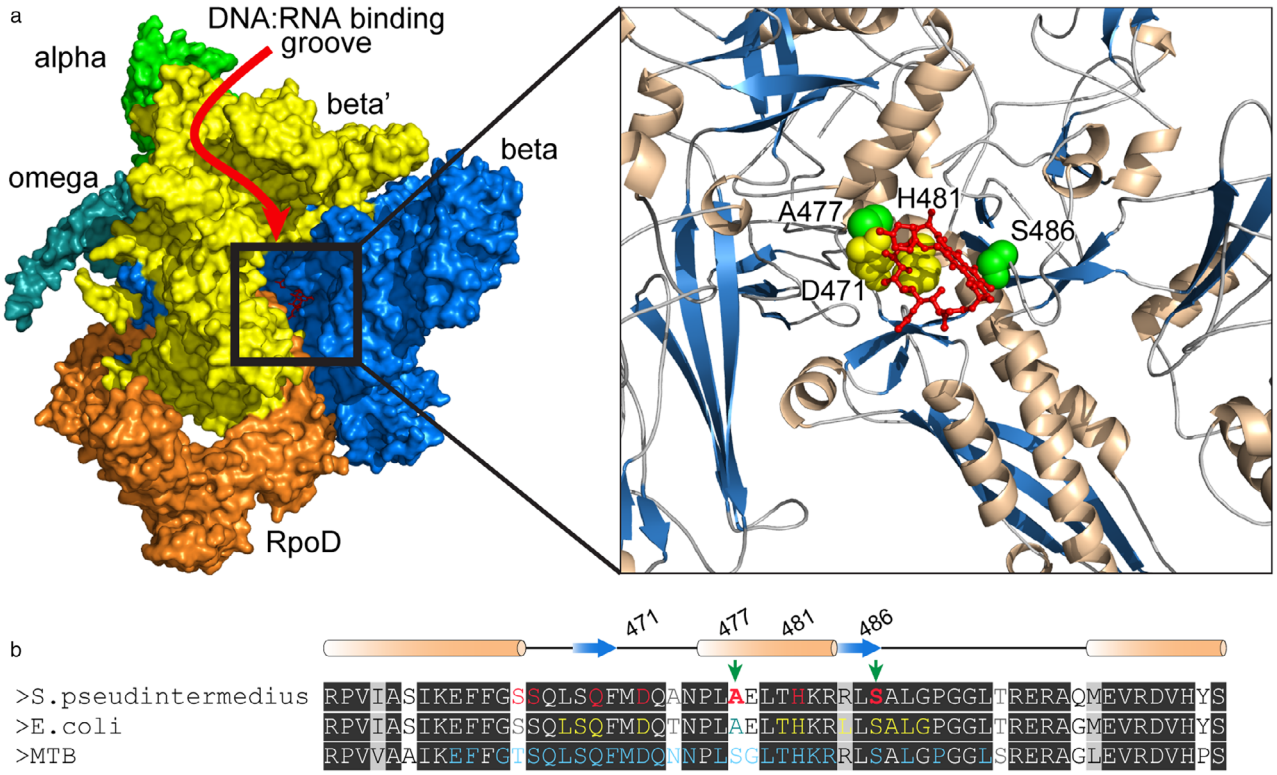

Figure 3. Causal mutations in the *rpoB* gene for antibiotic resistance in response to rifampicin (RFP) exposure.
(a) Surface view of the RNA polymerase complex (PDB:5UAC) which contains α, β, β′, Ω and RpoD subunits with the RFP docked to the RpoB, the β subunit (left), and a detailed schematic representation of interactions between the *Staphylococcus pseudintermedius* RpoB model and RFP. This highlights the mutation sites, A477 and S486, identified in this study and two additional major RFP resistance mutation sites, D471 and H481.
(b) Sequence alignment of the RFP resistance determining region (RRDR) of the RpoB proteins from *S. pseudintermedius*, *Escherichia coli* (E. coli) and *Mycobacterium tuberculosis* (MTB) with secondary structure shown above. Amino acids that are identical among the three species are shown in black background. The amino acids whose mutations are known to confer RFP resistance in each species are indicated in red, yellow and blue, respectively. The three major RFP resistance mutation sites are D471, H481 and S486. The two mutation positions which were identified in this study are indicated by green arrows, including A477 and S486.

Discussion

Genetic basis of antibiotic resistance in MDR-MRSP

The emergence of antibiotic resistance has become a serious issue in canine skin infections. Of the *S. intermedius* group isolates cultured between 2017 and 2019, 49% were found to be MR. Based on MIC results according to the CLSI standards, these isolates are resistant to six categories of antibiotics.[21] We have identified the ARGs responsible for all of them, and we also found the *sat4* gene, which confers the resistance to nucleoside antibiotics (Table 2). Interestingly, these ARGs are located in operon clusters with insertion element proteins, topoisomerase, conjugal transfer protein, recombinase and insertion-sequence (IS) elements (Figure 1). A similar association of these ARGs with transposons has been observed previously in 12 MDR-MRSP strains isolated worldwide,[35] suggesting that transposon cassettes have facilitated the spread of ARGs. It is of note that previously identified MDR-MRSP strains contain five major ARGs [*aphA3, sat, aadE, erm(B), dfr*],[35] while we identified a total of nine in this study. This suggests that the newly identified MDR-MRSP stains might have been selected recently against multiple classes of antibiotics and acquired additional ARGs. Our genome analysis provides a catalogue of ARGs in MDR-MRSP isolates from the southeastern US.

Multiple time-kill assays revealed that RFP acts in a time-dependent, bacteriostatic manner against canine MDR-MRSP at clinically relevant concentrations

For MDR-MR strains, RFP has become an attractive therapeutic option as very few choices for treatment are available. However, little is known about the appropriate use of this drug in dogs for the treatment of staphylococcal pyoderma. An understanding of killing properties and pharmacokinetics is necessary to design appropriate dosing regimens in order to use RFP judiciously.[36] Previous studies have evaluated RFP at <32 × MIC, and observed time-dependent responses, bactericidal activity and rapid bacterial regrowth.[10,37] *Staphylococcus aureus* has shown similar regrowth in RFP time–kill studies ranging from 1 to 8 × MIC, which is further reinforced by the behaviour of the *S. aureus* ATCC "control" strain in this study.[38-40] However, these concentrations are significantly below the plasma RFP levels in treated dogs, which is 600–1,000 × MIC. To address this discrepancy, we investigated the kill-curve and the development of RFP resistance at clinically relevant concentrations (32–1,024 × MIC), and discovered that the inhibitory response was considered time-dependent and bacteriostatic according to CLSI guidelines.[41]

RFP resistance developed rapidly under intermediate concentrations due to causal mutations in the RRDR region of the *rpoB* gene

Resistance to RFP is well-characterized in the *rpoB* gene of many species (e.g. *M. tuberculosis, E. coli* and *S. aureus*).[15] Mutations are enriched in RRDR, which is further divided into three clusters. Mutations within clusters II and III are more significant in *M. tuberculosis* and *E. coli*,[33] whereas most *S. aureus* mutations occur in cluster I. For *S. aureus*, high-level RFP resistance is associated with mutations at codons 468 and 481 (in *S. aureus* coordinates), and H481Y is the most common mutation.[17,33,34] Less is known about *rpoB* mutations in dogs and *S. pseudintermedius*, with only 10 known mutations in seven codons. In these reports, H526R is the most common, followed by less prevalent positions 508, 509, 513, 516, 522, 526 and 531.[17] In our study, all three resistant strains have a single point mutation in the RRDR region of the *rpoB* gene. M1R and M3R have the same single G-to-A mutation resulting in an S486L change, and they belong to the high-level resistance category with extremely high postexposure MIC at >32 μg/mL. The structural analysis showed that S486 is involved in interacting directly with RFP. M2R has a single G-to-A mutation 17 bp upstream, causing an A477V mis-sense mutation. The M2R strain is in the low-level resistant category with a MIC of 4 μg/mL, suggesting that this mutation is less effective compared to S486L in the resistance consequences. This is consistent with our structural model in which A477V may affect the RFP-binding pocket indirectly. Both mutations were found previously in *S. aureus*,[33] and the S486L has been documented in *S. pseudintermedius*.[17] The A477V has not been reported in *S. pseudintermedius* before.

The *rpoB* single mutation is the only mutation in M2R and M3R genomes, whereas M1R has three additional changes, including one Phe-to-Leu mis-sense mutation in a hypothetical protein, one Asn-to-Ser mutation in an aminopeptidase P family protein (Table 3), and the loss of a 16 kb plasmid. Because the S486L mutation is sufficient to drive the RFP resistance and no functional relevance of other changes were discovered, we speculate that these are randomly occurring mutational events and plasmid loss, without a role in RFP resistance in M1R.

Dose recommendations for RFP monotherapy in canine pyoderma

Monotherapy with RFP is not commonly recommended as a consequence of the rapid development of resistance during and following treatment in people and dogs, although this also occurs with combination therapy.[17,33,42,43] However, veterinarians are utilizing this drug in cases of MDR-MR pyoderma when no other choices are available. A recent retrospective study of 32 MDR-MR staphylococci cases found that oral RFP monotherapy was effective in 72% of all cases with a dose range of 4–10 mg/kg twice daily; however, five of 11 dogs that had skin cultures following RFP therapy on this dosage had developed RFP resistance.[18] A similar study discovered 90% efficacy in 20 dogs with pyoderma receiving 5 mg/kg twice daily for 10 days, yet these were not evaluated for the development of RFP resistance.[19] These reports indicate good efficacy of RFP for MDR-MR pyoderma, even as a sole therapy.

Under the suggested RFP dose range of 5–10 mg/kg,[44] a mean oral dose of 5.9 ± 1.1 mg/kg corresponds to plasma RFP concentrations ranging from 600 to 1,000 × MIC.[20] Our *in vitro* study was designed to encompass the entire range of clinical relevant concentrations, and we found that resistance did not occur at concentrations > 256 × MIC. Based on our current results, when RFP is selected for oral treatment when no other choice is available, we recommend a minimum dose of 6 mg/kg per day for the treatment of MDR-MRSP pyoderma to prevent the development of RFP resistance. A higher dose (10 mg/kg per day) might be prudent to minimize the emergence of RFP resistance, although this could potentially result in a great number of more severe adverse effects.[45] The therapeutic approach ideally would include the concurrent use of topical antimicrobial therapy (e.g. daily to every other day chlorhexidine),[46] which is effective in both MS and MR staphylococci.[47]

Conclusions

In conclusion, based on this study, RFP acts in a time-dependent and bacteriostatic fashion against canine MDR-MRSP. Resistance can develop rapidly following exposure to RFP, even at concentrations ranging from 32 to 256 × MIC, and RFP resistance is mediated by point mutations in the *rpoB* gene. The degree of RFP resistance is related to the location of the mutations, with S486L producing high-level resistance and A477V low-level resistance. We identified nine ARGs in these MDR-MRSP isolates in this study, compared to five ARGs in a report in 2015. Under these circumstances where few antibiotic options remain, we recommend RFP to be considered at a >6 mg/kg total daily dose, based on the

development of RFP resistance observed in our data. Future studies are warranted to better understand the use of RFP in veterinary practice as antibiotic choices become more limited.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1 The gene composition of the plasmid pAUM1_2.

Résumé

Contexte – Les résistances aux antibiotiques sont de plus en plus importantes pour les dermatites canines à *Staphylococcus pseudintermedius*. Le traitement à la rifampicine (RFP) est envisagé seulement pour les MDR-MRSP (*S. pseudintermedius* résistant à la méticilline et multi résistant).

Hypothèses/Objectifs – Déterminer une dose optimale de RFP pour le traitement des MDR-MRSP sans induire de résistance à RFP et identifier les mutations en cause pour la résistance antimicrobienne.

Matériels et methods – Le temps d'élimination a été réalisé pour une souche contrôle et trois MDR-MRSP à six concentrations cliniquement importantes [32 à 1,024 × MIC (minimum inhibitory concentration)]. Le séquençage de tout le génome et l'analyse bio-informatique ont été réalisés dans les souches résistantes développées dans cette étude.

Résultats – Les analyses génomiques ont identifié neuf gènes de résistances antimicrobiennes (ARGs) dans les souches MDR-MRSP, qui étaient responsable de résistance à sept classes d'antibiotiques. L'activité RFP contre les quatre souches était compatible avec une réponse bactériostatique et temps-dépendante. La résistance à RFP a été observée pour six des 28 tests de temps d'élimination incluant les concentrations 64 x MIC des souches à MDR-MRSP1 à 24 h, 32x MIC des MDR-MRSP2 à 48h, 32 × MIC des MDR-MRSP3 à 48 h et 256 × MIC des MDR-MRSP3 à 24 h. Les analyses de mutation de génome dans ces souches résistantes à RFP ont découvert les mutations en cause dans la région codant pour le gène *rpoB*.

Conclusions et importance Clinique – Une étude a montré que 6 mg/kg per os résultaient en des concentrations plasmatiques de 600-1000 x MIC de *S. pseudintermedius*. Basé sur nos données, cette dose pourrait atteindre la MIC minimum (x512) pour prévenir le développement de résistances à RFP ; ainsi, nous recommandons une dose journalière minimum de 6 mg/kg pour les pyodermites MDR-MRSP quand des options antibiotiques limitées sont disponibles.

Resumen

Introducción – la resistencia a los antimicrobianos es una preocupación creciente en la dermatitis canina por *Staphylococcus pseudintermedius*. El tratamiento con rifampicina (RFP) se considera solo en *S. pseudintermedius* resistente a meticilina y resistente a múltiples fármacos (MDR-MRSP).

Hipótesis/Objetivos – determinar una dosis de RFP óptima para el tratamiento de MDR-MRSP sin inducción de resistencia a RFP e identificar mutaciones causales de resistencia a los antimicrobianos.

Materiales y métodos – Se realizaron ensayos de tiempo de eliminación en un aislado de control y tres aislados MDR-MRSP a seis concentraciones clínicamente relevantes [32 a 1,024 × MIC (la concentración inhibitoria mínima)]. La resecuenciación del genoma completo y el análisis bioinformático se realizaron en las cepas resistentes desarrolladas en este ensayo.

Resultados – el análisis genómico identificó nueve genes de resistencia a los antimicrobianos (ARGs) en los aislados de MDR-MRSP, que son responsables de la resistencia a siete clases de antibióticos. La actividad de RFP contra los cuatro aislamientos fue consistente con una respuesta bacteriostática dependiente del tiempo. Se observó resistencia a la RFP en seis de los 28 ensayos de eliminación temporal, incluidas concentraciones de 64 × MIC en aislados de MDR-MRSP1 a las 24 h, 32 × MIC en MDR-MRSP2 a las 48 h, 32 × MIC en MDR-MRSP3 a las 48 h y 256 × MIC en MDR-MRSP3 a las 24 h. Los análisis de mutaciones de todo el genoma en estas cepas resistentes a RFP descubrieron las mutaciones causales en la región codificante del gen rpoB.

Conclusiones y relevancia clínica – un estudio ha demostrado que 6 mg/kg por vía oral dan como resultado concentraciones plasmáticas de 600-1.000 × CMI de *S. pseudintermedius*. Según nuestros datos, esta dosis debería alcanzar la CMI mínima (× 512) para prevenir el desarrollo de resistencia a la RFP; por lo tanto, recomendamos una dosis diaria mínima de 6 mg/kg para el tratamiento de la pioderma causada por MDR-MRSP cuando hay opciones limitadas de antibióticos disponibles.

Zusammenfassung

Hintergrund – Die Antibiotika Resistenz gewinnt zunehmend an Bedeutung bei der *Staphylococcus pseudintermedius* Dermatitis des Hundes. Eine Behandlung mit Rifampicin (RFP) wird nur in Betracht gezogen, wenn es sich um einen Methicillin-resistenten und multiresistenten *S. pseudintermedius* (MDR-MRSP) handelt.

Hypothese/Ziele – Es war das Ziel, eine optimale Dosierung von RFP zur MDR-MRSP Behandlung ohne die Auslösung einer RFP Resistenz zu bestimmen und verursachende Mutationen der antimikrobiellen Resistenz zu identifizieren.

Materialien und Methoden – Es wurden bei einem Kontrollisolat und bei drei MDR-MRSP Isolaten Time-kill Assays bei sechs klinisch relevanten Konzentrationen [32 bis 1,024 x MIC (minimale Hemmstoffkonzentration)] durchgeführt. Eine Gesamtgenom Sequenzierung und eine bioinformatische Analyse wurde bei den resistenten Stämmen, die bei diesem Assay entstanden, durchgeführt.

Ergebnisse – Durch die Genom Analyse wurden neun Gene antimikrobieller Resistenz (ARGs) bei MDR-MRSP Isolaten identifiziert, die für die Resistenz gegenüber sieben Antibiotikaklassen verantwortlich waren. Die RFP Aktivität gegenüber den vier Isolaten war konsistent mit einer Zeit-abhängigen und bakteriostatischen Antwort. Eine RFP Resistenz wurde bei sechs der 28 Time-Kill Assays beobachtet, dabei handelte es sich um die Konzentrationen 64 x MIC bei MDR-MRSP1 Isolaten bei 24h, 32 x MIC bei MDR-MRSP2 bei 48h, 32 x MIC bei MDR-MRSP3 bei 48h und 256 x MIC bei MDR-MRSP3 bei 24h. Eine Genom-weite Mutationsanalyse bei diesen RFP-resistenten Stämmen enthüllte die verursachenden Mutationen in der Kodierungsregion des *rpoB* Gens.

Schlussfolgerungen und klinische Bedeutung – Eine Studie hat gezeigt, dass 6 mg/kg *per os* in einer Plasmakonzentration von 600-1.000 x MIC von *S. pseudintermedius* resultiert. Basierend auf unseren Daten, sollte diese Dosis eine minimale MIC (x512) erreichen, um die Entstehung einer RFP Resistenz zu verhindern; daher empfehlen wir eine minimale tägliche Dosis von 6 mg/kg für die Behandlung einer MDR-MRSP Pyodermie, wenn nur limitierte antibiotische Optionen zur Verfügung stehen.

要約

背景 – 犬のStaphylococcus pseudintermedius由来膿皮症では，抗菌薬耐性が問題となっている。メチシリン耐性および多剤耐性S. pseudintermedius（MDR-MRSP）に対してのみリファンピシン（RFP）による治療が検討されている。

仮説・目的 – 本研究の目的は、MDR-MRSP治療においてRFP耐性を誘発しない最適なRFP投与量を決定し，抗菌薬耐性の原因となる変異を特定することであった。

材料と方法 – 対照分離株1株およびMDR-MRSP分離株3株を対象に，臨床的に適切な6濃度（32～1,024×MIC（最小発育阻止濃度））でTime-kill assay法を実施した。また，Time-kill assay法で得られた耐性株を対象に，全ゲノム再配列決定およびバイオインフォマティクス解析を実施した。

結果 – ゲノム解析の結果，MDR-MRSP分離株には9つの抗菌剤耐性遺伝子（ARG）が同定され，これらは7クラスの抗菌剤に対する耐性を担っていた。4株すべてに対するRFP活性は，時間依存的な静菌反応と一致していた。RFP耐性は28種のTime-kill assayのうち6種で認められ，MDR-MRSP1株では24時間後に64×MIC，MDR-MRSP2株では48時間後に32×MIC，MDR-MRSP3株では48時間後に32×MIC，MDR-MRSP3株では24時間後に256×MICの濃度であった。

結論と臨床的妥当性 – 経口投与6 mg/kgで血漿中濃度がS. pseudintermediusの600～1,000×MICになるという研究結果がある。我々のデータに基づけば，この用量はRFP耐性発現を防ぐ最小MIC（512×MIC）を達成するはずである。したがって，限られた抗生物質の選択肢しかない場合のMDR-MRSP膿皮症に対する治療には，1日あたりの最小用量である6 mg/kgを推奨する。

摘要

背景 — 抗生素耐药性是犬假中间型葡萄球菌皮炎中越来越受到关注的问题。利福平(RFP)治疗仅考虑用于耐甲氧西林和多重耐药的假中间型葡萄球菌(MDR-MRSP)。

假设/目的 — 在不会诱导RFP耐药的前提下，确定MDR-MRSP治疗的最佳RFP剂量，并确定抗菌药物耐药的突变原因。

材料和方法 — 在6个临床相关浓度[32-1,024×MIC（最小抑菌浓度）]下，针对对照分离株和3株MDR-MRSP分离株进行时间-杀灭试验。对本试验开发的耐药菌株进行全基因组重测序和生物信息学分析。

结果 — 基因组分析在MDR-MRSP分离株中鉴定出9个抗菌药物耐药基因(ARGs)，它们是对7类抗生素耐药的原因。全部4株分离株的RFP活性与时间依赖性和抑菌反应一致。28次时间-杀菌试验中有6次观察到RFP耐药，包括在24h时MDR-MRSP1分离株的浓度为64×MIC，在48h时MDR-MRSP2的浓度为32×MIC，48h

时MDR-MRSP3为32×MIC，24h时MDR-MRSP3为256×MIC。对这些RFP耐药菌株进行全基因组突变分析，发现了rpoB基因编码区是突变原因。
结论和临床相关性 — 一项研究表明，6 mg/kg经口给药导致假中间型链球菌的血浆浓度为600-1,000×MIC。基于我们的数据，该剂量应达到最小MIC(×512)，以防止发生RFP耐药；因此，当可用的抗生素选择有限时，我们建议MDR-MRSP脓皮病治疗的最小日剂量为6 mg/kg。

Resumo
Contexto – A resistência a antimicrobianos é uma preocupação crescente na dermatite canina causada por *Staphylococcus pseudintermedius.* O tratamento com rifampicina (RFP) é apenas considerado em casos de *S. pseudintermedius* multirresistente e resistente à meticilina (MDR-MRSP).
Hipótese/Objetivos – Determinar a dose ideal de RFP para o tratamento de MDR-MRSP sem indução de resistência à RFP e identificar as mutações causadoras de resistência a antimicrobianos.
Materiais e métodos – Os ensaios de tempo de eliminação (*time-kill)* foram realizados em um isolado controle e três isolados MDR-MRSP em seis concentrações clinicamente relevantes [32 a 1.024 × MIC (a concentração inibitória mínima)]. O resequenciamento de todo o genoma (*whole-genome resequencing)* e a análise de bioinformática foram realizados nas cepas resistentes desenvolvidas neste ensaio.
Resultados – A análise genômica identificou nove genes de resistência antimicrobiana (ARGs) em isolados MDR-MRSP, que são responsáveis pela resistência a sete classes de antibióticos. A atividade de RFP contra todos os quatro isolados foi consistente com uma resposta bacteriostática tempo-dependente. A resistência a RFP foi observada em seis dos 28 ensaios *time-kill,* incluindo concentrações 64×MIC em isolados MDR-MRSP1 em 24 h, 32×MIC em MDR-MRSP2 em 48 h, 32× MIC em MDR-MRSP3 em 48 h e 256×MIC em MDR-MRSP3 em 24 h. As análises de mutação em todo o genoma (*whole genome)* nessas cepas resistentes a RFP descobriram as mutações causais na região codificadora do gene *rpoB*.
Conclusões e relevância clínica – Um estudo mostrou que 6 mg/kg por via oral resulta em concentrações plasmáticas de 600-1.000 × MIC de *S. pseudintermedius*. Com base em nossos dados, esta dose deve atingir o MIC mínimo (×512) para evitar o desenvolvimento de resistência a RFP; portanto, recomendamos uma dose diária mínima de 6 mg/kg para o tratamento de piodermite MDR-MRSP quando há opções limitadas de antibióticos disponíveis.