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Virulence properties of Staphylococcus delphini strains isolated from domestic pigeons

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Summary

Virulence properties (biofilm formation, antibiotic susceptibility, inducible clindamycin resistance, extracellular protease-lipase productions and presence of virulence genes) and genetic-relatedness of 18 Staphylococcus delphini strains (8 group A and 10 group B), isolated from domestic pigeons, were investigated. All strains were susceptible to vancomycin, oxacillin, cefoxitin, novobiocin, sulphamethoxazole/trimethoprim, gentamicin, teicoplanin, amoxycillin/clavulanic acid, rifampicin, cephazolin, linezolid, imipenem, chloramphenicol and tobramycin. However, 15, 13, 12 and 6 strains were found to be resistant to erythromycin, tetracycline, clindamycin and penicillin G, respectively. Although 12 strains showed constitutive resistance, inducible clindamycin resistance was detected in 3 strains by D-test. In addition, ermABC genes related to inducible or constitutive resistance were detected in 9 strains (ermA), in 5 strains (ermC) and in one strain (ermB). Biofilm formation results demonstrated that 9/18 strains showed high adherence to microplate surfaces in tryptic soy broth (TSB) supplemented with 1% sucrose. None of the strains harbored virulence genes, including enterotoxins, toxic-shock syndrome toxin, exfoliative toxins, heamolysins, methicillin-resistance, set1, lukE-lukD leukocidin and Panton-Valentine Leukocidine (PVL). Pulsed-field gel electrophoresis (PFGE) analysis revealed that there were two main clusters with 75% homology and only 3 strains showed 100% homology. In addition, 5 strains have strong proteolytic activity and 8 strains showed lipase activity. To our knowledge, this is the first study to investigate the virulence properties and genetic-relatedness of S. delphini strains isolated from pigeons in Turkey.

Keywords: Staphylococcus delphini, biofilm, enterotoxin genes, pigeon, PFGE

Staphylococci are one of the most examined pathogens in humans and animals. All avian species seem to be susceptible to staphylococcal infections, the predominant causative agent being *S. aureus* leading to inflammation of the skin of foot or pododermatitis that is commonly referred to as “bumblefoot” (12). In fact, pigeons serve as potential vehicles for the transmission of opportunistic and zoonotic pathogens to humans. Mostly, *S. intermedius* is seen as a member of the nasopharyngeal flora of pigeons (17). Except for *S. aureus*, the identification and differentiation of coagulase-positive staphylococci are very complicated. Sequence similarities of 16S rRNA genes of *S. delphini*, *S. intermedius*, *S. schleiferi* subsp. *coagulans* and *S. pseudintermedius* are > 99% and this method is insufficient to discriminate these closely related species (8, 39). *S. delphini* was first described by Varaldo et al. (42) and it was isolated from purulent skin lesions of dolphins. Moreover, *S. pseudintermedius* was isolated from clinical and necroscopy specimens from a cat, a dog, a horse and a parrot in 2005 and described as a new staphylococcal species (8). These two species, which are closely related to *S. intermedius*, could be misidentified by phenotypic tests (31). Recently, Sasaki et al. (32) described a multiplex-PCR (mPCR) method to distinguish *S. aureus*, *S. delphini*, *S. hyicus*, *S. schleiferi*, *S. intermedius* and *S. pseudintermedius* groups A and B, by targeting thermonuclease (*nuc*) gene locus with 99.8% sensitivity and 100% specificity. This easy and precise mPCR prevents misidentification of closely related coagulase-positive staphylococcal species.

S. intermedius, like *S. aureus*, has many virulence properties, such as exfoliative toxins, enterotoxins, leukotoxin, extracellular enzymes and alpha-beta haemolysin productions, biofilm formation and antibiotic resistance (14, 15, 41). However, there are few studies about the identification of *S. delphini* strains

isolated from various sources and examination of their virulence properties (1, 4).

The aim of this study was to determine virulence properties and genetic-relatedness of *S. delphini* strains isolated from domestic pigeons in Izmir, Turkey. For this purpose, antibiotic susceptibility, biofilm formation, presence of virulence genes and extracellular enzyme productions were investigated.

Material and methods

Samples for the isolation of staphylococci were collected by swabs from the nasopharynx of healthy domestic pigeons in Izmir-Turkey. The swabs were transported in Stuart transport medium (Oxoid, Basingstoke, UK) to the laboratory, then inoculated onto blood agar plates (Oxoid). Staphylococcal strains were identified by Gram staining, catalase and coagulase tests. Furthermore, the strains were identified at the species level by partial 16S rRNA gene sequencing by ABI 3130*xl* Genetic Analyzer using primers described by Mora et al. (26) and applying mPCR (32).

Susceptibility testing was performed by agar disc diffusion according to the Clinical and Laboratory Standards Institute (7) for the following antibiotics: vancomycin (30 µg), cefoxitin (30 µg), oxacillin (1 µg), penicillin G (10 U), gentamicin (10 µg), erythromycin (15 µg), rifampicin (5 µg), linezolid (30 µg), cephazolin (30 µg), levofloxacin (5 µg), chloramphenicol (30 µg), sulphamethoxazole/trimethoprim (25 µg), teicoplanin (30 µg), clindamycin (2 µg), amoxycillin/clavulanic acid (30 µg), ofloxacin (5 µg), kanamycin (30 µg), tobramycin (10 µg), imipenem (10 µg), novobiocin (5 µg) and tetracycline (30 µg) (Oxoid). D-test was also carried out to determine inducible clindamycin resistance using erythromycin and clindamycin discs (23).

Genomic DNA was isolated based on the protocol described previously (38). The presence of the genes responsible for the production of methicillin-resistance (*mecA*) (22), exfoliative toxins (*eta* and *etb*) (20), toxic-shock syndrome toxin (*tst*) (5), α- and β-haemolysins (*hla* and *hlb*) (29), staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed* and *see*) (20), *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sep*, *seq* (2), *sem*, *sen*, *seo* (19), *seu* (27), staphylococcal exotoxin like protein-1 (*set*1) (29), *lukE-lukD* leukocidin (19), PVL encoding gene (24), macrolide, lincosamide and streptogramin (MLS) resistance determinant genes (*ermA*, *ermB* and *ermC*) (25) were searched by PCR. The reactions were performed in 50 µL reaction mixture containing 1.2 U *Taq* DNA polymerase (Fermentas), 5 µL of 10 × reaction buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20, and 1.5 mM $MgCl_2$), 10 µM of each of the primers, 0.2 mM each of the four dNTPs (Fermentas), and 5 µL of genomic DNA as template. Next, PCR products were resolved in 1.5% (w/v) agarose gel in 1 × TAE buffer. PCR experiments were repeated twice for each strain. In the study, *S. aureus* subsp. *aureus* NCTC (National Collection of Type Cultures) 10652 for *sea*, *S. aureus* subsp. *aureus* NCTC 10654 for *seb*, *S. aureus* subsp. *aureus* NCTC 10655 for *sec*, and *S. aureus* subsp. *aureus* NCTC 10656 for *sed* were used as positive controls. *S. aureus* N315, positive for *tst*, *sem*, *sen*, *seo* and *seu*, was kindly supplied by Dr. Teruyo Ito and Prof. Keiichi Hiramatsu from Juntendo University Japan and used in the experiments.

Biofilm forming properties of *S. delphini* strains were determined quantitatively in microtiter plates using TSB (Merck, Darmstadt, Germany) supplemented with 1% (w/v) sucrose and brain heart infusion broth (BHI, Merck) (35). Optical densities (OD) were determined at 590 nm by microplate reader (Varioskan® Flash, Thermo, Finland) and OD > 0.5 values were accepted as biofilm formers. In the biofilm tests, *S. epidermidis* strain YT-55 was used as a positive control (37). Moreover, the adherence of the strains to glass tube surfaces was investigated by tube adherence test (6).

Genetic-relatedness of *S. delphini* strains was determined by PFGE analysis. Agarose plugs were prepared as described previously (10). Bacterial DNA in agarose plugs was digested with 30U *Sma*I (Fermentas, Vilnius, Lithuania) overnight and they were run in 1% (w/v) pulsed-field certified agarose (Bio-Rad) with 5-40 s pulse time, 6 V/cm, 120° angle, at 14°C for 22 h using the CHEF-Mapper PFGE system (Bio-Rad). After electrophoresis, the gel was stained with 5 µg/mL ethidium bromide and visualized with VersaDoc 4000MP image analyzer (Bio-Rad). The obtained band patterns were analyzed using BIO-PROFIL Bio-1D++ software (Vilber Lourmat, Marne-la-Vallée Cedex, France) at 11% homology coefficient. The similarity between the isolates was determined automatically by specifying the formula of Nei and Li (28). The clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA) (Vilber Lourmat).

Extracellular lipase and protease productions were determined on the media containing substrates specific for the enzymes (36). For the detection of lipase activity, Tween-20 and Tween-80 containing agar media were briefly used. Skim milk agar, milk agar and casein agar were used for the determination of protease activity. After spotting the strains on the plates, they were incubated at 37°C for a minimum of 3 days. The growth of bacteria from the inoculation site or halo formation was accepted as enzyme production. Halo formation due to proteolytic activity on casein agar was determined after treatment with 5% (v/v) trichloroacetic acid (Riedel-de Haën, Seelze, Germany).

Results and discussion

In this study, 73 samples collected from the nasopharynx of domestic pigeons were examined. Eighteen isolates were identified as staphylococci by biochemical and phenotypic tests and they were further identified as *S. delphini* at the species level by mPCR (32) and partial 16S rRNA sequencing. Results from mPCR revealed that 8 isolates belonged to *S. delphini* group A and 10 isolates belonged to *S. delphini* group B. Partial 16S rRNA gene sequencing of the strains (GenBank accession numbers HQ450212-16 and HQ452501-13) showed 99% maximum identity with *S. delphini* strain ATCC 49171 (GenBank accession no. NR_024666.1). However, partial 16S rRNA gene sequences of *S. delphini* strains also showed 99% maximum identity with *S. pseudintermedius* strains

(ATCC 49051, ATCC 46052, ATCC 51874, LMG 22220 and LMG 22219^T with GenBank accession numbers FJ858975.1, FJ536213.1, FJ536212.1, AJ780977.1 and AJ780976.1, respectively). Taken together, DNA sequencing data indicated that 16S rRNA gene sequencing of these closely related coagulase-positive staphylococcal strains was not discriminative. Therefore, the discriminatory power of mPCR was found higher in distinguishing these strains. In this study, *S. delphini* strains did not give any amplification in either monoplex or mPCR using primers described for the identification of other coagulase-positive staphylococci (32).

Agar disc diffusion zone diameters were evaluated according to zone diameter interpretive standards for *S. aureus* (7) and the results revealed that all strains were susceptible to vancomycin, oxacillin, cefoxitin, novobiocin, sulphamethoxazole/trimethoprim, gentamicin, teicoplanin, amoxycillin/clavulanic acid, rifampicin, cephazolin, linezolid, imipenem, chloramphenicol and tobramycin. On the other hand, the majority of the strains ($n = 15$) were resistant to erythromycin. In addition, 13, 12 and 6 of the strains were found to be resistant to tetracycline, clindamycin and penicillin G, respectively. Although two *S. delphini* strains (GS-99 and GS-104) showed resistance to ofloxacin and levofloxacin, only one strain (GS-104) was resistant to kanamycin. In addition, constitutive and inducible clindamycin resistance was detected by D-test. Inducible MLS resistance was detected in 3 *S. delphini* strains (GS-35, GS-87 and H3-2) (fig. 1) and constitutive MLS resistance, which are resistant to both erythromycin and clindamycin, was found in 12 strains. Inducible and constitutive clindamycin resistance of *S. delphini* strains were first described in this study. Furthermore, 3 strains (GS-11, GS-55 and GS-90) were susceptible to both antibiotics. Erythromycin resistance methylase (*erm*) genes are genetic determinants for constitutive and inducible clindamycin resistance (21).

Fig. 1. Positive result of D-test on *S. delphini* strain GS-87. E, erythromycin disc (15 µg); Da, clindamycin disc (2 µg)

The presence of *ermABC* genes was searched by PCR and the results revealed that 8 strains (GS-18, GS-35, GS-48, GS-57, GS-63, GS-67, GS-99 and H3-2) contained the *ermA* gene. Four strains (GS-6, GS-87, P8-1 and P12-1) gave a positive result for *ermC* and only one strain (GS-104) was found to contain both *ermA* and *ermC* genes. Additionally, only the *S. delphini* strain P20-1 was positive for *ermB*. Moreover, the presence of *ermABC* genes were searched by PCR and in order to control the desired amplification of the genes, the amplification products were sequenced directly with the primers used in PCR. The obtained DNA sequences match with *S. aureus erm* genes in the GenBank database. In fact, the presence of *ermB* gene in staphylococci is very rare (25). However, in this study, one *S. delphini* strain (P20-1) was positive for *ermB* gene with constitutive MLS resistance. Previous studies related with *erm* genes in *S. aureus* indicated that *ermA* gene was more common in methicillin-resistant *S. aureus* than *ermC* gene (25). Moreover, *ermA* and *ermC* genes were shown to be responsible for erythromycin resistance in 98% of 428 *S. aureus* isolates in Denmark (43). Summing up this data, most of the strains were found to be resistant to erythromycin, tetracycline and clindamycin. The reason for the high antibiotic resistance could be due to the use of high dosages of these antibiotics during treatment and the protection of domestic pigeons against infections. *S. intermedius* pigeon isolates showed a combined resistance to tetracycline and minocycline (18), while Schwarz et al. (33) reported tetracycline resistance in 14/23 and erythromycin resistance in 13/23 of *S. intermedius* strains isolated from pigeons. In addition, Futagawa-Saito et al. (13) reported antimicrobial susceptibility of 62 *S. intermedius* strains isolated from domesticated and non-domesticated pigeons and the results showed that 19.4%, 16.1% and 9.7% of the strains were resistant to lincomycin (derivative of clindamycin), erythromycin and tetracycline, respectively. Based on the results, Futagawa-Saito et al. (13) suggested that prior exposure to antimicrobials in domesticated pigeons might be selected for *S. intermedius* clones with multi-antimicrobial resistance phenotype.

In the survey of virulence genes, *S. aureus* specific primers were used. Since, there has been no report related to the enterotoxin genes of *S. delphini* strains and other virulence genes. PCR results indicated that none of the strains harbored enterotoxins, toxic-shock syndrome toxin, exfoliative toxins, heamolysins, methicillin-resistance, staphylococcal exotoxin like protein-1, *lukE-lukD* and PVL genes. In this study, 26 virulence genes were examined but none of them was detected in the strains. In a recent report, Sledge et al. (34) isolated enterotoxigenic *S. delphini* strains from farmed mink kits. In that report, the production of staphylococcal enterotoxins A and E were detected by ELISA test, whereas only the presence of *see* was

found, and *sea*, *seb*, *sec* and *sed* genes could not be amplified by PCR. The amplified *see* showed 91% sequence similarity with *S. aureus see* gene (34). On the basis of the results, there might be DNA sequence similarities of *S. delphini* virulence genes with *S. aureus*, but in this study it could be not detected among the tested genes.

Biofilm is a multicellular complex, formed by microorganisms that are attached to a surface and embedded in a matrix, consisting of exopolysaccharides. Biofilm formation of *S. delphini* strains was tested using two different media. Although 9/18 strains showed high adherence to plate surfaces in TSB supplemented with 1% sucrose, none of the strains were detected as biofilm formers in BHI (fig. 2). Moreover, weak adherence to plate surfaces was obtained in 3 strains (GS-87, GS-104 and P8-1). In fact, both genetic and environmental factors – especially pH, presence of O_2, ionic strength, surface proteins and nutrient availability – can affect biofilm formation of bacteria on the surfaces (16). In this study, TSB supplemented with sucrose increased or stimulated biofilm formation of 50% of the strains. However, 6 strains did not form biofilm in either media (fig. 2). Tube adherence results showed that strong biofilm forming strains also formed biofilm on glass tube surfaces. However, 2 non-biofilm forming strains (GS-6 and GS-57) slightly adhered to glass tube surfaces. The reason for this might be the hydrophilic nature of glass surfaces. Biofilm forming abilities of *S. intermedius* pigeon isolates were previously examined by Futagawa-Saito et al. (14). They examined 62 isolates and determined absorbance values ranging from 0.04 to 0.79 at 490 nm. However, in this study the absorbance values of biofilm formers were higher than the previously reported values (fig. 2).

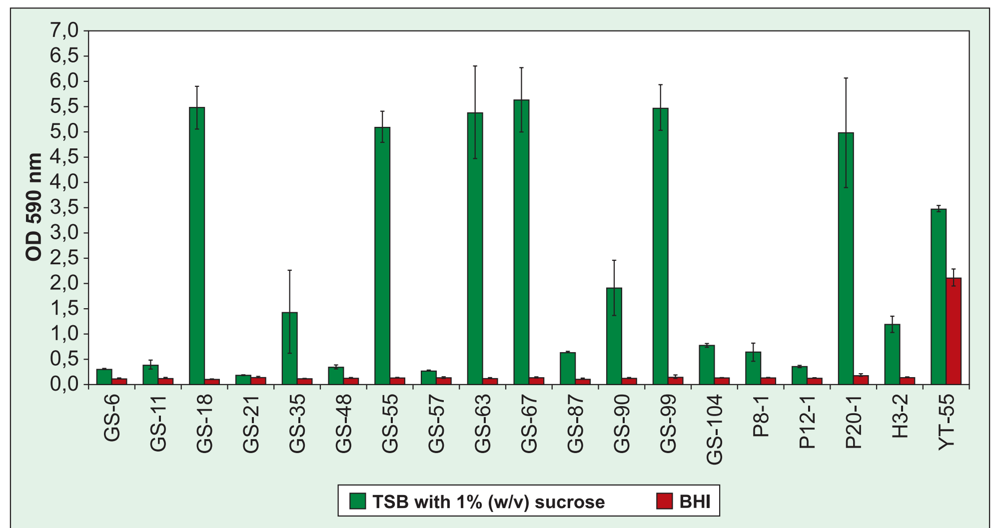

Fig. 2. Biofilm formation by *S. delphini* strains in TSB supplemented with 1% (w/v) sucrose and BHI. Bars indicate the mean of triplicate measurements and biofilm forming *S. epidermidis* YT-55 strain was used as a positive control in the test

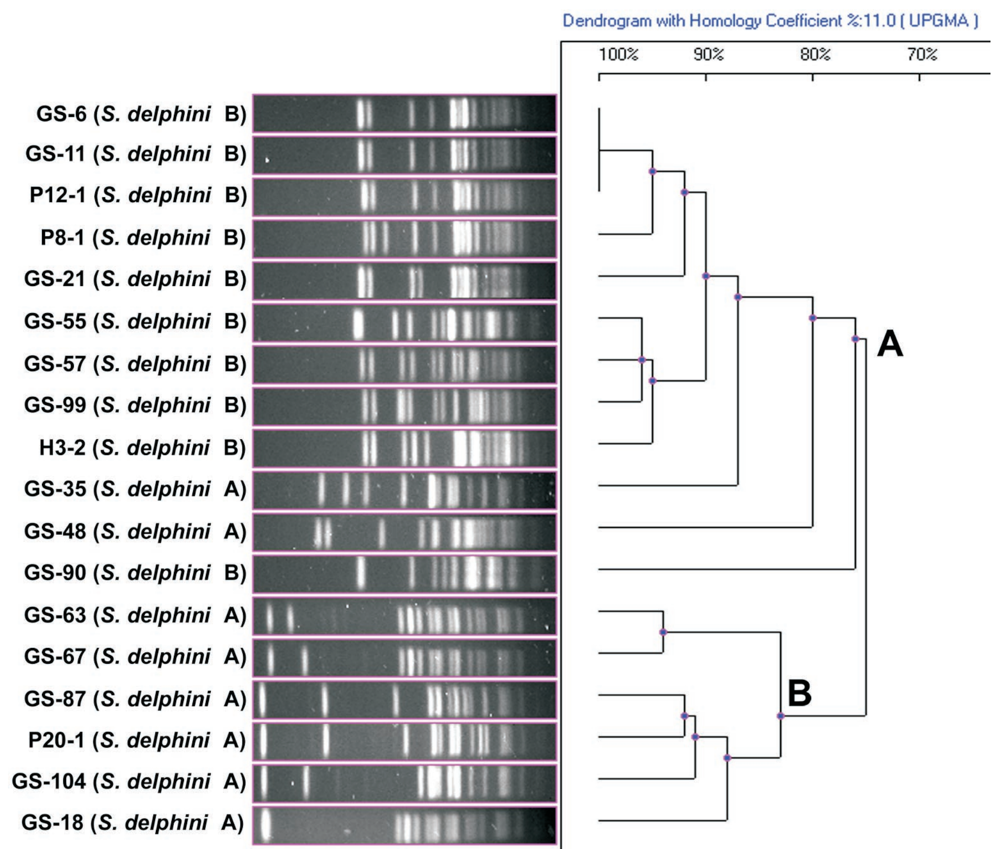

Fig. 3. Dendrogram of PFGE band patterns showing the genetic-relatedness of 18 *S. delphini* group A and group B strains

PFGE analysis was shown to be a useful tool for epidemiologic typing and determining the genetic-relatedness of staphylococci. Digestion of DNA with *Sma*I resulted in 8-15 fragments producing two main clusters (A and B) with 75% homology. Although cluster B consisted of *S. delphini* group A strains, cluster A contained both group A and mainly group B strains. Only 3 strains (GS-6, GS-11 and P12-1) isolated from

the same cage showed 100% homology with indistinguishable band patterns (fig. 3). However, antibiotic susceptibility of GS-11 was different from GS-6 and P12-1. The homology among the other strains, which were isolated from the same and different cages, varied from 76% to 96%. In addition, 4 *S. delphini* group B strains (GS-55, GS-57, GS-99 and H3-2) isolated from 3 different cages showed more than 95% homology. PFGE results indicated that there was no unique clonal type among the examined domestic pigeons. Interactions of pigeons among others and sharing the same cage and water sources could contribute to the widespread of these bacteria.

Extracellular enzyme production by staphylococci is one of the important virulence factors. Especially protease and lipase productions may help disease progression during the infection process by degrading tissues (9). In this study, 5 strains (GS-18, GS-55, GS-57, GS-99 and H3-2) showed proteolytic activity by forming clear zones on milk agar, skim milk agar and casein agar plates. In addition, 6 strains (GS-6, GS-11, GS-21, P8-1, P12-1 and P20-1) showed mild proteolytic activity on the tested media. In the case of lipase activity, 8 strains (GS-18, GS-35, GS-48, GS-63, GS--67, GS-87, GS-104 and P20-1) were positive both on Tween-20 and Tween-80 containing media. Only the *S. delphini* strain GS-18 had strong extracellular protease and lipase activity. Recently Futagawa-Saito et al. (14) also observed protease activity in 11/62 *S. intermedius* pigeon isolates. The results in this study support the previous findings that protease production of bacteria may help their infectious abilities in pigeons.

In summary, there has been no report related to the infectious abilities of *S. delphini* strains in humans. The close relationship of this bacterium with *S. intermedius* could lead to misidentification of the isolates. Especially in wounds caused by dogs, birds or other animals and in zoonotic infections, the presence of *S. delphini* strains should be examined to elucidate the infectious abilities. Most of the *S. intermedius* and *S. pseudintermedius* strains have been isolated from healthy and diseased dogs (3, 11, 30, 40). In addition, the presence of *S. delphini* in the normal flora of animals is questionable. Further studies are needed to fully elucidate the importance and infectious abilities of *S. delphini* isolates. In conclusion, *S. delphini* is one of the predominant microorganisms in the nasopharyngeal flora of domestic pigeons. Most probably, because of the misidentification of *S. delphini* with *S. intermedius* and *S. pseudintermedius*, the occurrence of this bacterium has not been reported in pigeons, clinical and environmental sources.

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