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Original Research Article

PARTICIPATION AND DRUG RESISTANCE OF COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM CASES OF PYODERMA AND OTITIS EXTERNA IN DOGS

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Abstract: Staphylococcus *pseudintermedius* is considered as a major pathogen in dogs, typically involved in skin and ear infections. Other staphylococci, as well as ß-hemolytic streptococci, *Pseudomonas aeruginosa* or yeast-like fungi of the genus *Malassezia* also play an important role in inflammation. Because of this diversity, an appropriate choice of antimicrobial agent(-s) can be difficult. A total of 474 tests were performed (including 255 pyoderma and 219 *otitis externa cases*). In the case of pyoderma, 82.4% of skin lesions were caused by stap hylococci. Co-infections with ß-hemolytic streptococci (17.3 %), *Malassezia* sp. (15.7 %) and *P. aeruginosa* (4.3 %) were also recorded. For external ear infections, the share of staphylococci in inflammation was lower (44.8 %) than that of *Malassezia* sp. (58 %). Relatively frequent co-infections with ß-hemolytic streptococci (18.8 %) and P. aeruginosa (7.8 %) were also noted. A total of 308 susceptibility tests for coagulase-positive staphylococci were performed (210 and 98 for skin and *otitis externa*, respectively). In ≥ 86 % of cases, amoxicillin potentiated with clavulanic acid, cephalexin and fluoroquinolones effectively inhibited the growth of all bacteria *in vitro*. A total of 25 isolates (24 *S. pseudintermedius* and one *S. aureus*) were considered as methicillin-resistant. The *mecA* gene was identified in 100 % of those strains but only 44 % of the isolates additionally carried the *blaZ* gene. All *mecA*-positive staphylococci were multidrug-resistant, mainly to all ß-lactams, fluoroquinolones, linkozamides, macrolides as well as sulfonamides but always susceptible to mupirocin. Overall, multidrug-resistance varied between 6 % (*otitis externa*) and 9 % (inflamed skin) and may become a significant problem in the future.

Key words: pyoderma; otitis externa; dog; staphylococci; multidrug resistance; *mecA*, mupirocin

Introduction

Staphylococcal infection is one of the most common diseases diagnosed in dogs, since it is commonly seen as a secondary cause of skin changes. Staphylococcal diseases are most often described for multifactorial skin changes throughout the body as various types of purulent dermatitis (pyoderma) (1) or a disease focused on the dog's ear called otitis externa (OE) (2).

Pyoderma occurs in the state of reduced animal immunity or anatomical predisposition (skin folds, etc.) and is often accompanied by other dermatological problems as a complication of the primarily underlying disease: ectoparasites (genera of *Sarcoptes*, *Cheyletiella*, *Demodex*, etc.), ringworm (dermatophytes), allergy (e.g. atopic dermatitis, flea allergic dermatitis or food allergies), internal diseases (mainly hormonal disorders) and sebaceous adenitis (3).

Otitis externa is characterized by inflammation of the external ear canal as a combination of primary factors (parasitic e.g. *Otodectes cynotis*, allergic, etc.), predisposing factors (e.g. ear canal conformation, overproduction of cerumen, moisture, floppy ears, etc.), narrowed ear canals (e.g. hyperplasia, stenosis, foreign bodies, etc.)

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and secondary factors (e.g. bacterial or yeast-like fungi infections, etc.) (4).

Coagulase-negative strains of *Staphylococcus* sp. as well as *Staphylococcus pseudintermedius* are usually normal inhabitants of the dog's skin and mucosa. However, a large number of them in connection with an innate predisposition (e.g. age, breed, steroids, short coat) under favorable environmental conditions for the proliferation causes the appearance of skin inflammation (5). The coagulase test differentiates coagulase-positive strains of *Staphylococcus aureus*, isolates belonging to the *S. intermedius* group (SIG; which is divided into three clusters: *S. intermedius*, *S. pseudintermedius* and *Staphylococcus delphini*) and *S. schleiferi* subspecies *coagulans* from *S. epidermidis* and other coagulase-negative species (6). *Staphylococcus pseudintermedius* is the most commonly isolated staphylococcus in dogs (31 – 68 % of cases, even up to 100% in puppies). *Staphylococcus aureus* and *Staphylococcus schleiferi* subsp. *coagulans* are isolated much less frequently (up to 10 % and 4%, respectively). It appears that these staphylococcal species have evolved separately through adaptation to their respective natural hosts and differ in various aspects concerning ecology, population structure and evolution of antibiotic resistance (7). Additional predispositions for increasing the amount of *S. pseudintermedius* on the skin are breed (8) or atopic dermatitis (9). Gram negative rods of the *Pseudomonas* genus or *Enterobacteriaceae* family (*Escherichia coli*, *Proteus*, etc.) as well as Gram positive environmental rods are definitely less frequently isolated. Among other non-bacterial pathogens, that may be diagnosed in routine cultures, yeast-like fungi (*Malassezia* sp., *Candida* sp., etc.) and dermatophytes (*Microsporum* sp., *Trichophyton* sp., etc.) occur most often (10, 11).

Staphylococcus pseudintermedius and other staphylococci have usually low drug resistance. Then, with a properly selected antimicrobial drug and duration of treatment, anti-inflammatory therapy has a positive effect. Failure in the treatment of pyoderma and/or otitis externa in dogs can be caused by an inadequate selection of the first-line antibiotics (natural resistance), inappropriate dosage (≤ MIC), dose interval and duration or increasing antibacterial resistance to a given antibiotic (after a longer period of administration of the same antibiotic) (12).

The aim of the study was to determine the current participation of coagulase positive staphylococci to the formation of pyoderma and otitis externa in dogs and to estimate the current drug resistance of staphylococci isolates to selected antibacterial agents belonging to various groups of antibiotics, differing in chemical structure and composition.

Material and methods

Microorganisms were isolated from clinical cases of pyoderma and otitis externa (Labo-Wet, Szczecin, Poland), with a particular focus on staphylococci and yeast-like fungi. The research was carried out in the period from January 2017 to December 2018.

The research material consisted of swabs with transport medium or deep skin scrapings (transfer to the growth media within < 24 h). Each sample was cultured on Columbia agar base with 5% defibrinated sheep blood (GRASO Biotech, Starogard Gdański, Poland) as well as some selective media (Mannitol Salt Agar for staphylococci, Edwards Medium (modified) with 7% defibrinated sheep blood for streptococci, MacConkey agar for Gram negative rods and Sabouraud agar supplemented with Tween80 for yeast-like fungi; OXOID, Argenta, Poznań, Poland). The cultures were incubated at +37°C up to 96 h and then evaluated by categorizing bacteria and fungi. Due to the requirement of a longer culture period, the research did not include dermatophytes.

Preliminary division into Gram positive and Gram negative bacteria or yeast-like fungi was determined on the basis of morphological characteristics of the colonies, growth on individual culture media and the Gram staining method with subsequent microscopic examination.

Gram positive catalase-positive cocci capable of rapid growth on Chapman's medium were preliminarily classified as staphylococci. For each isolate, the tube coagulase test (Biomed, Cracow, Poland) was performed to detect free coagulase. In order to preliminarily distinguish *Staphylococcus aureus* from other coagulase-positive staphylococci, a clumping factor - "bound coagulase" test (Staphaurex™ Plus Latex Agglutination Test, Remel, OXOID, Argenta, Poznań, Poland) was performed. Additionally, selected phenotypic traits of coagulase-positive staphylococci were tested: VP test (Voges-Proskauer for acetoin; Mikrolatest®, Erba Mannheim, Brno, Czech Republic), acid production from D-mannitol as well as D-trehalose

(anaerobically) and arginine dihydrolase (ADH) tests (GRASO Biotech, Starogard Gdański, Poland) (13, 14). Growth on chromogenic media (Chromagar *Staphylococcus aureus*, GRASO Biotech Starogard Gdański, Poland), sensitivity to polymyxin B, presence and type of hemolysis as well as colony shape and color determined an additional internal control of staphylococcal differentiation. However, there is no "gold standard" to differentiate phenotypically similar coagulase-positive staphylococci, especially *S. pseudintermedius* and *S. intermedius*. Therefore, a multiplex-PCR (M-PCR) method for species identification of seven coagulase-positive staphylococci (*S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S.delphini* group A and B, *S. schleiferi* subsp. *coagulans*, *S. hyicus*) and one coagulase-negative staphylococcus (*S. schleiferi* subsp. *schleiferi*) was used for all isolated staphylococci strains by targeting the thermonuclease (*nuc*) gene locus according to Sasaki et al. (15). Briefly, a single colony of each strain was suspended in 200 µl of 0.9% NaCl and then the DNA was extracted using the genesig Easy DNA/RNA extraction kit (Primerdesign Ltd, United Kingdom). Supernatants were used as crude DNA extracts for M-PCR. As an internal control of expected band size (electrophoresis on a 1.0% agarose gel), DNA belonging to two reference strains (*S. aureus* ATCC 25923 and *S. pseudintermedius* ED99) was also used.

Gram positive catalase-negative cocci capable of rapid growth on Edwards medium with blood (usually with a strong β hemolysis) were classified as streptococci. To confirm this, a latex agglutination test for the identification of streptococcal Lancefield's groups was used (OXOID, Argenta, Poznań, Poland).

G-negative rods were initially differentiated by growth type on MacConkey agar. Non-lactose fermenting strains were additionally differentiated by the oxidase test (Microbiologics Inc., OXOID, Argenta, Poznań, Poland), while lactose fermenters on ECC chromogenic agar (GRASO Biotech, Starogard Gdański, Poland). For the genus *Pseudomonas*, the ability to produce characteristic odor and dye diffusing into agar was also observed as well as significant drug resistance. Yeast-like fungi were isolated on Sabouraud agar supplemented with Tween80 (GRASO Biotech, Starogard Gdański, Poland).

A detailed analysis of the drug resistance was performed only on staphylococci. Sensitivity to specific groups of antibiotics and antimicrobial agents was tested by the diffusion-disc method according to the VET01-A4 (16) and M100-S27 (17) recommendation. Commercial discs (OXOID, Argenta, Poznań, Poland) used in the present study were saturated with the following antimicrobial agents: amoxicillin with clavulanic acid (20+10 µg), enrofloxacin (5 µg), marbofloxacin (5 µg), ciprofloxacin (10 µg), gentamicin (10 µg), polymyxin B (300 U) trimethoprim with sulphametaxazole (1:19; 25 µg), cefalexin (30 µg), linkomycin with spectinomycin (109 µg), clindamycin (2 µg), neomycin (30 µg) and orbifloxacin (5 µg). For the detection of methicillin resistance, oxacillin (1 µg; resistance with the zone of inhibition ≤ 17 mm recommended for *S. pseudintermedius*; and cefoxitin (30 µg; a surrogate for oxacillin recommended for *S. aureus*, ≤ 21 mm) were used (17). In addition, mupirocin discs (5 µg and 200 µg; OXOID, Argenta, Poznań, Poland; no zone at 200 µg = high level mupirocin resistance) were utilized only in the case of all multidrug-resistant strains (17). Plates of Mueller-Hinton agar (GRASO Biotech, Starogard Gdański, Poland; + 2% of NaCl only for oxacillin) culture medium were used. Incubation was carried out at +35±2 °C (2 x 6 discs on 90 mm plates) and +30±2 °C (only for oxacillin and cefoxitin; testing at temperatures above +35°C may not detect Methicillin-resistant staphylococci) for 18 ÷ 24h. The zones were measured to the nearest millimeter and examined carefully in good light to detect colonies within the zone. In the case of the observed resistance to β-lactam antibiotics, the presence of the *mec*A, *mec*C and *blaZ* genes has also been tested according to Ruzauskas et al. (18).

Results

Over the period considered, a total of 474 samples were examined, including 255 swabs or skin scrapings and 219 swabs from the canine external ear canal. The results are summarized in Tables 1 and 2. In the cases of the S+1, S+2, S+3 and "no STAPH, growth" schemes, particular numbers given for individual pathogens (underlined values) do not refer to the actual number of tests. They only indicate a quantitative contribution in combination with another microorganism.

In the case of pyoderma, the vast majority of skin lesions were caused by staphylococci (82.4%), with more than half of the cases (51%) alone. In the present study, β-hemolytic

streptococci have never been the primary cause of skin lesions. Along with staphylococci, they caused 44 infections (17.3% of cases). Yeast-like fungi were rarely the sole cause of skin changes (only 1.6% of all cases), while their frequent coexistence with staphylococci was found (n = 36, which represented 14.1% of cases) and only one case in mixed infection without staphylococci was observed. "Aseptic" cases accounted for a slightly lower proportion of all samples (12.8%), in which no growth of either bacteria or yeast-like fungi was found despite 96 h of incubation. In these cases, allergies or dermatophytes could not be confirmed or excluded. Of the remaining bacteria belonging to the group of opportunistic microorganisms, the greatest importance was attributed to the *Pseudomonas* spp. (a total of 11 cases; 4.3%).

In the case of *otitis externa*, the share of staphylococci in inflammation was almost half lower (44.8%), of which only 11% of cases of staphylococci presence were the sole cause of changes in the ear (nearly five times less than in the case of skin lesions). On the other hand, the share of yeast-like fungus increased dramatically (58%, almost four times), with up to 26% of the primary cause. It was also the dominant microorganism that complicated wounds together with coagulase-positive *Staphylococcus* sp. (27.4%) or other species (n=10, i.e. 4.6%). Among other microorganisms, the share of the pyogenic rods (*Pseudomonas* sp.) increased significantly. They where present either alone (4.6%) or in mixed growth with staphylococci (3.2%) or other opportunistic bacteria and / or yeast-like fungi (4.6%). β-hemolytic streptococci still constituted a significant proportion of cases (18.8%), sporadically occurring alone (1.4%), but more often with coagulase-positive *Staphylococcus* (11.9%) or other microorganisms (5.5%). In 16% of samples, there was no growth of microorganisms under aerobic conditions after 96 h incubation, which was the basis for ending the test with negative results (marked as "sterile" samples).

Table 1: The participation of staphylococci, yeast-like fungi and other microorganisms in the formation of pyoderma in dogs

type of growth	no growth	Y-L	STAPH	S+1				S+2					S+3					no STAPH, growth:			
				STR	G(-)	G(+)	Y-L	STR	PSE	G(-)	G(+)	Y-L	STR	PSE	G(-)	G(+)	Y-L	PAS	STR	PSE	Y-L
n	33	4	130	19	6	5	21	23	4	8	2	13	2	3	3	2	2	4	4	4	1
				51 (20)				25 (9.8)					4 (1.6)								
(%)	(12.8)	(1.6)	(51)	80 (31.4)														8 (3.2)			
				210 (82.4)																	
total:	255 (100%)																				

S+1, S+2, S+3 *Staphylococcus* growth in addition to one, two or three other microorganisms, respectively n – number of individuals
Y-L yeast-like fungi STAPH - *Staphylococcus* spp. STR - *Streptococcus* spp. PSE - *Pseudomonas* spp. G(-) – Gram negative rods
G(+) – Gram positive rods PAS - *Pasteurella* spp.

Table 2: The participation of staphylococci, yeast-like fungi and other microorganisms in the formation of otitis externa in dogs

type of growth	no growth	Y-L	STAPH	S+1				S+2					S+3				no STAPH, growth:				
				STR	PSE	G(-)	Y-L	STR	PSE	G(-)	G(+)	Y-L	STR	G(-)	G(+)	Y-L	STR	PSE	Y-L	G(-)	G(+)
n	34	58	24 (11)	11	2	1	35	10	3	3	4	20	5	3	2	5	15	20	10	2	2
				49 (22.4)				20 (9.1)					5 (2.3)								
(%)	(16)	(26)		74 (33.8)													29 (13.2)				
				98 (44.8)																	
total:	219 (100%)																				

S+1, S+2, S+3 *Staphylococcus* growth in addition to one, two or three other microorganisms, respectively n – number of individuals
Y-L yeast-like fungi STAPH - *Staphylococcus* spp. STR - *Streptococcus* spp. PSE - *Pseudomonas* spp. G(-) – Gram negative rods
G(+) – Gram positive rods

Table 3: The drug resistance of coagulase-positive staphylococci isolated from pyoderma cases in dogs

antimicrobial agent		coagulase-positive staphylococci (other than *S. aureus*) n = 200*			Total	*Staphylococcus aureus* n = 10			Total
		S	I	R**		S	I	R	
Amoxicillin / Clavulanic acid	n	182	n/a	18	200	9	n/a	1	10
	%	91		9		90		10	
Enrofloxacin	n	172	3	25	200	9	-	1	10
	%	86	1.5	12.5		90	-	10	
Marbofloxacin	n	161	3	22	186	9	-	1	10
	%	86.6	1.6	11.8		90	-	10	
Ciprofloxacin	n	165	3	22	190	9	-	1	10
	%	86.8	1.6	11.6		90	-	10	
Gentamicin	n	148	18	23	189	6	3	1	10
	%	78.3	9.5	12.2		60	30	10	
Polymyxin B	n	166	34	-	200	-	-	10	10
	%	83	17	-		-	-	100	
Trimethoprim with sulfamethoxazole	n	137	21	24	182	9	-	1	10
	%	75.3	11.5	13.2		90	-	10	
Cephalexin	n	158	6	18	182	6	2	1	9
	%	86.8	3.3	9.9		66.7	22.2	11.1	
Clindamicin	n	78	22	46	146	2	1	1	4
	%	53.4	15.1	31.5		50	25	25	
Lincomycin with spectinomycin	n	43	25	13	81	-	2	-	2
	%	53.1	30.9	16		-	100	-	
Neomycin	n	29	10	11	50	2	1	1	4
	%	58	20	22		50	25	25	
Mupirocin	n	18	-	-	18***	1	-	-	1***
	%	100	-	-		100	-	-	
Cefoxitin	n	n/a	n/a	n/a	n/a	9	n/a	1	10
	%					90		10	
Oxacillin	n	182	n/a	18	200	n/a	n/a	n/a	n/a
	%	91		9					

S – susceptible I – intermediate R – resistant n – number of individuals n/a – not applicable
* - S. pseudintermedius n=196; *S. schleiferi* subsp. coagulans n = 4; *S. intermedius* and *S. delphini* were not detected ** - *S. pseudintermedius* in all cases *** - only multidrug-resistant strains

Table 4: The drug resistance of coagulase-positive staphylococci isolated from *otitis externa* cases in dogs

antimicrobial agent		coagulase-positive staphylococci (other than *S. aureus*) n = 96*			Total	*Staphylococcus aureus* n = 2			Total
		S	I	R**		S	I	R	
Amoxicillin / Clavulanic acid	n	90	n/a	6	96	2	-	-	2
	%	93.8		6.2		100		-	
Enrofloxacin	n	87	2	7	96	2	-	-	2
	%	90.6	2.1	7.3		100	-	-	
Marbofloxacin	n	85	2	7	94	2	-	-	2
	%	90.4	2.2	7.4		100	-	-	
Ciprofloxacin	n	82	1	7	90	2	-	-	2
	%	91.1	1.1	7.8		100	-	-	
Gentamicin	n	76	8	8	92	1	1	-	2
	%	82.6	8.7	8.7		50	50	-	
Polymyxin B	n	70	26	-	96	-	-	2	2
	%	72.9	27.1	-		-	-	100	
Trimethoprim with sulfamethoxazole	n	65	5	15	85	2	-	-	2
	%	76.5	5.9	17.6		100	-	-	
Cephalexin	n	85	-	5	90	1	-	-	1
	%	94.4	-	5.6		100	-	-	
Lincomycin with spectinomycin	n	15	8	1	24	-	-	-	0
	%	62.5	33.3	4.2		-	-	-	
Clindamicin	n	10	3	7	20	2	-	-	2
	%	50	15	35		100	-	-	
Neomycin	n	15	10	11	36	1	-	-	1
	%	41.7	27.8	30.6		100	-	-	
Orbifloxacin	n	34	2	9	45	1	-	-	1
	%	75.6	4.4	20.0		100	-	-	
Mupirocin	n	6	-	-	6***	n/a	n/a	n/a	n/a
	%	100	-	-		n/a	n/a	n/a	
Cefoxitin	n	n/a	n/a	n/a	n/a	2	-	-	2
	%					100	-	-	
Oxacillin	n	90	n/a	6	96	n/a	n/a	n/a	n/a
	%	93.8		6.2					

S – susceptible I – intermediate R – resistant n – number of individuals n/a – not applicable
* - S. pseudintermedius n=95; *S. schleiferi* subsp. coagulans n = 1; *S. intermedius* and *S. delphini* were not detected
** - *S. pseudintermedius* in all cases *** - only multidrug-resistant strains

Table 5: Frequency of selected genes among multidrug-resistant strains of coagulase-positive staphylococci

PCR	multi-drug resistant staphylococci (n=25)			
	S. pseudintermedius		*S. aureus*	
	skin	ear	skin	ear
mecA	18/18 (100%)	6/6 (100%)	1/1 (100%)	0 (0%)
total:	24/24 (100%)		1/1 (100%)	
	25/25 (100%)			
mecC	0/18 (0%)	0/6 (0%)	0/1 (0%)	0 (0%)
total:	0/24 (0%)		0/1 (0%)	
	0/25 (0%)			
blaZ	8/18 (44.4%)	2/6 (33.3%)	1/1 (100%)	0 (0%)
total:	10/24 (41.7%)		1/1 (100%)	
	11/25 (44%)			

Among all the above mentioned microorganisms, detailed biochemical and molecular tests were conducted only on staphylococci. In the present study, coagulase-negative staphylococci (including *Staphylococcus schleiferi* subsp. *schleiferi*) occurred very rarely (< 5% of cases during the whole research) and, if detectable, they usually grew as single colonies and for this reason they were ignored in routine research. The vast majority of staphylococci, that were isolated due to their abundant and homogeneous growth, were coagulase-positive (but clumping factor negative), VP-positive, ADH-positive and D-trehalose positive. Mannitol was not fermented (or a weak and delayed fermentation of this sugar occurred; similar observations were made on Mannitol Salt Agar) and always with double-zone hemolysis on sheep blood agar (without pigmentation of colony). All such isolates were sensitive to polymyxin B and grew on Chromagar as mixed violet-blue colonies. After electrophoresis, band size was always at 926 bp (similar to *S. pseudintermedius* ED99 internal control). Taking into account biochemical traits and molecular results (M-PCR), *S. pseudintermedius* was predominant both in the pyoderma (93.33%; Table 3) and *otitis externa* (96.94%; Table 4) cases. Only few β-hemolytic strains (4.76% and 2.04%, respectively) were additionally positive in the clumping factor test, VP-positive, both D-mannitol and D-trehalose positive and ADH-negative (or showed a weak result after 48 h incubation). In addition, they were always resistant to polymyxin B and grew on Chromagar as dark-pink colonies (colony color was in accordance with the recommendations for *S. aureus* given by the manufacturer of the chromogenic media). After M-PCR, all such isolates had a 359-bp amplification fragment similar to the *S. aureus* ATCC 25923 reference strain. These strains were considered to be *Staphylococcus aureus* (Tables 3 and 4). Finally, only 1.91% of the skin and 1.02% of the ear isolates of staphylococci had completely different traits: both sugars were not fermented, while VP and ADH were positive. Colonies on blood sheep agar were β-hemolytic (without pigmentation), clumping factor was negative, sensitivity to polymyxin B was noted and each strain grew on Chromagar as light pink colonies. Without an internal control, band size of M-PCR was estimated at ~ 500 ÷ 550-bp. Both biochemical traits and the size of amplification fragments indicated the presence of *S. schleiferi* subsp. *coagulans*.

For each coagulase-positive *Staphylococcus* isolate, a susceptibility test was carried out for various antimicrobial agents (antibiotics, chemotherapeutics, sometimes with the second component). A total of 308 susceptibility tests were performed, including 210 for skin lesions (Table 3) and 98 for *otitis externa* (Table 4).

In > 90% of cases, regardless of the site from which the *Staphylococcus* strain was isolated, amoxicillin (semi-synthetic β-lactam antibiotic, aminopenicillin) potentiated with clavulanic acid effectively inhibited the growth of these bacteria *in vitro*. Slightly worse activity but also at a high level (86% for the skin isolates and 90 ÷ 91% for the ear isolates), was noted for chemotherapeutics

from the second generation fluoroquinolone group (ciprofloxacin, enrofloxacin and marbofloxacin). However, in 10 ÷ 12% of cases the above-mentioned antimicrobial agents proved ineffective *in vitro*. Another β-lactam antibiotic - cephalexin (first-generation cephalosporin) was also characterized by high efficacy (nearly 87% for the skin and 94% for the ear isolates). Similar results were obtained for other antibiotics: gentamicin (aminoglycoside; 78% and 82% respectively) and one of the polymyxins: Polymyxin B (~ 73% for the ear and 83% for the skin). About 75% of the isolates were sensitive to the third generation of synthetic fluoroquinolone - orbifloxacin (mainly targeted along with antifungal posaconazole for *otitis externa*) and a chemotherapeutic trimethoprim potentiated with sulfametaxazole. The remaining antimicrobial agents were moderately effective.

A total of 24 *S. pseudintermedius* isolates (18 from skin and 6 from ear) were fully resistant to oxacillin (no doubtful results) and considered as MRSP. One *S. aureus* isolate was resistant to cefoxitin and considered as MRSA. This observation was confirmed by PCR tests. Similar to oxacillin and cefoxitin, the *mecA* gene was identified in those 25 out of 308 coagulase-positive strains (8.1 % of total strains) (Table 5; reference strains of *S. pseudintermedius* ED99 and S. aureus ATCC 25923 were *mecA*-negative). Among them, only 44% of the isolates additionally carried the *blaZ* gene encoding penicillinase that is capable of inactivating penicillin(s) by hydrolysis of the beta-lactam ring (19) (*S. pseudintermedius* ED99 was positive and *S. aureus* ATCC 25923 was negative). The rare *mec*C gene has never occurred.

All *mecA*-positive staphylococci were not only to methicillin-resistant but also multidrug-resistant, mainly to all β-lactams, fluoroquinolones, linkozamides, macrolides as well as some antimicrobial agents like sulfonamides. However, these strains were intermediate (predominant) or susceptible to linkomicin with spectinomicin, aminoglycosides and additionally tested tetracyclines. Coagulase-positive staphylococci other than *S. aureus* were always susceptible or intermediate to Polymyxin B. Resistance of dog's *S. aureus* strains to Polymyxin B was confirmed. However, each multidrug-resistant strain was always susceptible to mupirocin and the size of a clear zone of inhibition was always ≥ 20 mm.

Discussion

In the present study, the prevalence of coagulase-positive staphylococci was significantly higher when material was taken from the skin rather than the ear. In the case of pyoderma, the observed proportion of *Staphylococcus* infection was similar to other papers from all over the world. Ruzauskas et al. (18) reported that 76.8% of samples were positive for coagulase-positive staphylococci isolated from sick dogs in Lithuania. In Canada, *S. pseudintermedius* was even isolated from 78% of even healthy dogs (20). Slightly lower prevalence (65.2%) was noted in Portugal (21). In older reports, coagulase-positive staphylococci as well as *S. schleiferi* subsp. *schleiferi* were also isolated from 88% of the cases of inflamed dog's skin (22) and 76.9% of the cases of canine otitis externa (23). In healthy dogs, the carrier prevalence of coagulase-positive staphylococci is estimated at 69% (24) to 87.4% (25).

Besides methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has become a worldwide problem in small animal veterinary medicine (26). Methicillin resistance of both staphylococci is due to the presence of the *mecA* gene which encodes penicillin binding protein 2a (PBP2a) that significantly lowers affinity to all β-lactam antimicrobials (27) and often other classes. The *mecA* gene is located within the chromosome mobile element called the 'staphylococcal chromosomal cassette' (SCCmec). The SCCmec element can be easily transferred between different staphylococcal species (6). Thus, some cases of pyoderma or otitis externa seem to be extremely difficult to treat. Moreover, direct and indirect transmission of *S. pseudintermedius* (including MRSP) may occur between a carrier (even a healthy dog) and humans or other pets (28).

The prevalence of MRSP colonization or contamination has been studied in various dog populations in different countries, with rates of 0–7% in dogs with skin diseases (6). In the present study, the occurrence of *mecA*- and coagulase-positive strains varied between 6 (otitis externa) and 9 % (inflamed skin). In other studies, MRSP was detected in 5/189 (2.6%) (29) and 8/221 (3.6%) (20) of healthy dogs. Griffeth et al. (22) reported that 17% and 8% of affected dogs and 0% and 3% of isolates from healthy dogs were

MRSA and MRSP, respectively. Moreover, most of those MRSP strains also contained a wide range of different antimicrobial drug resistance genes, making them resistant to almost all classes of commonly used antimicrobial agents, even those that are not routinely used to treat staphylococcal infections like amikacin (30). A similar situation was observed in the present study.

Although a few of mupirocin-resistant *S. aureus* strains in human (31) and mupirocin-resistant *S. pseudintermedius* in dogs (32) were isolated, the mechanism of action of mupirocin differs from other clinical antibiotics, rendering cross-resistance to other antibiotics unlikely. In the present study, all multi-drug resistant strains were susceptible to mupirocin, and zones of growth inhibition (> 19 mm at the 5 µg disc) were in agreement with Creagh and Lucey (33). Mupirocin (mixture of several pseudomonic acids) is an antibiotic originally isolated from *Pseudomonas fluorescens* and is structurally unrelated to any other antibiotic (34). Pseudomonic acid is capable of binding to the isoleucyl t-RNA synthetase in *Staphylococcus* sp., resulting in a total inhibition of their protein synthesis (35). Unfortunately, after intravenous or oral administration, mupirocin is rapidly metabolized to monic acid, which has no antibacterial activity. Therefore, only topical treatment of bacterial skin infections (as ointments or creams) or nano-carriers remain available (34).

Conclusions

Routinely used antimicrobial drugs are still effective against the most commonly isolated staphylococci responsible for the development of pyoderma or otitis externa in dogs. However, since 2006, not only MRSA but also MRSP has emerged as a significant animal health problem in veterinary medicine. Such cases (~10%; *mecA*-positive strains), as well as the occurrence of naturally multi-drug resistant *Pseudomonas aeruginosa* (multidrug efflux pumps) or coexistent yeast-like fungi of the genus *Malassezia* (resistance to antibacterial drugs) make initial treatment ineffective and, without the diagnosis of disease etiology, may easily turn into chronic infections. For this reason, clinical cases of pyoderma and otitis externa should be tested more frequently in the laboratory.

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VKLJUČENOST IN ODPORNOST NA ANTIBIOTIKE STAFILOKOKOV, POZITIVNIH NA KOAGULAZO, IZOLIRANIH IZ PRIMEROV PIODERME IN VNETJA ZUNANJEGA UŠESA PRI PSIH

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Povzetek: Bakterijea *Staphylococcus pseudintermedius* so ene od pomembnejših patogenih bakterij pri psih in so običajno prisotne pri okužbah kože in ušes. Pomembno vlogo pri teh vnetjih pa imajo običajno tudi drugi stafilokoki, ß-hemolitični streptokoki, *Pseudomonas aeruginosa* ali kvasovkam podobne glive iz rodu *Malassezia*. Zaradi raznolikosti povzročiteljev vnetja je primerna izbira protimikrobnih zdravil lahko težavna. V študiji je bilo skupno opravljenih 474 testov (vključno z 255 primeri pioderme in 219 primeri vnetja zunanjega ušesa). V primeru pioderme so 82,4 % kožnih vnetij povzročili stafilokoki. Zabeležene so bile tudi sočasne okužbe z ß-hemolitičnimi streptokoki (17,3 %), *Malassezia* sp (15,7 %) in *P. aeruginosa* (4,3 %). Pri okužbah zunanjih ušes je bil delež stafilokokov v vnetjih nižji (44,8 %) kot pri *Malassezia sp.* (58 %). Opažene so bile tudi sorazmerno pogoste sookužbe z ß-hemolitičnimi streptokoki (18,8 %) in *P. aeruginosa* (7,8 %). Opravljenih je bilo skupno 308 testov občutljivosti za stafilokoke, pozitivne na koagulazo (210 za kožo in 98 za vnetje zunanjega ušesa). V manj kot 86 odstotkov primerov je amoksicilin, z dodatkom klavulanske kisline, cefaleksina in fluorokinolonov *in vitro* učinkovito zaviral rast vseh bakterij. Skupaj 25 izolatov (24 *S. pseudintermedius* in 1 *S. aureus*) je veljalo za odporne proti meticilinu. Gen *mecA* je bil identificiran v vseh sevih, vendar je le 44 % izolatov imelo gen *blaZ*. Vsi *mecA*-pozitivni stafilokoki so bili odporni na več zdravil, večinoma na vse ß-laktame, fluorokinolone, linkozamide, makrolide in sulfonamide, vendar so ostali dovzetni za mupirocin. Na splošno je odpornost na več zdravil znašala med 6 % (vnetje zunanjega ušesa) in 9 % (vnetje kože), kar lahko v prihodnosti povzroča velik problem pri zdravljenju tovrstnih okužb.

Ključne besede: pioderma; vnetje zunanjega ušesa; pes; stafilokoki; odpornost na več zdravil; *mecA*; mupirocin