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RESEARCH ARTICLE

Isolation and Genetic Characterization of *Staphylococcus haemolyticus* from Cats

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ABSTRACT

Staphylococcus haemolyticus is widespread in humans and animals. The aim of this study was to characterize *S. haemolyticus* isolates from sick and healthy cats according to their antibiotic properties and biofilm formation. A total of 80 *S. haemolyticus* isolates from 36 healthy and 20 sick cats were investigated. All the isolates from the sick as well as from the healthy animals were multidrug resistant at the genetic level, whereas 82.5% showed phenotypic resistance. Almost all the isolates were methicillin-resistant *S. haemolyticus* (MRSH) at the genotypic and phenotypic levels of 98.75% and 96.25%, respectively. Moreover, the most frequently observed phenotypic resistances were those for erythromycin (87.5%) and penicillin (93.75%), and the isolates harboured genes involved in resistance to penicillin – *bla*Z (97.5%), aminoglycosides – *aac(6')Ie-aph(2")Ia* (92.5%), macrolide-lincosamide-streptogramin – *erm*B (98.75%) and *erm*C (72.5%), and tetracyclines – *tet*(K) (98.75%) and *tet*(M) (100%). The percentage of biofilm-positive strains in healthy and sick cats amounted to 92.68% and 97.44% for the microtiter plate test (P=0.4962) and 71.17% and 56.41% for the Congo red agar assay (P=0.5164), respectively. Statistically significant associations were observed when the owner's occupation was connected with healthcare, both human or veterinary, there was previous hospitalization of the owner, or cats were kept with other animals in the household. MRSH strains isolated from companion animals were frequently multidrug resistant, although they remained susceptible to antibiotics used in mainly human medicine.

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INTRODUCTION

Differentiation between coagulase-negative *Staphylococcus* (CNS) is still a problem in the field of medicine. There are numerous staphylococcal species (54 species reported in the list of prokaryotic names with standing in nomenclature, LPSN, www.bacterio.net/staphylococcus) and the predominant are the CNSs. Among diagnosticians and medical practitioners, there is often still a conviction regarding the low pathogenicity of those bacteria, although there has been an increase in the number of studies that focus on the virulence and antibiotic resistance of several CNS species (Krzymińska *et al*., 2015). CNS is also recognized as a cause of hospital-acquired opportunistic and healthcare-related pathogen worldwide, and in a hospital setting, *Staphylococcus haemolyticus* has gained attention as the second most frequently encountered CNS after *S. epidermidis* (Panda and Singh, 2018). *S. haemolyticus* is an emerging and important human pathogen causing serious infections, which can be involved in endocarditis, urinary tract infections, septicaemia, peritonitis, and wound, bone and joint infections (Krzymińska *et al.*, 2015). Multidrug-resistant strains of *S. haemolyticus* also pose a serious problem in veterinary medicine. Due to the possibility of transmission between animals, owners, and veterinary staff, animals, especially pets, can act as reservoirs of multidrug-resistant strains of *S. haemolyticus* (Kizerwetter-Świda *et al*., 2019).

S. haemolyticus isolates are saprophytic bacteria with the ability to colonize human and animal skin and mucosal membranes (Bierowiec *et al*., 2019; Pain *et al*. 2019). In humans, staphylocooci have a predilection to colonize specific areas of the body, such as the axillae and inguinal and perineal areas. The predominant body areas colonized in animals are less understood, but

staphylococci have been isolated from nostrils, skin, ears, teats and reproductive tracts (Ruzauskas *et al.*, 2015).

The *S. haemolyticus* genome is very plastic, which could result in frequent genomic rearrangements, phenotypic diversification, and the acquisition of antibiotic resistance (Barros *et al*., 2012). There is a very high similarity (even 99.95%) of *mec*A gene sequences harboured by *S. aureus*, *S. haemolyticus*, and *S. epidermidis*, which could be connected with the frequent interspecies transfer of the *mec*A gene (Czekaj *et al.*, 2015). Among all the CNS species, *S. haemolyticus* has the highest level of antimicrobial resistance against both β-lactam antibiotics and glycopeptides, which limits therapeutic options in human medicine (Fredheim *et al*., 2009). Methicillin-resistant S. *haemolyticus* (MRSH) strains isolated from companion animals are also frequently resistant to some classes of critically important antimicrobials, although they remain susceptible to antibiotics used in exclusively human medicine (Ruzauskas *et al.*, 2014).

Due to our currently limited knowledge about *S. haemolyticus* isolates from cats, the aim of the study was to characterize these bacteria according their antibiotic properties and biofilm formation.

MATERIALS AND METHODS

Sampling and identification: In this study, were investigated *S. haemoliticus* isolates that were collected from 673 cats and identified from 2013-2019 in the Department of Epizootiology and Clinic of Birds and Exotic Animals, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences. The swabs from cats were collected from the conjunctival sacs, nares, anus, skin (groin) and additionally, from the wound or skin with pathological changes in sick cats. The sampling procedure and initial identification was described previously (Bierowiec *et al.*, 2019). Isolates were confirmed by a PCR based on primers specific for *gro*ESL (Seng *et al*., 2017). In addition, 21 of the isolates were confirmed as *S. haemolyticus* using BLAST analysis of the 16S RNA PCR product (Lane, 1991). The obtained sequences were identified by comparison with sequences available in the GenBank database using the BLAST search algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers of isolated strains are MK446917-MK446933, MK680826, MK680827.

Antibiotic resistance: All the isolates of *S. haemolyticus* were screened for antibiotic susceptibility using both disc diffusion and MIC (Sensititre, Staphylococcus MIC plates, Thermo Fisher Scientific, Waltham, MA) methods. Antimicrobial resistance phenotyping of isolates was performed and interpreted according to the Clinical and Laboratory Standards Institute M100-S28 and CLSI VET08 ED4:2018 (http://vet01s.edaptivedocs.info). Tigecycline and fusidic acid data were interpreted according to the protocol used in a recent study (Coutant *et al.*, 1996; Brink *et al*., 2012). The double-disc diffusion test (D-test) was performed on all isolates to detect inducible clindamycin resistance (Spode Coutinho *et al*., 2010). The interpretation of the test was as follows: a flattening of the inhibition zone around the clindamycin disc and near the erythromycin disc indicated that erythromycin induced clindamycin resistance (inducable resistance to macrolides-lincosamides-streptogramin B - iMLSB). The constitutive resistance to macrolides-lincosamides-streptogramin B (cMLSB) phenotype was characterized by erythromycin and clindamycin resistance. The resistance only to macrolides and streptogramins B (MSB) phenotype was characterized by clindamycin susceptibility and erythromycin resistance, with a negative D-test.

Antibiotic resistance genotypes of isolates were identified using PCR. The presence of genes involved in resistance to penicillin, aminoglycosides, β-lactamase, glycopeptides, macrolide-lincosamide-streptogramin, tetracyclines, fusidic acid and mupirocin was determined using PCR using conditions and positive controls as previously described (Bierowiec *et al*., 2017). The resistance of *S. haemolyticus* isolates to 3 or more classes of antimicrobial agents was interpreted as multidrug resistance.

Biofilm production: Strains were tested for slime production by the Congo red agar (CRA) (Arciola *et al*., 2002) method and microtiter plate (MTP) test (Ploneczka-Janeczko *et al.*, 2014). *S. epidermidis* PCM 2532 was used as a positive control. The interpretation of bacterial growth on CRA was conducted according to a colorimetric scale (Arciola *et al*., 2002). The interpretation of the MTP method was as follows. The cut-off of the MTP test was established according to the mean for the negative control (0.245) – noninoculated tryptic soy broth (TSB, Tryptone Soya Broth, Oxoid Ltd., Wade Road Basingstoke, United Kingdom) – plus two standard deviations (0.08). The biofilm formation capability was considered positive at a cut-off level of 0.398. For the positive biofilm formers, the classification criteria were established as follows: weak biofilm formers: $0.398 < A570 \leq 0.796$ (2 × cut-off level); medium-positive biofilm formers: $0.796 < A570 \leq 1.592$ (4 × cut-off level); and strong biofilm formers: $A570 > 1.592$ (>4 × cut-off level). The MTP procedure was performed three times, with four replicates for each isolate. A standard PCR technique was used for *icaA* (Wojtyczka *et al*., 2014) and *bap* (Tormo *et al.*, 2005). genes with the positive controls being *S. epidermidis* PCM 2532 and *S. epidermidis* AIR08630, respectively.

Statistical methods: Statistical analysis was carried out using the R statistical package (v2.11.1). The characteristics of the cats and data regarding the medical history and condition of the environment where the cats were kept were compared with scores of antibiotic resistance and biofilm formation properties of *S. haemolyticus* isolates. The data were analysed using the Shapiro-Wilk test, the Wilcoxon test, the Kruskal–Wallis test, 2 × 2 contingency tables and bootstrapped chi-squared tests. $P<0.05$ was considered indicative of a statistically significant association.

RESULTS

A total of 80 *S. haemolyticus* strains were included in this study. The strains were treated as separate *S.*

haemolyticus strains even when isolated from the same host if there were differences in antibiotic resistance or biofilm formation properties. The strains were isolated from the conjunctival sacs (n=26; healthy cats n=13; sick cats n=13), nares (n=21; healthy cats n=9; sick cats n=12), anus (n=11; healthy cats n=7; sick cats n=4) or skin (n=22; healthy cats n=10; sick cats n=12) from animals kept in the Wrocław' city area between 2013 and 2019 (Table 1). In the sick cat group, most of the cats (92.86%) had clinical signs of conjunctivitis and sneezing, but only two cats had clinical signs of conjunctivitis or dermatitis. Nearly half of the cats had upper respiratory tract signs, and of these, at least one of the *S. haemolyticus* strains was isolated from the conjunctival sacs or nares and from seven cats in both anatomical localizations. Almost all of the isolates from the sick cats (95.12%) were obtained from animals with clinical signs of conjunctivitis and sneezing. *S. haemolyticus* strains were isolated from the conjunctival sacs and nares in 17.95% of cases or from only one localization: 15.38% from the conjunctival sacs or 12.82% from the nares. In all cases, *S. haemolyticus* strains were solitary *Staphylococcus* species isolated from the nares or conjunctival sacs. Nevertheless, no correlation was observed between the frequency of *S. haemolyticus* strain isolation and their anatomical localization in the sick or in healthy cats. In any of the cats under investigation, clinical signs were observed that could suggest other infections, such as chlamydophila infection or viral feline infections, including herpesvirus infection or calicivirus infection.

There were some statistically significant features connected with the care and characteristics of the cat from which the bacterial strains were isolated. In the sick cat group, *S. haemolyticus* strains were most frequently isolated when cats were kept in catteries (P<0.001), were pure breed (P=0.006; OR=0.18; CI=0.04-0.64%), were younger than 6 months (P<0.001) and were kept with other animals (P=0.0078). Healthy cats were most frequently colonized with *S. haemolyticus* when they were kept in households where one of the owners was working in healthcare (P=0.045), had been hospitalized during the last year (P=0.013) or was working in veterinary healthcare (P=0.01). Additionally, colonization with a *S. haemolyticus* strain occurred significantly more frequently in cats that have not been previously treated (P=0.019; OR=11.12; CI=1.34 -51.8%) and in cats that have not been kept with other animals that have been treated previously (P<0.001; OR=0.06; CI=0.01-0,23%).

The antimicrobial resistance profiles of *S. haemolyticus* isolates obtained via PCR were verified by testing each isolate with a suitable antimicrobials (Table 2; Table 3). All the strains from the sick as well as from the healthy animals were multidrug resistant at the genetic level, whereas 82.5% showed phenotypic resistance: 69.23% from the healthy and 92.68% from the sick cats (P=0.03; OR=0.2; CI=0.03-0.87%). Almost all *S. haemolyticus* strains were methicillin resistant at the genotypic and phenotypic levels of 98.75% and 96.25%, respectively. *S. haemolyticus* strains from healthy cats were significantly more frequently resistant to clindamycin (P<0.001), ciprofloxacin (P<0.001; OR=4.54; CI=1.22-21.28%), fusidic acid (P<0.01161; OR=7.48; CI=1.46-74.56%), quinupristin/dalfopristin (P=0.03568) and trimethoprim (P=0.04256; OR=5.73; CI=1.07-58.3%) than strains from sick cats. Additionally, isolates from healthy animals more frequently harboured *fus*B (P<0.001; OR=19.38; CI=0.26-8.69%) and *van*A (P<0.001; OR=15.27; CI=0.2-69.04%) genes than isolates from sick animals, whereas *erm*C (P<0.001; OR=0.18; CI=0.05-0.61%) and *tet*(L) (P<0.001; OR=0.03; CI=0.005-0.1%) were detected in sick cats more often than in healthy cats. All the investigated *S. haemolyticus* strains showed susceptibility to tigecycline and linezolid and did not harbour the *van*B or *mup*A genes, whereas all of them harboured the *tet*(M) gene. The vancomycin MIC results of two isolates were 8 mg/mL, and these two isolates were grouped as vancomycin-intermediate strains.

Three types of MLSB resistance were observed, at 50% iMLSB (15.38% in healthy and 82.93% in sick cats), 11.25% cMLSB (23.08% in healthy and zero in sick cats) and 26.25% MSB (41.03% in healthy and 12.2% in sick cats). The most MLSB induction (67.14%) was caused by the ermB/C genes (87.5% = iMLSB, 55.56% cMLSB and 28.57% = MSB). *Erm*B caused mostly MSB (61.9%), whereas the *erm* A/B/C genes caused 7.5% iMLSB, 11.11% cMLSB and 9.52% MSB.

Of the 80 *S. haemolyticus* isolates investigated, 92.5% were confirmed to form a biofilm on a polystyrene plate after 24 hours, and 65% of them changed CRA colour after 48 hours of growth. Nevertheless, none of the isolates harboured the *bab* or *ica*A genes. The percentage of biofilm-positive strains in the healthy and sick cats amounted to 92.68% and 97.44% according to the MTP test (P=0.4962) and 71.17% and 56.41% on CRA (P=0.5164), respectively (Table 4).

Table 1: Demographic characteristics of the cats colonized with *S. haemolyticus*

	Category				Sex		Breed		Age								
	Multiple feline (%)	Single feline (%)	Pure breed cats (%)	Feral cats (%)0	Female (%)	Male (%)	Cross-breed (%)	Breed (%)	≤6 (month)			7–36 (month)			≥37 (month)		
									%	x−	σ	%	x−	σ	%	x−	σ
Healthy cats	22.2	25	44.4	8.3	52.9	47.2	38.9	61.1	16.7	3.3	2.3	58.3	17.1	8.3	25	68.1	29.8
Sick cats	10	10	80	-	75	25	15	85	65	3.2	0.9	30	23	13	5	5	0

Table 2: Percentage of antimicrobial resistance in *S. haemolyticus* strains isolated from clinically healthy and sick cats.

	P (%)	AUG (%)	OX (%)	CN (%)	TOB (%)	AMP (%)	DA (%)	E (%)	TET (%)	SXT (%)	C (%)	CIP (%)	MAR (%)	FC (%)	RD (%)
Healthy cats	38.	15.4	20.5	15.4	12.8	38.5	43.6	79.5	18	12.8	2.6	23.1	23.1	12.8	0
Sick cats	97.6	82.9	95.1	12.2	9.8	97.6	80.5	95.1	97.6	100	0	4.9	4.9	0	4.9

P=penicillin; AUG=amoxicillin-clavulanate; OX=oxacillin; CN=gentamicin; TOB=tobramycin; AMP - ampicilin; DA=clindamycin; E=erythromycin; TET=tetracycline; SXT=trimethoprim/sulfmethoxazole; C=chloramphenicol; CIP=ciprofloxacin; MAR=marbofloxacin; FC=fusidic acid; RD=rifampin.

Table 3: Percentage of antibiotic resistance genes in *S. haemolyticus* strains isolated from clinically healthy and sick cats

	blaZ (%)	*mecA* (%)	*aac** (%)	*ermA* (%)	*ermB* (%)	*ermC* (%)	*tet*(K) (%)	*tet*(L) (%)	*tet*(M) (%)	*tet*(O) (%)	*fusB* (%)	*vanA* (%)	*vanB* (%)
Healthy cats	84.2	97.4	94.9	18	100	56.4	97.4	15.4	100	43.6	33.3	28.2	0
Sick cats	100	100	100	7.3	97.6	87.8	100	87.8	100	63.4	2.4	2.4	0

* *aac(6')Ieaph(2")Ia* .

Table 4: Comparing of biofilm-producing tests for *S. haemolyticus* strains in healthy and sick cats

Method	CRA			MTP			
	Strains unable to produce slime [%]	Week slime producer strains [%]	Slime producer strains [%]	Strains unable to produce slime A570<0.398 [%]	Week slime producer strains 0.398<A570≦0, 796 [%]	Medium slime producer strains 0.796<A570 ≦1.592 [%]	Strong slime producer strains A570>1.592 [%]
Healthy cats	7.7	69.2	23.1	0	30.8	56.4	12.8
Sick cats	0	53.7	46.3	4.9	29.3	56.1	9.7
All cats	3.8	61.3	35	2.5	30	56.3	11.3

CRA = Congo red agar; MTP = microtiter plate.

DISCUSSION

Recently, the significance of *S. haemolyticus* as a human pathogen associated with hospital settings has risen (Chang *et al*., 2018). However, in veterinary clinical practice, it is sometimes difficult to determine if isolation of a CNS indicates a true infection or species contamination. Therefore, analysis of the occurrence frequency and the characteristics of opportunistic bacteria can simplify the management of infections in patients.

A previous report showed that the occurrence of *S. haemolyticus* is significantly higher in sick cats than in healthy cats (Bierowiec *et al*., 2019), although the current study showed that the characteristics of isolates are very similar regardless of the clinical state of the host and that MRSH is widespread in cat population. The high resistance of the isolates in the current study was not surprising as such high rates have been observed in pets previously (Ruzauskas *et al*., 2015). As well the tendency of rising resistance in *S. haemolyticus* in humans has been observed for the last few decades (Czekaj *et al.*, 2015).

MRSH isolates demonstrated resistance to the antimicrobial classes recognized as critically important for humans – fluoroquinolones, macrolides, and aminoglycosides (Ruzauskas *et al.*, 2015). Such a tendency in resistance in staphylococci of animal origin is widely observed (Ruzauskas *et al*., 2014; Ruzauskas *et al*., 2015; Bierowiec *et al.*, 2017). How was shown in the study by Szemraj *et al.* (2019) there is widespread prevalence and accumulation of genes encoding the MLSB resistance among CNS strains in opportunistic pathogens that might become a gene reservoir for bacteria with superior pathogenic potential. Nevertheless, there are some differences in the *erm* genes composition depending on host. In the current study, where were only animal *S. haemoliticus* strains under investigation, the *erm*B gene was dominant while the same gene was not detected in isolates of human origin in Poland (Szemraj *et al.*, 2019). Phenotypic resistance to vancomycin was not found among the isolates of *S. haemolyticus* in current study, but a few isolates harboured a *van*A gene, and two isolates showed intermediate resistance to vancomycin, which in *S. aureus* is due to a cross-linked and thickened cell wall matrix that sequesters and limits glycopeptide penetration (Berger-Bachi and McCallum, 2006). Although vancomycin is not used in veterinary settings, a rising resistance in staphylococci of animal origin has been observed (Abd El-Aziz *et al*., 2018).

The ability to form biofilms is thought to be an important mechanism involved in catheter-related bloodstream infections and in other device-associated diseases (Pain *et al.*, 2019). Although none of the isolates under investigation harboured *ica*A genes, almost all of the strains had biofilm formation properties. Similar results have been obtained previously (Panda and Singh 2018). Indeed, it has been was established that the genetic background for biofilm formation in *S. haemolyticus* is clearly different from that commonly found in *S. epidermidis* and that *S. haemolyticus* forms mainly polysaccharide intercellular adhesin-independent biofilms (Fredheim *et al*., 2009). Additionally, none of the isolates harboured a biofilm-associated protein (*bap*) gene, which mediates attachment to polystyrene and the accumulation phase of biofilm production (Tormo *et al.*, 2005). While the presence of the *bap* gene was demonstrated in nosocomial human isolates of *S. haemolyticus* (Potter *et al.*, 2009), such isolates seem to be both less investigated and less widespread (Kern and Perreten, 2019). Nevertheless, in other staphylococci of cat origin, the *bap* genes were previously identified (Ploneczka-Janeczko *et al*., 2014).

The high level of similarity of the antibiotic resistance and biofilm formation properties of *S. haemolyticus* isolates from healthy and sick animals suggests that the upper respiratory tract infections in the cats under investigation were likely of an endogenous origin or that the conjunctivitis was caused by other pathogens. Previous studies showed that, the composition of the bacterial flora was similar in both healthy and sick cats (Kielbowicz *et al.*, 2015).

Risk factors have been previously observed to be characteristic for the occurrence of *S. aureus* in healthy cats (Soares Magalhaes *et al.*, 2010; Bierowiec *et al*., 2019). The identification of a contact with owners who work in healthcare and/or veterinary healthcare or had been previously hospitalized as a risk factor in this study suggests that many of the *S. haemolyticus* strains in animals could have a hospital origin. Coagulase negative staphylococci, especially *S. epidermidis* and *S. haemolyticus*, are typically on touch surfaces in hospital wards, also in Poland (Rożańska *et al*., 2017; Pain *et al*., 2019). The previous treatment of other animals in the same group is another factor previously connected with *S. aureus* colonization in healthy cats (Bierowiec *et al.*, 2019). However, in current study, colonization was more frequently observed in cats that had no contact with

veterinary settings during the previous year than in cats that had veterinary contact. In the current study, the first time was presented data suggested the influence of some individual characteristics of cats on the frequency of *S. haemolyticus* isolation. Colonization was most typical in pure-breed, young, clinically sick kittens, but this observation should be further investigated. Similarly, in a previous report on *Staphylococcus* colonization in dogs, the presence of other animals in the households favoured an increased incidence rate of methicillin-resistant staphylococci (Han *et al.*, 2016).

Conclusions: The current study has shown that MRSH colonized healthy as well as sick cats. Predominately, MRSH isolates are phenotypically and genetically multidrug-resistant strains; thus, further studies investigating the dissemination of antibiotic resistance determinants would benefit from considering the possible role of reservoirs of CNSs in their distribution. Mostly the same risk factor previously observed in animals colonized with *S. aureus* was identified for *S. haemolyticus* in cats. Further studies should especially focus on possible *S. haemolyticus* sources for pets in healthcare settings. MRSH carriers are a potential source for the possible transmission of MRS for their offspring, other animals, and humans who come in contact with them. Therefore, these isolates should not be ignored in a standard bacteriological investigation.

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