AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

Changes in the Population of Methicillin-Resistant *Staphylococcus pseudintermedius* and Dissemination of Antimicrobial-Resistant Phenotypes in the Netherlands

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Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), which is often multidrug resistant (MDR), has recently emerged as a threat to canine health worldwide. Knowledge of the temporal distribution of specific MRSP lineages, their antimicrobial resistance phenotypes, and their association with clinical conditions may help us to understand the emergence and spread of MRSP in dogs. The aim of this study was to determine the yearly proportions of MRSP lineages and their antimicrobial-resistant phenotypes in the Netherlands and to examine possible associations with clinical conditions. MRSP was first isolated from a canine specimen submitted for diagnostics to the Faculty of Veterinary Medicine of Utrecht University in 2004. The annual cumulative incidence of MRSP among *S. pseudintermedius* increased from 0.9% in 2004 to 7% in 2013. MRSP was significantly associated with pyoderma and, to a lesser extent, with wound infections and otitis externa. Multilocus sequence typing (MLST) of 478 MRSP isolates yielded 39 sequence types (ST) belonging to 4 clonal complexes (CC) and 15 singletons. CC71 was the dominant lineage that emerged since 2004, and CC258, CC45, and several unlinked isolates became more frequent during the following years. All but two strains conferred an MDR phenotype, but strains belonging to CC258 or singletons were less resistant. In conclusion, our study showed that MDR CC71 emerged as the dominant lineage from 2004 and onward and that less-resistant lineages were partly replacing CC71.

Staphylococcus pseudintermedius is an important pathogen in dogs and cats and is sporadically associated with human infections (1–3). Methicillin-resistant *S. pseudintermedius* (MRSP) has recently emerged in different parts of the world (4–6). MRSP is resistant to all beta-lactam antimicrobials and is generally resistant to three or more antimicrobial classes (multidrug resistant [MDR]), which poses a challenge to therapy in veterinary medicine. Recent genomic analyses indicated that antimicrobial resistance evolved through acquisition of mobile genetic elements and through mutation, reinforcing the notion that antimicrobial use can coselect for antimicrobial-resistant strains (7).

Most MRSP infections in dogs are surgical wound infections, pyoderma, and otitis externa but also include pneumonia, urinary tract infections, and, to a lesser extent, other infections (8). Furthermore, it has become clear that companion animals play an important role in the epidemiology of MRSP, as humans can become transient carriers of MRSP after contact with their colonized dogs (9).

The prevalence of MRSP colonization in dogs depends on the population under study as is summarized by Weese and Van Duijkeren (8). In a recent study in the United Kingdom, no MRSP was isolated from dogs visiting veterinary practices (10); however, MRSP isolates were found in 0.8% of canine clinical samples in a German study and in 0.28% of those from another United Kingdom study (11, 12). In Germany, 7.4% of dogs were found to be MRSP positive at admission to veterinary clinics (13). In the Netherlands, prevalence data are not available although MRSP has been identified in household dogs (14).

Clonal lineages of MRSP have been disseminated globally. Multilocus sequence typing (MLST) has shown that sequence type 71 (ST71) is a dominant clonal lineage that has spread successfully in Europe and Asia, whereas ST68 has spread mainly in North America (15). A limited number of MRSP STs have been identified that are reflected in the dissemination of a few dominant clonal lineages (5, 15–17). Isolates belonging to ST71 and ST68 often contain genes that confer resistance to multiple antimicrobials in addition to the *mecA* gene on the staphylococcal cassette chromosome *mec* element (SCC*mec*), which encodes methicillin resistance (15, 18).

In this study, we investigated the molecular epidemiology of MRSP lineages in the Netherlands by analyzing data for *S. pseudintermedius* isolates obtained from dogs over a period of 20 years. This allowed us to study the yearly changes in the proportion of

Received 13 May 2015 **Returned for modification** 10 June 2015
Accepted 6 November 2015

Accepted manuscript posted online 18 November 2015

Citation Duim B, Verstappen KM, Broens EM, Laarhoven LM, van Duijkeren E, Hordijk J, de Heus P, Spaninks M, Timmerman AJ, Wagenaar JA. 2016. Changes in the population of methicillin-resistant *Staphylococcus pseudintermedius* and dissemination of antimicrobial-resistant phenotypes in the Netherlands. J Clin Microbiol 54:283–288. doi:10.1128/JCM.01288-15.

Editor: B. W. Fenwick

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01288-15.

MRSP versus *S. pseudintermedius*, the association with clinical conditions, and the dynamics of their antimicrobial-resistant phenotypes.

MATERIALS AND METHODS

Strain isolation and identification. *S. pseudintermedius* isolates were obtained from clinical specimens submitted from 2004 to 2013 to the Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University, the Netherlands. All included specimens were from clinical infections and not from screening procedures. The specimens were cultured on sheep blood agar (bioTRADING, the Netherlands), and after overnight incubation at 37°C, presumptive colonies were identified as *S. pseudintermedius* by colony morphology, Gram-staining, tests for catalase and coagulase, and by using mannitol (bioTRADING, the Netherlands). Strains were confirmed as *S. pseudintermedius* with PCR-restriction fragment length polymorphism (RFLP) analysis with MboI digestion of the *pta* gene (2). All MRSP isolates were stored at −80°C in brain heart infusion (BHI) with 50% glycerol.

Susceptibility to 14 antimicrobials belonging to 9 antimicrobial classes, including beta-lactams (amoxicillin-clavulanic acid, ampicillin, and ceftiofur), tetracyclines (tetracycline), lincosamides and/or macrolides (clindamycin and erythromycin), amphenicol group (chloramphenicol), diaminopyrimidines-sulfonamides combinations (trimethoprim-sulfamethoxazole), aminoglycosides (gentamicin, kanamycin, and amikacin), fluoroquinolones (enrofloxacin), fusidic acid (fusidic acid), and rifamycins (rifampin), was tested by an agar diffusion method using Neo-Sensitabs (Rosco, Denmark). The categories, susceptible (S), intermediate (I), and resistant (R), were defined on the basis of the breakpoints recommended by the Dutch Committee on Guidelines for Susceptibility Testing (20). Intermediate susceptibility values were scored as resistant phenotypes. Since 2000, adjustments in breakpoints according to the recommendations of the manufacturer (Rosco, Denmark) were implemented (http://www.rosco.dk/default.asp?mainmenu=20). An isolate was classified as multidrug resistant when it was resistant to ≥3 antimicrobial classes (19). Microbial resistance (R) and susceptibility (S) data were assigned in resistance profiles. When resistance was found for a combination of beta-lactams and fluoroquinolones, of beta-lactams and aminoglycosides, or of beta-lactams and trimethoprim-sulfamethoxazole, isolates were determined as methicillin-resistant by *mecA* real-time PCR (21).

Retrospective data analysis on clinical MSSP and MRSP isolates. In the case of multiple samples from a patient, the first isolate of methicillin-susceptible *S. pseudintermedius* (MSSP) or MRSP was included in the study. Trends in the yearly proportion of MRSP were assessed using the Cochran-Armitage test for trend. Associations of MRSP and MSSP with clinical conditions (skin/pyoderma, wound/injury, arthritis, otitis, cystitis, or throat/respiratory infection) were assessed using univariate logistic regression by using the numbers of other diseased as reference groups. The associations were expressed as odds ratios (OR) providing their 95% confidence intervals (CI); *P* values of <0.05 were considered significant.

Genotypic characterization of MRSP. MRSP isolates were typed using MLST targeting seven genes as described previously (22). In a slightly adapted protocol, two freshly grown colonies were suspended in 500 μl Tris-EDTA (TE) buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) with 20% Chelex 100 molecular biology grade resin (Bio-Rad, Veenendaal, the Netherlands) incubated at 99°C for 10 min and centrifuged for 1 min at 20,000 × *g*. The PCR mixture (20 μl) consisted of 5 μl DNA lysate, 2× GoTaq Hot Start Green master mix (Promega Benelux BV, Leiden, the Netherlands), 1 μM each of forward and reverse primer, and molecular-grade water. PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol and were subsequently sent for sequence analysis (BaseClear, Leiden, the Netherlands). Sequences were assembled using BioNumerics v7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). Sequence types were assigned by comparison with the allele sequences present in the PubMLST database (http://pubmlst.org/spseudintermedius). New types were assigned by the curator Vincent Perreten. Trends in the yearly occurrence of MLST clonal complexes were assessed using the Cochran-Armitage test for trend; *P* values of <0.05 were considered significant.

Population structure of MRSP. The software goeBURST, which uses the same clustering rules for linking STs as eBURST, was used to infer the relatedness of STs (23, 24). Clonal complexes (CCs) were defined as the predicted founder STs and linked single locus variants (SLVs).

TABLE 1 Number and proportion of methicillin-resistant and methicillin-susceptible *S. pseudintermedius* from dogs

Year	No. MSSP	No. MRSP	MRSP proportion (%)[a]
2004	1,045	1	0.95
2005	1,066	1	0.09
2006	970	9	0.92
2007	955	49	4.88
2008	985	54	5.20
2009	845	73	7.95
2010	918	51	5.26
2011	974	73	6.97
2012	968	81	7.79
2013	1,077	86	7.38
Total	9,803	478	4.65

[a] Linear slope = 0.0919; $P = <0.001$.

RESULTS

Retrospective data analysis on clinical MSSP and MRSP isolates. Between 2004 and 2014, a total of 9,803 MSSP and 478 MRSP isolates, all first isolates, were isolated from dog samples. Canine *S. pseudintermedius* accounted for 95% of the total number of clinical *S. pseudintermedius* isolates at the VMDC. The number of isolated canine MRSP isolates showed a significantly increasing trend (linear slope = 0.0919 and $P = <0.001$) and an increase in the yearly MRSP proportion from 0.9% in 2004 to 7% in 2013, with a slight decrease in 2010 (Table 1).

Association of MSSP and MRSP with clinical conditions. Descriptively, the majority of MSSP isolates were obtained from cases of otitis externa (32.1%), and the majority of MRSP were obtained from cases of pyoderma (43.7%). MRSP isolates were significantly more likely to be obtained from cases of pyoderma (OR = 5.1), wound infection (OR = 4.3), or arthritis (OR = 3.3) than MSSP isolates (Table 2). Conversely, MRSP isolates were significantly less likely to be obtained from cases of otitis externa (OR = 0.5) or throat/respiratory infections (OR = 0.5) than MSSP isolates (Table 2).

Distribution of allelic profiles. Out of the 478 isolates typed with MLST, 39 unique STs were identified (Table 3). Most isolates ($n = 292$) belonged to ST71. Other predominant STs were ST258 ($n = 49$), ST45 ($n = 33$), ST261 ($n = 30$), and ST265 ($n = 17$). Another 14 STs were each represented by <10 isolates, and 20 STs were represented by only a single isolate (ST68, ST118, ST282, ST307, ST333, ST337, ST341, ST343, ST344, ST345, ST347, ST348, ST349, ST351, ST352, ST353, ST382, ST383, ST388, ST389) (Table 3). ST71 was first identified in 2004, increasing steadily since then, but stabilizing from 2007 and onward. In 2006, only ST71 and ST45 were found, but in the following years the number of STs increased with the introduction of 24 previously undisclosed STs (Fig. 1; see also Table S1 in the supplemental material).

Population structure of MRSP. Clustering of the 39 unique STs by the goeBURST algorithm showed that their allelic profiles

TABLE 2 *S. pseudintermedius* isolated from clinical specimens of dogs

Clinical group	No. MSSP strains (n = 9,803)	No. MRSP strains (n = 478)	Odds ratio for methicillin resistance	*P* value	95% CI
Skin/pyoderma	1,129	209	5.1	<0.001	4.2–6.2
Wound/injury	844	95	4.3	<0.001	3.5–5.1
Arthritis	102	16	3.3	<0.001	1.9–5.6
Otitis	3,146	92	0.5	<0.001	0.4–0.6
Cystitis	221	14	Not significant		
Throat/respiratory infection	487	11	0.5	0.010	0.3–0.8
Unknown	3,874	1	ND[a]		

[a] ND, not determined.

are only distantly related to each other and that they are grouped in four CCs and 14 singletons. The CC consisted of allelic profiles with five or more allele matches, while singletons (i.e., STs) were unrelated to any other within the SLV collection. The founders in the CCs were assigned as ST71, ST68, ST45, and ST258, respectively (Fig. 2). The largest CC with founder ST258 consisted of 17 STs. This CC solely represents isolates that emerged after 2007 (Table 3). There were statistically significant annual trends in the occurrence of clonal complexes. The proportion of CC71 showed a significantly decreasing trend (linear slope = −0.0748; P = <0.001), whereas the proportion of CC258 showed a significantly increasing trend (linear slope = 0.598; P = <0.001).

To determine the clonal relationships of the sequence types obtained in this study together with the entries in the global PubMLST *S. pseudintermedius* database, all entries available on September 2015 were clustered using the same goeBURST procedure. This showed 6 major CC, 23 smaller groups with no candidate founder, and 200 singletons. The branches are connected with a higher locus variant level to show the relations of all STs (see Fig. S1 in the supplemental material). Currently, six major *S. pseudintermedius* clonal lineages are present (CC45, CC71, CC68, CC258, CC240, and CC84). The largest is CC258, which has 129 STs with founder ST258.

Distribution of antimicrobial resistance. The majority of MRSP isolates were resistant to either five or six antimicrobial classes. Only three MRSP isolates (CC258) were resistant to two antimicrobial classes and were not considered MDR. Three MRSP isolates (CC71 and CC45) were resistant to eight antimicrobial classes. The resistance profiles were highly diverse (see Table S2 in the supplemental material). Combining the susceptibility data of drugs from the same antimicrobial group identified common resistance profiles (Table 4). This clustered the profiles into 6 major groups (see Table S3 in the supplemental material). Profiles with resistances to seven antimicrobial classes were found in CC71 (n = 47) and CC45 (n = 31) isolates and in three isolates with single STs. CC258 isolates were susceptible to enrofloxacin and were resistant to five or six antimicrobial classes. These profiles contained either resistance for tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, or gentamicin (see Table S2). The majority of the less-resistant CC258 isolates and single ST isolates were isolated after 2010. Furthermore, the MDR MRSP population showed important dynamics in antimicrobial resistances over the years.

TABLE 3 MRSP clonal complexes and different sequence types

Clonal complex	Sequence types
71	71, 123, 382
45	45, 179, 282
258	258, 118, 261, 265, 277, 307, 312, 334, 336, 342, 343, 346, 349, 350, 351, 383, 389
68	68, 29
Singletons	196, 298, 333, 335, 337, 339, 341, 344, 345, 347, 348, 352, 353, 388

DISCUSSION

In order to develop measures to prevent and control the dissemination of resistant *S. pseudintermedius*, insights into the spread of resistant strains and into the dynamics of antimicrobial resistance are needed. Among the diagnostic specimens studied here, the cumulative incidence of MRSP increased to a relatively stable 7% in 2013. We estimated that our isolates originated from approximately 50% of the companion animal clinics present in the Netherlands. It cannot be excluded, however, that there has been a submission bias over the years, as there has been intense communication about MRSP issues to clinics via professional veterinary journals in parallel to presentations at meetings and communications from the diagnostic laboratory to the practitioners themselves after recovery of MRSP from their patient's sample. Practitioners may therefore have been keen to submit samples when empirical antimicrobial therapy failed, which in turn may have resulted in an increased detection of MRSP. However, the yearly isolated number of MSSP isolates and the total number of submissions in a year were constant, which indicate that there was no general change in sample submissions. The number of clinics submitting MRSP-positive canine samples increased (data not shown), which supported our observation that MRSP was emerging in the Dutch dog population.

The preferred method to predict *mecA*-associated resistance in *S. pseudintermedius* isolates is to test for oxacillin susceptibility (25). As oxacillin was not included in our routine susceptibility testing, we used other selection criteria to predict *mecA*-associated resistance. To evaluate our method of selection, we performed a prospective study for 6 weeks, including all isolated *S. pseudintermedius* isolates (n = 200) in this period. No discrepancy was found between phenotypic resistance determination and *mecA* PCR in this study (data not shown). It is therefore unlikely that we missed many MRSP isolates in our study. Nevertheless, we cannot exclude an underestimation of the actual proportion of MRSP due to our selection method.

We showed that MRSP was significantly more likely to be associated with pyoderma, wound infection, or arthritis, whereas MSSP was associated with otitis externa. Whether these associations reflect, for example, the selective pressure of previously used

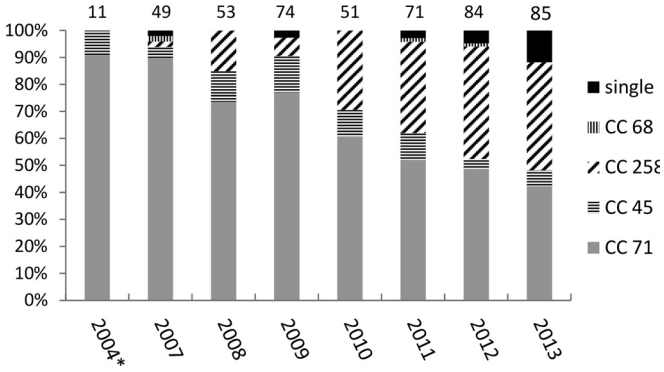

FIG 1 Distribution of MRSP clonal complexes per year. *, Combined numbers obtained from 2004 to 2006. The top numbers indicate the total number of strains.

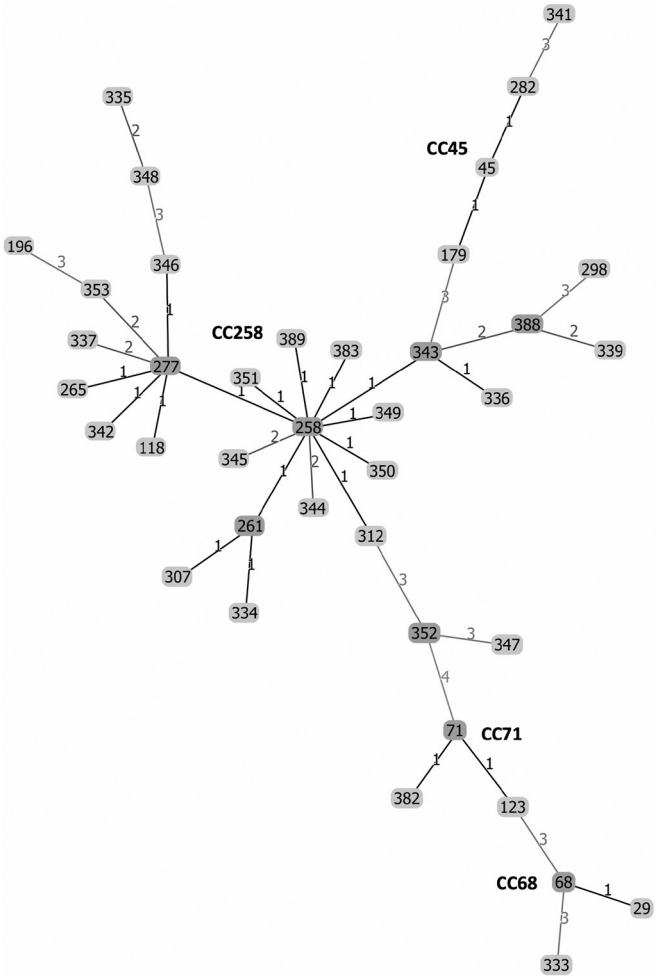

FIG 2 Clonal relationships of MLST sequence types in the Netherlands (2004 to 2013). goeBURST analysis in which the branches are connected with a double locus variant level to show the relation of STs.

antimicrobials in the patients or different strain characteristics is unknown.

The increase in MRSP over the years was dominated by CC71, which is a widespread multiresistant MRSP lineage (15, 26). Diversion of ST71 was visible in two single locus variants: ST123 with a nucleotide difference in the *sar* locus and ST382 with a nucleotide difference in the *cpn60* locus. The relatedness of MRSP CCs was identified by clustering STs with the BURST algorithm that assigned the STs in four CCs with closely related STs. A similar type of clustering has been observed in *Staphylococcus aureus*, in which single nucleotide differences were proposed to define a group within a ST (27). GoeBURST analysis of all STs present in the *S. pseudintermedius* MLST database confirmed that 478 Dutch isolates belong to four CCs with the same predicted founder assignments (CC71, CC258, CC68, and CC45) and to numerous unlinked STs. The recently observed emergence of MRSP in six clonal lineages (7) raised questions about which mechanisms shape the genetic diversity of MRSP isolates. Recombination, rather than nucleotide substitution, has been suggested as the driver of the diversity in MRSP populations (15). This is in line with recent studies showing the acquisition of resistance genes by incorporation of mobile elements in the genome (7). Furthermore, in the very successful CC71 lineage, SCC*mec* types II and III predominate (15, 28), but other types are emerging (7, 29).

Initially, MRSP isolates were resistant to eight or seven antimicrobial classes tested in our test panel. Rifampin resistance was occasionally observed in the studied population. This resistance results from induced *rpoB* mutations during antimicrobial treatment, and our observation indicates that this resistance is not maintained in the population (30). Almost all CC71 and CC45 isolates were resistant to fluoroquinolones, which have been described to be present in successful MDR MRSP clones (7). However, whether this resistance is a key factor to success is unclear, as since 2008, the emerging CC285 lineage and most singletons were susceptible to fluoroquinolones. MRSP strains resistant to four to six antimicrobial classes carried different combinations of resistances for aminoglycosides, tetracycline, and chloramphenicol. This may result from the fact that these resistances are associated with mobile elements, Tn*5405*, Tn*5801*, Tn*916*, and IS*256* or IS*1272*-like elements (7, 18), and with the multiple genes that can confer resistance to lincosamides and/or macrolides. For example, *ermB* has been shown to be associated with Tn*917* and Tn*5405* (7, 18). The exact gene flow conferring lincosamide and/or macrolide resistance is unclear, but the number of identified mobile elements in MRSP may indicate the major role of transmission of resistance genes in the success of the CC71 and CC285 lineages. These lineages showed a stepwise gain and loss of phenotypic resistances over a period of 10 years that did not influence their spread. For methicillin-resistant *Staphylococcus aureus* (MRSA), it has been shown that differential gene expression was important in the evolution of MRSA ST8 virulence and spread in the community (31). To date, the molecular determinants underlying the success of CC71 and CC258 are unknown, although Osland et al. suggested that ST71 strains were better adapted to produce biofilms (32). However, the flexible availability of antimicrobial resistance genes in MRSP may, as it has been suggested before, be a strong selection factor for the dissemination of major clonal lineages (7).

In conclusion, our study showed that MDR CC71 emerged as the dominant lineage from 2004 and onward and that other less-resistant MRSP lineages were partly replacing CC71 in later years. Furthermore, the MDR MRSP population showed important dynamics in antimicrobial resistances over the years.

TABLE 4 Distribution of common antimicrobial-resistant phenotypes

Resistant for antimicrobial classes (no.)	Resistance profile (no.)	Resistant phenotype[a]	No. of strains	Years	MRSP CC/ST (no.)
7	3	RRRRRRSSR	82	2008–2013	CC71 (47), CC45 (31), ST196 (1), ST335 (1), ST341 (1)
6	7	RRRRSRSSR	58	2008–2013	CC71 (57), CC45 (1)
6	5	RRRRRSSSR	45	2007–2013	CC71 (41), CC45 (1), CC258 (2), ST335 (1)
6	9	RRRSRRSSR	16	2010–2013	CC258 (16)
5	12	RRRSRSSSR	90	2008–2013	CC258 (83), CC71 (3), ST347 (1), ST298 (3)
5	8	RRRRSSSSR	73	2008–2013	CC71 (71), CC45 (1)

[a] Contains resistance (R) or susceptibility (S) in the order: β-lactam, lincosamide and/or macrolide, aminoglycoside, enrofloxacin, tetracycline, chloramphenicol, fusidic acid, rifampin, and trimethoprim-sulfamethoxazole. Isolates were considered to be resistant to lincosamide and/or macrolide when they were resistant to either clindamycin or erythromycin and they were considered to be resistant to aminoglycoside when resistant to kanamycin, amikacin, or gentamicin.

ACKNOWLEDGMENTS

We thank the personnel of the veterinary clinics for their cooperation. We thank Aldert Zomer and Lapo Mughini-Gras of the Faculty of Veterinary Medicine, Utrecht University, for their support in analyzing the resistance data and for the statistics. We thank Vincent Perreten of the Institute of Veterinary Bacteriology of the University of Bern, as curator of the MLST website developed by Keith Jolley at the University of Oxford.

The Royal Dutch Veterinary Association (KNMvD) provided funding. This was also a self-funded project.

We declare no conflict of interest.

FUNDING INFORMATION

The Royal Dutch Veterinary Association (KNMvD) provided funding to Engeline van Duijkeren.

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