



## SEQUENCE ANALYSIS OF *siet* gene IN *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM CANINE PYODERMA

Prashantha M. K.; Shambulingappa B E\*; Sundareshan S;  
Kotresh A. M.; Rudresh B H; Madhavaprasad C. B. and Arun S. J.

Veterinary College Shimoga (KVAFSU, Bidar), Karnataka

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### ABSTRACT

A study was conducted on isolation, identification and characterization of staphylococcal organisms associated with the canine pyoderma. The bacteriological processing of the samples resulted in the recovery of 95 staphylococcal isolates and 18 other bacterial isolates. On culture, staphylococci were the most predominantly (n=95, 75.39%) isolated organisms. Based on *nuc* gene-based PCR, out of 95 staphylococcal isolates obtained, 82 (86.1%) of the isolates were found belonging to *S. pseudintermedius*. And out of 82 *S. pseudintermedius* isolates, *siet* gene was detected in 69 (86.1%) isolates. *S. pseudintermedius* was found to be predominant bacterial pathogen responsible for pyoderma in dogs.

Two PCR products which were amplified for *nuc* gene and one each of *siet* and *mec A* gene were sent for commercial sequencing and the sequence alignment revealed 99.23 per cent, 99.14 per cent, 98.98 per cent and 100 per cent, similarities with the corresponding genes of *S. pseudintermedius* respectively.

**Keywords:** *Staphylococcus pseudintermedius*, canine pyoderma, *siet* gene, sequence analysis, virulence gene.

### INTRODUCTION

Skin disease in dogs is one of the most common problems encountered in small animal practice and accounts for 20 per cent of the total cases (Sischo *et al.*, 1989). Among the skin problems of different etiologies, bacterial skin infections are most common in small animal veterinary practice (Hiller, 2009). Most of the pyoderma cases in dogs are associated with *Staphylococcus* species, especially *S. intermedius* is one of the major causative agents of canine bacterial skin infections, such as otitis externa, pyoderma and abscesses (Kloos and Bannerman, 1994). Guardabassi *et al.* (2004) reported that being the normal inhabitant bacteria of skin coat, *Staphylococcus* alone or along with other bacteria frequently invade the debrided skin causing pyoderma;

*Staphylococcus intermedius* is an opportunistic bacterial pathogen causing various diseases in dogs. Staphylococcal strains designated up to 2005 as *S. intermedius* species are currently assembled into the so-called *S. intermedius* group (SIG), consisting of *S. intermedius*, *S. pseudintermedius* and *S. delphini* (Sasaki *et al.*, 2007). It is the *S. pseudintermedius*, and not *S. intermedius*, is the species of the *S. intermedius* group (SIG) that colonizes and causes infections in dogs and cats (Perreten *et al.*, 2010). The novel species *S. pseudintermedius* is the most significant of the SIG from a clinical point of view. Being an important canine opportunistic pathogen often isolated from dermatitis, otitis and other secondary infections (Sasaki *et al.*, 2007), the SIG particularly *S. pseudintermedius*, has been implicated as a common cause of pyoderma in dogs (Becker *et al.*, 2005).

\*Corresponding author: [shambu71@gmail.com](mailto:shambu71@gmail.com)

It is difficult to differentiate *S. intermedius* from *S. pseudintermedius* during routine diagnostic procedures, but the vast majority of canine isolates are *S. pseudintermedius*. It has therefore been proposed to report all strains belonging to the SIG from dogs as *S. pseudintermedius*; unless genomic investigations prove that the strain belongs to other related species of SIG (Devriese *et al.*, 2009). The most common cause of pyoderma in dogs is the coagulase-positive *S. pseudintermedius* (previously misidentified as *Staphylococcus intermedius*) (Jones *et al.*, 2007). *Staphylococcus pseudintermedius* is one of the most common pathogens isolated from skin and post-operative infections in dogs and cats and can also occasionally cause infections in humans (Stegmann *et al.*, 2010). Hence the present study was undertaken with an objective to isolate *Staphylococcus* species from canine pyoderma in Shivamogga region of Karnataka and an attempt was made to study the sequencing of the *Staphylococcus pseudintermedius* by targeting *siet* gene responsible for its virulence.

#### **MATERIAL AND METHODS:**

The study was conducted in the Department of Veterinary Microbiology, Veterinary College, Shivamogga, a constituent institute under Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka State.

#### **Isolation of bacterial agents from samples collected from canine pyoderma cases:**

Clinical cases of canine pyoderma presented to teaching veterinary clinical complex Shivamogga were used for collecting samples in this study. The veterinarians who were practicing in the study area (in and around Shivamogga) who volunteered to send from their respective district polyclinic veterinary dispensaries/Veterinary hospitals were also used in the study. All the cases of pyoderma such as papules, pustules, erythema, alopecia, pruritus and epidermal collarettes were selected as subjects for bacterial culture and antimicrobial sensitivity assay.

#### **Culturing of samples for Isolation of bacteria:**

The clinical material collected using sterile swabs from the lesions was initially inoculated in BHI broth and incubated for 12-24 hrs at 37°C, Primary identification of bacterial agents were carried out based on colony morphology, and Gram's staining. A

loopful of the inoculum was then streaked on to pre-prepared mannitol salt agar and MacConkey agar petri-plates and incubated for 24hrs at 37°C and examined as per the standard procedure described by Cruickshank *et al.* (1975).

#### **Identification of bacterial agents by phenotypic methods**

##### **Grams staining and cultural characters**

Gram's staining kit, which contained Crystal violet, Gram's iodine, Decolourizer and Safranin was procured from M/s Hi-Media, Mumbai. Staining of all the culture isolates was carried out as per the instructions mentioned in the kit and differentiated as gram positive and gram negative and documented.

On the agar plates colony morphology and lactose fermenter/non fermenters were recorded. Grams staining of the pure colonies so obtained on the plates were carried out and the primary identification of bacterial isolates were done based on colony morphology. Then the selective plating of the isolates was carried out for further confirmation. Then relevant biochemical tests were carried out for gram positive and gram-negative isolates so obtained as per the standard procedure (Collee *et al.*, 1989) as follows.

##### **Biochemical tests**

The recovered isolates confirmed by Gram's staining were further subjected to biochemical tests such as coagulase and catalase tests to confirm coagulase positive staphylococci and the aerobic bacterial isolates and other isolates were confirmed by conventional biochemical tests like oxidase and IMVIC tests.

##### **Detection of virulence gene (*siet* gene) using PCR Procedure**

The isolates were subjected to PCR for detection of *siet* gene responsible for its virulence.

The PCR was carried out using published primers and the procedure described by Ananda Chitraet *et al.* (2018) as shown in below table-1. The reaction mixture of 25 µl each was prepared in 0.2 ml thin-walled PCR tubes placed in mini cooler as shown below (Table-2).

**Table 1 Oligonucleotide sequences of *S. pseudintermedius* *siet* gene primers.**

Name of the primer	Primer sequence 5'–3'	Product size (bp)
Staph <i>siet</i> - F	TGCGGGTCCTCA ATCTTTAAC	465
Staph <i>siet</i> - R	CTTTCAACTCTGCACGCAATC	

**Table 2: Details of the contents of PCR mixture for *siet* gene-based PCR**

Reagents (Concentration)	Volume
Master mix (Ampliqon Taq DNA Polymerase Master Mix RED,2x)	12.5 µL
Staph <i>siet</i> -F (12.5 µL/ml)	1 µL
Staph <i>siet</i> -R (12.5 µL/ml)	1 µL
Template (Staphylococcal DNA)	3 µL
Nuclease Free Water	7.5 µL
TOTAL	25 µL

After mixing the contents, tubes were centrifuged to collect the contents in the bottom. The amplifications were performed in a thermal cycler (Biorad T 300) and the thermal conditions were set as detailed below (AnandaChitra et al., 2018).

- Initial denaturation at 94° C for 3 min
- 30 cycles of denaturation at 94° C for 30 sec, annealing at 60° C for 30 sec, and extension at 72° C for 30 sec
- Final extension at 72° C for 5 min
- Hold at 4° C

After completion of PCR reaction, 3µl of the amplified product was loaded onto a pre prepared 1.5-2 per cent agarose gel (prepared in 1x TAE buffer) containing ethidium bromide at the concentration of 1 µl/10ml. marker (DNA ladder), positive control, negative control and no template control was also loaded onto one well each, and gel was made to run in a gel electrophoresis unit using a 1x TAE buffer. Later gel was read under gel documentation unit and the images were captured using gel documentation system; Gel Doc XR (Bio-Rad., U.S.A).

#### Reference strain

One of the PCR positive samples which were commercially sequenced (Eurofins Genomics India Pvt Ltd.) and the sequencing results obtained were edited using Mega version X software and then

compared with sequences deposited in NCBI. These samples which showed 100% similarity with the corresponding partial gene sequence of *mecA* of *S. pseudintermedius* were used as positive reference strains in the study.

#### Analysis of nucleotide sequence and submission to GenBank

The nucleotide sequence data obtained were edited by Bio-edit/ Mega version X software before they were submitted to GenBank. The sequence data was further analyzed by BLAST and Clustal method with Weighted residue weight table software program. MegAlign of DNA STAR was used for further analysis of nucleotide sequences. The edited partial nucleotide sequences of *nuc*, *siet* and *mecA* genes of *S. pseudintermedius* isolates were submitted to BankIt-Gen bank of NCBI (<http://www.ncbi.nlm.nih.gov>) and accession numbers were obtained.

### RESULTS AND DISCUSSION

In the present study, staphylococci were confirmed by biochemical tests and all the staphylococcal isolates were catalase and coagulase positive. In this study the total number of staphylococcal isolates obtained was 95 (75.5%) out of 126 samples collected and processed.

#### Molecular Identification and sequencing of *S. pseudintermedius*

Ninety five staphylococcal isolates confirmed phenotypically were further subjected for the PCR

targeting *nuc* gene for the identification of *S. pseudintermedius*.

#### **Detection *siet* gene in *Staphylococcus pseudintermedius***

PCR targeting *siet* gene was carried out for the *S. pseudintermedius* isolates (n=82) which were confirmed up to species level by PCR. Of these, 69 isolates were found to be positive for the presence of *siet* gene which yielded amplicons of the size corresponding to 465 bp in the DNA ladder (Plate.1 Table-3). One of the PCR products was sent for the commercial sequencing (Eurofins Genomics India Pvt Ltd). The chromatogram and the nucleotide sequence obtained by sequencing were analyzed and edited using Mega version software. Further the edited nucleotide sequences were compared with deposited sequences of NCBI using BLAST tool and the sequence showed 100% sequence similarity with corresponding partial *siet* gene sequence of *S. pseudintermedius* (Fig.1).

The results are supported by the work done by Bannoehr and Guardabassi (2012) who reported that *S. pseudintermedius* was the most prevalent coagulase-positive staphylococci inhabitant of the skin and mucosa of dogs and cats. It was also the major bacterial pathogen isolated from canine infections. Similar work done by Ruzauskaset *al.* (2016) reported in a study that 192 samples (76.8%) of the 250 samples collected from dogs tested positive for *Staphylococcus* species. The percentage was higher in non-treated animals (89.5%).

#### **Detection of exfoliative toxin (*siet*) gene**

*S. pseudintermedius* produces enzymes such as coagulase, protease, thermonuclease and toxins, including haemolysins, exfoliative toxins and enterotoxins (Ross Fitzgerald *et al.*, 2009; Zakouret *al.*, 2011). Exfoliative toxin is a virulence factor involved in canine pyoderma, because the exfoliative toxin gene can mainly be found among *S. (pseud)intermedius* isolated from skin infections. Dogs injected with purified exfoliative toxin develop clinical signs such as erythema, exfoliation and crusting, which are signs of canine pyoderma (Lautz *et al.*, 2006; Iyori *et al.*, 2010).

In this present study out of 82 samples tested, *siet* gene was detected in 69 isolates and the percentage was

84.14 per cent and the amplicon was confirmed as *siet* gene (partial) in *Staphylococcus pseudintermedius* by sequencing and analysis by BLAST tool.

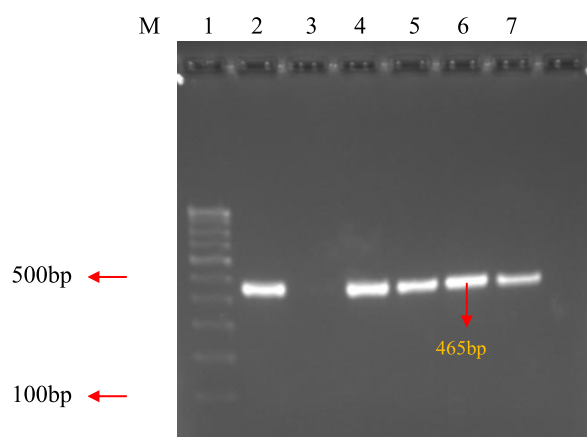
Lautz *et al.* (2006) developed a PCR technique which specifically amplifies a 359 bp portion of the *siet* gene. They detected that only 62 per cent of the *S. intermedius* from skin and wound infections were *siet* positive.

Sareyyupoglu *et al.* (2013) reported a similar study in which *siet* gene was detected by PCR in all (100%) of the *S. intermedius* isolates. Following sequencing of the *siet* amplicon of a single *S. intermedius* isolate, the sequence gave a 100 per cent 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 per cent identical to that of SIET sequence. Recently Ruzauskaset *al.* (2016) reported in a study that the *siet* gene encoding exfoliative toxin was detected in 69 per cent of the *S. pseudintermedius* isolates.

Results are supported by the studies on canine *S. pseudintermedius* isolates by Yoon *et al.* (2010) in Korea, Garbaczet *al.* (2013) in Poland and Elhaniet *al.* (2015) Tunisia. Dogs injected with purified SIET develop clinical signs such as erythema, exfoliation and crusting, which are signs of canine pyoderma (Terauchi *et al.*, 2003). Futagawa *et al.* (2009) identified a novel exfoliative toxin in *S. pseudintermedius* isolates from canine pyoderma, and presented its homology with other recognized staphylococcal exfoliative toxins, exfoliative toxicity in neonatal mice, and prevalence in canine pyoderma. *S. aureus* exfoliative toxins are extremely specific serine proteases and function as 'molecular scissors' during skin infection and cleave desmosomal cadherins only in the superficial layers of the skin, which is directly responsible for the clinical manifestation of staphylococcal scalded skin syndrome in human. Recent reports demonstrated that 3 to 4 per cent of methicillin sensitive *S. aureus* (MSSA) strains carry the *eta* (exfoliative toxin A) or *etb* gene (exfoliative toxin B) (Megevand *et al.*, 2010; Sila *et al.*, 2009). whereas around 10 % of methicillin resistant *S. aureus* (MRSA) were *eta* positive (Sila *et al.*, 2009).

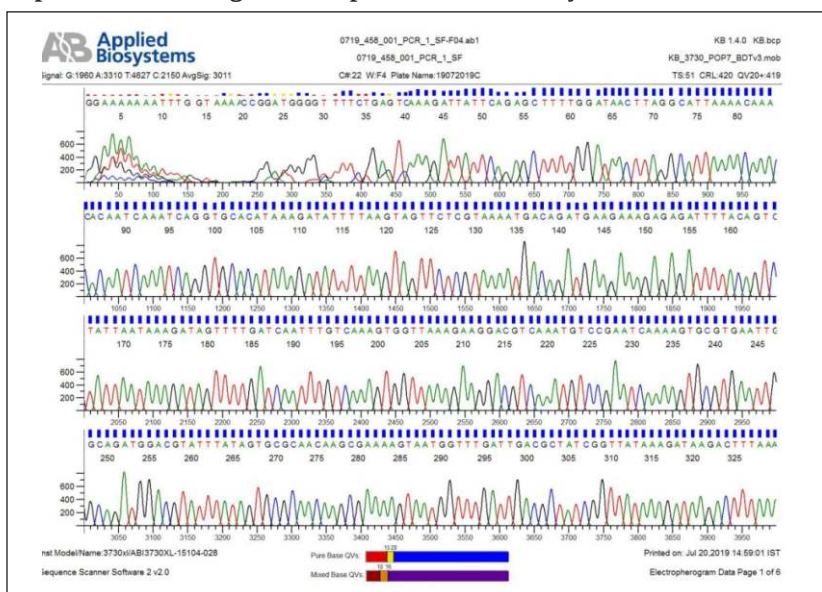
**Table 3: PCR based detection of *nuc*, *siet*, and *mecA* genes in staphylococcal isolates.**

SI. No.	No. of isolates subjected for PCR	Gene targeted	No. of isolates positive by PCR (percentage)
1	95	<i>nuc</i> gene	82 (86.31 %)
2	82	<i>siet</i> gene	69 (84.14 %)
3	82	<i>mecA</i> gene	03 (3.65 %)



Lane M : 100 bp DNA ladder  
 Lane 1 : Positive control (*S. pseudintermedius* isolate)  
 Lane 2 : Negative control (*S. aureus* DNA)  
 Lanes 3-6 : Test samples showing partially amplified product (465bp) of *siet* gene of the *S. pseudintermedius* isolates  
 Lane 7 : No template control

**Plate 1: Partial amplification of *siet* gene of *S. pseudintermedius* by PCR**



**Fig. 1: Chromatogram of *siet* gene obtained by sequencing with forward primer.**

## SUMMARY

The findings of the present study is summarized as follows.

- *Nuc*gene-based PCR detection can be used a reliable diagnostic tool for laboratory diagnosis of *S. pseudintermedius* infections and in this study, it detected 86.3% of the staphylococcal isolates as *S. pseudintermedius*.
- *Siet* gene encoding exfoliative toxin an important virulence factor associated with skin affections was detected in 72.6 per cent of the *S. pseudintermedius* isolates.

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