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

Keiko Futagawa-Saito,[1]* Tsukasa Sugiyama,[1] Sayaka Karube,[1] Naomi Sakurai,[2] William Ba-Thein,[3] and Tsuguaki Fukuyasu[1]

Center for Medical Sciences, School of Health Sciences, Ibaraki Prefectural University of Health Sciences, Inashiki,[2] and Department of Infection Biology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba,[3] Ibaraki, and Department of Animal Health 2, School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa,[1] Japan

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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 SmaI 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, Kanagawa, 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 described previously (5). An *S. intermedius* type strain from pigeons, JCM2422^T (8), was used as the quality control strain.

Genomic DNA preparation from *S. intermedius* and genotyping by ITS-PCR were done as described by Matsuhashi et al. (11) and Bes et al. (2), respectively. Probes used to detect *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′-TGTAAGCAGCAGAAA ATGGGG-3′ and 5′-GCCCGATAGGACTTCTTACAA-3′ for *lukS* and 5′-CCTGTCTATGCCGCTAATCAA-3′ and 5′-AGGTCATGGAAGCTATCTCGA-3′ for *lukF*. DNA amplifications were performed for 35 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 isolated rabbit leukocytes were suspended in phosphate-buffered saline containing 0.5% gelatin to get a concentration of 2.0×10^5 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

* Corresponding author. Mailing address: Department of Animal Health 2, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan. Phone: 81 (42) 754 7111. Fax: 81 (42) 768 2588. E-mail: saitohk@azabu-u.ac.jp.

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

Source of isolate	ITS-PCR type	No. of isolates	No. of isolates positive for:		Leukotoxicity titer[a]	
			lukS	*lukF*	Range	Mean[b]
Dogs	A	14	14	14	256–512	441
	B	16	16	16	256–1024	450
	C	4	4	4	512	512
	D	6	6	6	256–1024	512
	E	2	2	2	512–1024	724
	F	1	1	1	512	512
	G	1	1	1	256	256
Total		44	44	44	256–1024	466
Pigeons	H	5	5[c]	0	<2	<2
	I	11	11[c]	0	8–32	12.4
	J	1	1[c]	0	<2	<2
	K	5	5[c]	0	<2	<2
	L	40	40[c]	0	<2–8	<3
Total		62	62	0	<2–32	<3.6

[a] Leukotoxicity titer is the inverse of the last dilution that induced the flattening of cells in 95% of leukocytes.
[b] Calculated after logarithmic transformation.
[c] Weak reaction as depicted in Fig. 1.

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

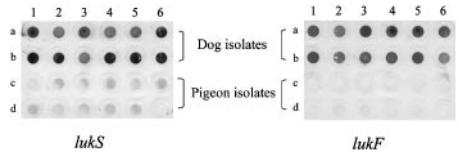

FIG. 1. Dot blot analysis of genomic DNA from *S. intermedius* isolates with *lukS* and *lukF* probes. Spots a1 and a2, ITS-PCR type A; a3 and a4, ITS-PCR type B; a5 and a6, ITS-PCR type C; b1 and b2, ITS-PCR type D; b3 and b4, ITS-PCR type E; b5, ITS-PCR type F; b6, ITS-PCR type G; c1 and c2, ITS-PCR 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.

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.

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