Prevalence of virulence factors in Staphylococcus intermedius isolates from dogs and pigeons

Keiko Futagawa-Saito*1, William Ba-Thein2, Naomi Sakurai3 and Tsuguaki Fukuyasu1

Address: 1Department of Animal Health 2, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa, 229-8501, Japan, 2Department of Molecular Microbiology/Immunology, Faculty of Allied Health Sciences, Thammasat University, Rangsit Campus, Pathum-thani, 12121, Thailand and 3Center for Medical Sciences, School of Health Sciences, Ibaraki Prefectural University of Health Sciences, 4669-2 Ami, Inashiki, Ibaraki, 300-0394, Japan

Email: Keiko Futagawa-Saito* - saitohk@azabu-u.ac.jp; William Ba-Thein - batheinw@yahoo.com; Naomi Sakurai - sakurai@ipu.ac.jp; Tsuguaki Fukuyasu - fukuyasu@azabu-u.ac.jp

* Corresponding author

Abstract

Background: Staphylococcus intermedius has been isolated from healthy dogs and pigeons as well as diseased dogs. Similar to Staphylococcus aureus, S. intermedius is known to carry many virulence factors but most of these factors remain to be studied. In this study, we examined 106 S. intermedius isolates (44 dog isolates and 62 pigeon isolates) for their hemolytic activity, biofilm formation, protease activity, and clumping factor and protein A production.

Results: Forty-three dog isolates (97.7%) and all pigeon isolates were hemolytic on sheep RBCs with a mean hemolytic titer of 336.7 and 47.32, respectively, whereas 43 dog isolates (97.7%) and 11 pigeon isolates (17.7%) exhibited a significant difference in their hemolytic activity on rabbit RBCs with a mean hemolytic titer of 11.04 and 3.76, respectively (p < 0.0005). The mean biofilm formation activity for dog isolates was 0.49, which was significantly higher than that (0.33) for pigeon isolates (p < 0.0005). Twenty-four dog isolates (54.5%) and 11 pigeon isolates (17.7%) were protease positive. Twenty-four dog isolates (54.5%) were clumping factor- and protein A- positive.

Conclusion: S. intermedius strains carrying the virulence factors examined in this study were more prevalent in dogs than pigeons.
ducing S. intermedius strains are more prevalent in dogs than pigeons [1,2].

Biofilm formation by S. aureus strains isolated from bovine mastitis has been reported [9]. Biofilm formation is considered to be one of the virulence factors in Staphylococci, which helps Staphylococci adhere to its target tissues, mainly implants and other foreign body materials, through adhesive mechanisms [10,11]. Microcolonies encased in extracellular polysaccharide of biofilm are protected from antimicrobial agents [12]. The biofilm formation in S. intermedius has not yet been investigated.

In this study, we have examined S. intermedius isolates from dogs and pigeons with regards to their hemolytic activity, biofilm formation, protease activity, and clumping factor and protein A production.

Results

Clumping factor and protein A production

Twenty-four dog isolates (24/44, 54.5%) and none of the pigeon isolates were positive for clumping factor and protein A.

Protease production

Protease production was significantly higher in dog isolates (24/44, 54.5%) than pigeon isolates (11/62, 17.7%) (p < 0.0005, Fisher’s exact test).

Hemolytic activity

With the exception of one dog isolate whose hemolytic titer was <2, all S. intermedius isolates (105/106, 99.1%), including 43 dog isolates (43/44, 97.7%) and all pigeon isolates, showed hemolytic activity on sheep RBC, and 54 S. intermedius isolates (54/106, 50.9%), including 43 dog isolates (43/44, 97.7%) and 11 pigeon isolates (11/62, 17.7%), showed hemolytic activity on rabbit RBC. Using sheep RBC, the mean hemolytic titer for dog isolates was 336.7 and that for pigeon isolates was 47.32 (p < 0.0005, t test). On rabbit RBC, the mean hemolytic titer for dog isolates was 11.04 and that for pigeon isolates was 3.76. There was a significant difference between the means of hemolytic titer on rabbit RBC for dog and pigeon isolates (Table 1, p < 0.0005, t test).

Biofilm formation

The range of biofilm formation activity for all S. intermedius isolates was 0.02 to 1.00. The mean of biofilm formation activity for dog isolates was 0.49, which was

### Table 1: Hemolytic activity and biofilm formation activity of Staphylococcus intermedius isolates from dogs and pigeons

| Animal (no. of isolates) | Hemolytic titer on sheep RBC (1/dilution) | Hemolytic titer on rabbit RBC (1/dilution) | Biofilm formation activity (absorbance value at 490 nm) |
|--------------------------|-------------------------------------------|--------------------------------------------|------------------------------------------------------|
|                          | Range | Mean | Range | Mean | Range | Mean |
| Dogs (44)                | <2–2048 | 336.7 | <2–32 | 11.04 | 0.02–1.00 | 0.49 |
| Pigeons (62)             | 8–128 | 47.32 | <2–16 | 3.76 | 0.04–0.79 | 0.33 |

a pair, b pair and c pair : p < 0.0005 (t test)

Note: Absorbance value at 490 nm is reported as biofilm formation activity

### Table 2: Association between hemolytic activity on rabbit RBC and biofilm formation activity in Staphylococcus intermedius isolates from dogs and pigeons

| Hemolytic activity on Rabbit RBC | Animal (no. of isolates) | Biofilm formation activity (absorbance value at 490 nm) |
|---------------------------------|--------------------------|------------------------------------------------------|
| Positive¹                      | Dogs (43) | Pigeons (11) | 0.02–1.00 | 0.48⁴ |
| Total                           | (54) | 0.02–1.00 | 0.49⁴ |
| Negative²                      | Dogs (1) | Pigeons (51) | 0.71 | 0.71 |
| Total                           | (52) | 0.04–0.79 | 0.28⁴ |

⁴Hemolytic activity ≥2; ⁵Hemolytic activity <2; ⁶after subtracting the absorbance value of blank (TSB with 0.25% glucose); d pair: p = 0.49; e pair and f pair: p < 0.0005
significantly higher than that (0.33) for pigeon isolates (Table 1, p < 0.0005, t test).

**Association between hemolytic activity and biofilm formation activity**

The association between hemolytic activity on rabbit RBC and biofilm formation activity is shown in Table 2. Regardless of the origin, the isolates with positive hemolytic activity had the mean biofilm formation activity of 0.49, whereas the isolates with negative hemolytic activity had significantly less biofilm formation activity with the mean activity of 0.29 (p < 0.0005). In addition, there was a significant difference in biofilm formation activity between hemolysis-positive and -negative pigeon isolates (0.52 vs. 0.28, p < 0.0005). However, there was no significant difference in biofilm formation activity between dog and pigeon isolates that had positive hemolytic activity on rabbit RBC (p = 0.49).

**Discussion**

*S. intermedius* isolates from dogs and pigeons have been reported to be genotypically distinguishable [2-4]. Here, we observed a difference in their virulence traits such as hemolytic activity, biofilm formation, protease activity, and clumping factor and protein A production.

Hemolytic activity of *S. intermedius* isolates from healthy and infected dogs and pigeons has been described previously [5,7,13]. The number of hemolysis-positive isolates in this study (99.1% on sheep RBC and 50.9% on rabbit RBC) is higher than that (88.2% on sheep RBC and 1.5% on rabbit RBC) in a previous study in which blood agar plate was used for assay [7]. This observed difference could be due to the fact that a sensitive microplate technique was used in our study to determine the hemolytic activity. On the other hand, the mean hemolytic titers on rabbit and sheep RBC for dog isolates were significantly higher than that for pigeon isolates. Since dog isolates also exhibited a very high leukotoxocity activity in a previous study [2], cytotoxin-producing *S. intermedius* strains seem to be prevalent among dogs.

Biofilm-forming *S. epidermidis* and *S. aureus* isolates have been recovered from hospitalized patients and non-hospitalized people [14], and instruments of dialysis [15], and bovine mastitis [9], and food and food processing environments [16], respectively. We tested a large number of *S. intermedius* isolates for their biofilm formability. Biofilm formation was significantly higher in the isolates from dogs than pigeons. Bacteria in biofilms are generally resistant to environmental stress [17], antibiotics [12], and phagocytosis by macrophage [18]. Therefore biofilm-forming *S. intermedius* isolates from dogs may have the potential to cause opportunistic and biomaterial-related infections.

Alpha-hemolysin, which is hemolytic on rabbit RBC [19], has been shown to be required for cell-to-cell interactions during biofilm formation in *S. aureus* [20]. Likewise, the association between biofilm formation and alpha-hemolysin production in *S. intermedius* was also observed in this study as the number of hemolysis-positive isolates was significantly higher than that of the hemolysis-negative isolates among the biofilm-forming *S. intermedius* isolates.

The accessory gene regulator (*agr*) of a two-component regulatory system in *S. aureus* is implicated in biofilm formation and alpha-hemolysin production [21]. In *S. intermedius*, an *agr*-like locus has also been identified by PCR [22], but the alpha hemolysin (*hla*) gene has not been reported. Therefore, it is of interest to further examine the production and regulation of virulence factors in *S. intermedius* strains.

*S. aureus* clinical isolates produce a variety of extracellular proteases [23]. Several in-vitro studies have suggested that extracellular protease is an important virulence factor in *S. aureus* [24,25]. Clumping factor promotes binding of fibrinogen and fibrin to the bacterial cell surface [26], and is shown to act as a virulence factor in experimental septic arthritis in *S. aureus* [27]. *S. aureus* isolates from patients with Kawasaki disease produce high levels of protein A [28], which is reportedly associated with inflammation of lungs [29]. More than half of *S. intermedius* isolates from dogs in this study produced protease and they were clumping factor and protein A positive. It is interesting to note that the clumping factor- and protein A- positive *S. intermedius* were isolated only from dogs. It is not known if protease, clumping factor, and protein A are associated with pathogenesis of *S. intermedius* infections in animals, but the carriage of these virulence factors indicate the pathogenic potential of the isolates. Besides, the production of many virulence traits tested in this study are susceptible or dependent on in-vitro conditions and it should cautious in interpretation of the virulence properties of *S. intermedius* isolates.

**Conclusion**

This study demonstrated that *S. intermedius* strains carrying tested virulence factors are more prevalent in dogs than pigeons.

**Methods**

**Bacterial strains**

*S. intermedius* isolates (n = 106), including 44 isolates from dogs and 62 isolates from pigeons, were used in this study. Isolation and identification of *S. intermedius* isolates were done as described previously [1]. *S. aureus* RN4220 [30] and *S. hyicus* JCM2423T [5] were used respectively as a positive control and a negative control in
the hemolytic activity assay. *S. epidermidis* ATCC35984 was used as a positive control in the quantitative assay of biofilm formation.

**Clumping factor and protein A assay**

Simultaneous detection of clumping factor and protein A was performed as described previously by Essers et al. [31]. *S. intermedius* isolates were cultured on brain heart infusion agar plate (Becton, Dickinson and Company, MD, USA) for 18 h at 37°C. A mixture of one drop each of culture (approximately 10⁸ cfu) and saline was mixed with PS latex (Eiken, Tokyo Japan). Agglutination that occurred within one minute while stirring was considered a positive reaction.

**Protease activity**

Protease activity was determined on casein agar plates following the procedure described by Bjorklind et al. [32]. The production of protease was recognized as a clear zone or a broad zone of precipitation around the bacterial streak [32,33].

**Assay for hemolytic activity**

Hemolytic assay was performed by the microplate method [19] using sheep and rabbit erythrocytes (RBCs). A culture supernatant of overnight-grown bacteria at 37°C in brain heart infusion broth (Becton, Dickinson and Company, MD, USA) was used. Two-fold dilutions of the culture supernatant in PBS (pH 7.0) containing 0.1% bovine serum albumin (BSA) (50 µl each) were mixed with 50 µl of 1% RBC in PBS in a 96-well microtiter plate. The microtiter plate was incubated at 37°C for 1 h with gentle shaking and, for sheep RBC, further incubated at 4°C for 1 h without shaking. The microtiter plate was centrifuged at 600 × g for 5 min. The hemolytic activity titer was defined as the inverse of the last dilution that caused complete hemolysis. The isolates with hemolytic titer ≥2 were considered positive for hemolytic activity.

**Quantitative assay for biofilm formation**

The assay was performed as previously described [9,34] with some modifications. Bacteria were cultivated overnight in trypticase soy broth, TSB, (Becton, Dickinson and Company, MD, USA) containing 0.25% glucose. Each culture was diluted 1:200 in the same broth. The cell suspension (200 µl) was inoculated into each well of sterile 96-well polystyrene tissue culture plates (Becton Dickinson Labware, NJ, USA) and incubated at 37°C for 16 h. The wells were washed twice with 200 µl of PBS (pH 7.4) and stained with 100 µl of 0.1% safranin-O solution per well for 30 s. After removal of the staining solution, the wells were washed once again with PBS. Then, 100 µl of a 97% ethanol-3% ether solution was added to each well and mixed. The absorbance of the adherent biofilm was measured at 490 nm in a microplate reader (Model 680, Bio-Rad, CA, USA) and the absorbance value was expressed as the biofilm formation activity. The results were reported after subtracting the reading for a blank (TSB plus 0.25% glucose, without bacterial cells) from the experimental readings. Each assay was performed in triplicate.

**Authors' contributions**

KFS conceived of the study, carried out all the experimental work and drafted the manuscript. WBT participated in analysis and interpretation of data, and wrote the final manuscript. NS participated in analysis and interpretation of data. TF participated in the study design and coordination. All authors read and approved the final manuscript.

**Acknowledgements**

We would like to thank Drs. M. Fukuyama, K. Furuhata, and K. Oonaka of the College of Environmental Health, Azabu University, Kanagawa, Japan, for their kind assistance in collecting the samples.

**References**

1. Futagawa-Saito K, Suzuki M, Ohsawa M, Ohsima S, Sakurai N, Ba-Thein W, Fukuyasu T: Identification and prevalence of an enterotoxin-related gene, se-int, in *Staphylococcus intermedius* isolates from dogs and pigeons. J Appl Microbiol 2004, 96:361-366.
2. Futagawa-Saito K, Sugiyama T, Karube S, Sakurai N, Ba-Thein W, Fukuyasu T: Prevalence and characterization of leukotoxin-producing *Staphylococcus intermedius* isolates from dogs and pigeons. J Clin Microbiol 2004, 42:5324-5326.
3. Bes M, Saidi Slim L, Becharnia F, Meugnir H, Vandenbesch F, Etienne J, Frenery J: Population diversity of *Staphylococcus intermedius* isolates from various host species: typing by 16S-23S intergenic ribosomal DNA spacer polymorphism analysis. J Clin Microbiol 2002, 40:2275-2277.
4. Wakita Y, Shimizu A, Hajek V, Kawano J, Yamashita K: Characterization of *Staphylococcus intermedius* from pigeons, dogs, foxes, mink, and horses by pulsed-field gel electrophoresis. J Vet Med Sci 2002, 64:237-243.
5. Hajek V: *Staphylococcus intermedius*, a new species isolated from animals. Int J Syst Bacteriol 1976, 26:401-408.
6. Raus J, Love DN: Characterization of coagulase-positive *Staphylococcus intermedius* and *Staphylococcus aureus* isolated from veterinary clinical specimens. J Clin Microbiol 1983, 18:789-792.
7. Shimizu A, Kawano J, Kimura S: Biotyping of coagulase-positive *Staphylococcus aureus* and *Staphylococcus intermedius* strains isolated from various animals in Japan. Nippon Juigaku Zasshi 1986, 48:1227-1235.
8. Terauchi R, Sato H, Hasegawa T, Yamaguchi T, Aizawa C, Maehara N: Isolation of exfoliative toxin from *Staphylococcus intermedius* and its local toxicity in dogs. Vet Microbiol 2003, 94:19-29.
9. Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS: Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 2003, 92:179-185.
10. Rupp ME, Ullphani JS, Fey PD, Barsch K, Mack D, Lee J: Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. Infect Immun 1999, 67:2627-2632.
11. Gotz F: *Staphylococcus* and biofilms. Mol Microbiol 2002, 43:1367-1378.
12. Olson ME, Ceri H, Morck DW, Buret AG, Read RR: Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res 2002, 66:86-92.
13. Talan DA, Staatz D, Staatz A, Goldstein EJ, Singer K, Overturf GD: *Staphylococcus intermedius* in canine gingiva and canine-inflicted human wound infections: laboratory characterization of a newly recognized zoonotic pathogen. J Clin Microbiol 1989, 27:778-81.
14. Krepsky N, Rocha Ferreira RB, Ferreira Nunes AP, Casado Lins UG, Costa e Silva Filho F, de Mattos-Guaraldi AL, Netto-dosSantos KR: Cell surface hydrophobicity and slime production of Staphylococcus epidermidis Brazilian isolates. *Curr Microbiol* 2003, 46:280-286.

15. Chaieb K, Mahdouani K, Bakhrour A: Detection of icaA and icaD loci by polymerase chain reaction and biofilm formation by Staphylococcus epidermidis isolated from dialysate and needles in a dialysis unit. *J Hosp Infect* 2005, 61:225-230.

16. Moretro T, Hermansen L, Holck AL, Sidhu MS, Rudi K, Langsrud S: Biofilm formation and the presence of the intercellular adhesion locus ica among staphylococci from food and food processing environments. *Appl Environ Microbiol* 2003, 69:5648-5655.

17. Costerton JW, Stewart PS, Greenberg EP. *Bacterial biofilms: a common cause of persistent infections.* Science 1999, 284:1318-1322.

18. Shiau AL, Wu CL: The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-independent. *Microbial Immunol* 1998, 42:233-40.

19. Bhatki S, Muhly M, Fussle R: Correlation between toxin binding and hemolytic activity in membrane damage by *staphylococcal alpha-toxin*. *Infect Immun* 1984, 46:318-323.

20. Caiazzi NC, O'Toole GA: Alpha-toxin is required for biofilm formation by *Staphylococcus aureus*. *J Bacteriol* 2003, 185:3214-3217.

21. Vuong C, Saenz HL, Gotz F, Otto M: Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* 2000, 182:1688-1693.

22. Dufour P, Jarraud S, Vandenesch F, Greenwood T, Novick RP, Bes M, Etienne J, Lina G: High genetic variability of the agr locus in *Staphylococcus aureus* species. *J Bacteriol* 2002, 184:1180-1186.

23. Karlsson A, Arvidson S: Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor *sorA*. *Infect Immun* 2002, 70:4239-4246.

24. McDevitt D, Francois P, Vaudaux P, Foster TJ: Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol Microbiol* 1994, 11:237-248.

25. Palmqvist N, Joselfsson E, Tarkowski A: Clumping factor A-mediated virulence during *Staphylococcus aureus* infection is retained despite fibrinogen depletion. *Microbes Infect* 2004, 6:196-201.

26. Wann ER, Fehringer AP, Epezhukh YV, Schlievert PM, Bina P, Reiser RF, Hook MM, Leung DY: *Staphylococcus aureus* isolates from patients with Kawasaki disease express high levels of protein A. *Infect Immun* 1999, 67:4737-4743.

27. Normark BH, Normark S, Norby-Teigland A: Staphylococcal protein A inflames the lungs. *Nat Med* 2004, 10:780-781.

28. Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP: The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 1983, 305:709-712.

29. Bjorklind A, Arvidson S: *Staphylococcus aureus* by a latex agglutination test. *J Clin Microbiol* 1980, 12:641-643.

30. Bjorklind A, Arvidson S: Occurrence of an extracellular serine-proteinase among *Staphylococcus aureus* strains. *Acta Pathol Microbiol Scand B* 1977, 85:277-280.

31. Arvidsson K: Rapid and reliable identification of *Staphylococcus aureus* by a latex agglutination test. *J Clin Microbiol* 1980, 12:641-643.

32. Bjorklind A, Arvidson S: Occurrence of an extracellular serine-proteinase among *Staphylococcus aureus* strains. *Acta Pathol Microbiol Scand B* 1977, 85:277-280.

33. Arvidsson K: Hydrolysis of casein by three extracellular proteolytic enzymes from *Staphylococcus aureus*, strain V8. *Acta Pathol Microbiol Scand B* 1973, 81:538-544.

34. Heilmann C, Gerke C, Perdreau-Remington F, Gotz F: Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect Immun* 1996, 64:277-282.

---

**Publish with BioMed Central and every scientist can read your work free of charge**

*BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime.*

Sir Paul Nurse, Cancer Research UK

Your research papers will be:
- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp