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 y*) 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 y* (5%). Significant association (p = 0.0175) was found between the occurrence patterns of genes *hlg y* 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; leukocidins.

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**Introduction**

*Staphylococcus pseudintermedius* is a 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 β 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...
S. intermedia 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-Cl 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 MgCl2, final dilution of 10× PCR buffer (200 mM Tris-Cl 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

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**Table 1. List of primers.**

| Gene | Name | Oligonucleotide sequence | Amplicon size | Annealing temperature | Reference |
|------|------|--------------------------|---------------|-----------------------|-----------|
| 16S  | 27f  | 5’AGAGTTTGATCGTCTCAAG3’ | 1512pb        | 52°C                  | [26]      |
|      | 1492r| 5’GGTACGTTACGACT3’      |               |                       |           |
| sea  | Sea-f| 5’GCAGGGAACAGCTTTAGGC3’ | 521pb         | 50°C                  | [27]      |
|      | Sea-r| 5’GTTCTGTAAGATGTGAACACG3’|              |                       |           |
| sec  | Sec-f| 5’CTCAAGAAGCTAGACATAAAGCTAG3’ | 271pb       | 55°C                  | [28]      |
|      | Sec-r| 5’CAGAATCCGAATTACATTACCC3’ |           |                       |           |
| tst  | Tst-f| 5’AAGCCCTTTGGCTCTGCG3’ | 445pb         | 55°C                  | [28]      |
|      | Tst-r| 5’ATCGAATTGGCCCATATTT3’ |              |                       |           |
| SIET | Siet-f| 5’ATGGAATAATTAGCCGATCTGG3’ | 359pb       | 55°C                  | [29]      |
|      | Siet-r| 5’CCATTACCTTTGGCTTGTGCG3’ |            |                       |           |
| EXI  | Exi-f| 5’TAAAGCATGCAACATATATAATAGAGGAGAATATTAAAAAAGCAA3’ | 350pb      | 55°C                  | [14]      |
|      | Exi-r| 5’TCTGGATCCCTCCCTCTTGTTAAATTCGCTTCTTTTTTCAAAGTCTTC3’ | 535pb      | 55°C                  | [14]      |
|      | Exi-fn| 5’CAATTAGACCTTCCACATGCG3’ |            |                       |           |
| Luk F-I | Luf-f| 5’CCCTGTCATTGCCGCTTAACAA3’ | 572pb       | 55°C                  | [15]      |
|      | Luf-r| 5’AGGTCATGGAAAGCTCTCGA3’ |              |                       |           |
| Luk S-I | Lus-f| 5’TGAAGCAGCAGAAAAATGGGG3’ | 503pb       | 55°C                  | [15]      |
|      | Lus-r| 5’GCCCGATAAGGACTTCTTACAA3’ |            |                       |           |
| hlg γ | hlg-f| 5’AATGCCTTATAGAAAATGC3’ | 935pb        | 55°C                  | [30]      |
|      | hlg-r| 5’CCATAGACGTCAGCAACGGAT3’ |            |                       |           |
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 MgCl₂, 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 MgCl₂ 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.

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

| Infection site    | Disease      | N° Isolate + (%) | sec + (%) | 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          | 1         | 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          |
| Rектum            | 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' 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' 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.

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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         | 0         | 0         | 0          | 1         | 1             | 1             | 0            |
| Pig              | 1               | 0         | 0         | 0         | 0          | 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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