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Original article

Genetic characterization of coagulase-positive staphylococci isolated from healthy pigeons

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Abstract

Coagulase-positive staphylococci (CoPS) are opportunistic veterinary pathogens, of which *Staphylococcus aureus*, *S. delphini* and *S. intermedius* can be isolated from pigeons. The biochemical identification of *S. delphini* and *S. intermedius* isolates may be incorrect, because of their phenotypic similarity. The purpose of the present study was to isolate and identify CoPS from domestic and feral pigeons and to determine their genetic relatedness by PFGE. A total number of 31 isolates of CoPS were obtained, 15 were identified as *S. delphini* group B, six as *S. aureus,* four as *S. delphini* group A, three as *S. intermedius* and three as *S. schleiferi* subsp. *coagulans*. The results indicate that S. *delphini* group B is the predominant CoPS species among pigeons studied. PFGE restriction patterns of *S. delphini* group A and *S. delphini* group B form separate clusters, demonstrating their genetic heterogeneity. Indistinguishable or very similar PFGE patterns observed among *S. delphini* group B isolates from domestic and feral pigeons confirm the possibility of CoPS transmission between these birds.

Key words: coagulase-positive staphylococci, *Staphylococcus delphini*, pigeons, PFGE

Introduction

Coagulase-positive staphylococci (CoPS) are widely distributed in the environment and are commonly known as opportunistic pathogens (Hermans et al. 2010). After the last changes in the taxonomy of *Staphylococcus* genus, coagulase-positive staphylococcal species are: *S. aureus*, *S. intermedius*, *S. delphini*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, *S. lutrae* and coagulase-variable *S. hyicus* (Parte 2014), of which the first three were most frequently reported as being isolated from pigeons up to date.

Up to 2005 *S. intermedius* was considered as a member of the normal microbiota of dogs, as well as their opportunistic pathogen. Based on the growth features, biochemical activities and DNA sequence analysis a novel staphylococcal species, *Staphylococcus pseudintermedius* was described (Devriese et al.

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2005). Finally, it was demonstrated that isolates previously phenotypically identified as *S. intermedius* in fact belonged to three distinct species, such as *S. intermedius*, *S. pseudintermedius* and *S. delphini*, which together represent the *S. intermedius* group (SIG) (Devriese et al. 2005, Bannoehr et al. 2007). Members of the SIG are closely related and their 16S rRNA gene sequences are in more than 99% identical. The conventional microbiological diagnostic tests fail to distinguish between these species (Devriese et al. 2005, Bannoehr et al. 2007, Bannoehr and Guardabassi 2012). Various molecular biology methods for discrimination between staphylococci belonging to the SIG have been described, such as sequencing of *sodA* or *hsp60* genes, and RFLP-PCR, but they are not suitable for routine laboratory diagnostics, since they are expensive and time consuming (Bannoehr and Guardabassi 2012). Proteomic method matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) could also be used for species identification within the SIG group, but it was demonstrated, that it is not sufficiently evaluated for these bacteria (Decristophoris et al. 2011). A relatively simple multiplex PCR, targeting the thermonuclease (*nuc*) gene, is now commonly used for differentiation of CoPS important in veterinary medicine, including SIG (Sasaki et al. 2010). This method allows to distinguish between *S. aureus*, *S. pseudintermedius*, *S. intermedius*, *S. hyicus*, *S. schleiferi* subsp. *coagulans*, *S. delphini* group A and *S. delphini* group B.

S. delphini was initially isolated from a purulent skin lesions in dolphins (Voraldo et al. 1988). Subsequently, this species was also found in a wide range of animals, including horses, mink, domestic pigeons, and recently it was isolated from donkeys (Hesselbarth and Schwarz 1995, Futagawa-Saito et al. 2006, Futagawa-Saito et al. 2007, Sledge et al. 2010, Gharsa et al. 2014). The phylogenetic analysis of *nuc* gene sequences revealed, that this species is formed by two genetically distinct groups (A and B) (Sasaki et al. 2007). Moreover, DNA-DNA hybridization results showed, that *S. delphini* group A isolates were distinguished from *S. delphini* group B, *S. intermedius*, and *S. pseudintermedius* isolates. The *nuc* gene sequences analysis showed that isolates belonging to *S. delphini* group B are more related to the reference *S. pseudintermedius* LMG 22219(T) than to *S. delphini* LMG 22190(T). However, phenotypic properties to differentiate *S. delphini* group A, *S. delphini* group B, and *S. pseudintermedius* were not found (Sasaki et al. 2007). Mustelidae such as minks, ferrets, and badgers are regarded as natural hosts of *S. delphini* group A (Guardabassi et al. 2012). *S. intermedius* is rarely isolated, and it was recovered only from pigeons to date, moreover in the vast majority from feral pigeons (Bannoehr et al. 2007, Sasaki et al. 2007). Sparse literature data indicate that *S. intermedius* was isolated only from feral pigeons. In contrast, most of the *S. delphini* isolates were obtained from domestic pigeons or Mustelidae (Bannoehr et al. 2007, Sasaki et al. 2007, Sudagidan and Aydin 2012).

The purpose of the present study was to isolate and identify CoPS from posterior nares (*choanae*) and cloaca of healthy pigeons, and to determine their genetic relatedness using pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Sampling and bacterial identification

Samples were collected from healthy pigeons from August to December 2012. Feral pigeons originated from Birds' Azylum of City Zoological Garden in Warsaw (n = 12). Domestic birds originated from Birds' Azylum of City Zoological Garden (n=4) and from pigeon lofts in the area of Warsaw (n = 32). The total number of feral and domestic pigeons was 12 and 36, respectively. Two swabs were collected from each pigeon, from posterior nares and from cloaca. The total number of swabs tested was 96. Each swab was put in Mueller-Hinton broth (bioMerieux) containing 6.5% NaCl and incubated at 37°C for 24 hours. Then 10 µl of this culture was inoculated on Columbia agar with 5% sheep blood (bioMerieux). Colonies of staphylococci were evaluated on the basis of colony morphology and hemolytic activity. Bacterial cell morphology was analyzed using the Gram staining method. All staphylococci were tested using tube test for coagulase production and for catalase production according to standard procedures.

Genomic DNA isolation

A single colony of each isolate was transferred to Ependorff tube containing 25 µl lysostaphin (100 mg/ml, Sigma) and incubated at 37°C for 10 minutes. Subsequently, 25 µl of proteinase K (20 mg/ml, A&A Biotechnology) and 75 µl of Tris-HCl buffer were added to the bacterial suspension which was incubated at 37°C for 10 minutes. Proteinase K was inactivated by incubation at 97°C for 5 minutes. Then the mixture was centrifuged for 5 min at 15 000 rpm. The supernatant, containing the isolated DNA, was used as a template in PCR reactions.

Table 1. The number of CoPS isolates from feral and domestic pigeons and the area of isolation.

Type of pigeon	Species	Number of the isolates and the place of isolation		Total number of the isolates
		Posterior nares	Cloaca	
Feral pigeons n = 12	*S. delphini* group B	6	3	9
	S. delphini group A	–	–	–
	S. aureus	–	2	2
	S. intermedius	2	–	2
	S. schleiferi subsp. *coagulans*	1	1	2
Domestic pigeons n = 36	*S. delphini* group B	3	3	6
	S. delphini group A	1	3	4
	S. aureus	2	2	4
	S. intermedius	1	–	1
	S. schleiferi subsp. *coagulans*	1	–	1

Table 2. The characterization of CoPS isolated from pigeons.

Pigeon designation	Type of the pigeon	Isolate	Place of isolation	The results of multiplex PCR
GD1[a]	Feral	GD1N	PN[b]	*S. delphini* B
		BGD1K	C[c]	*S. delphini* B
GD2		GD2K	C	*S. aureus*
GD3		GD3NA	PN	*S. delphini* B
		GD3NB	PN	*S. schleiferi*
GD4		GD4N	PN	*S. delphini* B
		GD4K	C	*S. schleiferi*
GD5		GD5N	PN	*S. delphini* B
		GD5K	C	*S. aureus*
GD7		GD7NB	PN	*S. intermedius*
GD9		GD9NA	PN	*S. delphini* B
GD10		GD10NA	PN	*S. intermedius*
GD11		GD11N	PN	*S. delphini* B
		GD11K	C	*S. delphini* B
GD12		GD12K	C	*S. delphini* B
GO2[d]	Domestic	GO2NA	PN	*S. aureus*
		GO2NB	PN	*S. intermedius*
		GO2K	C	*S. delphini* B
GO3		GO3NA	PN	*S. delphini* B
		GO3K	C	*S. delphini* B
GO4		GO4NA	PN	*S. schleiferi*
GO5		GO5K	C	*S. delphini* A
GO11		GO11K	C	*S. delphini* A
GO14		GO14K	C	*S. delphini* A
GO18		GO18KA	C	*S. aureus*
		GO18KB	C	*S. delphini* B
GO19		GO19N	PN	*S. delphini* B
GO27		GO27N	PN	*S. delphini* B
GO30		GO30KA	C	*S. aureus*
GO33		GO33N	PN	*S. aureus*
GO34		GO34N	PN	*S. delphini* A

a - GD1, feral pigeon number 1
b – PN, posterior nares
c – C, cloaca
d – GO2, domestic pigeon number 2

Multiplex PCR for identification of CoPS

A described by Sasaki et al. (2010) multiplex PCR with primers specific for seven staphylococci species (*S. aureus*, *S. intermedius*, *S. delphini* group A, *S. delphini* group B, *S. pseudintermedius*, *S. schleiferi,* and *S. hyicus*) was used for identification of CoPS. As positive controls the following reference strains *S. intermedius* CCUG 6520T, *S. schleiferi* subsp. *coagulans* CCUG 37248T, *S. delphini* (group A) CCUG 30107T, *S. delphini* (group B) P-27B, *S. pseudintermedius* CCUG 49543T and *S. aureus* ATCC 6938 were used. DNA amplification products were resolved in 1.5% agarose (Serva) by electrophoresis in Tris-Acetate-EDTA buffer, stained with ethidium bromide, visualized with UV light and analyzed using a VersaDoc Model 1000 Imaging System with Quantity One 4-4-0 software (Bio-Rad Laboratories).

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to the harmonised protocol for *S. aureus* (Murchan et al. 2003) with minor modifications. Briefly, overnight cultures in Brain-hart infusion broth (bioMerieux) were adjusted to OD_{600} 0.5 and incorporated into 2.0% agarose discs (SeaKem Gold, Lonza). After 18 hours lysis with lysostaphin (1 mg/ml, Sigma), lysozyme (100 mg/ml, Sigma) and RNase (10 mg/ml, Fermentas) at 37°C, discs were incubated with proteinase K (20 mg/ml, A&A Biotechnology) overnight at 50°C. Then the agarose discs were digested overnight at 25°C with *Sma*I (20 U/µl, Fermentas). The restriction fragments were separated using a CHEF-DR II System (BioRad) in 1% agarose. Gel images were analyzed by Gel Compare II version 4.6 (Applied Maths) and cluster analysis was performed by UPGMA using dice similarity coefficient with optimization set at 0.5% and position tolerance at 1.5%. Isolates were clustered using an 80% homology cut-off, above which isolates were considered to be closely related and assigned to the same PFGE cluster (Tenover et al. 1995).

Results

Isolation and identification of coagulase-positive staphylococci

A total of 31 CoPS isolates were obtained, 16 were isolated from domestic pigeons and 15 from feral pigeons. Based on the results of multiplex PCR reaction, 15 isolates were identified as *S. delphini* group B, six as *S. aureus*, four as *S. delphini* group A, three as *S. intermedius* and three as *S. schleiferi* subsp. *coagulans*. The number of CoPS isolates and the place of isolation are presented in Table 1 and Table 2.

Staphylococci were isolated more frequently from feral pigeons, 15 isolates were obtained from 12 feral pigeons, wherein in case of two birds *S. delphini* group B isolates were cultured from posterior nares and cloaca of each bird (for details see Table 2). Additionally, *S. aureus* was found in cloaca and *S. delphini* group B was obtained from posterior nares of the same specimen from feral pigeon. Likewise, both *S. delphini* B and *S. schleiferi* subsp. *coagulans* were found in another feral pigeon.

Pulsed-field gel electrophoresis

All *S. delphini* group B (n = 15) and *S. delphini* group A (n = 4) isolates were both subjected to determination of genetic similarity using PFGE technique. After *Sma*I digestion of their total DNA, 12 different pulsotypes were obtained. Using a cut-off of 80% similarity all isolates except one were grouped into four clusters (Fig. 1). Two dominant clusters contained *S. delphini* group B isolated from feral and domestic pigeons and two, less numerous clusters consisted of *S. delphini* group A isolated only from domestic birds. Additionally, single isolate (GO34N) was not genetically related to the other isolates. Two *S. delphini* group B isolates with indistinguishable PFGE patterns were recovered from both sampling places of three pigeons (isolates GO3K and GO3NA, GD1N and GD1K, GD11N and GD11K). Moreover, 100% homology in PFGE patterns were observed between isolates obtained from two feral pigeons and one domestic (isolates GD1N and GD1K from feral one pigeon, GD5N from feral pigeon, GO2K from domestic pigeon), from one feral and one domestic (isolates GO27N, GD12K) and from two feral pigeons (isolates GD9NA, GD3NA).

Six obtained *S. aureus* isolates showed high level of heterogeneity in PFGE (Fig. 2). Two distinct clusters were observed. One consisted of two isolates obtained from domestic pigeons (isolates GO30KA andGO33N), while the other one comprised of two isolates isolated from feral and domestic pigeons (isolates GD2K and GO18KA). Two other *S. aureus* isolates showed PFGE patterns less related to the other isolates. The number of isolated *S. intermedius* and *S. schleiferi* subsp. *coagulans* was too small to perform a detailed analysis of their genetic relatedness. However, similarity of their PFGE pulsotypes was clearly visible (Fig. 3).

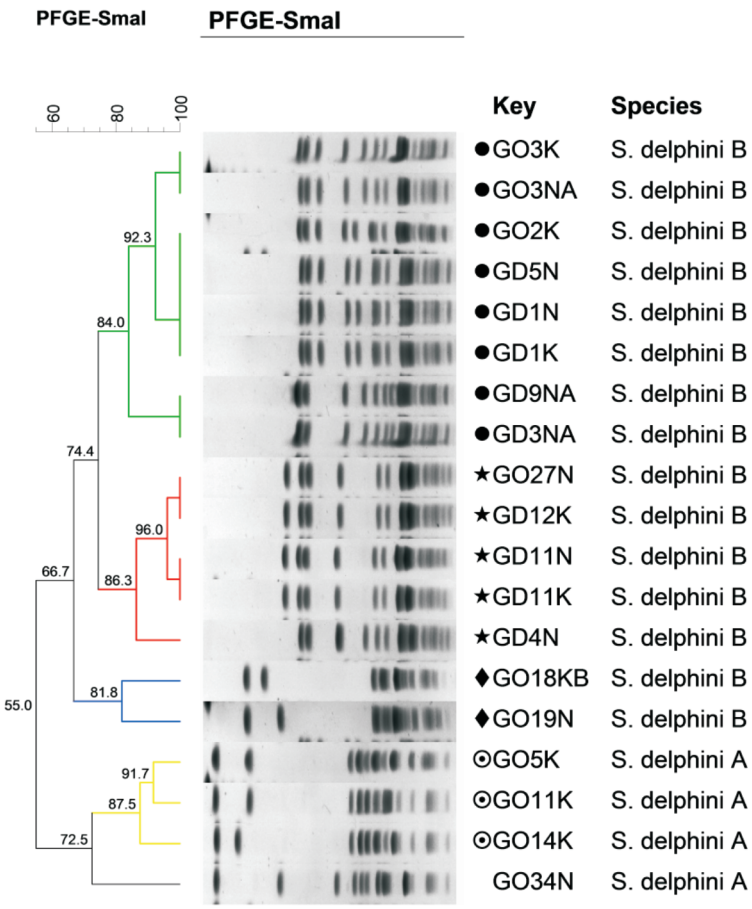

Fig. 1. Dendrogram showing the level of similarity of *Sma*I restriction PFGE patterns of the genomic DNA of *S. delphini* group A and group B isolates.

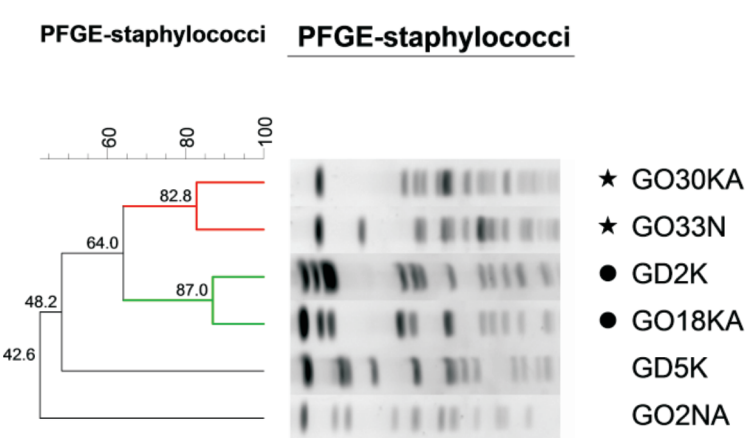

Fig. 2. Dendrogram showing the level of similarity of *Sma*I restriction PFGE patterns of the genomic DNA of *S. aureus* isolates.

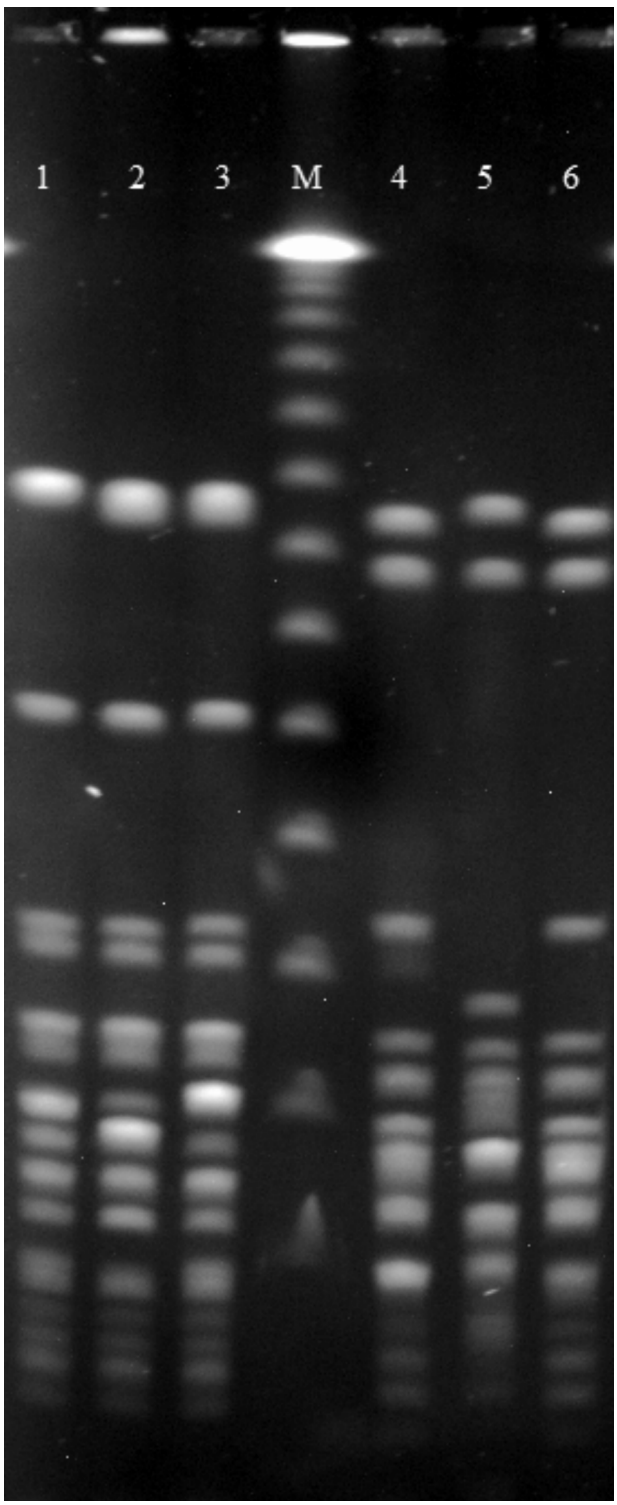

Fig. 3. PFGE patterns of chromosomal DNA digested with *Sma*I of *S. intermedius* (lines 1-3) and *Staphylococcus schleiferi* subsp. *coagulans* (lines 4-6) isolates from pigeons. M, Lambda Ladder PFG Marker (BioLabs).

Additionally, in two pigeons, two staphylococcal species were found. Both, *S. aureus* and *S. delphini* group B were obtained from cloaca of one domestic pigeon. Likewise, *S. delphini* group B and *S. aureus* were isolated from posterior nares and cloaca of one feral pigeon, respectively. Moreover, in one case three CoPS were obtained from one domestic bird, *S. aureus* and *S. intermedius* from posterior nares and *S. delphini* group B from cloaca.

Discussion

Members of the SIG as well as *S. aureus* are considered as the major pathogenic staphylococcal species that play a significant role in a multitude of pathological conditions in many animals species (Hermans et al. 2010). Currently the only available data refer to *S. aureus* as an ethological agent of staphylococcal infections in pigeons and there is no data regarding the SIG in these animals (Hermans et al. 2010). *S. delphini* as a coagulase-positive species should be regarded as an opportunistic pathogen. Similar to *S. aureus* and *S. pseudintermedius*, *S. delphini* produces a variety of virulence factors, including enzymes (coagulase, thermonuclease, proteases) and toxins (hemolysins, leukocidins, enterotoxins) (Sledge et al. 2010, Ben Zakour et al. 2012, Sudagidan and Aydin 2012, Gharsa et al. 2014). The results of this research showed that *S. aureus* and *S. delphini* may be isolated from healthy pigeons. Further research concerning the occurrence of CoPS in healthy and diseased pigeons are planned. Available literature data concerning the prevalence of CoPS among pigeons are limited (Hesselbarth and Schwarz 1995, Futagawa-Saito et al. 2006, Futagawa-Saito et al. 2007). It should be emphasized that previous publications describing *S. intermedius* as frequently isolated from pigeons, are probably not reliable (Futagawa-Saito et al. 2006, Futagawa-Saito et al. 2007). Although, Wakita et al. (2002) proved, that some of the staphylococci isolated from pigeons, produced similar PFGE patterns, regardless of their geographical origin, which probably resulted from the presence of various CoPS species. Nowadays it is known that phenotypic methods do not allow the correct recognition of species within the SIG, thus the reliability of these studies is questionable. Sasaki et al. (2007) identified *S. intermedius* isolates in feral pigeons only and the majority of *S. delphini* isolates were isolated from domestic pigeons with the exception of one *S. delphini* isolate obtained from feral pigeon (Sasaki et al. 2007). In other publication, *S. intermedius* and *S. delphini* group A and B were recovered from pigeons, but the authors did not provide information on the groups of the pigeons (Sasaki et al. 2010). *S. delphini* group A and *S. delphini* group B were isolated from nasopharynx of healthy domestic pigeons in Turkey (Sudagidan et al.

2012). However, *S. intermedius* was not isolated during this research.

A well-known phenomenon is the occurence of CoPS in healthy hosts. *S. aureus* has been isolated from the nares of healthy humans, pigs and chickens (Hermans et al. 2010). Likewise, healthy dogs may be carriers of *S. pseudintermedius* in the nares and the anus (Bannoehr et al. 2007). Thus we have examined swabs from posterior nares and cloaca of pigeons to study the possibility of colonization of these two sites. More CoPS isolates were obtained from posterior nares (17) than from cloaca (14) and *S. delphini* group B was the predominant species.

The results obtained in PFGE confirmed the genetic heterogeneity between *S. delphini* group A and *S. delphini* group B and their PFGE patterns formed two distinct clusters. Genetic diversity of *S. delphini* group group B and A was also observed by Sudagidan and Aydin (2012).However, some PFGE patterns in our study of *S. delphini* group B isolated from feral and domestic pigeons were indistinguishable or very similar, suggesting their genetic relatedness and the possibility of strains transmission. In addition, the results indicated that isolates with identical PFGE patterns may colonize the posterior nares and cloaca of the same bird. It could be also assumed that the transmission of CoPS between pigeons and pigeon breeders may be possible. Nevertheless, confirmation of this hypothesis requires further studies.

In conclusion, the results presented in this work confirm the few published data indicating that *S. delphini* group B is the predominant staphylococcal species among feral and domestic pigeons. The current study also showed the occurrence of other coagulase-positive staphylococci in pigeons and that the same or different species of CoPS may colonize both posterior nares and cloaca of the same healthy bird.

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