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Characterization of *Staphylococcus pseudintermedius* Isolates from Skin Infections of Dogs

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ABSTRACT

Background: *Staphylococcus pseudintermedius* is a part of the canine skin microflora and an opportunistic pathogen. It plays a central role in canine pyoderma, otitis and surgical wound infections. These conditions correlate with virulence genes distributed in the bacterial genome. These genes determine strain variability on typing, in turn aiding epidemiological surveillance. The aim of this study was to isolate, identify and characterize *Staphylococcus pseudintermedius* (SP) and Methicillin resistant *Staphylococcus pseudintermedius* (MRSP) from dogs with skin infections in Chennai, India.

Methods: SP and MRSP positive isolates were identified by multiplex PCR for *nuc* and *mecA* genes respectively. Characterization of the isolates for virulence genes responsible for biofilm formation (*icaA, icaD*), cell wall adherence (*SpsO, SpsK, SpsP, SpsQ, SpsF*), toxins (*ExpA, ExpB, SIET, Sel, Se-int, LukS, LukF*) and gene regulation (*Agr, SarA*) was performed.

Result: Out of 275 samples, 120 SP and 8 MRSP positive isolates were identified. Only one isolate could be typed as SCC*mec* Type V whereas other MRSP isolates were non typeable. Agr typing of MRSP isolates revealed type II in 7 isolates and type III in one isolate. Our study revealed that there was no significant difference in the detection of virulence genes between MSSP and MRSP.

Key words: MRSP, SCC*mec* typing, *Staphylococcus pseudintermedius*, Virulence genes.

INTRODUCTION

Staphylococcus pseudintermedius (SP) is a commensal bacterium of dogs and cats skin and mucous membranes. It is usually present in pharynx, rectum, nares, conjunctiva, perineum, perioral and axilla region. SP is responsible for pyoderma, otitis externa and post-operative infections in dogs. *S. pseudintermedius* can also colonize the nasal cavity of pet owners, veterinarians and animal handlers (Bannoehr and Guardabassi, 2012) and it is generally harmless in healthy individuals. However, individuals with compromised immune systems (*e.g.* HIV/AIDS, transplant and cancer patients) are more susceptible to SP infection and there have been reports of dermatitis and septicemia in human patients (Somayaji *et al*., 2016). Consequently *S. pseudintermedius* and especially methicillin resistant (MRSP) strains, are of public concern as potentially emerging zoonotic bacteria (Paul *et al*., 2011). Contamination with *S. pseudintermedius* in the environment in animal hospitals and households has been reported (Laarhoven *et al*., 2011; van Duijkeren *et al*., 2011) enabling re-infection and transfer of antimicrobial resistance genes (Frank *et al*., 2009).

The pathogenic potential of the organism can be explored through study of virulence factors such as cell adhering factors, toxins and enzymes. Particular genomic regions like the *OriC* in SP, controls virulence genes for adherence like *Sps*K, *Sps*P, *Sps*Q, *Sps*L, *Sps*M, *Sps*G and *Sps*J genes. After adherence, epithelial colonization can be attributed to biofilm formation coded by the *ica* operon (Gerke *et al*., 1998). Following colonization, bacterial cell survival is dependent on toxin production for defense. Hence, this study was aimed to isolate, identify and determine the distribution and frequencies of various virulence factors in SP and MRSP isolates from skin infections of dogs in Chennai, India.

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MATERIALS AND METHODS

Isolation and Identification

Skin swabs from pyoderma, otitis, allergy, demodicosis, dermatitis and other cases (hypothyroidism *etc*.) were collected during September 2017 to February 2018 from dogs presented to Dermatology ward of Teaching Veterinary Clinical Complex of Madras Veterinary College, Chennai, India. SP and MRSP isolation and identification were carried out as described previously (Ananda Chitra *et al.,* 2016).

Virulence genes detection

Molecular characterization for the presence of different virulence genes was performed by conventional PCR and

primer details along with reference for these genes are given in Table 1. PCR was performed in a reaction volume of 10 µl containing approximately 100-150 ng of genomic DNA, 5 pmol of each primer in 2X master mix (Ampliqon, Denmark). Cycling conditions were 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at appropriate temperature for 30 s, extension at 72°C for 30/45 s and a final extension cycle of 5 min at 72°C. PCR products were loaded on a 2% agarose gel for electrophoresis, visualized with ethidium bromide and documented.

Typing of MRSP isolates

The SCC*mec* cassette of MRSP isolates was typed using previously described multiplex PCR methods developed by Zhang *et al.* (2012) and Perreten *et al.* (2010) for type II-III and VII. *Agr* typing was carried out using published primers (Ananda Chitra *et al*., 2015) and sequencing by Sanger's sequencing technique.

RESULTS AND DISCUSSION

Isolation and Identification

A total of 275 swab samples were collected from various skin infections of dogs. Predominant samples (n=164) were collected from superficial bacterial folliculitis (pyoderma), followed by otitis (n=38), equal number of demodicosis and

Table 1: Details of the primers used for identification and characterization of SP isolates.

Gene	Primer sequences	References	Annealing Temp (^{0}C)	Product size (bp)
mecA	F-CAAACTACGGTAACATTGATCGC	Ananda Chitra *et al.,* 2015	60	210
	R-GCCTATCTCATATGCTGTTCCT			
Nuc	F-AAACACCGAGTAATACGCCG	Ananda Chitra *et al.,* 2015	60	780
	R-TTTAGCGTTCCCAAATGTTCAG			
Agr A	F-CCTATCCGGGAAATTGGCTTT	Ananda Chitra *et al.,* 2015	60	850
	R-CGGACAATGTATTCCTTGATACGA			
Agr BD	F-GGATGAGAATAATGTAATCCCTTTGAC	Ananda Chitra *et al.,* 2015	60	946
	R-AACAACCAATCACAACAGTTAGG			
SarA	F- TGGCTGTTACAAGAGTCAAGG	This study	55	279
	R – TCAGTACTGTACGTTCATCGTTT			
Se-int	F-TTGTTACGCCACCATACATACA	This study	55	357
	R-CTTTAGCAGACCATACGCTAGAC			
ExpA	F-GCGCGTCCTTCTGATCCAGAACT	Walther *et al*., 2012	55	394
	R-AACGTCCCCCTTTACCTACGTGAAT			
ExpB	F-CGCCTGGCGTATATGCTAAA	This study	55	473
	R-GCCGCTTTGCCATCTTTATTAG			
SIET	F-TGCGGGTCCTCAATCTTTAAC	Ananda Chitra *et al.,* 2016	60	465
	R-CTTTCAACTCTGCACGCAATC			
LukF	F-TTGGAAGTTACCGCCAACA	Ananda Chitra *et al.,* 2016	55	300
	R-AGCAGAAAATGGGGCGTT			
LukS	F-CCTGTCTATGCCGCTAATCAA	Futagawa-Saito *et al.,* 2004	55	572
	R- AGGTCATGGAAGCTATCTCGA			
Sel	F –TGCCTGAGGGAACAGATAGA	This study	55	344
	R – GTCGCTCATACGGTGTTCTT			
icaA	F CTTCCGACCATACTGGCATATT	This study	55	499
	R GGATAGTCGGCACACTGTTT			
icaD	F- ACCATCGTTAATGCCTTCTTTC	This study	55	167
	R- GCGCACATTCGGTGTTATT			
SpsQ	F-AACCTGCGCCAAGTTTCGATGAAG	Moodley *et al.,* 2009	55	820
	R-CGTGGTTTGCTTTAGCTTCTTGGC			
SpsF	F-AGTGGAAGCAACAGTTGAACGC	Bannoehr *et al.,* 2011	55	508
	R- TGGACCTACTTGGCTACCACCA			
SpsO	F-GGTAGTGTATCAGTGCTAATAGGAGCC	Bannoehr *et al.,* 2011	55	604
	R-TTGACAAATCAGTAGCTGATGCATC			
SpsK	F-ATTTCACAAGGGAACGCACATG	Bannoehr *et al.,* 2011	55	500
	R-TGAGCCGCACGTCTATTCTGAA			
SpsP	F-CAGGAGGACTAGGGTAATGTTCC	Bannoehr *et al*., 2011	55	277
	R-GCAAAACTTGGCGTGTTTACAAG			

dermatitis cases (n=23 each). Out of 275 samples, 128 (46.5%) samples were found to be positive for SP by amplifying 780 bp amplicon of SP specific thermonuclease gene in PCR as shown in Fig 1. The prevalence of SP infection in various skin infections is given in Fig 2. SP has also been identified at varying rates with 26.6% incidence in Lithuanian dogs (Ruzauskas *et al*., 2016), 59% Chennai, India (Ananda Chitra *et al*., 2016) and 59.6% in Brazil (Scherer *et al.,* 2018).

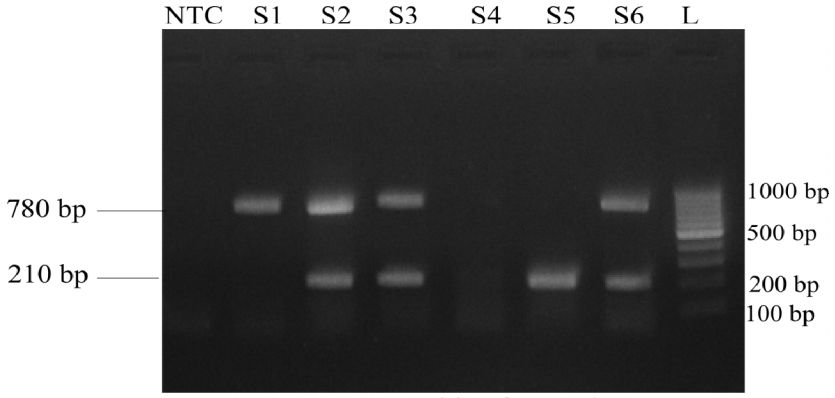
Among the 128 SP, eight isolates (6.25%) were identified as MRSP by detecting *mecA* gene (Fig 1). Six out of eight isolates were from pyoderma cases and two were from otitis cases. Higher prevalence of MRSP infection with 48% in China (Wang *et al*., 2012), 40.5% in Canada (Beck *et al*., 2012) and 28% in Chennai, India (Ananda Chitra *et al*., 2016). However, low MRSP prevalence rates have also been recorded such as 2% in Sweden (SWEDRES-SVARM, 2016) and 13.4% in Canada (Saab *et al.,* 2018).

Detection of genes encoding cell wall associated proteins

The ability of bacteria to colonize and to cause infection is initiated by attachment to the host cells using surface proteins or cell wall anchored proteins. *Sps*K gene was present in all the SP isolates in this study and the same was observed in various studies (Bannoehr *et al*., 2011; Phumthanakorn *et al*., 2017). Prevalence of various virulence genes in MSSP and MRSP isolates are given in Fig 3 and 4 respectively.

*Sps*F gene was detected in 38/128 (29.68%) isolates which varied from 17.7% (Phumthanakorn *et al*., 2017) to 71.4% (Latronica *et al*., 2014) worldwide. *Sps*O was the one which was present in the least number of isolates (11/128=8.6%) in this study. It has been reported that the *Sps*O gene was detected in 28.6% of isolates by Latronica *et al*. (2014) and 40% by Phumthanakorn *et al*. (2017).

SP organism has two orthologues (*Sps*P and *Sps*Q) of staphylococcal protein A. *Sps*Q was more frequently present in 47/128 (36.71%) isolates than *Sps*P gene (36/128=28.12%). Both the orthologues (*Sps*P and *Sps*Q) were identified in 23 strains of SP and 69 isolates were negative for both the genes. In general, incidence of detection of cell wall protein genes was in a greater number of MRSP isolates than MSSP isolates. Phumthanakorn *et al*. (2017) and Latronica *et al*. (2014) reported the concomitant presence of *Sps*P and *Sps*Q genes in SP isolates whereas, Bannoehr



Lane S1,S2,S3,S6 : positive for 780 bp *nuc* gene, S2, S3, S4, S6 : positive for 210 bp *mecA* gene, L is a 100 bp ladder, NTC is Negative control.

Fig 1: Agarose gel electrophoresis showing the results of PCR amplified products of *nuc* and *mecA* genes of SP isolates.

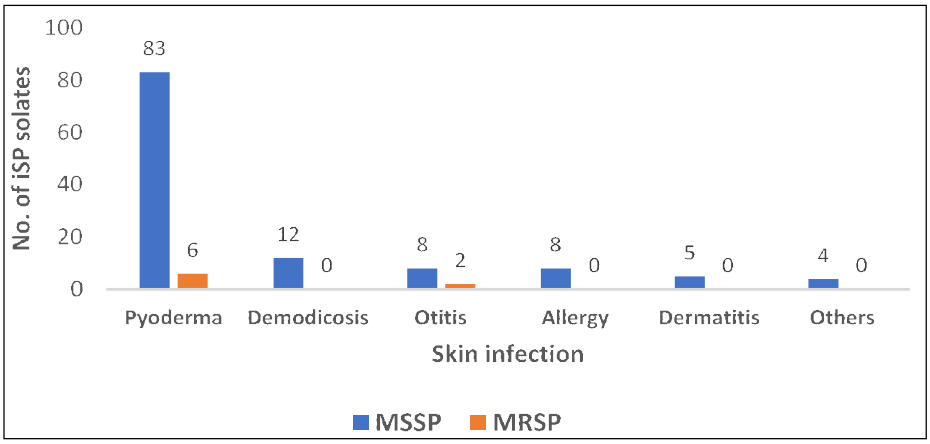


Fig 2: Case wise prevalence of MSSP and MRSP isolates

et al. (2011) reported the differential presence of genes with more prevalence of *Sps*Q genes (60%) than *Sps*P (40%) as seen in the present study.

Detection of Biofilm forming genes

Staphylococci species have *ica* operon which contains *icaADBC* genes for biofilm formation and regulatory function. The *icaA* gene was present in 69/128 isolate (53.9%) and *icaD* gene was present in 86/128 (67%) SP isolates. Higher prevalence of *icaD* than *icaA* gene observed in the present study is contrary to the presence of 75.7% *icaD* and 77.9% *icaA* genes in SP isolates of Canada and USA reported by Singh *et al.* (2013). However, transcriptome analysis of SP isolates showed that MSSP isolates had an increased ability to form biofilm under acidic circumstances through up-regulation of the entire *arc* operon (Couto *et al*., 2016).

Detection of virulence regulatory genes

All staphylococcal species examined to date have been shown to encode AIP (Auto-inducible protein) peptides that are unique to each species based on *AgrD* gene. Accessory gene regulator genes were detected in all SP isolates in this study. *Agr* typing of 5 MSSP isolates were previously reported in India and type I and III AIP were produced by two strains of each and one isolate produced type II AIP (Ananda Chitra *et al*., 2015). Type IV *agr* AIP was the majority type found in MRSP isolates of USA (Black *et al*., 2009) whilst type III was predominantly seen in MRSP than MSSP isolates in Portugal (Couto *et al*., 2016). The present study identified Agr II in seven MRSP isolates and Agr III type in one MRSP isolate.

SarA is a DNA-binding protein, which binds to the *agr* promoter region affecting control of several virulence genes in *S. aureus*. *SarA* like gene is also present in SP and it was detected in 5/8 (62.5%) and 43/120 (35.8%) of MRSP and MSSP strains respectively. To the best of our knowledge, there is no available literature either to support or contradict this report. However, Couto *et al*. (2016) reported that higher expression of transcription of regulatory genes in MRSP isolate than MSSP isolate and, in MSSP isolates *agrD* regulatory genes had higher transcriptional expression.

Detection of exfoliative toxin genes

Exfoliative toxins (ET) - serine proteases, produced by staphylococci are involved in cutaneous infections of mammals. All the isolates of this study were detected to have *SIET* gene which concurs with previous studies (Ananda Chitra *et al*., 2016; Couto *et al*., 2016; Melter *et al*., 2017). In the present study, *ExpA* isoform of ET was detected in more number (14.84%) of isolates than *ExpB* isoforms (6.25%) as opposed to 4.7% *ExpA* and 9.52% *ExpB* in another study (Walther *et al*., 2012). The occurrence of *ExpB* was found to be 23.2% of isolates from dogs with superficial pyoderma and 6.1% of SP isolates from healthy dogs while *ExpA* gene was detected in 23.3% of SP isolates from canine pyoderma (Iyori *et al*., 2010).

Detection of Panton-Valentine Leucocidin (PVL) Like Toxin (Luk-I) in SP isolates

Panton-valentine Leucocidin (PVL) found in certain strains of *S. aureus* is a bi component-LukS-PV and LukF-PV, pore forming leukotoxin that causes leukocyte damage and tissue

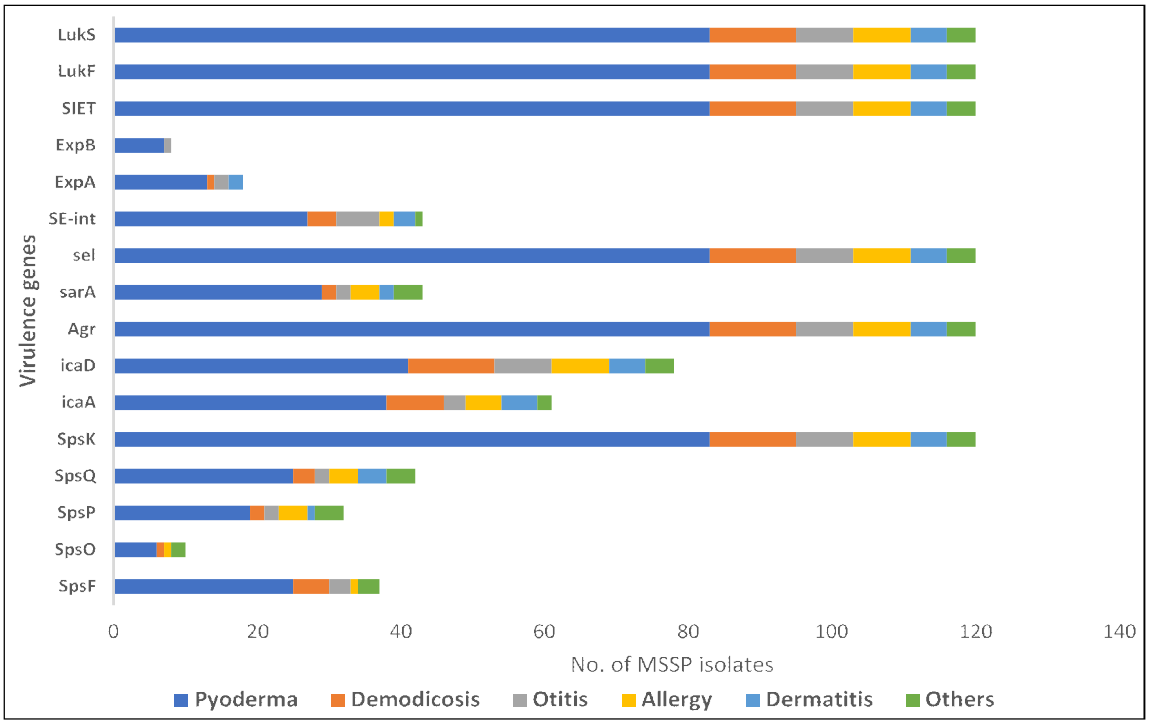


Fig 3: Prevalence of virulence genes in MSSP isolates.

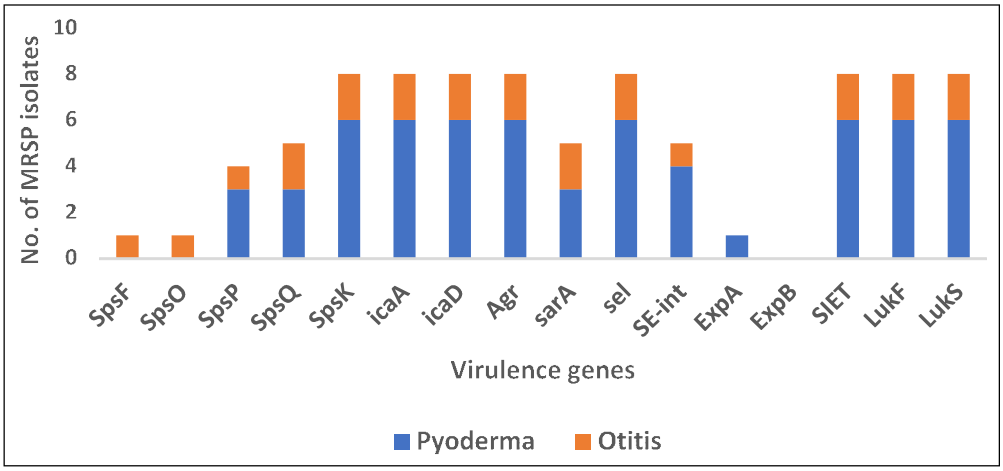


Fig 4: Prevalence of virulence genes in MRSP isolates.

necrosis (Gillet *et al.,* 2002). A similar bi component leukotoxin Luk-I, encoded by two genes, *LukS/F*, was detected in SP. All the SP isolates characterized in the present study as well as in the previous study (Ananda Chitra *et al*., 2016) possessed *Luk-I* genes. In other studies, 96.2% and 29.4% prevalence were reported (Melter *et al*., 2017; Ruzauskas *et al*., 2016). *Luk-I* gene was highly expressed in the MRSP isolate than MSSP isolate under transcriptome analysis (Couto *et al*., 2016).

Detection of enterotoxin *se-int* and superantigen like toxin (*sel*) genes in SP isolates

Staphylococcal enterotoxins (SE) are pyrogenic proteins associated with food poisoning and toxic shock syndrome. They are considered as superantigens as they bind to class II MHC molecules on antigen presenting cells and stimulate large populations of T cells releasing a cytokine bolus leading to an acute toxic shock. *S. pseudintermedius* produces two unique SEs - SECcanine which is an SEC variant (Cardona *et al*., 2006) and the other one is SE-int (Futagawa-Saito *et al*., 2004). In the present study, *se-int* gene was detected in 37.5% SP isolates where as 100% *se-int* gene prevalence was reported by Couto *et al*. (2016), Melter *et al*. (2017) and Futagawa-Saito *et al*. (2004). In another study, 37.5% and 75.9% of SP isolates from pyoderma and healthy dogs were found to possess *se-int* gene respectively (Tanabe *et al*., 2013).

In the present study, superantigen like protein (*sel*) gene was detected in all the SP isolates. 73.4% of the SP isolates from skin infection of Czech Republic was identified with *sel* gene (Melter *et al*., 2017).

SCCmec typing of MRSP isolates

In the present study, out of eight MRSP strains, only one isolate was identified as having SCC*mec* Type V by using multiplex PCR. Other seven strains were not typeable by the multiplex PCR methods. A 31% of the MRSP isolates from Italy were reported to be non-typeable (Gronthal *et al*., 2017).

SCCmec V has gained prominence in the UK (Maluping *et al*., 2014), Europe and North America (Perreten *et al*., 2010). In Asia, type V is dominant in Thailand, South China and Korea (Chanchaithong *et al*., 2014; Feng *et al*., 2012) and type II–III in Japan and North China (Ishihara *et al*., 2016; Wang *et al*., 2012). Therefore, *SCCmec* dissemination in Thailand and South China display a closer genetic relationship with Korea than Japan and North China.

CONCLUSION

This study showed the prevalence of various virulence genes in SP and MRSP in Chennai, India. Our study also revealed that there was no significant difference in the detection of virulence genes between MSSP and MRSP. However, it is possible that all the studied virulence factors may work in concert with other virulence factors to worsen skin infections of dog. Further studies are required to identify prevalent clonal types in this region to aid in molecular epidemiology.

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Ethical approval

Ethical approval was not required for this study as there were no invasive procedures. However, oral consent from the owners and complete confidentiality of the results were maintained.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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