Article

Nasal carriage of methicillin-resistant *Staphylococcus pseudintermedius* in dogs treated with cephalexin monohydrate

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Abstract — This study aimed to investigate the nasal carriage of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) in dogs treated with oral cephalexin monohydrate. Ten dogs with superficial pyoderma were monitored longitudinally for carriage of MRSP for up to 1 year after treatment; the strains were typed and antibiograms were determined. Methicillin-susceptible *S. pseudintermedius* (MSSP) was recovered prior to treatment in all dogs and could be isolated after 12 months in 1 dog. Methicillin-resistant *Staphylococcus pseudintermedius* was detected within 1 week of treatment in all dogs, and 3 clones represented by ST45, ST112, and ST181 were consistently present for up to 12 months after treatment. All MRSP isolates were resistant to at least 7 common antimicrobials. Oral cephalexin monohydrate treatment selected for strains of multi-resistant MRSP, which were still present after 1 year.

Résumé — Portage nasal de *Staphylococcus pseudintermedius* résistant à la méthicilline chez les chiens traités à l'aide de céphalexine monohydrate. Cette étude visait à étudier le portage nasal de *Staphylococcus pseudintermedius* résistant à la méthicilline (SPRM) chez les chiens traités à l'aide de céphalexine monohydrate par voie orale. Dix chiens ayant une pyodermie superficielle ont été surveillés dans une étude longitudinale pour le portage de SPRM pendant jusqu'à un an après le traitement; les souches ont été typées et des antibiogrammes ont été réalisés. *Staphylococcus pseudintermedius* susceptible à la méthicilline (SPSM) a été récupéré avant le traitement chez tous les chiens et pouvait être isolé jusqu'à 12 mois chez un chien. *Staphylococcus pseudintermedius* résistant à la méthicilline a été détecté une semaine après le traitement chez tous les chiens et 3 clones représentés par ST45, ST112 et ST181 étaient continuellement présents jusqu'à 12 mois après le traitement. Tous les isolats de SPRM étaient résistants à au moins sept antimicrobiens communs. Le traitement à la céphalexine monohydrate par voie orale a été choisi pour les souches multirésistantes de SPRM qui étaient toujours présentes après un an.

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Introduction

Staphylococcus pseudintermedius is a commensal bacterium on canine mucosa and skin that also can cause canine dermatitis. In rare cases it can opportunistically infect humans and contribute to detrimental outcomes such as septicemia, sinusitis, and dog bite wound infection (1–3). Systemic cephalexin administration is the primary choice of empirical therapy for canine superficial pyoderma (4); however, the use of antibiotics may encourage an increased frequency of resistant strains, resulting in recurrent infection or increased risk of bacterial zoonotic transmission to owners and veterinarians (5).

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Antimicrobial resistant strains can be selected following exposure to antimicrobials. According to the selective pressure concept, antibiotic resistant strains may persist depending on the relative genetic fitness of resident susceptible and resistant strains (6). Methicillin-resistant *S. pseudintermedius* (MRSP), can be increasingly detected after routine antibiotic treatment for canine dermatitis, and these MRSP also express resistance to other beta-lactam drugs, namely, penicillins and cephalosporins (7). Previously, emergence of MRSP from dogs with a history of treatment for dermatitis was observed in a longitudinal study and was shown to be the source of contamination for in-contact animals and the environment within the same household (8). Methicillin-resistant *S. pseudintermedius* has been reported to persist on dog's skin for more than 6 mo after antibiotic administration, and increased detection of MRSP during treatment seems to be common (9). However, changes in the *S. pseudintermedius* population and the duration of persistence of MRSP strains on dog skin following antimicrobial treatment still needs to be determined in index dogs. This study aimed to determine changes in the *S. pseudintermedius* population in dogs after treatment with cephalexin and to evaluate

Table 1. Age, gender, breed, and treatment for the 10 dogs and the number of *Staphylococcus*-like colonies recovered at each collection time

					Log (CFU/swab)					
					Pre-treatment		Treatment		Follow-up	
Dog	Age	Gender	Breed	Other treatments[a]	MSA[c,e]	MSA + Oxa[d]	MSA[e]	MSA + Oxa	MSA[e,f]	MSA + Oxa
1	2 y	M	Beagle	2% Chlorhexidine	2.54	ND	2.31	1.31	3.05	2.44
2	8 mo	F	Mixed	Herbal cream[b]	2.84	ND	2.7	1.52	2.95	2.65
3	9 mo	F	Mixed	Herbal cream	2.48	ND	2.56	1.64	3.64	3.65
4	1 y	M	German shepherd	2% Chlorhexidine	2.75	ND	2.82	0.9	2.87	2.88
5	1.5 y	F	Mixed	2% Chlorhexidine	2.35	ND	2.45	1.22	2.65	2.12
6	10 mo	F	English cocker spaniel	None	2.56	ND	2.46	1.32	2.95	2.44
7	1 y	M	Pug	None	2.25	ND	2.56	1.64	2.65	2.77
8	1.5 y	M	Beagle	2.5% Benzyl peroxide	2.29	ND	2.54	1.02	2.4	2.55
9	9 mo	M	Mixed	Herbal cream	2.36	ND	2.51	1.54	2.96	ND
10	1 y	M	Mixed	2.5% Benzyl peroxide	2.9	ND	2.89	1.33	3.54	ND

[a] Other treatments apart from oral cephalexin.
[b] Local herbal product containing custard apple seeds and other Thai herbal ingredients recommended for localized dermatitis.
[c] Mannitol salt agar.
[d] Mannitol salt agar containing oxacillin, 0.5 μg/mL.
[e] Numbers of *Staphylococcus*-like colonies between the 3 groups were significantly different (Kruskal-Wallis test; $P = 0.007$).
[f] Numbers of *Staphylococcus*-like colonies in the follow-up group were greater than in the pre-treatment and treatment groups (Wilcoxon Signed Ranks test; $P = 0.005$ and $P = 0.013$). ND — not detectable; M — male; F — female; CFU — colony-forming units. Chlorhexidine and benzoyl peroxidase were shampoos.

the persistence of the resistant population in a longitudinal study.

Materials and methods

Animals and treatment

This study was approved by the Chulalongkorn University Institutional Animal Care and Use Committee (permit number 113/56). Owner's permission was obtained through a consent form. Between 2011 and 2013, 10 dogs from different households were recruited on a voluntary basis by the Dermatological Unit at the University's Small Animal Teaching Hospital. Inclusion criteria for the dogs were generalized superficial pyoderma indicated by the presence of primary and secondary lesions including erythema, papules, pustules, or epidermal collarets, and having no previous treatment with any drugs. All subjects were treated with cephalexin monohydrate (Sialexin; Siam Pharmaceutical, Bangkok, Thailand), 22 to 30 mg/kg body weight (BW), PO, q12h for 2 mo and with topical therapy in most cases (Table 1). The dose and duration of treatment were prescribed by the veterinary dermatologist.

Bacterial collection

Each dog was sampled at 3 times: i) prior to treatment with antibiotics (Pre-treatment group); ii) 1 wk after the start of treatment (Treatment group); and iii) 6 to 12 mo after the onset of treatment (follow-up group). Dog 9 was sampled at both 6 and 12 mo after treatment. Up to 4 samples from the same dog were collected over the duration of the study, depending on the cooperation of the animal owners.

Isolation and identification of *S. pseudintermedius*

Samples were collected using sterile cotton swabs inserted at least 0.5 cm into the left rostral nares of the dogs. The tip of the cotton swab was added to 1 mL of 0.85% normal saline in a microcentrifuge tube, then vigorously mixed and kept at 4°C for no longer than 2 h before it was cultured for bacteria. Ten-fold serial dilutions were prepared as described in the ISO6888-1 guideline (10), and 100 μL of each dilution was plated onto mannitol salt agar (MSA) (Difco, Paris, France), and onto MSA containing 0.5 μg/mL oxacillin (Sigma-Aldrich, St. Louis, Missouri, USA) (MSA-O) (11). The plates were incubated at 37°C for 24 h and at 35°C for 48 h, respectively. Colonies of staphylococci that were pink, round, convex, smooth and 0.1 to 0.3 mm in diameter were counted on 2 plates per dilution series containing approximately 20 to 200 colonies and the average number was used to calculate the colony-forming units (CFU)/swab.

At the highest serial dilution plate with visible growth of bacterial colonies, 3 suspected staphylococcal colonies were selected from MSA-O plates for species identification. In the case of no bacterial growth on MSA-O, pink colonies were collected from MSA without oxacillin. *Staphylococcus pseudintermedius* from either MSA-O or MSA was identified by routine primary biochemical tests, the tube coagulase test and secondary biochemical properties, with confirmation by amplification of the *nuc* gene by polymerase chain reaction (PCR) (12,13). After identification, non-staphylococci and coagulase negative staphylococci were excluded from the experiment. *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923^T, *S. pseudintermedius* CVMC [Chulalongkorn University Veterinary Microbiology (CUVM), canine strain] 0108, *S. intermedius* CVMP (CUVM pigeon strain) 0309, *Staphylococcus delphini* CVMP 0109 and *Staphylococcus schleiferi* subsp. *coagulans* CVMC 0208 were used as control strains. One *S. pseudintermedius* isolate per dog per time of collection, comprising 10 isolates from prior to treatment, 10 isolates from the first week, and 11 isolates from follow-up dogs, were used for susceptibility testing and molecular typing.

Susceptibility testing and MRSP detection

All *S. pseudintermedius* isolates were assessed for susceptibility against 8 antimicrobials by the disk diffusion method including 1 μg oxacillin (OXA), 200 μg mupirocin (MUP), 15 μg erythromycin (ERY), 2 μg clindamycin (CLI), 30 μg

Table 2. Genotypic and antibiogram profiles of coagulase-positive staphyococci (CoPS) serially isolated from 10 dogs before treatment (0 month) until a maximum of 12 months after treatment.

Dog	CoPS	PFGE type	ST	Antibiogram	SCC*mec*	Time of occurrence (months) 0	1[a]	6	8	12
1	MSSP	A			Neg	■				
	MRSP	F	45	OXA-ERY-CLI-GEN-DOX-SXT	NT		■	■		
2	MSSP	A			Neg	■				
	MRSP	F	45	OXA-ERY-CLI-GEN-DOX-SXT	NT		■		■	
3	MSSP	B			Neg	■				
	MRSP	F	45	OXA-ERY-CLI-GEN-DOX-SXT	NT		■	■		
4	MSSP	B			Neg	■				
	MRSP	C	112	OXA-ENR-ERY-CLI-GEN-DOX-SXT	A1		■		■	
5	MSSP	D			Neg	■				
	MRSP	C	112	OXA-ENR-ERY-CLI-GEN-DOX-SXT	A1		■		■	
6	MSSP	E			Neg	■				
	MRSP	H	181	OXA-ENR-ERY-CLI-GEN-DOX-SXT	V		■			■
7	MSSP	G			Neg	■				
	MRSP	H	181	OXA-ENR-ERY-CLI-GEN-DOX-SXT	V		■		■	
8	MSSP	I			Neg	■				
	MRSP	C	112	OXA-ENR-ERY-CLI-GEN-DOX-SXT	A1		■			■
9	MSSP	G			Neg	■				■
	MRSP	C	112	OXA-ENR-ERY-CLI-GEN-DOX-SXT	A1		■	■		
10	MSSP	J			Neg	■				■
	MRSP	F	45	OXA-ERY-CLI-GEN-DOX-SXT	NT		■			

CoPS — coagulase-positive staphyloccci; MSSP — methicillin-sensitive *S. pseudintermedius;* MRSP — methicillin-resistant *S. pseudintermedius;* PFGE — pulsed-field gel electrophoresis; ST — sequence type in multilocus sequence typing; NT — non-typable; Neg — negative.
1[a]: the samples were collected on the 7th day after onset of treatment. OXA — oxacillin; ERY — erythromycin; GEN — gentamicin; CLI — clindamycin; DOX — doxycycline; SXT — sulphamethoxazole; ENR — enrofloxacin.
A grey block indicates the presence of the clones at the time of sampling. All dogs had CoPS at each sampling time. Three samples were obtained from each dog, except for dog 9 samples were obtained.

doxycycline (DOX), 10 μg gentamicin (GEN), 5 μg enrofloxacin (ENR), and 25 μg sulfamethoxazole/trimethoprim (SXT). The protocol was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, Vet01-A4 (14). *Staphylococcus aureus* ATCC 25923^T was used as the standard control. Isolates were confirmed as MRSP by oxacillin resistance (15) and possession of the *mecA* gene (16).

Molecular typing

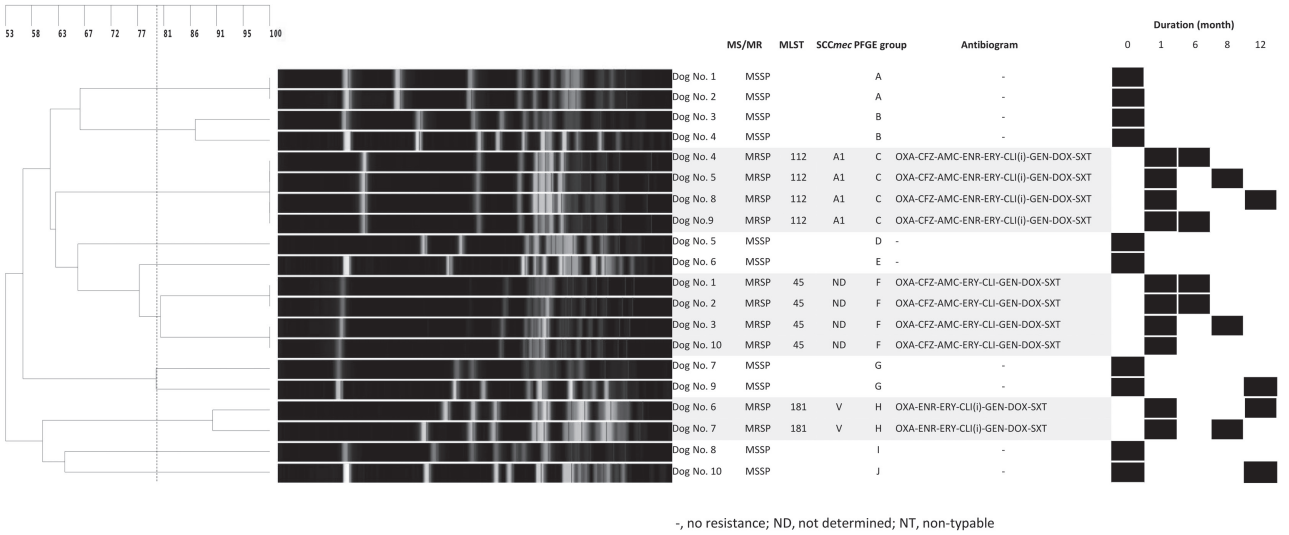
SCC*mec* of MRSP isolates were classified by the presence of the *mec* complex class and the type *ccr* complex by PCR (17). The DNA fingerprints were obtained for strain typing using *Cfr*9I-pulsed-field gel electrophoresis (PFGE) with the CHEF-DRIII apparatus (Bio-Rad, Hercules, California, USA), with a voltage of 6 V/cm and a switch time 0.5 to 15 s for 18 h and 20 to 25 s for 5 h (18). A The *Xba*I-digested chromosome of *Salmonella* Braenderup H9812 was used as a standard marker for normalization, and a dendrogram was constructed using Gene Directory software (Syngene, Frederick, Maryland, USA) with UPGMA and setting at 1.0% position tolerance. A PFGE group was defined as clustering with an 80% similarity in pattern. Multilocus sequence typing (MLST) was performed to determine the sequence type (ST) of MRSP strains by amplification and sequencing of 7 housekeeping genes (*ack, cpn60, fdh, pta, purA, sar,* and *tuf*), and analysis with the PubMLST database (http://pubmlst.org/spseudintermedius/) (19).

Statistical analysis

Statistics 17 for Microsoft Windows (SPSS Inc.; Chicago, Illinois, USA) was used for all analyses. Category comparison for number of colonies cultured among groups (Pre-treatment, Treatment, and Follow-up) was done using the Kruskal-Wallis test. Differences between the numbers of colonies cultured at each observation were analysed using the Wilcoxon signed-ranks test. Values of $P < 0.05$ were statistically significant.

Results

All selected dogs were presumptively diagnosed with generalized superficial pyoderma. After 2 mo of administration of cephalexin, all dogs had normal skin without the need for additional antibiotic or steroid therapies throughout the time of observation. All dogs had *S. pseudintermedius* isolated at each sampling time (Tables 1 and 2). On MSA, the numbers of CFU of staphylococcus-like colonies among the 3 groups



Supplementary Figure. Illustration of genotypic clustering of *S. pseudintermedius* serially isolated from 10 nasal carriage samples. – No resistance; ND – Not detectable by two panel multiplex PCR; NT – Non-typable.
Black block indicates appearance of the strain at the time of observation.

were significantly different (P = 0.007). Furthermore, the CFU for the dogs at follow-up were significantly greater than for the pre-treatment (P = 0.005) and treatment (P = 0.013) samples (Table 1). Only MSSP was isolated from all dogs before treatment, and dogs 9 and 10 also had MSSP isolated at 12 mo after treatment. Twelve MSSP isolates, including 10 from all dogs before treatment and 2 from Dog 9 and Dog 10 at 12 mo after treatment, were included for the PFGE fingerprint analysis. A total of 19 MRSP were selected from all dogs at the 1st week after treatment and the follow-up period (6 to 12 mo after treatment) (Table 2). All MRSP isolates were characterized by SCC*mec* typing, MLST, and DNA fingerprint analysis. A dendrogram from DNA fingerprint analysis of 31 *S. pseudintermedius* isolates illustrated with other characteristics and time of isolation is presented as a supplementary figure. Isolates from the same dog having an identical PFGE pattern, sequence type (ST), SCC*mec* type, and antibiogram are shown as 1 representative pattern.

By PFGE typing, 12 MSSP isolates clustered into 9 groups and 19 MRSP isolates clustered into 3 groups based on the 80% similarity cut-off. Typing by MLST identified 3 STs of MRSP including ST45, ST112, and ST181. MRSP ST181 contained SCC*mec* V (MRSP ST181-V), and ST112 carried non-typable SCC*mec* with a class A *mec* complex and type 1 *ccr* complex (MRSP ST112-A1). Multiplex PCR could not identify the SCC*mec* type of MRSP ST45 (MRSP ST45-ND). Antibiograms of MRSP are presented in the supplementary figure (available from the author) and Table 2.

Discussion

In previous studies, risk factors associated with increased detection of MRSP included frequent visits to veterinary clinics, prolonged hospital stays, and having a breeding bitch in the same household — but the effects of administration of antimicrobials have not been consistent (20–22). Thus, this longitudinal study assessed the dynamic population change of *S. pseudintermedius* between pre-treatment and drug-use, as well as the persistence of MRSP after treatment. Samples were taken from the nose, as this site is known to be an important source of staphylococcal carriage and contamination for other hosts (9). The careful selection of animals in the study may explain why untreated dogs had no resistant strains detected, which differed from previous reports (9,21). In our study, *S. pseudintermedius* could be a microbial marker for selection of antimicrobial resistant strains.

The use of MSA allowed growth of all staphylococci with pink colonies that could be used to differentiate them from other genera (12). The MSA-O agar was used to screen the staphylococci with the methicillin resistance trait, thus the bacterial number tentatively represented the MRSP number (15). The increased number of colonies of staphylococci on MSA found in the follow-up samples compared to pre-treatment might have arisen from co-colonization with MRSP and pre-existing MSSP strains. Adaptation mechanisms of bacterial strains in their ecological niche in the canine nose following antibiotic treatment have not been investigated. In all treated dogs, MSSP appeared to be replaced by MRSP as the dominant coagulase-positive *Staphylococcus* by the first week after treatment. Hence cephalexin treatment rapidly drove an increase in MRSP, consistent with the selective pressure theory for staphylococcal populations (23). This result confirms that selection of MRSP during treatment occurs frequently in the nasal environment (9). Moreover our follow-up demonstrated maintenance of a high level of persistence for 6 to 12 mo in this longitudinal study, which was concordant with the results of a previous cross-sectional study (9).

Pulsed-field electrophoresis typing is an approved genetic classification tool, and gave results consistent with the MLST results. The findings confirmed that clones of MSSP were genetically different from MRSP. The MLST and PFGE analysis confirmed that the persistent MRSP in follow-up dogs was the

same clone in all cases (8). The frequency of specific MRSP clones in an individual could be explained by a selective pressure phenomenon exerted by pre-existing resistant strains during antimicrobial exposure.

Isolates ST45-ND, ST112-A1, and ST181-V were shown to be multi-resistant to at least 5 additional antimicrobial classes. SCC*mec* of MRSP ST45 was not specifically identified in this study, but ΨSCCmec_{57395} is commonly associated with this ST in Thailand and Israel (11,23,24). Additionally, ST45, ST112, and ST181 were previously reported as clones shared between dogs and owners (11). Our study showed that MRSP could be detected in healthy convalescent dogs, and that MLST and SCC*mec* typing were useful to study the molecular epidemiology of the infection.

In conclusion, we demonstrated that oral cephalexin treatment of 10 dogs with pyoderma was associated with selection of MRSP clones with multidrug resistance. We observed a rapid onset of selective pressure and maintenance of MRSP for up to 12 mo after treatment.

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