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Molecular detection of exfoliative toxin in *Staphylococcus intermedius* isolates from dogs with pyoderma*

Barış SAREYYÜPOĞLU[1], H. Kaan MÜŞTAK[1], Zafer CANTEKİN[2], K. Serdar DİKER[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, 06110, Diskapi, Ankara; [2]Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, Serinyol, Antakya, Turkey.

Summary: *Staphylococcus aureus* and *S. intermedius* are considered as the most significant species causing skin infections in dogs. The aim of this study was to develop a Polymerase Chain Reaction technique for the detection of *siet* (*S. intermedius* exfoliative toxin) gene encoding exfoliative toxin in *S. intermedius* and to investigate its presence in *S. intermedius* isolates from dogs with pyoderma. A total of 41 isolates (35 *S. intermedius*, 4 *S. aureus*, one *S. capitis* subsp. *ureolytica* and one *S. chromogenes*) from dogs with pyoderma were included in the study. Original primers specifically amplifying 145 bp of *siet* gene and 182 bp of *agr* (accessory gene regulator) gene locus of *S. intermedius* were designed in the study. *agr* gene was detected in all *S. intermedius* isolates, but not in other isolates. *siet* gene was detected in all *S. intermedius* isolates. *siet* Polymerase Chain Reaction assay was found to be specific since no amplifications were observed with *siet* negative *S. intermedius* and other bacterial control strains. Rapid and reliable detection of staphylococci causing skin lesions in dogs and their virulence markers like *siet* gene will provide important data for clinical practice to manage the disease more effectively by means of treatment and prevention.

Key words: Canine pyoderma, exfoliative toxin, *siet* gene, *Staphylococcus intermedius.*

Piyodermalı köpeklerden izole edilen *Staphylococcus intermedius*'larda eksfoliatif toksin varlığının moleküler olarak belirlenmesi

Özet: *Staphylococcus aureus* ve *S. intermedius*'un köpeklerde deri enfeksiyonlarına neden olan en önemli türler olduğu kabul edilmektedir. Bu çalışmada, *S. intermedius*'un eksfoliatif toksinini kodlayan *siet* (*S. intermedius* eksfoliatif toksin) genini belirlemek için bir Polimeraz Zincir Reaksiyonu (PCR) tekniği geliştirilmesi ve piyodermalı köpeklerden izole edilen *S. intermedius* izolatlarında bu genin araştırılması amaçlandı. Piyodermalı köpeklerden izole edilen toplam 41 izolat (35 *S. intermedius*, 4 *S. aureus*, bir *S. capitis* subsp. *ureolytica* ve bir *S. chromogenes*) çalışmaya dahil edildi. Bu çalışmada, *siet* geninin 145 bp'lik ve *S. intermedius*'un *agr* (accessory gene regulator) geninin 182 bp'lik bölümünü spesifik olarak çoğaltan özgün primerler tasarlandı. *agr* geni diğer stafilokok türlerinde değil sadece *S. intermedius* izolatlarında tespit edildi. Bütün *S. intermedius*'larda *siet* geni saptandı. Diğer kontrol suşlarında ve *siet* negatif *S. intermedius* suşunda amplifikasyon görülmediği için *siet* PCR yöntemi spesifik bulundu. Köpeklerde deri lezyonu oluşturan stafilokokların ve bu stafilokokların virulens özelliklerinden *siet* geninin hızlı ve güvenilir olarak belirlenmesi, tedavi ve korunma anlamında hastalıkla daha etkili mücadele için klinik olarak önemli bilgiler sunacaktır.

Anahtar sözcükler: Eksfoliatif toksin, köpek, piyoderma, *siet* geni, *Staphylococcus intermedius*

Introduction

Staphylococci are commonly isolated in routine veterinary diagnostics. Among the various coagulase-positive staphylococci which could be recovered from diseased and healthy dogs, *Staphylococcus intermedius* and *S. aureus* have been considered as the most significant species (8, 14). *S. intermedius*, first described by Hajek (12), is the most common cause of skin infections in dogs, with canine pyoderma being caused almost exclusively by *S. intermedius* (16, 37). It has also been isolated from a number of other carnivores, horses, ruminants and birds (7, 13, 18, 22, 23). In single cases, *S. intermedius* appears to be also responsible, as a zoonotic pathogen, for canine-inflicted human wound infections and invasive infections in immune compromised patients (28, 31).

Similar to *S. aureus*, *S. intermedius* produces many virulence factors such as protease, coagulase, clumping factor, enterotoxins, exfoliative toxin, leukotoxin, and hemolysins (10, 11, 12, 22, 29). It has been reported that the enterotoxin- and/or leukotoxin-producing *S. intermedius* strains are prevalent in dogs (10, 11). Terauchi et al. (29) revealed that *S. intermedius* D-52 isolated from canine pyoderma produced an exfoliative toxin (SIET). The

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molecular mass of SIET has been estimated to be 30 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); this toxin differs from *S. aureus* exfoliative toxins (i.e. ETA, ETB, and ETC) and *Staphylococcus hyicus* exfoliative toxins (SHETA and SHETB) in molecular weight and antigenicity. Furthermore, SIET causes exfoliation in 1-day-old chickens, suckling hamsters, and 3-week-old dogs, in contrast to the respective effects of ETs and SHETs (29). The *siet* gene, located on the chromosomal DNA, consists of a coding region of 990 bp specifying a polypeptide of 330 amino acid residues (30). Until recently, bioassays have been used to detect SIET, while molecular approaches have been searched to detect genes encoding exfoliative toxins (6). A Polymerase Chain Reaction (PCR) assay, for amplification of *siet* gene, has been developed by Lautz et al. (19).

In this study a PCR technique for the detection of *siet* gene encoding exfoliative toxin in *S. intermedius* was developed and used for the investigation of the presence of this gene in *S. intermedius* isolates from dogs with pyoderma.

Materials and Methods

Bacterial strains: A total of 41 *Staphylococcus* isolates (35 *S. intermedius*, 4 *S. aureus*, one *S. capitis* subsp. *ureolytica* and one *S. chromogenes*) from dogs with pyoderma, obtained in years 2005-2006 were used in this study. s*iet* positive and negative *S. intermedius* strains, and an ETA/ETB positive *S. aureus* strain were kindly provided by Prof. Christoph Laemmler (Institut fur Pharmokologie und Toxikologie, Justug-Liebig-Universitat Giessen, Germany). A *S. intermedius* strain was also provided by Dr. Karsten Becker, (Institut für Medizinische Mikrobiologie, Universität Münster). Bacterial strains from culture collection of Department of Microbiology (Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey) used as negative control in specificity tests for PCR analysis were *Escherichia coli* AVMC96-134, *Pseudomonas aeruginosa* AVMC95-61, *Pasteurella multocida* AVMC88-33, *Klebsiella pneumoniae* AVMC87-28, *Serratia marcescens* AVMC95-17, *Enterococcus faecalis* AVMC99-87, *S. aureus* AVMC96-122, *S. intermedius* AVMC97-132, *S. hyicus* AVMC97-117, *S. chromogenes* AVMC97-119, and *S. schleiferi* AVMC99-28. In addition, staphylococcal strains like *S. aureus* RSKK95-044, *S. chromogenes* RSKK95-051, *S. hyicus* RSKK95-050, *S. hominis* RSKK95-050 from national type culture collection (Refik Saydam Hıfzısıhha Merkezi, Ankara, Turkey) were also included in the study. All bacterial strains were stored at -70ºC until used.

Phenotypic characteristics of Staphylococcal isolates: Isolates were cultured on Columbia agar with 5% (vol/vol) sheep blood and incubated at 37ºC for 24 h. Suspected colonies were Gram stained and investigated by conventional biochemical tests and Microbact Staph 12S Identification System (Oxoid, MB1561).

Preparation of DNA samples: A modified phenol-chloroform extraction method was performed for DNA extraction (4). Briefly, 1 ml of bacterial culture that had been replicating overnight in Brain Heart Infusion Broth (Oxoid, CM1135) was centrifuged for 4 min at 2500×*g* in sterile microcentrifuge tubes. After washing once in TE buffer (10 mM Tris, 1mM EDTA, pH 7.8), the pellet was incubated for 1 h at 37°C in 400 µl of TE buffer containing lysostaphin on a specimen basis. At the end of that period, 375 µl of STE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA), 20 µl of sodium dodecyl sulfate (SDS) and 5 µl of 10% proteinase K (20 mg/ml) were added to the mixture and incubated for 1 h at 56°C. Phenol-chloroform extraction was then performed as described by Sambrook et al. (24). Isolated DNA was kept at -20°C until use.

Primers used in the study: The *siet* (Genbank no: AB099710) and *agr* (accessory gene regulator) gene locus (Genbank no: AY557375) sequence data were obtained from GenBank and used for designing the oligonucleotide primers specific to *siet* and *S. intermedius*. Primers specifically amplifying a 684 bp portion of *femA* gene of *S. aureus* (32) and 420 bp of 16S rDNA of staphylococci (27) were used for molecular confirmation of *S. aureus* strains or whether the bacterial strain belongs to *Staphylococcus* genus or not, respectively. All oligonucleotide primers were synthesized by Operon (Cologne, Germany) (Table 1).

Table 1. Oligonucleotide primers used in this study.
Tablo 1. Çalışmada kullanılan oligonukleotid primerler.

Gene	Primer	Oligonucleotide sequence (5'-3')	PCR products	Reference
femA	Fem1	CTTACTTACTGCTGTACCTG	684 bp	Vannufel et al. (1995)
	Fem2	ATCTCGCTTGTTATGTGC		
agr	Sint1	AAATCCCTGCTGAGTTGTTAGAAG	182 bp	This study
	Sint2	ACTATCCCGAAGATGAGAAGAATG		
siet	Siet1	AGCGTTAATAGTCCGGGTGG	145 bp	This study
	Siet2	CGGCTGGTGCTGAAATGTAG		
16S rDNA	16s1	CAG CTC GTG TCG TGA GAT GT	420 bp	Strommenger et al. (2003)
	16s2	AAT CAT TTG TCC CAC CTT CG		

PCR and sequencing of siet gene: The reaction mixture of *siet* amplification contained 1 µl of each primer (10 pmol/µl), 0.5 µl of dNTP (10 mM, Fermentas, Vilnius, Lithuania), 2.5 µl of 10×Taq DNA polymerase buffer (Fermentas, Vilnius, Lithuania) with a final concentration of 2 mM $MgCl_2$, 0.2 µl of Taq DNA polymerase (5 U/µl, Fermentas, Vilnius, Lithuania) and 15.8 µl of sterile DEPC treated water (Fermentas, Vilnius, Lithuania). Amplification conditions for *siet* and *agr* genes were: initial denaturation at 94ºC for 3 min; 30 cycles of 94ºC for 30 s, 54ºC for 30 s, 72ºC for 30 s; and final extension at 72ºC for 3 min. Beside this amplification conditions were: initial denaturation at 94ºC for 3 min; 30 cycles of 94ºC for 1 min, 52ºC for 1 min, 72ºC for 1 min; and final extension at 72ºC for 7 min assigned for *femA* (32) and 16S rDNA genes (27).

PCR cleansing and sequencing of the *siet* amplicon of a single *S. intermedius* strain was performed by REFGEN (METU Teknokent, Ankara, Turkey). The partial sequence of the strain has been submitted to the GenBank database and accession number assigned as EU090231. A sequence comparison was carried out by using the database of the National Center for Biotechnology Information available under http://www.ncbi.nlm.nih.gov/BLAST.

Results

Molecular confirmation of isolates: Following PCR tests all isolates had 420 bp long specific sequence of 16S rDNA gene conforming that they all belong to *Staphylococcus* genus. *agr* and *femA* genes were respectively found in *S. intermedius* and *S. aureus* isolates, but not detected in other isolates. It was observed that isolate 17 which was determined as *S. aureus* phenotypically had both *agr* and *femA* genes.

PCR detection of siet gene: siet gene was detected in all (100%) of the *S. intermedius* isolates and isolate 17 designated as *S. aureus*; *S. chromogenes* and *S. capitis* subsp. *ureolytica* isolates and all 3 *S. aureus* isolates were *siet* negative. No amplification was observed with *siet* specific primers in PCR tests with DNAs obtained from *E. coli*, *P. aeruginosa*, *P. multocida*, *K. pneumoniae*, *S. marcescens*, *E. faecalis*, *S. aureus*, *S. intermedius*, *S. hyicus*, *S. chromogenes*, and *S.schleiferi*.

Sequencing of partial sequence of siet gene: Following sequencing of the *siet* amplicon of a single *S. intermedius* isolate, a partial sequence of *siet* gene of 145 bp long was obtained (Genbank no: EU090231). This sequence gave a 100% sequence similarity with the *siet* gene sequence in BLAST search. This sequence was translated into amino acid sequence and compared to the amino acid sequence of SIET. The amino acid sequence of the *S. intermedius* isolated was found to be 100% identical to that of SIET sequence.

Discussion and Conclusion

We aimed to develop a PCR technique for the detection of *siet* gene encoding exfoliative toxin in *S. intermedius* and used it for the investigation of the presence of this gene in *S. intermedius* isolates from dogs with pyoderma in this study.

Staphylococcal pyoderma is the most common skin disease in dogs. Canine pyoderma is caused almost exclusively by *S. intermedius* (12, 20). However, the increasing prevalence of *S. aureus* infections and the emergence of a new species like *S. schleiferi* force the veterinary community to become more vigilant to prevent zoonosis (33, 35). Other *Staphylococcus* species isolated from canine pyodermas and dermatitis were *S. hyicus*, *S. epidermidis*, *S. xylosus*, *S. simulans*, *S. hominis* (20, 21), and *S. chromogenes*, *S. sciuri*, *S. saprophyticus*, *S. capitis* (15), respectively. *S. intermedius* (85.4%) and *S. aureus* (9.8%) isolates from the dogs with pyoderma constituted the main material of this study.

Exfoliative toxins or epidermolysins are exotoxins produced by staphylococci causing skin lesions in humans and animals. ETA, ETB or both produced by *S. aureus* strains cause the staphylococcal scaled skin syndrome, characterized by the splitting of the epidermis and exfoliation (21). It was also shown that *S. aureus* produces a third exfoliative toxin ETC, which was isolated from a strain obtained from a horse phlegmon (25). Virulent *S. hyicus*, as causative agent of exudative epidermitis in pigs, produce exfoliative toxins, which differ in their amino acid sequence and were designated as SHETA and SHETB in Japan (26) and as ExhA, ExhB, ExhC and ExhD in Denmark (1). More recently, a fourth exfoliative toxin from *S. aureus*, designated ETD, and the exfoliative toxin SIET from *S. intermedius* were described by Yamaguchi et al. (34) and Terauchi et al. (29), respectively. Since *S. intermedius* has no toxin types like other skin-pathogenic staphylococci, we targeted the *siet* gene in particular.

In the present study, we developed a PCR assay using newly designed primers which specifically amplifies a 145 bp sequence of the *siet* gene encoding exfoliative toxin of *S. intermedius* isolates. In recent years, with its increasing advantages, PCR has been used widely in the field of both medical sciences and veterinary medicine for molecular identification of infectious agents and detection of their virulence markers. There are previous studies on investigation of exfoliative toxin genes by molecular methods such as PCR (17, 19) and multiplex PCR (2, 3). Lautz et al. (19) developed a PCR technique which specifically amplifies a 359 bp portion of the *siet* gene. They detected that only 62% of the *S. intermedius* from skin and wound infections were *siet* positive. Using this PCR assay we found that all *S. intermedius* isolates from dogs with pyoderma harbored the *siet* gene. Additionally, *siet* PCR

performed in our study was 100% specific since no amplifications were observed with negative control *S. intermedius* strains and other bacteria examined. Furthermore, we also confirmed our results with PCR assays performed with the primers designed by Lautz et al. (19). SIET, the exfoliative toxin from *S. intermedius* was described by Yamaguchi et al. (34), and Terauchi et al. (29). The nucleotide sequence of the SIET encoding gene consists of a coding region of 990 bp (30). Our partial sequence showed 100% homology with this gene.

In this study, we designed primers those specifically amplify the *agr* gene of *S. intermedius* and used them for molecular confirmation of *S. intermedius* isolates. These primers have specifically discriminated *S. intermedius* strains from other staphylococci. Furthermore, we tested our isolates by singleplex PCR tests with primers amplifying *femA* for molecular confirmation of *S. aureus* isolates, and also with primers amplifying a specific sequence of 16S rDNA gene in order to confirm the isolates whether they belong to *Staphylococcus* genus. Baron et al. (5) developed a multiplex PCR that could differentiate between *S. aureus* and *S. intermedius* that targets species-specific sequences in the *nuc* gene, which encodes thermonuclease in the two species. Lautz et al. (19) used this technique to discriminate their isolates. In a previous study of Ardic et al. (4), a multiplex PCR technique co-amplifying 16S rDNA portion of staphylococci and *femA* gene of *S. aureus* was developed. Interestingly, we detected both *femA* gene specific for *S. aureus* and *agr* gene specific for *S. intermedius* in one isolate, isolate 17. Although this isolate was identified as *S. aureus* with Microbact Staph 12S System, we believe the culture of the isolate could be contaminated with the cells of the related bacteria originated from the swab sample, or there could be a cross-contamination of DNA in either of the relevant PCR tests.

We believe that it could be a valuable approach to design specific primers which could be used to develop multiplex PCR techniques for the molecular detection and discrimination of important pathogens isolated from pet and companion animals. Recent studies showed that staphylococcal isolates from skin infections of dogs phenotypically identified as *S. intermedius* could indeed be *Staphylococcus pseudintermedius* (9, 36, 37). According to this new finding further investigations have to be done for the discrimination and confirmation of the two species. Molecular discrimination of *S. intermedius*, *S. pseudintermedius*, and *S. delphini* isolated from canine pyoderma with multiplex PCR could be a good start point.

As a conclusion, rapid and reliable detection of staphylococci causing skin lesions in dogs and their virulence markers like *siet* gene will provide important data for clinical practice to manage the disease more effectively by means of treatment and prevention.

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Address for correspondence:
Barış Sareyyüpoğlu
Department of Microbiology,
Faculty of Veterinary Medicine, Ankara University,
06110, Diskapi, Ankara, Turkey.
e-mail: sareyyupoglu@yahoo.com