Prevalence and Characterization of Leukotoxin-Producing
Staphylococcus intermedius in Isolates from Dogs and Pigeons

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Staphylococcus intermedius isolates from dogs (n = 44) and pigeons (n = 62) were categorized into 12 types by intergenic ribosomal DNA spacer polymorphism analysis. All isolates from pigeons were lukS positive and all isolates from dogs were lukS and lukF positive by dot blot analysis. The mean leukotoxicity titer for dog isolates was at least 129-fold higher than that for pigeon isolates.

Staphylococcus aureus strains produce several toxins, including single-component α-hemolysin and the bicomponent leukotoxins Panton-Valentine leucocidin (PVL) and γ-hemolysin (4). PVL is cytotoxic to human and rabbit polymorphonuclear cells, monocytes, and macrophages, and γ-hemolysin is cytolytic to mammalian erythrocytes (4, 7). PVL-producing S. aureus is strongly associated with skin infections, such as furuncles (14), and with lethal necrotizing pneumonia in young immunocompetent patients (6). An S. intermedius leukotoxin known as Luk-I has also been identified (15). Characterization and sequence analysis have shown that, similar to PVL, Luk-I is encoded as a lukI operon with two cotranscribed genes, lukS and lukF (referred to elsewhere as lukS-I and lukF-I, respectively), encoding LukS and LukF (15). Luk-I shows a strong leukotoxicity on various polymorphonuclear cells, but only a slight hemolytic activity on rabbit erythrocytes (15).

It has been shown by various genotyping methods, such as 16S-23S intergenic ribosomal DNA spacer polymorphism analysis (ITS-PCR), EcoRI ribotyping, and Smal pulsed-field gel electrophoresis, that S. intermedius strains are diverse and that the genotypes of S. intermedius isolates from dogs are distinct from those from pigeons (2, 3, 18). The enterotoxins and hemolysins are more prevalent among S. intermedius isolates from dogs than among those from pigeons (5, 17). The prevalence of leukotoxin in S. intermedius isolates from dogs and pigeons, however, has yet to be investigated.

Here, we have typed S. intermedius isolates from dogs and pigeons by ITS-PCR and have investigated the prevalence of the lukI operon by dot blot hybridization and the leukotoxic activity of the isolates. We also report the identification of a new leukotoxin gene, i.e., a lukS ortholog, in S. intermedius isolates from pigeons.

The study was carried out with 106 S. intermedius isolates recovered from healthy skin or infected sites of dogs and pigeons from four different prefectures in Japan (Chiba, Kanna-
gawa, Saitama, and Tokyo). Included were 44 isolates from dogs (8 healthy dogs, 23 dogs with pyoderma, and 13 dogs with otitis externa) and 62 isolates from pigeons (5 pigeons from a zoo, 10 domesticated pigeons, and 47 wild pigeons). Isolation and identification of S. intermedius isolates were done as des-
cribed previously (5). An S. intermedius type strain from pi-
gions, JCM2422T (8), was used as the quality control strain.

Genomic DNA preparation from S. intermedius and geno-
typing by ITS-PCR were done as described by Matsuhashi et al. (11) and Bes et al. (2), respectively. Probes used to de-
tect lukS and lukF in S. intermedius isolates were prepared by PCR using genomic DNA of a dog isolate, S. intermedius AV8004. The primers used were 5′-TGTAAACAGACGAAAA
ATGGGG-3′ and 5′-GCCCGATAGGACTTCTTACA-3′ for lukS and 5′-CGCGTGCTAGCGCCGCTAATA-3′ and 5′-
AGGTCTAGGAGCTATCTCGA-3′ for lukF. DNA amplifica-
tions were performed for 25 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Sequences of PCR products, such as lukS (503 bp) and lukF (572 bp), were confirmed with published sequences in a database (GenBank accession number X79188). Dot blot hybridization was done as described previously (10) by using the PCR probes, bacterial genomic DNA, and a DIG DNA labeling and detection kit (Roche).

The assay for leukotoxic activity was performed as described previously by Rainard et al. (16), with some modifications. The culture supernatants of bacteria grown overnight in brain heart infusion broth (Difco) were collected and stored frozen at −20°C until used. Freezing did not have a significant effect on the leukotoxic activity of the samples examined. Freshly iso-
lated rabbit leukocytes were suspended in phosphate-buffered saline containing 0.5% gelatin to get a concentration of 2.0 × 10⁶ cells/20 μl. Serial twofold dilutions (20 μl each) of the culture supernatant in phosphate-buffered saline containing 0.5% gelatin were done in a 96-well microtiter plate and were mixed with 20 μl (each) of leukocyte suspension and incubated at 37°C for 10 min in a moisturized chamber (12). The last
dilution that induced the flattening of cells, a feature of cytotoxicity, in 95% of leukocytes was determined under a phase-contrast microscope and confirmed by Giemsa staining. The leukotoxicity titer was the inverse of the last dilution (16).

*S. intermedius* isolates (*n* = 106) were categorized into 12 ITS-PCR types (A to L); isolates from dogs and pigeons were distributed into types A to G and types H to L, respectively (Table 1). The observed heterogeneity among our *S. intermedius* isolates is in agreement with the report of Bes et al. (2). Isolates from zoo pigeons and from domesticated pigeons were restricted to ITS-PCR types H and I, respectively. Isolates from infected and healthy dogs were genotypically not distinguishable by ITS-PCR typing (data not shown), similar to previous findings (1, 9, 13).

All 44 *S. intermedius* isolates from dogs (ITS-PCR types A to G) were *lukS* and *lukF* positive and exhibited very high cytotoxic activity on rabbit leukocytes, with a mean leukotoxicity titer of 466 (Table 1). Some randomly selected dog isolates (*n* = 11) also had similar levels of activity on human leukocytes (data not shown). There was no significant difference in leukotoxic activity between the dog isolates belonging to ITS-PCR types A to E or to different sources (healthy dogs, dogs with pyoderma, and dogs with otitis externa) (*P* = 0.52 or *P* = 0.77, respectively; Tukey-Kramer test). In contrast, 62 isolates from pigeons (ITS-PCR types H to L) were positive only for *lukS* by dot blot analysis (Fig. 1). Dot blot results were confirmed by PCR (data not shown). Furthermore, the mean leukotoxicity titer for pigeon isolates was <3.6, which was significantly lower than that for dog isolates (*P* < 0.0001; *t* test).

Sequence analysis of PCR-amplified *lukS* products from representative isolates from each ITS-PCR type showed that *lukS* of pigeon isolates had a lower homology (75 to 86% identity at the amino acid level), compared with that of dog isolates (98 to 100% identity), to the *lukS* probe from the dog isolate AV8004 used in dot blot hybridization. Accordingly, we considered the leukotoxin gene amplifiable by *lukS* primers in pigeon isolates to be a new ortholog. This low homology may explain the weak reactions observed for dot blot analysis of pigeon isolates (Fig. 1). From these observations, it is conceivable that the apparent difference in leukotoxicity between *S. intermedius* strains from dogs and from pigeons in this study is contributed by *lukF* and that the presence of both *lukF* and the *lukS* ortholog is required for maximal leukotoxic activity. However, our results do not eliminate the possibility that a gene for the second component was present in pigeon isolates but not detected in this study.

Summarizing, our results demonstrated that there was a significant difference in the leukotoxic activity between *S. intermedius* strains from dogs and from pigeons, with at least 129-fold-higher activity in strains from dogs, and that the *S. intermedius* strains recovered from infected dogs were not distinct from those from healthy dogs with regard to leukotoxin production and genotype by ITS-PCR typing.

**Nucleotide sequence accession number.** The *lukS* ortholog was assigned GenBank accession number AB185109.

### REFERENCES


10. Holeckova, B., E. Holoda, M. Fotta, V. Kalinacova, J. Gondof, and J. Grol-

### TABLE 1. Genotypic and phenotypic characteristics of *S. intermedius* isolates from dogs and pigeons

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>ITS-PCR type</th>
<th>No. of isolates</th>
<th>No. of isolates positive for:</th>
<th>Leukotoxicity titer*</th>
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<td></td>
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<td>16</td>
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<tr>
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<td>2</td>
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<td>512–1024</td>
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<td>44</td>
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<tr>
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<td>2–32</td>
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</table>

* Leukotoxicity titer is the inverse of the last dilution that induced the flattening of cells in 95% of leukocytes.

‡ Calculated after logarithmic transformation.

§ Weak reaction as depicted in Fig. 1.

![FIG. 1. Dot blot analysis of genomic DNA from *S. intermedius* isolates with *lukS* and *lukF* probes. Spots a1 and a2, ITS-PCR type A; a3 and a4, ITS-PCR type B; a5 and a6, ITS-PCR type C; b1 and b2, ITS-PCR type D; b3 and b4, ITS-PCR type E; b5, ITS-PCR type F; b6, ITS-PCR type G; c1 and c2, ITS-PCR type H; c3 and c4, ITS-PCR type I; c5, ITS-PCR type J; c6 and d1, ITS-PCR type K; d2 to d5, ITS-PCR type L; and d6, negative control (*Salmonella enterica* serovar Typhimurium). All *S. intermedius* strains were included in dot blot hybridization analysis, but only two representative, randomly selected strains for each ITS-PCR type, with the exception of four strains for ITS-PCR type L and one strain each for ITS-PCR types F and G, are shown.](http://jcm.asm.org/Download?file=346x667)


