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ORIGINAL ARTICLE

Contamination of the urban environment with excrements of companion animals as an underestimated source of *Staphylococcus* species posing a threat to public health

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ABSTRACT

The aim of the study was to assess the incidence, resistance, virulence, and genotypic characteristics of *Staphylococcus* spp. residing in the gastrointestinal tract of dogs and cats, as a group of animals causing potential contamination of the urban space. A high percentage of strains resistant to penicillin (58%), oxacillin (9%) and tetracycline (60%) were found. All isolates resistant to penicillin, kanamycin, or chloramphenicol carried genes responsible for individual resistance (*blaZ*, *aph(3′)-IIIa*, and *cat* (pC194)/*cat* (pC223), respectively. The *mec*A gene was detected in 45% of the oxacillin-resistant *Staphylococcus pseudintermedius* strains. The amplification of DNA fragments surrounding rare restriction sites analysis demonstrated high heterogeneity of genotypic profiles correlating with phenotypic resistance profiles. Multilocus sequence typing analysis classified the methicillin-resistant *S. pseudintermedius* strains as ST71, ST890, and the totally new ST1047. The presence of a high level of resistance among *Staphylococcus* strains may suggest a potential risk of transfer of these bacteria between companion animals and humans.

KEYWORDS

methicillin-resistant *Staphylococcus pseudintermedius*, antimicrobial resistance, multilocus sequence typing

INTRODUCTION

Contamination of the urban space with the faeces of dogs and cats may pose a serious risk of contamination with potentially dangerous human and animal pathogens like *Staphylococcus* spp., which can be aetiological agents of diseases on the one hand and contribute to the spread of multi-drug resistance (MDR) on the other. Currently, the assessment of the epidemiological status of public health, in addition to infections caused by methicillin-resistant *Staphylococcus aureus* strains, more often focuses on the importance of other methicillin-resistant or multi-drug resistant *Staphylococcus* species (Perreten et al., 2010). Both coagulase-positive and coagulase-negative species that were previously regarded as typical of companion animals are being increasingly recognised as an aetiological factor of human infections (Stegmann et al., 2010). This group of microorganisms comprises mainly the methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) species isolated from domestic and free-living carnivorous animals (Nowakiewicz et al., 2016; Ventrella et al., 2017). On the one hand, documented cases of isolation of MRSP and MDR *S. pseudintermedius* from lesions of predisposed groups, i.e. dog owners and veterinary staff, indicate direct

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contact with the animal as a route of infection (Sasaki et al., 2007; Frank et al., 2009). On the other hand, the more frequent cases of *S. pseudintermedius* isolation from people unrelated to pet animals (Frank et al., 2015) may indicate a different route of infection than direct contact, i.e. accidental contact with faeces discharged to the environment. Furthermore, as a nosocomial pathogen in veterinary medicine often compared to hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) in human medicine (Perreten et al., 2010), MRSP may play a role in the spread of the *mec*A gene and pet-acquired methicillin-resistant staphylococci (PA-MRS) carrying resistance determinants and can pose a threat to public health (Epstein et al., 2009). The presence of the *mec*A or *mec*C gene in *Staphylococcus* results in the elimination of β-lactams used in the treatment.

The aim of this study was to evaluate the risk of contamination of the environment by resistant and virulent *Staphylococcus* strains isolated from the gastrointestinal tract of companion animals (dogs, cats) living in urban areas and to analyse their genetic diversity.

MATERIALS AND METHODS

The material for the study consisted of 150 faecal samples collected from urban areas in Lublin (Poland) from November 2012 to May 2013. It mostly included faeces of dogs (n = 90) collected from different places where these animals live or play (walking places, pleasure grounds). Due to the behavioural habits of cats, samples from free-living cats (rectal swabs) were collected during a neutering campaign in an animal shelter (n = 60).

The samples (1 g of dog faeces and a swab from each cat) were incubated in 3 mL of buffered peptone water for 12 h at 37 °C, inoculated onto the selective medium Baird-Parker Lab Agar™ (Biocorp, Warsaw, Poland), and incubated at 37 °C for 24 h. One typical colony from each positive sample was taken for further analysis.

Identification to the genus *Staphylococcus* was based on micromorphology, a catalase test, a commercial test STAPHYtest 24 (Erba Lachema, Brno, Czech Republic), and a coagulase test with rabbit plasma (Biocorp, Warsaw, Poland).

Species identification of coagulase-negative *Staphylococcus* was carried out by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS) as described in a previous study (Nowakiewicz et al., 2016). Molecular confirmation of the identification of coagulase-positive isolates was carried out with by the use of PCR-RFLP according to Blaiotta et al. (2010).

The susceptibility of the isolated strains was evaluated in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI, 2012, 2013a, 2013b). The minimum inhibitory concentration (MIC) was determined by the broth microdilution method in relation to 12 agents with the following ranges tested: vancomycin (0.25–32 μg/mL), penicillin (0.12–64 μg/mL), ciprofloxacin (0.25–128 μg/mL), tetracycline (0.25–128 μg/mL), gentamicin (0.25–128 μg/mL), kanamycin (0.25–128 μg/mL), enrofloxacin (0.25–128 μg/mL), chloramphenicol (0.25–128 μg/mL), oxacillin (0.25–128 μg/mL), clindamycin (0.06–32 μg/mL), cefoxitin (0.25–128 μg/mL) and erythromycin (0.25–128 μg/mL) (Sigma-Aldrich, Germany). Reference *S. aureus* strains ATCC 29213 and ATCC 43300 were used for quality control.

The classification of MDR was performed on the basis of the criteria specified by Magiorakos et al. (2012) (resistance to at least one agent in 3 or more antimicrobial classes).

The bacterial isolates were tested for the presence of selected genes encoding resistance to methicillin (*mec*A, *mec*C), penicillin (*bla*Z), macrolides (*erm*A, *erm*C, *msr*A), tetracycline (*tet*L, *tet*K, *tet*M), chloramphenicol [*cat* (pC221), *cat* (pC194), *cat* (pC223)], and aminoglycosides [*aac(6′)-Ie-aph(2″)-Ia, aph(3′)-IIIa*] as well as genes encoding virulence factors: enterotoxins (*se*A, *se*B, *se*C, *se*D, *se*E), shock syndrome toxin (*tst*), and exfoliative toxin (*siet*). The primers and PCR conditions used in this study were as published previously (van de Klundert and Vliegenthart, 1993; Becker et al., 1998; Aarestrup et al., 2000; Martineau et al., 2000; Oliveira and de Lencastre, 2002; Strommenger et al., 2003; Lautz et al., 2006; Schnellmann et al., 2006; García-Álvarez et al., 2011).

Genotyping based on the ADSRRS (amplification of DNA fragments surrounding rare restriction sites) method was performed as previously described (Nowakiewicz et al., 2016). The PCR was carried out in a T100 Thermal Cycler (Bio-Rad, USA) in 25 μL of the reaction mixture consisting of 1 μL of ligation mix, 5 μL of Gold Taq Mix (Syngen Biotech, Wroclaw, Poland), and 50 pmol of each primer (Genomed, Warsaw, Poland). Electrophoresis of PCR products was carried out in 6% polyacrylamide gel (Sigma-Aldrich Germany). Electrophoretic profiles were fixed using GelDoc2000 (BioRad). BIO-1D++ 11.9 software (Vilber Lourmat, France) was used for cluster analysis of the strains (UPGMA method). The similarity index of the isolates was calculated using the Jaccard correlation coefficient option of the software with position tolerance and optimisation of 1%.

Multilocus sequence typing (MLST) was performed for the phenotypically oxacillin-resistant *Staphylococcus pseudintemedius* strains. Seven housekeeping genes (*tuf, cpn60, pta, purA, fdh, ack,* and *sar*) were sequenced according to the protocol described elsewhere (Bannoehr et al., 2007; Solyman et al., 2013). The PCR reactions were performed in a T Personal thermal cycler (Biometra GmbH, Göttingen, Germany) using Gold Taq MIX (Syngen Biotech, Wrocław, Poland). The BigDye® Direct Cycle Sequencing Kit (Applied Biosystems) was used for amplification of the PCR products. The PCR mixture consisted of 2 μL 2.5 × concentrated Ready Reaction Premix, 1 μL 5 × concentrated BigDye Sequencing Buffer, 0.25 μL of the primer (concentration of 5 pmol) and DNA amplicon (concentration of 50 ng), and sterile distilled water at a final volume of 10 μL. The sequencing reaction was performed in the following conditions: initial denaturation for 1 min at 96 °C and 25 cycles consisting of denaturation for 10 s at 96 °C, annealing for 5 s at 50 °C, and elongation for 4 min at 60 °C. An

ExTerminator kit (A&A Biotechnology, Gdynia, Poland) was used to purify the PCR product. The DNA sequence was read in a 3,500 Genetic Analyser (Thermo Fisher Scientific, USA). The MLST database maintained by the University of Oxford (http://pubmlst.org/spseudintermedius/) was used to determine the number of alleles. The new type detected in this study was approved by the curator of the PubMLST database. Sequences for selected strains were deposited in the international NCBI database (accession numbers from MK213243 to MK213270).

Statistical analysis of the results obtained was carried out using Statistica version 13.1 program (StatSoft Polska, Kraków, Poland). Statistically significant differences in the phenotypic resistance of *Staphylococcus* spp. strains between the host species were evaluated using the U Mann-Whitney test ($P < 0.05$).

RESULTS

Staphylococcus spp. were isolated from 118 faecal samples (83 from dogs and 35 from cats). All *Staphylococcus* strains isolated from dogs belonged to *S. pseudintermedius*. From the material obtained from cats *S. pseudintermedius* (n = 10), *Staphylococcus felis* (n = 20) and *Staphylococcus schleiferi* subsp. *schleiferi* (n = 5) were isolated.

Analysis of the resistance profiles showed a high percentage of *Staphylococcus* spp. resistant to β-lactams (oxacillin 9%, penicillin 58%) and tetracycline (60%) and totally susceptible to vancomycin (Table 1). *Staphylococcus schleiferi* subsp. *schleiferi* strains were resistant to penicillin, tetracycline, and cefoxitin and none of the *S. felis* strains showed resistance to any of the antimicrobials tested.

Multi-drug resistance was noted in 14% of all the *Staphylococcus* spp. isolates (Table 2). Statistically significant differences in the phenotypic resistance of *Staphylococcus* spp. strains between dogs and cats were demonstrated in relation to penicillin and tetracycline.

All penicillin-, kanamycin- or chloramphenicol-resistant isolates carried the *blaZ*, *aph(3′)-IIIa*, and *cat* (pC194)/*cat* (pC223) genes, respectively. Only five of the eleven oxacillin-resistant *S. pseudintermedius* strains (45%) contained genes related to methicillin resistance (*mec*A). The presence of genes determining resistance to tetracycline or gentamycin was detected in 71% and 83% of isolates that are phenotypically resistant to these antibiotics (Table 2).

None of the isolated strains harboured the virulence genes tested.

Genotyping of the *Staphylococcus* strains by the ADSRRS method was limited to isolates characterised by resistance to at least one agent. The ADSRRS analysis revealed high heterogeneity of the genotypic profiles (Fig. 1). *Staphylococcus pseudintermedius* strains isolated from dogs (n = 78) included 28 profiles and those from cats (n = 10) had 5 profiles. The *S. schleiferi* subsp. *schleiferi* isolates were classified into two profiles.

A high degree of similarity (> 80%), regardless of the host species, was exhibited by the isolates with phenotypic penicillin resistance (resistance profile No. I, genotypes: 9, 10, 11, 16, 12, 13, 14, 15) and with no susceptibility to penicillin and aminoglycoside antibiotics (gentamycin and/or kanamycin) (resistance profiles No. VIII, VII, VI; genotypes No. 21, 22, 23). Genotypes 25, 26, 27, 28, 29, and 30 characterised by the presence of the molecular resistance determinants *blaZ* and *tet*M (resistance profile III) exhibited a similar degree of similarity (> 80%) (Table 3, Fig. 1).

Based on the MIC value for oxacillin (> 0.5 µg/mL), 11 strains from the pool of 93 *S. pseudintermedius* strains were classified as MRSP, with only 5 exhibiting the presence of

Table 1. Percentage of antimicrobial resistance of *Staphylococcus* spp

Agent	*S. pseudintermedius* (n = 93)				*S. felis* (n = 20)				*S. schleiferi* subsp. *schleiferi* (n = 5)**		Total (n = 118)			
	MIC_{50}	MIC_{90}	R	%	MIC_{50}	MIC_{90}	R	%	R	%	MIC_{50}	MIC_{90}	R	%
P*	0.25	1	63	68	0.12	0.12	0	0	5	100	0.25	0.5	68	58
OX	0.25	16	11	12	0.25	0.25	0	0	0	0	0.25	0.25	11	9
CIP	0.25	0.25	5	5	0.25	0.25	0	0	0	0	0.25	0.25	5	4
TE	32	64	66	71	0.25	0.25	0	0	5	100	32	64	71	60
CN	0.25	0.25	6	6	0.25	0.25	0	0	0	0	0.25	0.25	6	5
K	0.5	64	7	8	0.5	1	0	0	0	0	0.5	1	7	6
ENR	0.25	0.25	5	5	0.25	0.25	0	0	0	0	0.25	0.25	5	4
C	4	8	2	2	8	8	0	0	0	0	4	8	2	2
CD	0.125	0.5	7	8	0.12	0.25	0	0	0	0	0.12	0.25	7	6
CEF	0.25	0.5	7	8	0.25	0.25	0	0	5	100	0.25	0.5	12	10
E	0.5	2	11	12	0.5	0.5	0	0	0	0	0.5	2	11	9

*P – penicillin, CIP – ciprofloxacin, TE – tetracycline, CN – gentamicin, K – kanamycin, ENR – enrofloxacin, C – chloramphenicol, OX – oxacillin, CD – clindamycin, CEF – cefoxitin, E – erythromycin.

**due to the small number of strains, MIC_{50} and MIC_{90} were not determined.

R/% – number/percentage of resistant strains.

Table 2. Resistance profiles of *Staphylococcus* strains

Species	No. of resistant strains		Resistance profiles	Resistance genes
S. pseudintermedius (n = 93)	78	I	P (n = 13)	*bla*Z (n = 13)
		II	TE (n = 6)	none
		III	P + TE (n = 42)	*bla*Z, *tet*M, *tet*K (n = 5)*bla*Z, *tet*M (n = 30)*bla*Z (n = 7)
		IV	TE + E (n = 5)	*tet*M (n = 5)
		V	P + TE + OX + CEF (n = 5)	*bla*Z, *tet*M (n = 5)
		VI	P + CD + K + TE + C + E (n = 1)	*bla*Z, *tet*M, *aph(3′)-IIIa*, *cat* (pC194), *cat* (pC223) (n = 1)
		VII	P + OX + CEF + CD + CN + K + TE + C (n = 1)	*bla*Z, *tet*M, *aph(3′)-IIIa*, *cat* (pC194), *cat* (pC223) (n = 1)
		VIII	P + OX + CN + K + CD + ENR + CIP + E (n = 5)	*bla*Z, *mec*A, *mec*C, *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *erm*C (n = 5)
S. schleiferi subsp. *schleiferi* (n = 5)	5	IX	P + CEF + TE (n = 5)	*bla*Z, *tet*M (n = 5)
S. felis (n = 20)	0		none	none

*P – penicillin, TE – tetracycline, E – erythromycin, OX – oxacillin, CEF – cefoxitin, CD – clindamycin, K – kanamycin, CN – gentamicin, C – chloramphenicol, ENR – enrofloxacin, CIP – ciprofloxacin.

*mec*A genes. All oxacillin-resistant isolates represented the MDR category.

Molecular analysis of these isolates performed using MLST classified 5 strains as MRSP ST71, one as ST890, and 5 as ST1047.

DISCUSSION

Pet animals are often underestimated as a potential source of zoonoses, in comparison with food-borne zoonoses; hence, scarce and limited investigations have been carried out in this field. The high rate of *Staphylococcus* spp. isolation from dogs and cats (i.e. 92% and 58%, respectively) in this study indicates a high degree of colonisation of not only the skin and mucous membranes of the oral and nasal cavities, which are commonly regarded as sites predisposed to *Staphylococcus* occurrence (Morris et al., 2006), but also the end portions of the gastrointestinal tract. The high percentage of isolated *S. pseudintermedius* demonstrated in this study may indicate a high risk of environmental contamination with this bacterial species.

In the present study, the *S. pseudintermedius* isolates exhibited the highest resistance to tetracycline and penicillin. Investigations conducted by Yoon et al., (2010) confirmed the high resistance of *S. pseudintermedius* strains to these groups of antibiotics (> 91.9%). The global trend of high resistance to tetracycline and penicillin of strains from the *Staphylococcus intermedius* group (SIG), which was observed and confirmed by the presence of at least one gene encoding the relevant type of resistance in this study, may result from the frequent use of these antibiotic groups in veterinary medicine (Beever et al., 2015). According to the ESVAC report of the European Medicines Agency from 2016 (European Medicines Agency, 2016), the highest sales of veterinary drugs in Poland were noted in the case of two antimicrobial classes of antibiotics: tetracyclines and penicillins.

The present study demonstrated that MRSP strains accounted for 12%, which is a similar value to that reported by Ventrella et al., (2017) (MRSP 12%) and Grönthal et al., (2017) (MRSP 14%).

While the detection of the *bla*Z gene confirmed the phenotypic penicillin resistance in all strains, only 45% of the oxacillin-resistant strains were carriers of the *mec*A gene. Two additional definitions have been proposed for oxacillin-resistant *S. aureus* strains without the *mec*A gene: BORSA (borderline oxacillin-resistant *S. aureus*), indicating resistance to methicillin and other β-lactams and regarded as borderline resistance associated with hyper-production of β-lactamases, and MODSA (modified *S. aureus*), i.e. resistance associated with the modification of protein PBP (Penicillin Binding Proteins) and induced by antibiotic selective pressure (Reygaert, 2013). Given the phylogenetic relatedness of the *S. aureus* and *S. pseudintermedius* species and the similar type of staphylococcal cassette chromosome (scc), the absence of genes encoding oxacillin resistance in *S. pseudintermedius* strains may result from similar mechanisms as those in *S. aureus*. Hyper-production of penicillinase by *S. pseudintermedius* was regarded as the cause of phenotypic resistance to methicillin among isolates without the *mec*A gene (Beever et al., 2015).

Oxacillin resistance in *Staphylococcus* spp. strains is often correlated with the phenomenon of multi-drug resistance (Grönthal et al., 2017), as confirmed in the present study. The presence of resistance genes *bla*Z, *tet*M, *aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*, *cat* (pC194), *cat* (pC223), *mec*A, *mec*C, and *erm*C in MRSP isolated from dogs and cats makes this group of animals a potential reservoir of bacteria that pose a threat to public health.

The MLST analysis of MRSP strains revealed that 5 strains isolated from cats represented ST71, i.e. one of the most common sequence types in Europe, also recorded in the countries of North America (Perreten et al., 2010). As demonstrated by Latronico et al., (2014), MRSP ST71 may

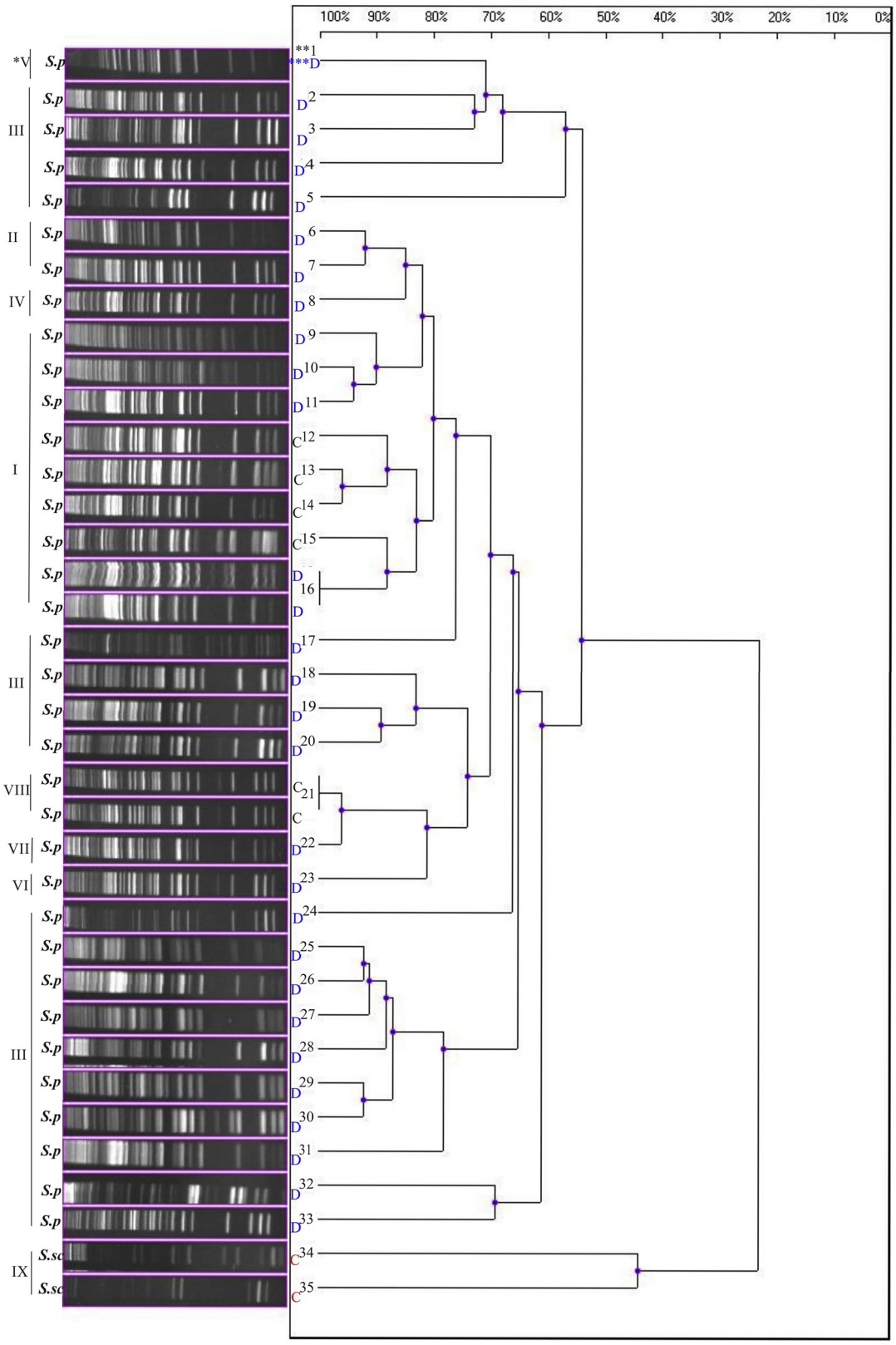

Figure 1. Representative electrophoretic profiles and dendrogram of similarity of *Staphylococcus* strains isolated from dogs and cats (BIO-1D++ 11.9 software). S.p – *S. pseudintermedius,* S.sc – *S. schleiferi* subsp. *schleiferi*; *phenotypic resistance profiles; **number of electrophoretic profile; ***D – dog, C – cat

Table 3. Occurrence of resistance and genotypic profiles of *Staphylococcus* strains

Species	Host	Resistance profiles (No. of strains)	ADSRRS profile (No. of strains)
S.p	dog	III (42)	2 (3), 3 (3), 4 (2), 5 (3), 24 (4), 25 (5), 26 (3), 27 (2), 28 (3), 29 (3), 30 (3), 31 (1), 18 (1), 19 (1), 20 (2), 32 (1), 17 (1), 33 (1)
	dog	V (5)	1 (5)
	dog	I (8)	9 (2), 10 (3), 11 (1), 16 (2)
	cat	I (5)	12 (1), 13 (2), 15 (1), 14 (1)
	dog	VI (1)	23 (1)
	dog	VII (1)	22 (1)
	cat	VIII (5)	21(5)
	dog	IV (5)	8 (5)
	dog	II (6)	6 (4), 7 (2)
S.sc	cat	IX (5)	34 (2), 35 (3)

S.p – *S. pseudintermedius*, S.sc – *S. schleiferi* subsp. *schleiferi*.

be able to adapt to human skin, indicating that this epidemic clone has a zoonotic potential. The risk related to the MRSP carrier state in companion animals is relatively high, which is evidenced by cases of human infections caused by methicillin-resistant *S. pseudintermedius* strains from this sequence type (Stegmann et al., 2010). A recent tendency, however, is the occurrence of other sequence types, including those so far not reported in Poland (Kizerwetter-Świda et al., 2017) and Europe (Duim et al., 2016). The present study has demonstrated the presence of *S. pseudintermedius* isolates representing two sequence types, ST890 and ST1047, which have not been detected in Poland before. Moreover, the ST1047 type has never been reported anywhere else in the world. The ST1047 *S. pseudintermedius* strains have been shown to exhibit lower resistance to antibacterial substances than the ST71 isolates, which is in line with the previous reports on the higher susceptibility of the less frequently isolated sequence strains in comparison with the ST71 or ST68, which are dominant in Europe (Duim et al., 2016; Perreten et al., 2010).

In the present study, none of the analysed *Staphylococcus* spp. strains had virulence genes. Similarly, Garbacz et al., (2013) did not show the presence of *se*A, *se*B, *se*D, *se*E, and *tst* genes in *S. pseudintermedius* isolated from dogs, but all their strains were carriers of the *siet* gene, and the *se*C gene was detected in 1.4% of the strains.

The lower genotypic diversity of the MRSP strains observed in the present study compared to the MSSP isolates suggests clonal expansion and regional selection of only the most successful clones. These results are similar to those reported by Perreten et al., (2010), who described only 18 different pulsed-field gel electrophoresis (PFGE) types among 103 MRSP strains. The association of resistance with genotype patterns among bacteria is a frequent subject of discussion. The occurrence of correlations between resistance patterns and genotypic profiles in different species of bacteria has been confirmed by Nowakiewicz et al., (2017). The results of our research indicate that the profile of resistance, especially when correlated with the type of resistance genes, can be associated with particular ADSRRS patterns.

In conclusion, contamination of the urban space with faeces from dogs and cats is a serious problem for public health. The high frequency of the MRSP strains and other *Staphylococcus* spp. carrying antimicrobial resistance genes indicates a need to include companion animals in drug-resistance monitoring programmes. Since humans and companion animals share the environment and many pets are often treated with antibiotics used in human medicine, the risk of transmission of determinants of resistance to broad-spectrum antibiotics of critical importance to humans is very high.

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