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Short Communication

Differentiation of *Staphylococcus pseudintermedius* in the *Staphylococcus intermedius* Group (SIG) by Conventional and Molecular Methods

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Abstract

Staphylococcus pseudintermedius is a member of the *Staphylococcus intermedius* group (SIG) comprising representatives with similar phenotypic characteristics. The purpose of this study was to optimise a method for accurate differentiation of *S. pseudintermedius* in clinical samples from dogs and cats based on PCR-RFLP analysis, following the key biochemical tests for SIG group assignment, and to determine the prevalence of *S. pseudintermedius* compared to other members of the SIG group. To implement this, 158 isolates from dogs and 17 from cats, 4 reference strains - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *Staphylococcus delphini* ATCC 49171, *Staphylococcus aureus* ATCC 25922 and 5 field strains of *S. aureus* were analysed. The generated PCR-RFLP profile *(pta* gene, *MboI* restriction enzyme) clearly differentiated *S. pseudintermedius* within the SIG group by having two distinctive bands of 213 and 107 bp, respectively. The data showed a predominance of *S. pseudintermedius* in clinical samples from dogs, while no *S. pseudintermedius* were found among the cat's samples. In conclusion, due to the atypical biochemical reactions found in some *S. pseudintermedius* isolates (14.6%), precise identification should be based on an extensive phenotypic assay together with a molecular biological method or the latter alone.

Keywords: *Staphylococcus pseudintermedius, Biochemical markers, PCR-RFLP analysis, differentiation, Staphylococcus intermedius Group*

Staphylococcus intermedius Grubundaki (SIG) *Staphylococcus pseudintermedius*'un Geleneksel ve Moleküler Yöntemlerle Ayırdedilmesi

Öz

Staphylococcus pseudintermedius, benzer fenotipik özelliklere sahip temsilciler içeren *Staphylococcus intermedius* grubunun (SIG) bir üyesidir. Bu çalışmanın amacı, köpek ve kedilerden elde edilen klinik örneklerde SIG grubu ataması için temel biyokimyasal testleri takiben PCR-RFLP analizine dayanarak *S. pseudintermedius*'un doğru şekilde ayırdedilmesi için bir yöntemi optimize etmek ve SIG grubunun diğer üyelerine kıyasla *S. pseudintermedius*'un yaygınlığını belirlemekti. Bu amaçla, köpeklerden elde edilen 158 ve kedilerden elde edilen 17 izolat, 4 referans suş - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *Staphylococcus delphini* ATCC 49171, *Staphylococcus aureus* ATCC 25922 ve *S. aureus*'un 5 saha suşu analiz edildi. Oluşturulan PCR-RFLP profili *(pta* geni, *MboI* kısıtlama enzimi), *S. pseudintermedius*'u sahip olduğu 213 ve 107 bp'lik iki farklı bantla SIG grubu içinde belirgin şekilde ayırdetti. Veriler, köpeklerden alınan klinik örneklerde *S. pseudintermedius*'un baskın olduğunu gösterirken, kedi örneklerinde *S. pseudintermedius* belirlenmedi. Sonuç olarak, bazı *S. pseudintermedius* izolatlarında bulunan atipik biyokimyasal reaksiyonlar (%14.6) nedeniyle kesin tanı, kapsamlı bir fenotipik test ile birlikte kullanılacak moleküler biyolojik bir yöntem veya sadece moleküler yönteme dayanmalıdır.

Anahtar sözcükler: *Staphylococcus pseudintermedius, Biyokimyasal markerler, PCR-RFLP analizi, Ayırdetme, Staphylococcus intermedius Grubu*

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INTRODUCTION

Staphylococcus spp. are Gram-positive bacteria causing a variety of opportunistic infections in humans, domestic and wild animals [1]. Usually, the pathogenicity of these bacteria is associated with the production of coagulase enzyme, on the basis of which they are defined as coagulase-positive and coagulase-negative, respectively. The following coagulase-positive/variable species are essential for veterinary medicine -*Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus schleiferi* subsp. *coagulans*, *Staphylococcus hyicus*, *Staphylococcus lutrae*, *Staphylococcus delphini* and *Staphylococcus pseudintermedius* [2,3], while coagulase-negative ones are considered significant when isolated from sterile body sites or in community-acquired infections [4].

Until recently, *S. intermedius* was thought to be commensal on the skin and mucous membranes of various animal species such as dogs, cats, pigeons, minks, horses, foxes, raccoons, goats and gray squirrels [5]. According to Wang et al.[6] *S. intermedius* is the predominant bacterial agent responsible for the etiology of the superficial pyoderma and soft tissue infections in dogs. With the development of advanced molecular methods, in particular DNA sequencing technologies, a group of researchers have clarified that the isolates identified as *S. intermedius* by phenotype methods actually a compound of three species - *S. intermedius*, *S. pseudintermedius* and *S. delphini*, which form together the *S. intermedius* group (SIG) [2]. Subsequently, based on DNA-DNA hybridization techniques and multilocus sequence typing (MLST), it was found out that the main bacterium responsible for the etiology of skin infections in dogs, cats and humans was practically *S. pseudintermedius*, and *S. intermedius* colonised mainly pigeons. Thus, the earlier scientific publications describing *S. intermedius* are now regarded as reports on *S. pseudintermedius* [7-9]. In addition, the clinical significance of *S. delphini* isolated from different animal species is considered greater than previously thought [10,11].

The misidentification of the SIG group representatives is due to the high degree of similarity in their phenotypic characteristics, as well as the existence of different biotypes of *S. intermedius* showing 50 to 65% DNA homology, which can be different species such as *S. delphini* and *S. pseudintermedius* [8]. On the other hand, genetic methods for differentiating microbial species based on the 16S rRNA gene are useless in these staphylococci because of the established gene similarity of over 99% [2,12]. The literature overview reveals several genetic approaches for the identification of *S. pseudintermedius* such as - PCR-RFLP analysis, nuc-PCR, sodA /hsp60 sequencing, and MALDI-TOF-MS analysis [3,8,13,14].

The purpose of this study was to optimise a method for accurate identification of *S. pseudintermedius* in clinical samples from dogs and cats based on the standard polymerase chain reaction with subsequent restriction of amplified fragments - PCR-RFLP analysis, following the key biochemical tests for SIG group assignment, and to determine the prevalence of *S. pseudintermedius* compared to other members of the SIG group.

MATERIAL and METHODS

Bacterial Strains

One hundred and seventy five isolates relevant to the SIG group - from dogs (n=158) and cats (n=17), 4 reference strains as controls - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *S. delphini* ATCC 49171, *S. aureus* ATCC 25922, as well as 5 field *S. aureus* strains of cow, sheep, chicken, dog and rabbit origin were included in the study. Clinical specimens from dogs included ear discharge (n=48), skin lesions (n=42), ocular discharge (n=29), wound discharge (n=17), urine (n=8), material of oral tongue and gingival lesions (n=11) and synovial fluid (n=3). Cat isolates originated from skin lesions (n=9), urine (n=5), and ear discharge (n=3). Samples were cultured on trypticase soy agar (Fluka, India) supplemented with 5% defibrinated sheep blood and on MacConkey agar (NCIPD, Bulgaria; HiMedia, India). The plates were incubated under aerobic conditions at 37°C for 24-48 h. The initial identification of significant staphylococcal species was based on Gram's staining, production of catalase and cytochrome oxidase enzymes, colonial characteristics and pigmentation, presence of complete haemolysis and the tube coagulase test with lyophilised rabbit plasma (NCIPD, Bulgaria).

Biochemical Profile

The biochemical profile of the isolates was determined on the basis of the following key tests: acetoin production in liquid medium with glucose, detection of β-galactosidase (4 mg disks, HiMedia, India), sensitivity to polymyxin B (300 U/disk, Oxoid, UK), utilisation of mannitol on mannitol-salt agar ((NCIPD , Bulgaria), of trehalose and maltose in liquid media (MkB Test, Slovak republic).

The tests were carried out in accordance with the manufacturer's instructions and the general rules for aseptic work in the microbiology laboratory [15].

Additionally, reference strains from the SIG group as well as selected clinical isolates were analysed with a semi-automatic BioLog phenotype identification system (BioLog, USA) with GenIII microplates according to the manufacturer's instructions. Briefly, wells were loaded with 100 µL of bacterial suspensions at a density of 90-98% determined on a turbidimeter (BioLog, USA) and the plates incubated at 33°C for 20-24 h.

PCR-RFLP Analysis

DNA was isolated using a QIAamp commercial DNA mini kit (Quiagen, GmbH, Germany) following the Gram-positive bacteria protocol requiring pre-treatment of the bacterial

stock with buffer containing 20 mg/mL lysozyme, 20 mM Tris HCl with pH 8.0, 2 mM EDTA and 1.2% Triton. DNA quality was determined spectrophotometrically at A260/A280 on a Gene Quant 1300 apparatus (Healthcare Bio-Sciences AB, Sweeden). The extracts were stored at -20°C until the start of the experiments.

PCR was performed with the primers targeting amplification of a 320 bp fragment of the phosphoacetyl transferase *(pta)* gene. The sequences of primers as follows: pta_f1, AAA GAC AAA CTT TCA GGT AA and pta_r1, GCA TAA ACA AGC ATT GTA CCG [13]. The PCR reaction adjusted in a final volume of 20 µL was as follows: primers 0.25 µM, 1 x PCR buffer, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, Taq polymerase 0.5 U/rxn (Canvax, Spain) and DNA 1 µL. The thermal conditions were adjusted in a gradient PCR machine included: initial denaturation at 95°C for 2 min followed by 30 cycles consisting of denaturation at 95°C for 60 sec, annealing at 53°C for 60 sec, elongation at 72°C for 60 sec and final amplification for 7 min at 72°C. Subsequently, 10 µL of amplified product were subjected to restriction with 1 µL of *MboI* (10 U/µL) according to the manufacturer's protocol (Canvax, Spain). The restricted products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide.

RESULTS

Bacterial isolates determined as suspicious for *S. pseudintermedius* based on their characteristics in the conventional microbiological tests until PCR-RFLP analysis was performed are presented in *Table 1*. The table shows that most (85.44%) of the isolates exhibit a typical *S. pseudintermedius* profile expressed in the presence of β-haemolysis, a positive test for proving free plasma coagulase within 2-4 h, negative test for acetoin production and β-galactosidase positivity (ONPG test), sensitivity to polymyxin B, acidification of mannitol, trehalose and maltose. Atypical reactions were found in 23 (14.6%) strains with respect to the presence of hemolysins and acetoin production in the VP test. These isolates were respectively γ-haemolytic (2.5%) and VP positive (12.0%). *Table 1* also shows the biochemical characteristics of the reference strains included in the study. The coagulase-positive isolates of cats exhibited a variability in VP and ONPG assays and were interpreted as resistant to polymyxin B, which was not in line with the profile for *S. pseudintermedius*.

The *S. intermedius* ATCC 29663, *S. delphini* ATCC 49171 and *S. pseudintermedius* LMG 22219 reference strains, and the two clinical isolates tested were successfully identified with the BioLog commercial system. The substrates used as carbon sources by the reference strains of the SIG group and two of the isolates identified as *S. pseudintermedius* with *MboI* restriction analysis are shown in *Table 2*. It is obvious that 14 of the substrates are completely utilised from the five strains tested. Only six substrates - stachyose, D-aspartic acid, D-serine, p-hydroxy-phenylacetic acid, D-malic acid and γ-amino-butryric acid, were not metabolised by the species tested, whereas no metabolic activity was recorded against D-cellobiose, D-raffinose, D-melibiose, D-salicin, 3 methyl glucose, L-fucose, L-rhamnose, bromo-succinic acid and β-hydroxy-D, L-butyric acid with exception of one of the strains showing borderline activity. The profile of *S. pseudintermedius* strains differs only in their inability to use seven substrates as a carbon source, unlike *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 and, with respect to four substrates, borderline activity or lack thereof in clinical isolates was recorded.

The results of the PCR-RFLP analysis are presented on *Fig. 1*. The restriction profile of the dog strains suspected to be *S. pseudintermedius* consisted of two bands at 213 bp and 107 bp, respectively. *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 did not exhibit the *MboI* restriction site, whereas the tested *S. aureus* strains showed a restriction profile with a band of 160 bp. Amongst the cat isolates, the *MboI* restriction profile specific for *S. pseudintermedius* was not established.

DISCUSSION

In routine bacteriological examinations, pathogenic staphylococci are frequently identified to the level of coagulase activity and no further identification is undertaken. On the other hand, the incorrect differentiation of

Table 1. *Biochemical profile of the isolates from dogs suspected to be S. pseudintermedius and the reference strains from the SIG group determined on the basis of key assays*

Bacterial Isolates and Reference Strains	Hemolysis	Coagulase	Acetoin	β-galactosidase	Polymyxin B	Mannitol	Trehalose	Maltose
S. pseudintermedius n=98	β	+	-	+	S	+	+	+
S. pseudintermedius n=19	β	+	+*	+	S	+	+	+
S. pseudintermedius n=4	ϒ*	+	-	+	S	+	+	+
S. pseudintermedius n=37	β	+	-	+	S	-	+	+
S. pseudintermedius LMG 22219	β	+	-	+	S	-	+	+
S. intermedius ATCC 29663	β	+	+	+	S	+	+	+
S. delphini ATCC 49171	β	+	-	-	S	+	+	+

*+ = positive reaction/test (including weakly positive and delayed positive reaction); - = negative reaction/test; S/R = sensitivity/resistance; * atypical reactions*

Table 2. *Biochemical profile data of S. intermedius ATCC 29663, S. delphini ATCC 49171, S. pseudintermedius LMG 22219, and two S. pseudintermedius clinical isolates (#1 and # 2) determined on the basis of 71 carbon sources in the BioLog GenIII plate*

Substrates	ATCC 29663	ATCC 49171	LMG 22219	#1	#2	Substrates	ATCC 29663	ATCC 49171	LMG 22219	#1	#2
Dextrin	+	+	B	B	B	Glycyl-L-Proline	+	+	+	-	B
D-Maltose	+	+	+	B	B	L-Alanine	+	+	+	+	+
D-Trehalose	+	-	+	+	+	L-Arginine	B	+	-	-	-
D-Cellobiose	B	-	-	-	-	L-Aspartic Acid	B	+	B	-	-
Gentiobiose	+	-	-	-	-	L-Glutamic Acid	+	+	+	B	B
Sucrose	+	+	+	+	+	L-Histidine	+	+	+	B	B
D-Turanose	+	+	+	-	+	L-Pyroglutamic Acid	B	+	-	-	-
Stachyose	-	-	-	-	-	L-Serine	+	+	+	+	+
D-Raffinose	B	-	-	-	-	Pectin	+	+	B	B	B
α-D-Lactose	+	+	+	+	+	D-Galacturonic Acid	+	+	B	B	-
D-Melibiose	B	-	-	-	-	L-Galactonic Acid Lactone	+	+	B	-	-
B-Methyl-D-Glucoside	+	-	+	+	+	D-Gluconic Acid	+	+	+	+	+
D-Salicin	B	-	-	-	-	D-Glucuronic Acid	-	+	-	B	-
N-Acetyl-D-Glucosamine	+	+	+	B	B	Glucuronamide	-	+	-	B	B
N-Acetyl-β-D-Mannosamine	+	+	-	-	-	Mucic Acid	B	+	-	-	-
N-Acetyl-D-Galactosamine	-	+	-	B	-	Quinic Acid	B	+	-	-	-
N-Acetyl Neuraminic Acid	+	+	+	+	+	D-Saccharic Acid	B	+	B	-	-
α-D-Glucose	+	+	+	+	+	p-Hydroxy-Phenylacetic Acid	-	-	-	-	-
D-Mannose	+	+	+	B	B	Methyl Pyruvate	+	B	B	+	B
D-Fructose	+	+	+	+	+	D-Lactic Acid Methyl Ester	-	B	B	-	-
D-Galactose	+	+	+	+	+	L-Lactic Acid	+	+	+	+	+
3 Methyl Glucose	B	-	-	-	-	Citric Acid	-	+	B	-	-
D-Fucose	+	-	-	-	-	α-Keto-Glutaric Acid	+	+	+	B	B
L-Fucose	B	-	-	-	-	D-Malic Acid	-	-	-	-	-
L-Rhamnose	B	-	-	-	-	L-Malic Acid	B	+	+	B	B
Inosine	+	B	+	B	+	Bromo-Succinic Acid	-	-	B	-	-
D-Sorbitol	-	+	B	-	-	Tween 40	+	+	-	-	-
D-Mannitol	+	+	+	+	+	γ-Amino-Butryric Acid	-	-	-	-	-
D-Arabitol	+	-	-	-	-	α-Hydroxy-Butyric Acid	+	+	+	B	B
Myo-Inositol	B	+	-	-	-	β-Hydroxy-D, L-Butyric Acid	-	B	-	-	-
Glycerol	+	+	+	+	+	α-Keto-Butyric Acid	+	+	+	B	B
D-Glucose-6-PO4	+	+	+	+	+	Acetoacetic Acid	+	+	B	B	B
D-Fructose-6-PO4	+	+	+	+	+	Propionic Acid	+	+	+	-	-
D-Aspartic Acid	-	-	-	-	-	Acetic Acid	+	+	+	B	B
D-Serine	-	-	-	-	-	Formic Acid	-	+	+	B	B
Gelatin	-	+	B	-	-						

B*=borderline activity*

coagulase-positive staphylococci is quite possible, especially for the representatives of the SIG group, due to their similar biochemical properties. The precise determination of these staphylococci is important for clinical practice with regard to the choice of antimicrobial agent associated with the correct interpretation of the minimum inhibitory

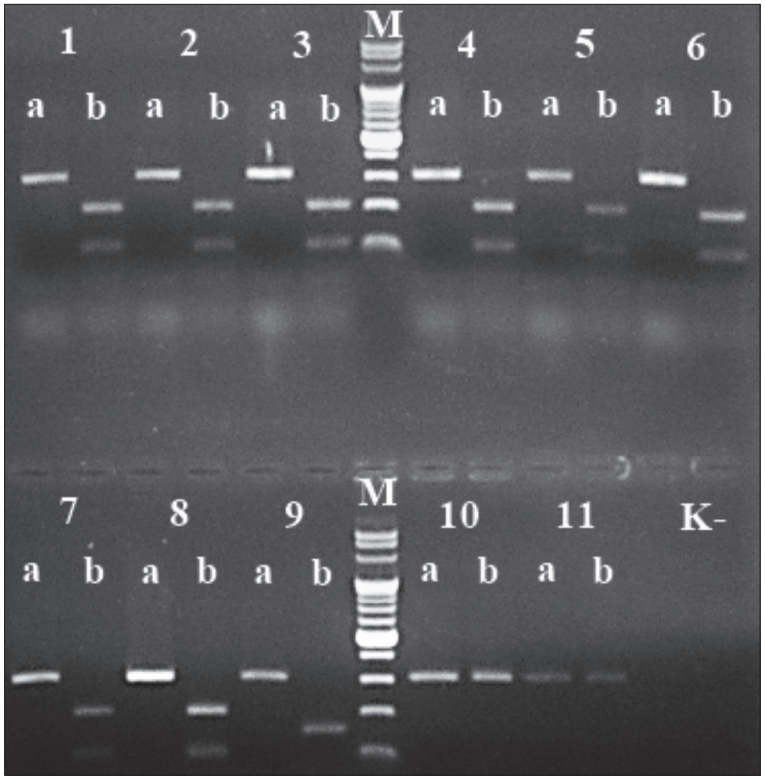

Fig 1. MboI restriction profile of *pta* PCR products in 2% agarose gel stained with ethidium bromide. Legend: 1-7 - *S. pseudintermedius* isolates; 8 - *S. pseudintermedius* LMG 22219; 9 - *S. aureus* ATCC 25922; 10 - *S. intermedius* ATCC 29663; 11 - *S. delphini* ATCC 49171; a - *pta* PCR product (320 bp); b - *pta* PCR-MboI product; M - molecular marker, 100 bp; K- negative control

concentrations and the resulting outcome of therapy [1,8,16], and because of the established zoonotic potential of *S. pseudintermedius* and *S. intermedius* [11].

In the present study, the isolates with a biochemical profile suspected for *S. pseudintermedius* were selected based on key biochemical markers for primary differentiation of clinically relevant staphylococci in veterinary medicine [15]. Key markers for the differentiation of the three staphylococcal species included in the test panel were the VP test and the ONPG test, which clearly categorised the reference strains. However, atypical reactions were found in *S. pseudintermedius* isolates relative to the production of acetoin in which *S. pseudintermedius* and *S. intermedius* profiles did not differ. The BioLog GenIII phenotypic identification system identified reference strains *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 with the highest probability of 1.000, whereas *S. pseudintermedius* LMG 22219 and both clinical isolates with a probability of 0.555 - 0.569, confirming the great biotype diversity of this bacterial species [8]. In this regard, we disagree with Devriese [2] who claims that there is no commercial kit available to identify *S. intermedius* and *S. delphini*. In addition, the updated BioLog system software includes the metabolic profile of *S. pseudintermedius*, which enables accurate identification at the phenotypic level. The carbon sources that did not detect metabolic activity in the reference strain and the clinical isolates compared to S. *intermedius* ATCC 29663 and *S. delphini* ATCC 49171 were N-acetyl-β-D-mannosamine, myo-inositol, L-arginine, L-pyroglutamic acid, mucic acid, quinic acid and Tween 40. Borderline activity or lack thereof in one of the clinical isolates was found against pectin, D-galacturonic acid, L-galactonic acid lactone and acetoacetic acid. With respect to the other biochemical reactions, detailed comparisons with other data published on *S. pseudintermedius* cannot be made due to the different substrates incorporated in the commercially used identification systems - BioLog in our study, API 50CH and API STAPH (BioMerieux), and STAPH-ZYM (Rosco) in the research of Devriese [2].

The PCR-RFLP method on which accurate identification of *S. pseudintermedius* isolates was based in this study was originally developed by Bannoehr et al.[13], and has been used successfully in other countries [17,18]. The method was also reproduced in our laboratory after optimisation of temperature parameters for hybridisation of primers at amplification of pta-PCR. The most distinct amplification products of the *pta* gene were visualised at 54°C, and the optimum denaturation, annealing and elongation time for each cycle was determined to be 45 s. Subsequent *MboI* analysis of PCR products of 320 bp clearly differentiates *S. pseudintermedius* isolates having a restriction site resulting in two bands of 213 and 107 bp, respectively. The restriction profile of *S. aureus* ATCC 25922 as well as the five *S. aureus* field strains differed by one visible band of about 160 bp in the agarose gel, which actually consists of two closely spaced bands - 156 and 164 bp, which are not established in the routine electrophoresis [13]. *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 are not differentiated by this method as they do not have a restriction site and *MboI* products have not been realised.

The data in our study showed a predominance of *S.*

pseudintermedius in clinical samples from dogs. The species affiliation of the isolates found to be suspicious for *S. pseudintermedius* by conventional methods was confirmed by PCR-RFLP analysis. Only two skin lesion isolates showed no restriction site and could not be determined by the RFLP method. The involvement of *S. aureus* in infectious pathology in dogs in a previous study of ours was estimated at 16.1% [19]. All these data confirm the prevalence of *S. pseudintermedius* in dog specimens. With respect to cat isolates, *S. pseudintermedius* was not detected in any of the samples tested, which is consistent with the data of Bannoehr et al.[13], who found *S. pseudintermedius* in only one of the 14 cat isolates tested. Due to the limited number of feline isolates analysed, definitive conclusions regarding the prevalence of *S. pseudintermedius* in this animal species in our country cannot be made. Obviously, its prevalence ranges from 3.08% in healthy cats to 11.97% in sick cats, values found in Poland in a large animal study conhort [20]. The other isolates did not show a restriction profile and their precise determination will be the subject of future studies based on other modern approaches.

In conclusion, the results of this study demonstrate the usefulness of key biochemical tests to target the SIG group members, but the precise differentiation of *S. pseudintermedius*, *S. intermedius* and *S. delphini* should be based on extensive phenotypic analysis together with molecular biological methods, or the latter alone.

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Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

N.R. designed, carried out the laboratory work, and wrote the paper; S.K., A.A., A.R - contributed in collecting clinical samples; S.S. assisted in molecular analysis and final revision of the paper.

REFERENCES

1. Velázquez-Guadarrama N, Olivares-Cervantes AL, Salinas E, Martinez L, Escorcia M, Oropeza R, Rosas I: Presence of environmental coagulase-positive staphylococci, their clonal relationship, resistance factors and ability to form biofilm. *Rev Argent Microbiol*, 49, 15-23, 2017. DOI: 10.1016/j.ram.2016.08.006

2. Devriese LA, Vancanneyt M, Baele M, Vaneechoutt, M, De Graef E, Snauwaert C, Cleenwerck I, Dawyndt P, Swings J, Decostere A, Haesebrouck F: *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int J Syst Evol Microbiol*, 55, 1569-1573, 2005. DOI: 10.1099/ijs.0.63413-0

3. Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami T, Fukata T, Hiramatsu K: Multiplex-PCR method for species identification of coagulase-positive staphylococci. *J Clin Microbiol*, 48, 765-769, 2010. DOI: 10.1128/JCM.01232-09

4. Szczuka E, Jabłonska L, Kaznowski A: Coagulase-negative staphylococci: Pathogenesis, occurrence of antibiotic resistance genes and *in vitro* effects of antimicrobial agents on biofilm-growing bacteria. *J Med Microbiol*, 65, 1405-1413, 2016. DOI: 10.1099/JMM.0.000372

5. Vandenesch F, Célard M, Arpin D, Bes M, Greenland T, Etienne J: Catheter-related bacteremia associated with coagulase-positive *Staphylococcus intermedius*. *J Clin Microbiol*, 33, 2508-2510, 1995.

6. Wang N, Neilan AM, Klompas M: *Staphylococcus intermedius* infections: Case report and literature review**.** *Infect Dis Rep*, 22, 5:e3, 2013. DOI: 10.4081/IDR.2013.E3

7. Bannoehr J, **Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AHM, Fitzgerald JR:** Population genetic structure of the *Staphylococcus intermedius* group: Insights into *agr* diversification and the emergence of methicillin-resistant strains. *J Bacteriol*, 189, 8685-8692, 2007. DOI: 10.1128/JB.01150-07

8. Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K: Reclassification of phenotypically identified *Staphylococcus intermedius* strains. *J Clin Microbiol*, 45, 2770-2778, 2007. DOI: 10.1128/JCM.00360-07

9. Fitzgerald JR: The *Staphylococcus intermedius* group of bacterial pathogens: Species re-classification, pathogenesis and the emergence of methicillin resistance. *Vet Dermatol*, 20, 490-495, 2009. DOI: 10.1111/J.1365-3164.2009.00828.x

10. Devriese LA, Hermans K, Baele M, Haesebrouck F: *Staphylococcus pseudintermedius* versus *Staphylococcus intermedius*. *Vet Microbiol*, 133, 206-207, 2009. DOI: 10.1016/j.vetmic.2008.06.002

11. Kizerwetter-Świda M, Chrobak-Chmiel D, Rzewuska M, Antosiewicz A, Dolka B, Ledwoń A, Czujkowska A, Binek M: Genetic characterization of coagulase-positive staphylococci isolated from healthy pigeons. *Pol J Vet Sci*, 18, 627-634, 2015. DOI: 10.1515/pjvs-2015-0081

12. Becker K, Harmsen D, Mellmann A, Meier C, Schumann P, Peters G, von Eiff C: Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *J Clin Microbiol*, 42, 4988-4995, 2004. DOI: 10.1128/JCM.42.11.4988-4995.2004

13. Bannoehr, J, Franco A, Iurescia M, Battisti A, Fitzgerald JR: Molecular diagnostic identification of *Staphylococcus pseudintermedius. J Clin Microbiol*, 47, 469-471, 2009. DOI: 10.1128/JCM.01915-08

14. Nisa S, Bercker C, Midwinter AC, Bruce I, Graham CF, Venter P, Bell A, French NP, Benschop J, Bailey KM, Wilkinson DA: Combining MALDI-TOF and genomics in the study of methicillin resistant and multidrug resistant *Staphylococcus pseudintermedius* in New Zealand. *Sci Rep*, 9:1271, 2019. DOI: 10.1038/s41598-018-37503-9

15. Markey B, Leonar, F, Archambault M, Cullinane A, Maguire D: Clinical Veterinary Microbiology. 2nd ed., 105, Mosby, Elsevier Ltd, 2013.

16. Chrobak D, Kizerwetter-Swida M, Rzewuska M, Moodley A, Guardabassi L, Binek M: Molecular characterization of *Staphylococcus pseudintermedius* strains isolated from clinical samples of animal origin. *Folia Microbiolol*, 56, 415-422, 2011. DOI: 10.1007/s12223-011-0064-7

17. Geraghty L, Booth M, Rowan N, Fogarty A: Investigations on the efficacy of routinely used phenotypic methods compared to genotypic approaches for the identification of staphylococcal species isolated from companion animals in Irish veterinary hospitals. *Ir Vet J,* 66:7, 2013. DOI: 10.1186/2046-0481-66-7

18. Sareyyüpoğlu B, Müştak HK, Cantekin Z, Diker KS: Methicillin resistance in *Staphylococcus pseudintermedius* isolated from shelter dogs in Turkey. *Kafkas Univ Vet Fak Derg*, 20, 435-438, 2014. DOI: 10.9775/kvfd.2013.10364

19. Rusenova NV, Rusenov AG: Detection of *Staphylococcus aureus* among coagulase positive staphylococci from animal origin based on conventional and molecular methods. *Mac Vet Rev*, 40, 29-36, 2017. DOI: 10.1515/macvetrev-2016-0095

20. Bierowiec K, Miszczak M, Biskupska M, Korzeniowska-Kowal A, Tobiasz·A, Rypula K, Gamian A: Prevalence of *Staphylococcus pseudintermedius* in cats population in Poland. *Int J Infect Dis*, 79, 70-71, 2019. DOI: 10.1016/j.ijid.2018.11.180