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Brief Original Article

Occurrence of toxin genes in *Staphylococcus pseudintermedius* from diseased dogs and other domestic and wild species

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Abstract

Introduction: *Staphylococcus pseudintermedius* is coagulase-positive species of the *Staphylococcus intermedius* group. It is an opportunistic pathogen that can cause infection in various parts of the body and has a zoonotic potential. Although studies on the pathogenicity and epidemiology of *S. pseudintermedius* are limited, it is known that this bacterium has several virulence factors, including toxins. These toxins can be classified into three main groups: pyrogenic toxins with superantigenic properties such as toxic shock syndrome toxin and staphylococcal enterotoxins, exfoliative toxins, and cytotoxins such as hemolysins and leukocidins.

Methodology: In this study, the occurrence of eight toxin genes (*sea*, *sec*, *tst*, *SIET, EXI, LuK F-I, Luk S-I,* and *hlg γ*) was examined by PCR in 58 isolates of *S. pseudintermedius* from four domestic animal species.

Results: All *S. pseudintermedius* isolates had at least one of the eight toxin genes. The predominant toxin genes were *Luk S-I* (95%), *Luk F-I* (91%), and *EXI* (91%), and the least prevalent gene was *hlg γ* (5%). Significant association (p = 0.0175) was found between the occurrence patterns of genes *hlg γ* and *Luk F-I*.

Conclusions: The frequent occurrence of these genes in *S. pseudintermedius* obtained from diseased animals indicates that these toxins may play an important role in the pathogenesis of infection among domestic animals.

Key words: *Staphylococcus intermedius;* virulence factors; enterotoxins; hemolysins; leucocidins.

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Introduction

Staphylococcus pseudintermedius is coagulase-positive species that was classified as *Staphylococcus intermedius* based on phenotypic characteristics. Recently, however, genotypic analyses revealed that it belongs to the *Staphylococcus intermedius* group (SIG), which comprises *S. intermedius*, *S. pseudintermedius*, and *S. delphini* [1,2]. It is the opportunistic bacteria that colonizes and clinically infects dogs and cats [3] and occasionally other animals [3,4], including humans [5]. Evidence of the zoonotic transmission of *S. pseudintermedius* from dogs to humans has been reported [5,6].

S. pseudintermedius can cause skin infections such as pyoderma, external ear infections, wound infections, and abscesses, postoperative wound infections, and infections involving tissue and body cavities [5]. The microorganism more often colonizes the anal and nasal vestibular regions in healthy dogs than the groin, armpits, foreskin, vagina, external auditory canal, or interdigital skin [7,8].

Staphylococcal toxins described to date can be classified into three main groups: pyrogenic toxin superantigens (PTSAgs), which comprise toxic shock syndrome toxin (TSST) and staphylococcal enterotoxins (SEs); exfoliative toxins; and cytotoxins such as leukocidins and hemolysins.

Pyrogenic toxin superantigens are exocellular proteins that are able to stimulate the polyclonal proliferation of T lymphocytes by forming a complex that binds the histocompatibility complex II to the variable portion of the T-lymphocyte ß or α receptor, resulting in the production of toxic levels of cytokines [9,10]. Eighteen staphylococcal enterotoxins and their variants are responsible for toxic food poisoning caused by *Staphylococcus aureus*. Other diseases such as mastitis, arthritis, atopic dermatitis, inflammatory bowel disease, Kawasaki disease and autoimmune diseases have been associated with enterotoxins [11,12]. *S. pseudintermedius* exfoliative toxin SIET and exfoliative toxin EXI possibly play a role in the pathogenesis of cutaneous canine lesions and chronic

otitis [13,14]. *S. intermedius* produces a bicomponent leukotoxin (Luk-I) similar to Panton-Valentine Leukocidin (PVL) of *S. aureus*, which is also encoded by two genes, *LukS-I* and *LukF-I* [15].

Most of the virulence factors have not been characterized in detail so far, especially in *S. pseudintermedius* isolated from animals. Therefore, the purpose of this study was to evaluate the presence of eight staphylococcal genes encoding pyrogenic toxins (*sea*, *sec*, and *tst*), exfoliative toxins (*SIET*, *EXI*), and cytotoxins (*Luk F-I, LuK S-I*, and *hlg γ*) in *S. pseudintermedius* isolated from various species of domestic and wild mammals.

Methodology

Clinical samples

A total of 100 clinical samples were collected from diseased animals in the Laboratory of Veterinary Microbiology at the Federal University of Mato Grosso, Brazil, between January 2012 and December 2013. Four domestic animal species were tested which included 50 dogs, four cats, one cow and one pig as well as a two wild species, namely one crab-eating fox (*Cerdocyon thous*) and one crab-eating raccoon (*Procyon cancrivorus*). Clinical samples were cultured and colonies were isolated and identified as *Staphylococcus* spp. according to morphological and staining characteristics [16].

DNA extraction

Before extraction of genomic DNA from the samples, the colonies were inoculated into brain heart infusion (BHI) broth and incubated overnight at 37°C with constant stirring. After centrifugation, the pellet was resuspended in lysis buffer (100 mM NaCl, 25 mM EDTA, 100 mM Tris-HCl pH 8.0, 0.5% SDS, and 1 ng proteinase K) and then processed for DNA extraction by phenol chloroform method, as previously described [17]. Resulting DNA was dissolved in ultrapure water and stored at −20°C until use.

Identification of S. pseudintermedius

Nucleotide sequencing of select gene fragments was used for identification of the clinical isolates. The extracted DNA samples were subjected to PCR amplification of a fragment of the 16S rRNA gene. Each reaction contained 10 ng of genomic DNA, 0.4 pmol of each primer, 0.2 mM dNTPs, 3 mM $MgCl_2$, final dilution of 10× PCR buffer (200 mM Tris-HCl pH 8.4, and 500 mM KCl), 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA), and ultrapure water in a final volume of 25 µL. The reactions were amplified on a MyCycler thermocycler (Bio-Rad Laboratories, Hercules, USA), with initial denaturation for 5 minutes at 95°C, followed by 30 cycles of denaturation for 45 seconds at 95°C, hybridization for 1 minute at 52°C, and extension for 1 minute and 30 seconds at 72°C, ending with a final extension cycle of 7 minutes at 72°C. Table 1 lists the oligonucleotide primers, the positive control, and the size of the amplicons used in this study. Agarose gel electrophoresis (1.0% agarose) of the PCR products was performed, followed by staining with GelRed (Biotium, Fremont, USA) at 10

Table 1. List of primers.

Gene	Name	Oligonucleotide sequence	Amplicon size	Annealing temperature	Reference
16S	27f	5'AGAGTTTGATCCTGGCTCAG3'	1512pb	52°C	[26]
	1492r	5'GGTTACGTTACGACT3'			
sea	*Sea*-f	5'GCAGGGAACAGCTTTAGGC3'	521pb	50°C	[27]
	Sea-r	5'GTTCTGTAGAAGTATGAAACACG3'			
sec	*Sec*-f	5'CTCAAGAACTAGACATAAAAGCTAGG3'	271pb	55°C	[28]
	Sec-r	5'TCAAAATCGGATTAACATTATCC3'			
tst	*Tst*-f	5'AAGCCCTTTGTTGCTTGCG3'	445pb	55°C	[28]
	Tst-r	5'ATCGAACTTTGGCCCATACTTT3'			
SIET	Siet-f	5'ATGGAAAATTTAGCGGCATCTGG3'	359pb	55°C	[29]
	Siet-r	5'CCATTACTTTTCGCTTGTTGTGC3'			
EXI	Exi-f	5'TAAGCATGCAATCATATAATGAGGAAGAAATATTAAAAAAGCAA3'	350pb	55°C	[14]
	Exi-r	5'TCTGGATCCTTCTTCTTGTAATTTAGCTCTTTTTTTCAAGTCTTC3'			
	Exi-fn	5'CAATAGACCTTCACATGCTG3'			
Luk F-I	Lukf-f	5'CCTGTCTATGCCGCTAATCAA3'	572pb	55°C	[15]
	Lukf-r	5'AGGTCATGGAAGCTATCTCGA3'			
Luk S-I	Luks-f	5'TGTAAGCAGCAGAAAATGGGG3'	503pb	55°C	[15]
	Luks-r	5'GCCCGATAGGACTTCTTACAA3'			
hlg γ	hlg -f	5'AATCCGTTATTAGAAAATGC3'	935pb	55°C	[30]
	hlg -r	5'CCATAGACGTAGCAACGGAT3'			

V/cm, and the results were viewed in a ChemiDoc XRS system using the Image Lab software (Bio-Rad Laboratories, Hercules, USA). The molecular weight markers were Lambda/HindIII (Ludwig Biotec, Alvorada, Brazil).

The PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited, Amersham, UK), and then used in the sequencing reaction, together with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) on an automated sequencer (ABI 3500 Genetic Analyzer, Applied Biosystems Foster City, USA). The sequences were compared with the GenBank database using a BLAST search on the NCBI server (http: www.ncbi.nlm.nih.gov/BLAST).

Detection of toxin genes by PCR

PCR reactions were conducted in a final volume of 20 µL of a mixture containing 10 ng of genomic DNA, 10 pmol of each primer (Table 1), 0.2 mM dNTPs, 1.25X dilution of 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% of gelatin), 0.5 U of Taq DNA polymerase (Sigma-Aldrich, St. Louis, USA), and ultrapure q.s. water for the *sec*, *tst*, *SIET, EXI, Luk F-I, Luk S-I*, and *hlg γ* genes, whereas 1.25 mm $MgCl_2$ was added to the reaction mixture for the *sea* gene. A ProFlex PCR System thermal cycler (Thermo Fisher Scientific, Waltham, USA) was used, with the initial denaturation performed at 95°C for 5 minutes, followed by 30 cycles of 30 seconds at 95°C, specific annealing for each gene (Table 1), extension for 30 seconds at 72°C, and the final extension for 7 minutes at 72°C. For the *EXI* gene, semi-nested PCR was performed using nested Exi-f and Exi-r primers under the same conditions as described above. The resulting products were analyzed as described above.

Statistical analysis

The Fischer's exact test was performed to examine the association between the occurrence of the toxin genes and the characteristics of the species and infection sites as well as the associations among the genes by the R software Version 3.3.1 (Lucent Technologies, New Jersey, USA) [18] with the Rcmdr package [19], where differences with $p < 0.05$ were considered significantly different.

Results

Fifty-eight isolates were identified as *S. pseudintermedius*. These isolates were from animals with different diseases (Table 2), mainly dermatitis (n = 21) and otitis (n = 21) from domestic animals (Table 2), and two isolates were from free-ranging animals such as crab-eating fox and crab-eating raccoon.

Each *S. pseudintermedius* isolate had at least one of the eight toxin genes, either alone or in combination, and the maximal number of toxin genes in one isolate was seven. Figure 1 illustrates the prevalence of toxin genes among the samples tested. Table 3 shows the number of positive isolates for each gene and their prevalence in each species. The most prevalent toxin gene among all the animals was *Luk S-I* (95%), and the least prevalent one was *hlg γ* (5%), whereas the toxin with the highest prevalence in dogs was *Luk S-I.* A

Table 2. Distribution of toxin genes in *S. pseudintermedius* isolates from various infection sites.

Infection site	Disease	N° Isolates	*sea*	*sec*	*tst*	SIET	EXI	Luk F-I	Luk S-I	hlg γ
			+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)
Mouth	Stomatitis	1	1	0	0	1	0	1	1	0
Cornea	Keratitis	1	0	0	0	1	1	1	1	0
Bone fracture	Osteomyelitis	2	0	1	1	2	1	2	2	0
Lymph node	Lymphadenitis	1	1	0	0	1	1	1	1	0
Abdominal fluid	Peritonitis	1	0	1	1	1	0	1	1	0
Cerebrospinal fluid	Meningitis	1	0	1	0	1	0	1	1	0
Ear	Otitis	18	4	9 (50)	8 (44)	16 (89)	8 (44)	16 (89)	17 (94)	1
Hair	Dermatitis	21	2	15 (71)	2	18 (86)	6 (29)	19 (90)	19 (90)	1
Lung	Pneumonia	3	0	1	0	3	1	3	3	0
Rectum	Diarrhea	1	1	0	1	1	1	0	1	1
Nasal mucosa	Sinusitis	1	0	1	0	1	0	1	1	0
Urine	Cystitis	7	1	4	1	7 (100)	3	7 (100)	7 (100)	0
Total		**58**	**10 (17)**	**33 (57)**	**14 (24)**	**53 (91)**	**22 (38)**	**53 (91)**	**55 (95)**	**3(5)**

significant association (p = 0.0175) among the toxin genes was found only between *hlg y*ˇ and *Luk F-I*.

Discussion

In this study, all the isolates had at least one of the eight toxin genes which is, in contrast to another study that showed the presence of at least one toxin gene in only 35% of the *S. aureus* isolates examined [20]. To date, *S. pseudintermedius* has not been studied for simultaneous detection of the eight toxins particularly in diseased animals. In the present study, we evaluated larger number of species and all the isolates were derived from animals with a clinically confirmed *S. pseudintermedius* infection. However, the large majority of samples were derived from dogs.

Prevalence of *sea*, *sec*, *tst*, and *EXI* genes differed considerably in previous studies. In present study, we observed higher prevalence compared and only *hlg y*ˇ gene showed low frequency [15,21-23]. These discrepancies may be due to the differences in the health status of the animals between studies or due to considerable genetic variability among the isolates; for example, gene *sea* is more prevalent in pyoderma isolates than those present on healthy skin. Further investigation of regulatory genes for Accessory Gene Regulator (*agr*) system is warranted to prove role of these toxins during *S. pseudintermedius* infection.

The high prevalence of genes *Luk S-I* (95%), *Luk F-I* (91%), and *SIET* (91%) observed in this study is similar to the data from other studies, where 98%, 100%, and 100% of canine isolates were found to be positive for these genes [15,22-25]. This high prevalence probably indicates their importance for active infection.

Conclusion

High prevalence of isolates positive for a significant number of toxin genes suggests that these toxins may play an important role in infection caused by *S. pseudintermedius*. *sec* gene was more frequently associated with cases of pyoderma that indicate its involvement in the development of pathology. Knowledge about the toxigenic profile of *S. pseudintermedius* strains help in understanding of the pathogenesis of infection caused by this microbe.

Figure 1. The total percentage of test-positive isolates for each toxin gene among the 58 *S. pseudintermedius* isolates from domestic and wild animals.

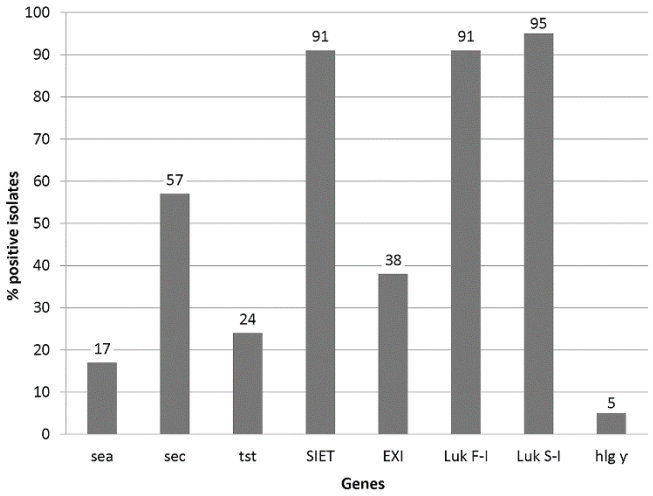

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Table 3. Distribution of toxin genes in *S. pseudintermedius* isolates from various animal species.

Species	No. of Isolates	*sea*	*sec*	*tst*	SIET	EXI	Luk F-I	Luk S-I	hlg y
		+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)	+ (%)
Dog	50	8 (16)	29 (58)	13 (26)	45 (90)	18 (36)	45 (90)	47 (94)	3
Cat	4	1	3	1	4	2	4	4	0
Cow	1	0	1	0	1	0	1	1	0
Pig	1	0	0	0	1	1	1	1	0
Crab-eating fox	1	0	0	0	0	1	1	1	0
Crab-eating raccoon	1	1	0	0	1	0	1	1	0
Total	**58**	**10 (17)**	**33 (57)**	**14 (24)**	**53 (91)**	**22 (38)**	**53 (91)**	**55 (95)**	**3(5)**

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