การตรวจหาและการกระจายตัวของกลุ่มยีนเอนเทอโรทอกซินและแอดฮีซินของเชื้อสแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียส ที่แยกได้จากสุนัข คน และสิ่งแวดล้อม

นางสาวนทิตา ภูมิธนากรณ์

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# DETECTION AND DISTRIBUTION OF ENTEROTOXIN AND ADHESIN GENES OF *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM DOG, HUMAN, AND ENVIRONMENT

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การศึกษาหาการกระจายตัวของกลุ่มยีนสร้างปัจจัยก่อความรุนแรงของเชื้อแบคทีเรียสแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียสที่แยกได้จากสุนัข คน และสิ่งแวดล้อม ได้แก่ เอนเทอโรทอกซินยีนและยีนที่ใช้สำหรับการเกาะติด ซึ่งอาจมีความเกี่ยวข้องกับการคงอยู่ ดำรงชีพ และการปรับตัวในคนและสิ่งแวดล้อม พบว่ามีการกระจายตัวของเอนเทอโรทอกซินยีนในเชื้อที่แยกได้จากคนจำนวน 12 ชนิดยีน จาก 17 ยีนซึ่งมากกว่าในสุนัขที่พบ 5 ชนิดและในสิ่งแวดล้อมจำนวน 3 ชนิดตามลำดับ สำหรับการตรวจหายีนที่ใช้สำหรับการเกาะติดของเชื้อสแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียส นั้นยังไม่มีวิธีที่ง่ายและสะดวก การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาชุดการตรวจกลุ่มยีนสำหรับการเกาะติดและใช้ในการตรวจหาการกระจายตัวของกลุ่มยีนนี้ โดยพบว่าตัวอย่างจากรอยโรคในสุนัขมียีน *spsP* และ *spsQ* มากกว่าเชื้อที่แยกได้จากตำแหน่งปกติในสุนัข อย่างมีนัยสำคัญทางสถิติ และยังพบการขาดหายไปในบางส่วนของยีน *spsR* จากตัวอย่างในคนเท่านั้น ซึ่งอาจเกิดขึ้นเพื่อตอบสองต่อการเกาะติดในคน เมื่อทำการทดสอบการเกาะติดของเชื้อ 5 สายพันธุ์ที่แยกได้จากคน สุนัขและสิ่งแวดล้อม ที่มีรูปแบบของยีนสำหรับการเกาะติดที่หลากหลายต่อเซลล์ผิวหนังชั้นนอกของคนและสุนัข พบว่าเชื้อที่มาจาก sequence type (ST) 45 ที่แยกได้จากสุนัข คนและสิ่งแวดล้อม แสดงความสามารถในการเกาะติดได้ดีกว่าเชื้อจาก ST อื่น โดยการเกาะติดไม่มีความสัมพันธ์กับจำนวนและชนิดของยีนสำหรับการเกาะติด การศึกษานี้ประสบความสำเร็จในการออกแบบชุดการตรวจยีนที่เกี่ยวกับการเกาะติดของเชื้อ สแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียส ด้วยวิธี multiplex PCR และค้นพบว่าแหล่งที่มาของเชื้อมีผลต่อชนิดและจำนวนของยีนที่ก่อความรุนแรง โดยเฉพาะเชื้อสแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียส ที่พบในเจ้าคน มียีนเกี่ยวข้องกับการก่อโรคสูงกว่าเชื้อที่พบในสุนัขและสิ่งแวดล้อม ซึ่งยีนเหล่านี้อาจเกิดจากการส่งผ่านจากชิ้นส่วนของยีนที่เคลื่อนที่ได้ และการปรับเปลี่ยนของยีนที่เกี่ยวข้องสำหรับการเกาะติดที่เซลล์ผิวหนังในแต่ละโฮสต์

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NATHITA PHUMTHANAKORN: DETECTION AND DISTRIBUTION OF ENTEROTOXIN AND ADHESIN GENES OF *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM DOG, HUMAN, AND ENVIRONMENT. ADVISOR: ASSOC. PROF. DR. NUVEE PRAPASARAKUL, D.V.M., Ph.D., D.T.B.V.P., CO-ADVISOR: ASST. PROF. DR. PATTRARAT CHANCHAITHONG, D.V.M., Ph.D., 109 pp.

To obtain the information of virulence factors associated with *Staphylococcus pseudintermedius* pathogenicity, host survival and adaptation, the study of staphylococcal enterotoxin (SE) genes and cell wall-associated (CWA) protein genes in *S. pseudintermedius* isolates from dogs, humans, and environment were performed. Human methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) harbored the hightes number of SE genes at 12 out of 17 genes compared to those from dogs at 5 and environmental isolates at 3 gene types. For CWA protein genes or *S. pseudintermedius* surface proteins (*sps*), there was no an easy and fast method to detect them. Therefore, this study develop the multiplex PCR assays (mPCRs) for detection of 18 *sps* genes. *spsP* and *spsQ* were more frequently detected in the canine isolates from infected sites than from carriage sites with statistically significant. The positive amplicons of *spsR* gene in three human isolates showed partial gene deletions. Adherence assays were performed to assess the ability of 5 MRSP isolates from different origins and to find the relation with CWA protein gene profiles. Three MRSP-ST45 from dog, human, and environment had the greatest ability to adhere to both canine and human corneocytes without the association to *sps* gene profiles. This study successfully developed the novel mPCR for *sps* genes detection. The association between sources and enterotoxin genes were hightlighted in the isolates from humans. The diversity of virulence genes in human isolates are assumed to transfer by mobile genetic elements and might associate with higher pathogenicity than dog, and environmental isolates. Moreover, the diversity of *sps* may be responsible for host adaptation.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ATCC | = | American Type Culture Collection |
| bp | = | Base pair |
| BAC | = | Bacterial adhesion counts |
| BLAST | = | Basic Local Alignment Search Tool |
| ° C | = | Degree Celsius |
| CDD | = | Conserved domain database |
| CWA | = | Cell wall-associated |
| DDBJ | = | DNA Data Bank of Japan |
| DNA | = | Deoxyribonucleic acid |
| ECM | = | Extracellular matrix |
| fg | = | Femtogram |
| *g* | = | Gravity (centrifuge) |
| h | = | Hour |
| LPXTG | = | Leucine-Proline-X-Threonine-Glycine |
| M | = | Molar |
| mg | = | Milligram |
| min | = | Minute |
| ml | = | Milliliter |
| $MgCl_2$ | = | Magnesium chloride |
| MLST | = | Multilocus sequence typing |
| mPCR | = | Multiplex polymerase chain reaction |

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| | | |
|---|---|---|
| MRSP | = | Methicillin-resistant *Staphylococcus pseudintermedius* |
| MSSP | = | Methicillin-susceptible *Staphylococcus pseudintermedius* |
| MUSCLE | = | Multiple Sequence Comparison by Log-Expectation |
| n | = | number |
| NCBI | = | National Center for Biotechnology Information |
| ng | = | Nanogram |
| *p* | = | Probability |
| PCR | = | Polymerase chain reaction |
| pg | = | Picogram |
| s | = | Second |
| SCC | = | Staphylococcal Cassette Chromosome |
| SIG | = | *Staphylococcus intermedius* group |
| *sps* | = | *Staphylococcus pseudintermedius* surface |
| SPSS | = | Statistical Package for the Social Sciences |
| ST | = | Sequence type |
| µl | = | Microliter |
| µg | = | Microgram |
| µm | = | Micrometer |

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# CHAPTER I

## 1. Introduction

*Staphylococcus pseudintermedius* is considered as a normal flora of skin and mucous membrane of dogs as well as an opportunistic pathogen that mostly associated with superficial canine pyoderma and canine otitis externa (Bannoehr and Guardabassi, 2012). Moreover, methicillin-resistant *S. pseudintermedius* (MRSP) and multidrug resistant have been reported worldwide which is emphasized the importance and awareness of antibiotics resistant distributing in veterinary field (Gómez-Sanz et al., 2011; Kadlec and Schwarz, 2012). Human can be occasionally colonized by *S. pseudintermedius* from dogs that is mostly found among veterinarians, and dog owner (Chanchaithong et al., 2014). *S. pseudintermedius* and/or MRSP also survive and contaminate in the environment such as household and veterinary hospital where are the important source of transmission, re-infection, and antimicrobial resistance gene transferring among pet and human in the same place (van Duijkeren et al., 2011). *S. pseudintermedius* was believed to colonize in human with no harm (Borjesson et al., 2015). However, the evidences of human infection by *S. pseudintermedius* were reported such as soft tissue infection in diabetes mellitus patients, dog-bite wound infection, implantable cardioverter-defibrillator infection associated with endocarditis, and bone marrow infection (Van Hoovels et al., 2006; Riegel et al., 2011; Savini et al., 2013; Borjesson et al., 2015; Viau et al., 2015). Although human cases were rarely reported, there seemed to be more life-threatening compared to those of canine infection (Riegel et al., 2011; Borjesson et al., 2015).

The knowledge of *S. pseudintermedius* survival and adaptation in human host is still unknown. According to *S. aureus* from human, the adaptation in new host such as poultry, swine and bovine is often associated with the acquisition or loss of mobile genetic elements (MGEs) containing immune evasion clusters, antibiotic resistance genes, and virulence genes (Price et al., 2012; Resch et al., 2013). Moreover, surface protein genes responsible for host tissue interaction are involved in the differential host-specific and adaptation (Uhlemann et al., 2012).

Virulence factors are associated with colonization, dissemination, metabolization, infection, and adaptation of *S. pseudintermedius* (Bannoehr and Guardabassi, 2012). *S. pseudintermedius* isolates from both healthy and clinical affected individuals harbor a diverse of specific virulence genes, including leukocidin genes (*lukS-I* and *lukF-I*), exfoliative gene (*siet*), exfoliative toxins of *S. pseudintermedius* (*expA* and *expB*), and *S. intermedius* enterotoxin gene (*si-ent*) (Futagawa-Saito et al., 2004; Gómez-Sanz et al., 2011; Garbacz et al., 2013; Gharsa et al., 2013). In addition, a variety of other staphylococcal enterotoxin genes (SE genes) exist, that vary between bacterial species and strains (Ortega et al., 2010). However, knowledge about SEs in *S. pseudintermedius* that may relate to pathogenicity is still limited.

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Regarding to *S. aureus*, most of staphylococcal enterotoxins (SEs) genes were MGEs associating with genetic transference during bacterial adaptation (Sung et al., 2008). These acquisition of virulent toxin implied host-specific activity. SE genes family are a group of similar conformation exotoxins (~20 types) including superantigen, toxic shock syndrome toxin, which has been widely

studied in *S. aureus* (Argudin et al., 2010). SEs are associated with food poisoning, toxic shock like syndrome, and atopic dermatitis in human (Argudin et al., 2010). Moreover, SEs are associated with bacterial survival by expression in each bacterial growth phase (Derzelle et al., 2009). SE genes are located on plasmid, bacteriophage, genomic islands, and pathogenicity Islands (Argudin et al., 2010). They were assumed to exchange between *Staphylococcus* spp. resulting their adaptation and evolution in other hosts (Podkowik et al., 2013). Not only *S. aureus*, but the other staphylococci species including *S. epidermidis* and *S. pseudintermedius* produce SE toxins (Gharsa et al., 2013; Podkowik et al., 2013). In contrast to *S. aureus*, SE genes are suggested to accelerate canine pyoderma and otitis but not a major virulence in clinical cases of *S. pseudintermedius* (Gharsa et al., 2013; Tanabe et al., 2013). The role of SE gene in host specific was elucidated in enterotoxin type C (*sec*) containing the nucleotide variant in different hosts, e.g. human, ovine, and canine (Marr et al., 1993; Argudin et al., 2010). SEC encoded by $sec_{canine}$ gene is the most frequently detected enterotoxin in canine clinical isolates suggested its unique host-specific for survival in respective host (Edwards et al., 1997). Therefore, *S. pseudintermedius* colonizing in different hosts may contain different enterotoxin genes.

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The diversification of a surface structure of bacteria also known as cell wall-associated (CWA) protein were associated with bacterial adaptation in different environment (Sung et al., 2008; Foster et al., 2014). These structures play an important role as the first critical step of bacterial-host interaction leading to colonization as commensal and infection as pathogen (Foster et al., 2014). Member of microbial surface component recognizing adhesive matrix molecule

(MSCRAMM) family such as adhesins is a predominant group of CWA proteins (Foster et al., 2014). Adhesins promote bacterial adhere to host protein. In *S. pseudintermedius,* there are 18 putative *S. pseudintermedius* surface protein genes names *spsA-spsR* (Bannoehr et al., 2011). The previous studies of adhesin showed that *sps* were geographic variation, and functional redundancy (Latronico et al., 2014). However, the distribution of CWA protein genes in isolates from different sources has not been investigated. Moreover, there are still no conventional method for *S. pseudintermedius* adhesin genes detection and most studies relied on high throughput technology required cost, time consume and expertise (Bannoehr et al., 2011; Ben Zakour et al., 2012). Therefore, this study aims to simplify the detection method for 18 adhesin genes belonging to *S. pseudintermedius* by a multiplex PCR method which can amplify many loci simultaneously (Edwards and Gibbs, 1994), and apply the new multiplex PCR with *S. pseudintermedius* isolates from dogs, humans, and environment, respectively.

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To confirm their adhesion ability, the adherence assay with host cell were usually performed in many aspects. The first adhesion target of staphylococci is host corneocytes cell (Bibel et al., 1982). The corneocytes are a dead, differentiated keratinocyte cell in cornified layer or stratum corneum and contain functions as a protective barrier of skin (Candi et al., 2005). Previous studies revealed that *S. pseudintermedius* had the ability to adhere both canine and human corneocytes answering their zoonotic potential (Woolley et al., 2008). However, the comparison of adhesion and their adhesin profiles were not well elucidated especially in *S. pseudintermedius* isolated from different sources.

The knowledge of genotypic diversity of *S. pseudintermedius* isolates from dogs, humans, and environmental sources are helpful to imply its pathogenicity and provide the basis understanding of molecular bacterial-host interaction and adaptation. The information can be applied in further study that may lead to improve monitoring and therapeutic strategy for *S. pseudintermedius* infection.



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**2. Objectives of study**

1. To detect and compare prevalence of staphylococcal enterotoxin genes in *S. pseudintermedius* isolates from dogs, humans, and environment

2. To develop a novel multiplex PCR that specific for adhesin gene detection of *S. pseudintermedius*

3. To determine on source-related adhesion ability of *S. pseudintermedius* isolates from dogs, humans, and environment to canine and human corneocytes by adherence assay

**3. Hypotheses of study**

1. *S. pseudintermedius* isolates from dogs, humans, and environmental harbor different types and number of staphylococcal enterotoxin and adhesin genes

2. Adhesin genes of *S. pseudintermedius* can be detected by newly developed multiplex PCR.

3. There is a direct relationship between type of corneocytes, sources of isolation and adhesin gene profiles.

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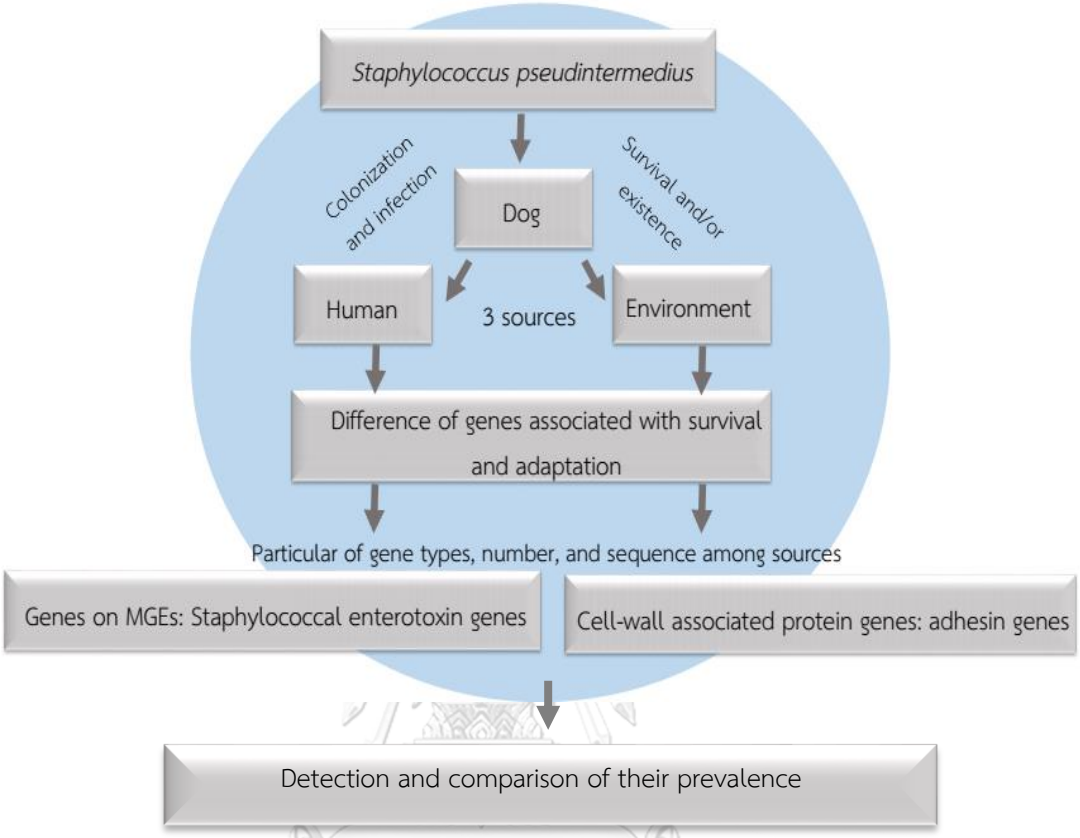
## 4. Conceptual framework

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## 5. Review literature

### 5.1 *Staphylococcus pseudintermedius*

*S. pseudintermedius* is a gram positive cocci bacteria found on dog's skin and mucous membrane (Bannoehr and Guardabassi, 2012). *S. pseudintermedius* is a member of coagulase-positive staphylococci (CoPS) base on the production of coagulase enzyme that can make clot blood (Devriese et al., 2005; Fitzgerald, 2009). The important CoPs of animal compose of *S. pseudintermedius, S. intermedius*, and *S. delphini* in *S. intermedius* group (SIG); *S. aureus*, *S. hyicus*, and *S. schleiferi* subsp. *coagulans* (van Duijkeren et al., 2011).

*S. pseudintermedius* had been previously identified as *S. intermedius.* Because of the improvement of molecular tools for example ribotyping and 16S-23S intergenic ribosomal DNA spacer polymorphism analysis (ITS-PCR) (Fitzgerald, 2009), they were introduced and used to reclassify *S. intermedius* from dog. Since 2005, it was recommended that *S. pseudintermedius* was the most frequently CoPS detected in dog instead of *S. intermedius* (Devriese et al., 2005).

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The prevalence of *S. pseudintermedius* in dog ranges from 0-100 % in various populations (Weese and van Duijkeren, 2010; Chanchaithong et al., 2014). The carriage sites in dog are dominant in nose, mouth, groin, and perineal area (Bannoehr and Guardabassi, 2012). The carrier status in dog is divided into 3 statuses consisting of non-carrier, intermediate, and permanent carrier which is similar to *S. aureus* in human (Paul et al., 2013).

The phenotypic characterization is identified base on colony morphology, gram staining, and biochemical tests such as coagulase test,

carbohydrate assimilation test, and mannitol fermentation (Chanchaithong and Prapasarakul, 2011). The molecular characterization for example multiplex PCR for *nuc* gene identification is useful for discriminating the species level in CoPS (Sasaki et al., 2010). The accuracy of species identification among CoPS is the important step that leads to the precision in antibiotic susceptibility interpretation.

*S. pseudintermedius* is an opportunistic bacterial pathogen. It is a major pathogen isolated from canine pyoderma, superficial bacterial folliculitis and otitis externa (Lyskova et al., 2007; Fitzgerald, 2009). Moreover *S. pseudintermedius* is associated with the infection in urinary tract, joint, surgical wound, and nosocomial infection. The risks of infection are increased in dog that has skin damage and allergic disease such as atopic dermatitis (Fitzgerald, 2009).

For epidemiology and evolution, the scheme of *S. pseudintermedius* specific multilocus sequence typing (MLST) has been used since 2013 (Solyman et al., 2013). Nowadays, there are more than 503 sequence types (STs) in the database (Santos et al., 2016). The successful clones are geographical distinct such as CC68 in North America, CC71 in Europe, CC45 and CC112 in Asia (Santos et al., 2016). In Thailand, the major clone is ST45 followed by ST112 and ST181, respectively (Chanchaithong et al., 2014).

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5.1.1 Methicillin-resistant *S. pseudintermedius*

Methicillin-resistant staphylococci (MRS) are the important antimicrobial resistance of staphylococci. They are classified into a group of staphylococci that resistant to penicillinase resistant penicillin. The methicillin resistance is caused by the expression of an alternative of penicillin binding protein 2a (PBP2a), the

non-target of penicillin drug, encoded by *mecA* gene (Kadlec and Schwarz, 2012). The affinity for beta-lactam antibiotic is decreased and the antibiotic can inhibit bacterial cell wall synthesis. Methicillin-resistant *S. (pseud) intermedius* (MRSP) was first reported in canine pyoderma case in USA in 1999 (Sasaki et al., 2010), and had been increasingly reported worldwide (Devriese et al., 2005; Ruscher et al., 2010). The MRSP is the serious and challenge problems in veterinary medicine, because MRSP can resist to all of β-lactam antibiotics which is the first tier drug for treatment of canine dermatitis, and trends to be a multi-drug resistance (MDR) (Kadlec and Schwarz, 2012). The resistant profiles of MDR were observed against tetracyclines, macrolides, lincosamides, chloramphenicol, aminoglycoside, trimetroprim, fluoroquinolones, rifampicin and the topical drug: mupirocin (Kadlec and Schwarz, 2012). These resistances lead to treatment failure, long hospitalization, and economic loss (Walther et al., 2012).

5.1.2 *S. pseudintermedius* in human

*S. pseudintermedius* has a zoonotic potential. The bacterial transfer and colonization in human are occasionally found in dog associated with people, such as veterinarian, staff in veterinary hospital, and dog's (Bannoehr et al., 2012) owner (Bannoehr and Guardabassi, 2012). Human *S. pseudintermedius* mostly found in nasal carriage at 0-5.5% (Walther et al., 2012). Both MRSP and MSSP could be found in human without obvious links to antimicrobial resistance (Walther et al., 2012). The risks of transmission were i) living with dog or dog patient in the same household ii) poor hygienic control after dog exposure iii) working in veterinary field (Beth A. Hanselman, 2009; Hanselman et al., 2009; van Duijkeren et al., 2011). Nevertheless, the time period and status of colonization

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in human are still unclear. Some reports suggested that it was contamination rather than colonization (van Duijkeren et al., 2011). Interestingly, Gómez-Sanz and colleague found some persistent *S. pseudintermedius* human carriers that had been colonized by *S. pseudintermedius* for 1 year (Gómez-Sanz et al., 2013).

In general, *S. pseudintermedius* causes no harm in healthy people (Borjesson et al., 2015). However, *S. pseudintermedius* infection in human have been reported in immunocompromised patients (**Table 1**). Not only the common case as dog bite wound, there were some severe and life-threatening case in human. The most common case were skin and soft tissue infections at lower limb of diabetes mellitus patient (Somayaji et al., 2016). Co-infection of MSSP/MRSP with other bacteria such as *Klebsiella pneumoniae* was also reported (Kuan et al., 2016). Interestingly, some patient had no history of dog exposure which reflected the importance of environmental contamination and human to human transfer (Viau et al., 2015).

The misdiagnosis of *S. pseudintermedius* as *S. aureus* should be concerned because they have different criteria for MIC values and disc diffusion susceptibility testing (Borjesson et al., 2015). The bacterial misidentification may lead to treatment failure and long hospitalization. The improvement of bacterial identification in the standard laboratory should be performed using new methods, for example, molecular identification of 16S rRNA and MALDI-TOF mass spectrometry identification (Borjesson et al., 2015).

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**Table** 1. Case reports of *S. pseudintermedius* human infection

| Clinical sign/infection site (immunocompromised disease/case) | Dog exposure | No. of cases | MSSP/ MRSP | Country | Reference |
|---|---|---|---|---|---|
| Implantable cardioverter-defibrillator (ICD) infection | N/A | 1 | MSSP | Belgium | (Van Hoovels et al., 2006) |
| Catheter-related bacteremia (Hemophilia B) | Yes | 1 | MSSP | Taiwan | (Chuang et al., 2010) |
| Rhinosinusitis/sinusitis | Yes | 2 | MRSP | Switzerland | (Stegmann et al., 2010) |
| ICD-related infection associated with endocarditis | Yes | 1 | MSSP | France | (Riegel et al., 2011) |
| Bone marrow infection (leukemia) | Yes | 1 | MRSP | Italy | (Savini et al., 2013) |
| Dog-bitted wound (Re-identified isolates in laboratory) | N/A | 13 | MRSP= 1, MSSP= 12 | Sweden | (Borjesson et al., 2015) |
| Leg and foot ulcer (DM) | Case 1=no, case 2=cat's owner | 2 | MRSP | Sweden | (Starlander et al., 2014) |
| Venous ulcers (DM) | No | 1 | MRSP | | |
| Septicaemia (DM) | No | 1 | MRSP | | |
| Sinusitis (multiple sinus surgery) | Yes | 4 | N/A | USA | (Kuan et al., 2016) |

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| Clinical sign/infection site (immunocompromised disease/case) | Dog exposure | No. of cases | MSSP/ MRSP | Country | Reference |
|---|---|---|---|---|---|
| Blood-filled blisters on foot (DM) | No | 1 | N/A | USA | (Viau et al., 2015) |
| Skin and soft tissue infection (DM, lymphoma, etc.) | Yes | 20 | MSSP= 18, MRSP= 2 | Canada | (Somayaji et al., 2016) |
| Nodular infection | | 1 | MSSP | | |
| Joint infection | | 1 | MRSP | | |
| Otitis externa | | 1 | MSSP | | |
| Bacteremia | | 1 | MSSP | | |
| Skin infection (ecthyma-like lesion at forehead) | Yes | 1 | N/A | UK | (Robb et al., 2017) |
| Surgical wound infection | No | 1 | MSSP | Spain | (Lozano et al., 2017) |
| Left lower extremity cellulitis | Yes | 1 | MSSP | | |
| Foot ulcer | Yes | 1 | MRSP | | |
| Nail exudate (cancer) | Yes | 1 | MSSP | | |

N/A, data not available, DM, diabetes mellitus, MSSP, methicillin-susceptible *S. pseudintermedius,* MRSP, methicillin-resistant *S. pseudintermedius*

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5.1.3 *S. pseudintermedius* in environment

*Staphylococcus* species can survive in the environment for a week or longer (Davis et al., 2012). Human and animal carriers can shed staphylococci into the environment around them via direct contact surface, shedding skin, sneezing, and gastrointestinal route (Laarhoven et al., 2011; Davis et al., 2012). The contamination of staphylococci in the environment associates with recolonization and re-infection in patient (Davis et al., 2012), and reflects for hygienic control for public health. The prevalence of MRSP in the household were reported at 18-44% in Netherland (Laarhoven et al., 2011; van Duijkeren et al., 2011). The contamination of *S. pseudintermedius* in the household rising dog was mainly found at dog's feeding and sleeping place (Laarhoven et al., 2011). Moreover dust particle was the maintenance source of *S. pseudintermedius* and/or MRSP (Laarhoven et al., 2011).

In veterinary hospital, the study of van Duijkeren and college in 2011 reported prevalence of environment MRSP at 14% (14/101) in 3 of 6 veterinary clinics after routine cleaning followed standard protocols (van Duijkeren et al., 2011). The other study showed the positive results of *S. pseudintermedius* in veterinary hospital (animal contact areas and human touch area) at 22.8% while 17.5% was MRSP (Bergstrom et al., 2012). However, PFGE pattern of environmental and dog isolates from the same hospital were distinct. They suggested that the exposure commonly found in inpatient dog and the origin of environmental isolates came from both hospital and community sources (Bergstrom et al., 2012). The importance of contamination was highlighted in the surgery room. The study of Fungwithaya et al., 2017 showed the presense of

MRSP in a surgery unit of one veterinary hospital (Fungwithaya et al., 2017). The positive sites were electric clipper and rebreathing circuit in preparation and surgery room, respectively (Fungwithaya et al., 2017). Therefore, the standard protocols for decontamination and monitoring of *S. pseudintermedius* in veterinary hospital should be conducted.

**5.2 Virulence genes**

5.2.1 Enterotoxin or superantigen gene

Staphylococcal enterotoxins (SEs) are the virulent proteins contained in the group of staphylococcal which widely studied in *S. aureus*. The SEs compose of staphylococci enterotoxin and toxic shock syndrome toxin including 5 classical SEs (SEA-SEE) and 18 new type of SEs or SE-like (SEG-SElU, SElV2, SElX) and 2 toxic shock syndrome toxin (TSST-1 and $TSST_{ovine}$) (Podkowik et al., 2013). The names base on the ability to cause emetic activity in primate model (Lina et al., 2004). If they have no emetic activity or there still not prove, the name of these toxins or genes becomes Staphylococcal enterotoxin-like or SE*l*s (Lina et al., 2004). The function of SEs is a potent gastrointestinal because of their properties in heat stable, low pH tolerant, proteolytic enzymes tolerant (Argudin et al., 2010). On the other hand, the other function is superantigen activity. The conformation of SEs can be presented on MHC class II on antigen presenting cell and introduces to T-cell receptor via specific subset at variable β-chain. On that site, superantigen can non-specific bind to that linkage resulting a large number of T-cell proliferation and release of chemokines and proinflammatory cytokine (Argudin et al., 2010).

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To date, the exact role of SE genes in *S. aureus* is still unclear. Some studies suggested that *S. aureus* containing many types of these gene might associate with their genetic fitness (Xu and McCormick, 2012). However, most of them are located on mobile genetic element (MGEs) i.e. bacteriophage, plasmid, and pathogenicity islands, which are not very stable in bacteria therefore the fitness cost cannot be answered overall of their roles. Nevertheless, Derzelle et al in 2009, provided evidence of the relation between bacterial survival and SE gene expression in *S. aureus* (Derzelle et al., 2009). Some of them expressed in concordance with their exponential and/or stationary growth phase under (*agr*) system suggested that the gene expression was required and also depended on stage of bacterial growth (Foster, 2009).

On the other hand, there were scarce study of these virulence genes in *S. pseudintermedius*. The $sec_{canine}$ was the most found in *S. pseudintermedius* isolated from dog because of its ability to adapt in new species (Edwards et al., 1997). *sec* was classified into *sec1, sec2, sec3*, $sec_{canine}$ and the other *sec* type based on the different hosts and the modified nucleotide sequences (Marr et al., 1993; Balaban and Rasooly, 2000). Nevertheless the presence of *sec* and other SE gene types in *S. pseudintermedius* were suggested as minor toxin in canine pyoderma that accelerate lesion with other toxin e.g. exfoliative toxin (Tanabe et al., 2013). The prevalence of SE genes in each study were different because of geography, origin of isolates and strain difference (Gharsa et al., 2013; Gómez-Sanz et al., 2013).

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5.2.2 Adhesin genes and cell wall-associated protein genes

Adhesin proteins are the surface structure or macromolecule of bacteria that binds a bacterium to a specific surface of host cell. This interaction is crucial as the earliest step of bacterial intracellular and extracellular colonization leading to infection (Soto and Hultgren, 1999; Geoghegan and Foster, 2015). These surface protein also known as cell wall-associated (CWA) proteins (Foster et al., 2014). The CWA proteins of staphylococci have a signal sequence at N-terminal as YSIRK-G/S motif and C-terminus as sorting signals, LPXTG motif (Bae and Schneewind, 2003). The CWA proteins are regarded as virulence factors and associate with bacterial colonization, survival, innate and adaptive immune evasion which promote adhesion to the extracellular matrix (ECM) of host cell such as fibronectin, fibrinogen, and collagen (Foster, 2009).

The most prevalence group is microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family (Foster et al., 2014). MSCRAMM promotes bacterial adhere to host ECM. The basic structures of MSCRAMM are characterized by containing at least 2 adjacent IgG-folded domains in the N-terminal region resulting in common ligand binding mechanism (Foster et al., 2014). This binding called dock, lock, and latch, respectively (Ponnuraj et al., 2003).

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Adhesins are widely studied in *S. aureus* both in genomic and proteomic. There are abundant CWA proteins up to 24 distinct types and redundancy of their functions that can bind to more than one matrix molecule (Vancraeynest et al., 2004; Geoghegan and Foster, 2015). Their names were assigned from their first discovery function or named in general for example

Clumping factor A and B (ClfA and ClfB), Serine–aspartate repeat protein C (SdrC), Fibronectin-binding proteins A (FnBPA) and B (FnBPB), Collagen-binding protein (Cna), and Protein A Spa (Geoghegan and Foster, 2015). Among MSCRAMM family, ClfB is the most important as a major adhesin in human nasal colonization (Xiang et al., 2012). ClfB bind to human-cytokeratin 10 which express on squamous epithelial cell (Wertheim et al., 2008). In addition, ClfB can bind to fribinogen-**α** associated with platelet activation and aggregation leading to endocarditis in rat model (Xiang et al., 2012). Moreover, anti-ClfB antibody cloud protect mice from *S. aureus* colonization and ClfB mutant *S. aureus* could not persist in human nasal cavity (Wertheim et al., 2008). Therefore, ClfB is now one of a vaccine candidate for *S. aureus* and be a target for *S. aureus* decolonization and medication (Xiang et al., 2012).

To date, there were 18 CWA protein genes called *S. pseudintermedius* surface A-R (*spsA-spsR*) that were found in *S. pseudintermedius* ED99 from whole genome sequencing (Bannoehr et al., 2011; Ben Zakour et al., 2012). Their location and function were elucidated with different sequence base number and protein structures. Some of them e.g. *spsA, spsF* located at *oriC* environ which was ~25 kb in size of the location downstream from an origin of replication containing distinct variable GC content and some MGEs (Bannoehr et al., 2011). Bannoehr and college, also provided sequence, structure, and function of *sps* and emphasized the role of MSCRAMM including *spsD*, *spsL,* and *spsO* by adherence assay to corneocytes, and host ECM (Bannoehr et al., 2011; Bannoehr et al., 2012). Moreover, their results showed the ability of *spsD* as the strongest binding gene and possible used as an antigen in vaccine against *S.*

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*pseudintermedius* (Bannoehr et al., 2012; Pietrocola et al., 2013). However adhesins were mainly studied and derived from one strain (ED99) of ST71. There might be strain and/or geographic variation of adhesins found in other *S. pseudintermedius* strain (Pietrocola et al., 2013).

**5.3 Host adaptation**

Staphylococci has the ability to colonize in the different host and environment and associated with a wide variety of animal and human diseases. Lowder and colleges investigated human-to-poultry host jump of *S. aureus* by whole genome sequencing analysis (Lowder et al., 2009). Phylogenetic analysis revealed that poultry clonal complex 5 e.g. ST5, ST1346 were originated from human clone. In addition, the representative poultry strain (ST5) had 5 distinct MGEs, including 2 prophages, 2 plasmids, and a staphylococcal pathogenicity islands (SaPI). One of prophage lacked the immune evasion cluster which responsible for human colonization. This prophage also distributed only in other poultry strains in this study. Moreover, this study showed a specific plasmid encoded a virulence factor in avian infection named thiolprotease that was not detected in human isolates. The presence of pseudogene were observed in putative cell-wall associated protein genes e.g. staphylococcal protein A. Taken together, these particular MGEs and mutation in surface protein genes leading to gene truncation indicated the presence of host-adaptation in poultry host jump *S. aureus*.

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For human-to-bovine host jump, it had been postulated that the loss of β-hemolysin converting prophages and the acquisition of new *mec*-negative SCC were associated with bovine-adapted (Resch et al., 2013). The presence of

this prophages in human isolates but absence in clinical mastitis bovine isolates might refer to the disadvantage of this element in bovine host. Moreover, the finding of a new cell-wall anchored contain LPXTG protein homolog to protein in *Geobacillus* spp. was suggested the importance role of surface protein genes in horizontal gene transfer and colonization in different hosts (Resch et al., 2013).

*S. aureus* ST398 is an importance livestock-associated that mostly found in swine as a major reservoir (Uhlemann et al., 2012). In general, genome of ST398 lacks of virulence factors, such as enterotoxins and phage-encoded toxins when compared to human *S. aureus* strain. The spread of *S. aureus* ST398 is not restrict from farm animal to human. Recently, the transfer of MSSA ST398 from human to human was reported in community household in Northern Manhattan (Uhlemann et al., 2012). This lineage caused a wide range of human infection and adapted to colonize in the household. From whole genome analysis of human-to-human *S. aureus* ST398, the difference of MSSA ST398 isolates from from animal and human were composed of difference of MGEs such as SaPI5 containing staphylococcal complement inhibitor (*scn*) that absence from pig isolates, insertion and deletion of surface protein genes and ability to adhere human skin receptors, and cluster of single nucleotide polymorphism (SNP) in core genome (Uhlemann et al., 2012). Therefore, core variable genome containing surface protein genes and virulence genes were associated with niche adaptation of staphylococci (Lindsay et al., 2006; Ben Zakour et al., 2008). In addition, mobile genetic elements (MGEs) and gene located on them in accessory genome were often exchanged both inter- and intraspecies resulting in adaptation in the new environment (Ben Zakour et al.,

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2008; Resch et al., 2013). Moreover, the genetic events in the genome, including combination of allelic diversification, acquisition and loss of gene function were lead to long term adaptation (Ben Zakour et al., 2008).



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# CHAPTER II

## PART I: STAPHYLOCOCCAL ENTEROTOXIN GENE DETECTIONS

**TITLE:** Detection of the Staphylococcal enterotoxin gene profile of methicillin-resistant *Staphylococcus pseudintermedius* isolates from dogs, humans and the environment

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### Introduction

*Staphylococcus pseudintermedius* can act both as a skin commensal and as a pathogen, causing canine dermatitis and otitis (Bannoehr and Guardabassi, 2012). Methicillin-resistant *S. pseudintermedius* (MRSP) are believed to have arise from clonal selection after routine antibiotic use, and increasingly have been reported worldwide (van Duijkeren et al., 2011). Moreover, *S. pseudintermedius* can transiently colonize humans, such as veterinarians and others who are closely associated with dogs (Bannoehr and Guardabassi, 2012; Borjesson et al., 2015), and can occasionally cause nosocomial infections such as sinusitis, soft tissue infection and endocarditis in humans (Van Hoovels et al., 2006; Starlander et al., 2014; Kuan et al.. 2016). Previous analysis of the distribution of *S. pseudintermedius* in veterinary teaching hospitals and households, along with molecular epidemiological evidence, revealed that the bacteria can persist in the environment, including on medical equipment, and can be transmitted between dogs and their owners or staff in veterinary hospitals, and vice versa (Laarhoven et al., 2011; Paul et al,. 2011; Fungwithaya et al., 2017). In contrast, there is currently no reported

evidence confirming the pathogenic potential of members of the species during colonization in the carrier hosts.

No significant relationship has been shown between the colonization of dog with MRSP or methicillin-sensitive *S. pseudintermedius* (MSSP) and canine mortality and bacterial virulence (Weese et al., 2012). *S. pseudintermedius* isolates from both healthy and clinical affected individuals harbor a variety of specific virulence genes, including leukocidin genes (*lukS-I* and *lukF-I*), exfoliative gene (*siet*), exfoliative toxins of *S. pseudintermedius* (*expA* and *expB*), and *S. intermedius* enterotoxin gene (*si-ent*) (Futagawa-Saito et al., 2004; Yoon et al., 2009; Iyori et al., 2010; Gómez-Sanz et al., 2011; Gharsa et al., 2013; Garbacz et al., 2013). In addition, a variety of other staphylococcal enterotoxin genes (SE genes) exist, that vary between bacterial species and strains (Ortega et al., 2010). To date there are still fewer types and less variety described amongst *S. pseudintermedius* SE genes than has been found in *S. aureus* (Gómez-Sanz et al., 2013a). Furthermore, knowledge about SEs in *S. pseudintermedius* that may relate to pathogenicity is still limited: the SE gene family products produced by human isolates of *S. pseudintermedius*, known as superantigens (e.g. SEA, SEB, SEC, and TSST-1 (Argudin et al., 2010), are involved in food poisoning and anaphylactic shock associated with septicemia, but other things lese are unknown.

Most studies to date have focused on the genetic characterization and antibiotic resistance profiles (antibiogram) of MRSP, but there have been few studies into the molecular epidemiology and ecology of MRSP from dogs, their owners and household or animal hospital environments. A previous study

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demonstrated that *S. pseudintermedius* could be transferred between dogs and humans, and vice versa, particularly where the high prevalence of MRSP in the human subjects provided indirect evidence for interspecies transmission (Soedarmanto et al., 2011; Chanchaithong et al., 2014). However, canine *S. pseudintermedius* is still a rare pathogen in humans compared to *S. aureus*, although its zoonotic potential in human patients has been demonstrated (Starlander et al., 2014; Borjesson et al.,. 2015; Somayaji et al., 2016). *S. pseudintermedius* infection in dogs is rarely life-threatening compared to the severe sickness recorded in humans. Thus, bacterial strains from different host origins are likely to play a crucial role in the pathogenicity, and this might be a reflection of their respective toxin gene profiles.

This study aimed to determine and compare the prevalence of members of the SE gene family among MRSP strains isolated from dogs, humans and the environment. The DNA fingerprint and staphylococcal cassette chromosome *mec* (SCC*mec*) type of MRSP form the different (host or environment) origins were analyzed together with their SE profile.

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**Materials and Methods**

1. Bacteria

Ninety-three MRSP isolates collected from dogs (n=43), humans (n=18) and the environment (n=32) during 2010–2013 were used to detect SE genes (Table 1). MRSP from dogs were isolated from their nasal cavity (n=18), groin (n=19), and areas of pyoderma (n=6) from patients attending the Outpatients Department (OPD) at the Veterinary Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2010-2012.

At the same time, human MRSP were simultaneously isolated from the nares of healthy veterinarians or the dog owners at the time of the physical examination of the animals (Chanchaithong et al., 2014). The isolates from the environment (floor, hand-touch sites and medical equipment) in the operating theatre and OPD were collected from the same hospital during 2011-2013 at operating room and outpatient department. The methods and criteria of environment sample collection were similar to those previously described (Fungwithaya et al., 2017). The isolates were classified to their species by biochemical tests (Chanchaithong and Prapasarakul, 2011) and multiplex PCR for the *nuc* gene (Sasaki et al., 2010). The methicillin resistance trait was classified by oxacillin disk diffusion method (CLSI, 2013), and *mec*A detection (Strommenger et al., 2003). This study was approved by the Faculty of Veterinary Science Biosafety Committee (CU-VET-BC number IBC1631004), Chulalongkorn University.

2. DNA extraction

The bacteria was grown on Tryptic Soy agar (Difco TSA, BD, USA) with 5% sheep blood and were incubated at 37° C for 24 h. Bacterial DNA was extracted using a commercial kit (Promega, USA) according to the manufacturer's instructions, with the minor modification, including lysostaphin (1 mg/ml) (Sigma-Aldrich, USA) and lysozyme (10 mg/ml) (Thermo Fisher Scientific, USA) during the cell lysis process.

3. Detection of SE genes

A total of 17 SE genes (*sea, seb, sec, sed, seg, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser* and *tst-1*) were detected by multiplex PCR (Omoe et al.,

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2005), and single PCR was used for *seu* with some modifications (Hwang et al., 2007). The 50 µl PCR mixture contained 1x reaction buffer, 200 µM dNTP, 1.5 mM MgCl2, 2.5 U of Taq polymerase (Promega, USA), 0.2 µM of forward and reverse primers, and 10-100 ng of DNA. The mixture was prepared on ice and immediately put into a T100 Thermal Cycler (Bio-rad, USA) at 94° C. PCR products were resolved in a 1.8-2 % (w/v) agarose gel and visualized under a UV transilluminator after staining with ethidium bromide. A representative PCR amplicon for each apparently positive SE gene was confirmed by DNA sequencing and thereafter used as the positive control for the PCR for that gene. The nucleotide sequences of the SE genes were compared with the data in GenBank (NCBI) using the BLASTn algorithm, and the sequences were submitted to GenBank and DDBJ. The accession numbers of positive genes are KP659467 (*seb*), KP659468 (*sec*), KP659469 (*sei*), KP659470 (*sek*), KP659471 (*sem*), KP659472 (*sep*), KP659473 (*seu*), KP659474 (*tst-1*), LC020109 (*sea*), LC020110 (*seq*), LC020111 (*seg*), LC020112 (*sel*), LC0201113 (*sen*), LC020114 (*seo*), LC209795 (*sed*), LC209796 (*sej*), and LC209797 (*ser*). Laboratory *S. aureus* strain 77.3 originally from human nasal carriage, and which had no SE genes, was used as a negative control. The PCRs were conducted in duplicate and the genes that did not amplify in the multiplex PCR were then examined using a single PCR for confirmation. The list of positive control strains is presented in **Table 2**.

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**Table 2.** Amplicon positive and negative control strains used in this study

| Strain | Positive gene and accession no. |
|---|---|
| *S. aureus* AQ55 | *sea* (LC020109), *seg* (LC020111), *tst-1* (KP659474) |
| *S. aureus* SC1 | *seb* (KP659467) |
| *S. aureus* SC2 | *sei* (KP659469), *sel* (LC020112), *sem* (KP659471) |
| *S. aureus* SC5 | *sek* (KP659470), *seo* (LC020114) |
| *S. aureus* ATCC 29213 | *seq* (LC020110), *sen* (LC0201113), *seu* (KP659473) |
| *S. aureus* ATCC BAA-1708 | *sed* (LC209795), *sej* (LC209796), *ser* (LC209797) |
| *S. pseudintermedius* AM25 | *sec* (KP659468) |
| *S. pseudintermedius* VB58 | *sep* (KP659472) |
| *S. aureus* 77.3 | Negative |

4. SCC*mec* typing and multilocus sequence typing (MLST)

The isolates from the environment with no SCC*mec* type and sequence type (ST) were subjected to SCC*mec* typing by the PCR method (Kondo et al., 2007; Descloux et al., 2008). The nontypeable SCC*mec* isolates were further identified using long-range PCR and restriction enzyme analysis with BSU-36I (New England Biolabs, USA) for Ψ-SCC*mec*$_{57395}$ (Perreten et al., 2013). MLST using the scheme for 7 genes was performed as previously described (Solyman et al., 2013). The sequences of the housekeeping genes were analysed and used to define the sequence type (ST) in the *S. pseudintermedius*MLST database (https://pubmlst.org/spseudintermedius/).

5. Pulsed-field gel electrophoresis (PFGE)

A total of 18 MRSP ST45- Ψ SCC*mec*$_{57395}$ were randomly selected for PFGE analysis using restriction enzyme Cfr9I (Thermo Fisher Scientific, USA). The isolates included six from humans, seven from dogs and five from the environment. The other STs shared by isolates from dogs and humans were 181-V (dog, n=4; human, n=4), 178-V (dog, n=1; human, n=1), 183-V (dog, n=2; human, n=2), and 112-A1 (dog, n=5; human, n=2). The isolates in MRSP ST182-V shared by dogs (n=3) and the environment (n=4) were included in the PFGE analysis. PFGE was performed followed the previously published protocol from the Centers for Disease Control and Prevention (https://www.cdc.gov/pulsenet/pathogens/pfge.html) (Murchan et al., 2003), with the minor modification of adding lysostaphin (1 mg/ml) (Sigma-Aldrich, USA) for lysis of cells in agarose plugs. Separation of the restricted DNA fragments was achieved using 6 V/cm and a switch time of 0.5–5 s for 18 h and 20–25 s for 5 h in a CHEF-DRIII apparatus (Bio-rad, USA) (Soedarmanto et al., 2011). The genetic relatedness of the strains was analysed by dendrogram construction using UPGMA in the GeneDirectory software (InGenius3 Syngene, UK), and setting a 1.5 % position tolerance. The *Xba*I (New England Biolabs, USA) digested chromosomal DNA of *Salmonella* Braenderup H9812 was used as a DNA ladder marker for gel normalization, and PFGE clusters were grouped at ≥ 80% similarity.

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6. Statistical analysis

The enterotoxin gene profile according to host origin was recorded by descriptive analysis. Differences in the frequency of SE genes among MRSP isolates obtained from dogs, humans, and the environment were analysed by

Fisher's exact test when the positive gene was observed in one or more sources amongst fewer than 5 isolates. For higher frequencies of occurrence, the $\chi^2$-test was performed. Statistical significance was accepted at the $p < 0.05$ level. The statistical analyses were performed using IBM SPSS statistics 22.0 software (IBM, Armonk, USA).

**Results**

1. SE genes detection

Of the seventeen SE genes, 12 (*sea, sec, seg, sei, sek, sel, sem, sen, seo, sep, seq*, and *tst-1*), 5 (*sec, sel, sem, seq*, and *tst-1*) and 3 (*sec, seq*, and *tst-1*) without *seb*, *sed*, *sej*, *ser*, and *sen* were detected in MRSP isolates from humans, dogs and the environment, respectively (**Table 3**). The SE gene profiles found in this study were diverse, including single and multiple (≥ 2) genes, and no SE genes detected (50.5 %; 47/93). Interestingly, diverse results were found amongst the human MRSP isolates in respect of gene type and number, and profiles compared to MRSP from dogs and the environment. The most prevalent SE gene detected in this study was *seq* (40.2 %) followed by *tst-1* (21.7 %), and *sem* (17.4 %). The other occurrences were *sea* (14.1 %), *sec* (16.3 %), *seg* (15.2 %), *sei* (15.2 %), *sek* (15.2 %), *sel* (5.4 %), *sen* (12 %), *seo* (13 %), and *sep* (3 %). The most commonly detected SE genes in MRSP from dogs, humans, and the environment, respectively, were *seq* (44.2 %; 19/43); *sem* (83.3 %; 15/18) and *tst-1* (83.3 %; 15/18); and *seq* (25.0 %; 8/32). Except for *seq*, the eleven SE genes detected in human MRSP were statistically significantly more common than amongst MRSP from dogs and the environment ($p< 0.05$; Fisher's exact test). The three SE genes *sec, seq*, and *tst-1* were found in MRSP

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strains from all the three sources, while *sek, sel* and *sem* were found in MRSP from dogs and humans but not in those from the environment.

**Table 3.** Information on the isolates and SE gene profiles

| Source | Year | MLST | SCC*mec* | No. of Isolates | SE genes profile | No. of isolates |
|---|---|---|---|---|---|---|
| Dog (n=43) | 2010-2012 | 45 | Ψ SCC*mec*$_{57395}$ | 23 | *seq* | 12 |
| | | | | | *seq, tst-1* | 1 |
| | | | | | *sek, seq, tst-1* | 1 |
| | | | | | None | 9 |
| | | 181 | V | 4 | None | 4 |
| | | 112 | A1 | 5 | None | 5 |
| | | 183 | V | 3 | *sem* | 1 |
| | | 182 | V | 3 | None | 1 |
| | | 121 | V | 1 | *tst-1* | 1 |
| | | 111 | A1 | 1 | None | 1 |
| | | 185 | Ψ SCC*mec*$_{57395}$ | 1 | None | 1 |
| | | 169 | II-III | 1 | *sec, sel, seq* | 1 |
| | | 178 | V | 1 | None | 1 |

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| Source | Year | MLST | SCC*mec* | No. of Isolates | SE genes profile | No. of isolates |
|---|---|---|---|---|---|---|
| Human (n= 18) | 2010-2012 | 45 | Ψ SCC*mec*$_{57395}$ | 6 | *tst-1* | 1 |
| | | | | | *sec, seg, sei, sek, sem, sep, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sem, sen, seo, seq, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sel, sem, sen, seo, seq, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sem, sen, seo, sep, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sel, sem, sen, seo, seq, tst-1* | 1 |
| | | 181 | V | 4 | *sea, sec, seg, sei, sem, sek, seo, seq, tst-1* | 1 |
| | | | | | *sea, seg, sei, sek, sem, sen, seo, seq, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sem, seo, seq, tst-1* | 1 |
| | | | | | *sea, seg, sei, sek, sem, sen, seo, seq, tst-1* | 1 |
| | | 183 | V | 2 | *seq* | 1 |
| | | | | | None | 1 |

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| Source | Year | MLST | SCC*mec* | No. of Isolates | SE genes profile | No. of isolates |
|---|---|---|---|---|---|---|
| | | 112 | A1 | 2 | *sea, sec, seg, sei, sem, tst-1* | 1 |
| | | | | | *sea, sec, seg, sei, sek, sel, sem, sen, seo, seq, tst-1* | 1 |
| | | 113 | Ψ SCC*mec*$_{57395}$ | 1 | *sea, sec, seg, sei, sek, sem, sen, seo* | 1 |
| | | 115 | V | 1 | *sea, sec, seg, sei, sek, sem, sen, seo,seq, sep, tst-1* | 1 |
| | | 110 | V | 1 | *sea, sec, seg, sei, sek, sem, sen, seo, seq, tst-1* | 1 |
| | | 178 | V | 1 | *sea, sec, seg, sei, sek, sem, sen, seo, seq, tst-1* | 1 |
| Environ-ment (n=32) | 2013 | 45 | Ψ SCC*mec*$_{57395}$ | 26 | *seq* | 5 |
| | | | | | *tst-1* | 1 |
| | | | | | *sec, seq* | 2 |
| | | | | | None | 16 |
| | | 182 | V | 4 | *seq* | 2 |
| | | | | | none | 2 |
| | | 496 | V | 2 | *seq* | 1 |
| | | | | | None | 1 |

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2. Clonal relatedness between molecular type and SE gene profiles

To analyse the role of host and source, the clonal relatedness of 46 MRSPs from 6 STs which harbored different SE gene profiles were selected. MRSP ST45-Ψ SCC*mec*$_{57395}$ was the only ST shared amongst the three isolation sources. Cluster analysis by PFGE showed 17 distinct electrophoretic types (A–Q) that mostly were in concordance with their ST and SCC*mec* type (**Figure 1**). Interestingly, five MRSP isolates from the environment belonging to ST45-ΨSCC*mec*$_{57395}$ with few or no SE genes were distinguishable from the ST45-ΨSCC*mec*$_{57395}$ MRSP isolates obtained from dogs and humans. In addition, the MRSP ST181-V, ST178-V, 183-V, and ST112-A1 isolates obtained from humans also showed a higher number of SE genes than the MRSP isolates obtained from dogs. On the other hand, MRSP ST 182-V shared by isolates from dogs and the environment were grouped in the same PFGE group (group Q), and they had a quite similar SE gene profile.

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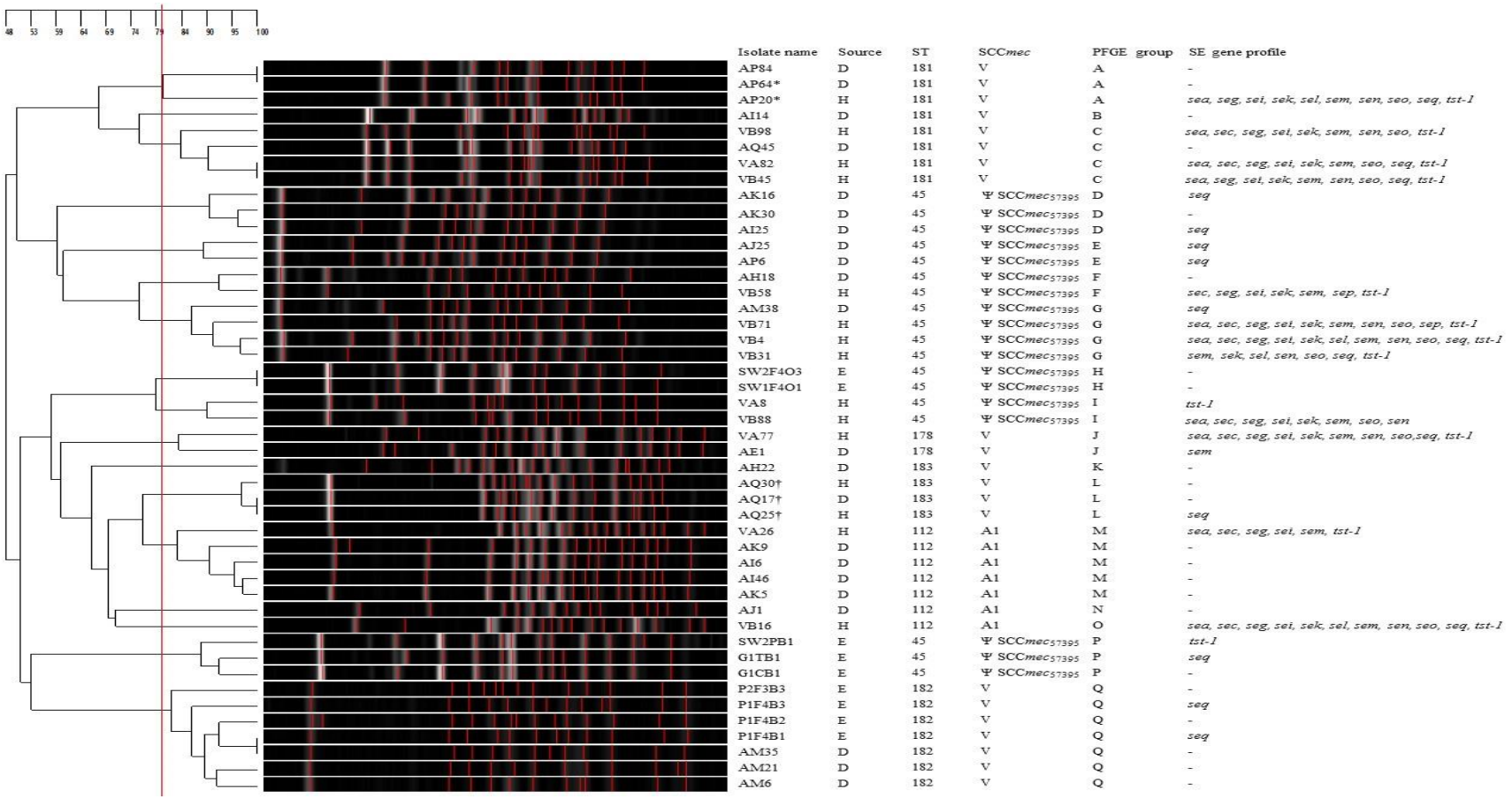


**Figure 1.** Dendrogram of 46 MRSP strains isolated from dogs, humans and the environment showing the PFGE cluster, ST, SCCmec type and their SE gene profiles. For PFGE, the cluster was grouped using UPGMA with a similarity coefficient at 1.5 of position tolerance and ≥ 80% cut-off. D, H, and E represent dogs, humans, and environment, respectively. *, †: isolates derived from the same household

**Discussion**

A higher prevalence of SE genes was found in MRSP obtained from humans compared to those isolated from dogs and environmental sources. This finding suggests that the isolation source rather than the clone type was the main risk factor for highly-pathogenic MRSP strains. Moreover, the isolates from the environment possibly represented contamination from dogs in the same area. In almost half the MRSP their genotypic characterization related to their pathogenic potential in relation to their recovery from lesions. Even though this study evaluated only a limited number of MRSP isolates (n=93), all had a potential connection between humans (owner or veterinarian) and dogs by their proximity and co-exposure within the hospital. The SE gene profiles of the human MRSP strains showed a higher variation in frequency and types compared with previous studies on canine *S. pseudintermedius* (Hwang et al., 2007; Yoon et al., 2010; Youn et al,. 2011; Tanabe et al., 2013). With respect to the variation in the SE profile, geographical and strain differences were probably the common causes of this variation. However, no study has investigated and compared the prevalence and distribution of virulence genes in MRSP strains from different sources (hosts and environmental sources).

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The SE gene profiles of the human MRSP strains showed a higher variation in frequency and types compared with previous studies on canine *S. pseudintermedius* (Yoon et al., 2010; Youn et al., 2011; Tanabe et al., 2013). With respect to the variation in the SE profile, geographical and strain differences were probably the common causes of this variation. The high frequency and types of SE genes in the MRSP isolated from humans was

possibly the result of their horizontal transmission via mobile genetic elements (MGEs) from other bacteria, especially *S. aureus* and coagulase negative staphylococci (CoNS) (Podkowik et al., 2013). *S. pseudintermedius* and/or MRSP may be either transient or persistent in the human nasal cavity, and therefore co-colonization and gene transfer possibly occurs in this niche (Gómez-Sanz et al., 2013b). The presence of MGEs, e.g. *S. aureus* pathogenicity islands (SaPIs) encoding superantigen, have been shown to be involved in intra- and inter-species transfer in *S. aureus* (Fitzgerald et al., 2001; Maiques et al., 2007; Alibayov et al., 2014). The phenomenon could mimic to that of human *S. epidermidis* strain FRI909 that acquired the *S. epidermidis* pathogenicity islands (SePI) containing *sec* and *sel* from *S. aureus* (Madhusoodanan et al., 2011). The process of SE acquisition in this SePI is still poorly understood and the transfer is assumed to have been via a bacteriophage (Madhusoodanan et al., 2011). Certain genes such as *sea, sek*, and *seq* are simultaneously located in the same *S. aureus* bacteriophages named ΦSa3ms and ΦSa3mw (Argudin et al., 2010). In our study, *sea, sek,* and *seq,* and *sek, seq,* and *tst-1* were detected in human isolates, and these also might be located on one or more bacteriophages. Evidence for SE and MGEs associated with colonization in different hosts was reported in *S. aureus* from ovine mastitis (Guinane et al., 2010). *S. aureus* ED133 ovine strain had SaPI and phage encoded variants of *sea, sec, sel,* and *tst-1* that differed from those in human strains, and were suggested to enhance activity in different hosts (Guinane et al., 2010). This study also detected some SE genes regarded as non-mobile genes on genomic islands e.g. *sei, seg* and *sem* (Gharsa et al., 2013), similar to a previous study (Argudin et al., 2010). The presence SE

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and their roles in other staphylococci are not known since they have been less studied and their horizontal transfer has not been proved (Madhusoodanan et al., 2011). The properties of SE genes and MGEs in *S. pseudintermedius* from different hosts still needs to be investigated.

In this study *seq* was the most common SE gene found from the three sources. This gene encodes a non-emetic toxin and is located on a bacteriophage or a *S. aureus* Pathogenic Island (SaPI) (Argudin et al., 2010). Expression of *seq* is found in every *S. aureus* growth phase and might be associated with the bacteriophage life cycle (Derzelle et al., 2009). However, the biological activity of *seq* of *S. pseudintermedius* is still not known. On the other hand, enterotoxin type C, especially variant canine ($sec_{canine}$), is believed to be a common specific SE gene of *S. pseudintermedius* isolates from dogs, especially from pyoderma lesions (Yoon et al., 2010). In contrast, this study found a low prevalence of *sec* (16.3 %), comprised a single canine isolate (1.1 %; 1/43) derived from the perineum, and was not recovered from a lesion. This may due to differences in geographical area and/or anatomical site, and in our study isolates with the *sec* gene are not necessarily specific to a canine source. The *sec* gene is also detected in dog and human strains of *S. intermedius* and *S. aureus* with 95-97% sequence homology, and is divided into at least 4 subtypes based on the nucleotide sequence: $sec_{canine}$ sequence and $sec_{1-3}$ variant sequences (Edwards et al., 1997). We found one different variant of *sec* in human strain VB16, whereas the other sources were identical for $sec_{canine}$ (**Figure 2**). The association of this finding with *S. pseudintermedius* adaptation in the human host needs to be clarified in future studies.

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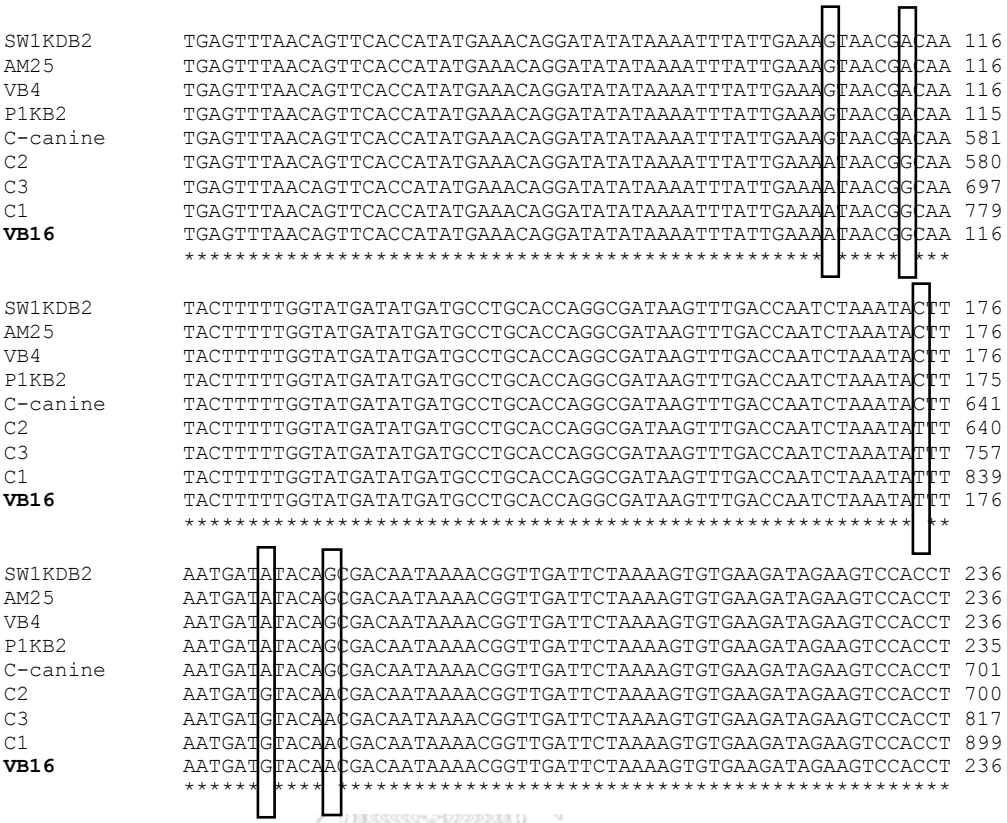


**Figure 2**. Partial *sec* sequence from representative dog, human, and environmental isolates aligned with the sec sequence in Genbank. The gene and accession number of reference strains are $sec_{canine}$ (U91526.1), *sec1* (X05815.1), *sec2* (DQ192646.1), *sec3* (X51661.1), respectively. The consensus sequences are highlighted in square boxes. The alignments were performed using MEGA 6.0.6 free software.

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In contrast to *S. aureus*, the roles of SEs in *S. pseudintermedius* have not been elucidated. Although there have been many studies on SEs and superantigens, the reason why *S. aureus* possess a large number and diversity of SEs is unclear (Xu and McCormick, 2012). The presence of SE genes is suggested to be related to immune evasion, as they function as an immunomodulator (Foster 2009) but without a high fitness cost to the bacteria because most are encoded on mobile genetic elements that do not persist in the bacteria (Xu and McCormick, 2012). These genes are expressed in different growth phases of bacteria, with and without the regulation of the enterotoxin gene cluster (*egc*) operon and *agr* system (Derzelle et al., 2009; Ortega et al., 2010). Although *S. aureus* possess the same SE genes, they can produce different amounts of toxin and so the existence of these genes alone is not always the (sole) disease-causing factor in human infections (Ortega et al., 2010).

MRSP ST45-Ψ SCC*mec*$_{57395}$ has been shown to be the most frequent clonal type in Thailand (Chanchaithong et al. 2014), and this was found in humans, dogs and the hospital environment. Thus, we used this major type together with the other 5 minor STs that were shared between dogs and humans, or dogs and the environment. The dog isolates appeared to be more diverse (ST) than human isolates, with human-associated MRSP lineages being dispersed in 5 STs and 7 PFGE types. MRSP clonal types ST45, 112, 178, 181 that were all isolated from the nares of veterinarians contained more virulence genes compared to the other sources, except for strains AQ25 and AQ30 from ST183 that were isolated from owners. Thus, veterinarians appear to have an

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increased risk of carrying highly pathogenic MRSP. The canine-associated MRSP lineages and those isolated from the environment had few or none SE genes. It is speculated that the sources of isolates might influent the finding.

The expression of the SE genes was not studied, and moreover, the exact origin of all environmental isolates could not be established. However, PFGE groups have greater discriminatory power compared to STs in MLST analysis (Melles et al., 2007). It can be assumed that these groups of isolates may be contaminants from dogs (ST182-PFGE type Q) because there was no human-environment sharing of PFGE groups in this study.

In conclusion, the enterotoxin genes of MRSP strains isolated from humans were highly diverse in terms of their type and frequency of SE gene distribution compared to MRSP strains isolated from dogs and the environment. This study implies that human MRSP have a tendency to have greater pathogenic potential than those from the other sources.

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# CHAPTER II

## PART 2.1: DEVELOPMENT OF MULTIPLEX PCR SETS FOR ADHESIN GENES DETECTION

**TITLE**: Development of a set of multiplex PCRs for detection of genes encoding cell wall-associated proteins in *Staphylococcus pseudintermedius* isolates from dogs, humans and the environment

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### Introduction

*Staphylococcus pseudintermedius* is regarded as a commensal and as an opportunistic pathogen causing canine pyoderma and otitis (Bannoehr and Guardabassi, 2012). *S. pseudintermedius* also can colonize the nasal cavity of humans, especially pet owners and veterinarians (Bannoehr and Guardabassi, 2012). Contamination with *S. pseudintermedius* in the environment in animal hospitals and households has been reported (Laarhoven et al., 2011, van Duijkeren et al., 2011), and this potentially represents an important source of re-infection and transfer of antimicrobial resistance genes (Frank et al., 2009). Although colonization in humans generally is transient and harmless in healthy individuals, there have been reports of dermatitis and septicemia in human patients (Somayaji et al., 2016). Consequently *S. pseudintermedius,* and especially methicillin resistant strains, are of public concern as potentially emerging zoonotic bacteria.

The ability of bacteria to colonize and to cause infection is initiated by attachment to the host cells using surface proteins or cell wall-associated

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proteins (Foster et al., 2014). Surface proteins are considered to be virulence factors associated with bacterial survival, immune evasion, biofilm formation and host-adaptation (Sung et al., 2008; Foster, 2009). Moreover, surface proteins of *S. aureus* are thought to be candidate antigens that can be targeted for vaccine development and immunotherapy (McCarthy and Lindsay, 2010). Based on analysis of the whole genome sequence of canine *S. pseudintermedius* ED99, 18 putative genes that encode cell wall-associated (CWA) proteins named *S. pseudintermedius* surface (*sps*) genes (*spsA* through to *spsR*) have been reported (Bannoehr et al., 2011). Their sequences encode components containing one or more characters of CWA proteins, including an N terminus signal sequence, repeat regions, and a LPXTG cell wall anchor motif at the C terminus; hence they became the prototypes for further study (Bannoehr et al., 2011). The surface proteins of *S. pseudintermedius* express binding activity to components of the host's extracellular matrix (ECM), including fibronectin, fibrinogen, and cytokeratin10 in a similar to those from *S. aureus,* suggesting that several homologs of CWA proteins are shared between them (Geoghegan et al., 2009). The sequences of the CWA proteins of *S. pseudintermedius* ED99 also showed the closest homology (25-57%) to various *S. aureus* surface proteins that are considered to be virulence factors (Bannoehr et al., 2011). For example, SpsD and SpsL with homology to fibronectin-binding proteins B of *S. aureus* can promote bacterial adhesion to canine corneocytes and the ECM, and assist in invasion of host epithelial cells: consequently they are necessary for colonization and pathogenicity (Bannoehr et al., 2012; Geoghegan and Foster, 2015; Pietrocola et al., 2015).

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To date there has been a lack of rapid tools for detecting the full suite of surface protein genes in *S. pseudintermedius*. Furthermore, it is not known if particular genes are responsible for colonization and infection in different hosts, because of different host-pathogen interactions. Knowledge of the distribution and occurrence of these genes among *S. pseudintermedius* isolates from different sources might help to understand basic aspects of their pathogenicity and adaptation to their roles in the carrier or infective stages. This study first aimed to develop a rapid tool to detect a number of genes encoding CWA protein by multiplex PCR, and then to determine their distributions and frequencies in *S. pseudintermedius* isolates from carriage and infected sites in dogs, nasal carriage of humans, and the environment of a veterinary teaching hospital.

**Materials and Methods**

1. Bacterial isolates

A total of 135 *S. pseudintermedius* isolates collected from dogs (n=70), humans (n=25) (Chanchaithong and Prapasarakul, 2011; Chanchaithong et al., 2014), and environmental sites (n=40) in a veterinary teaching hospital in Bangkok, Thailand were used in this study. *S. pseudintermedius* isolates from dogs were derived from carriage sites of dogs, including the nasal cavity and perineum (n=35), and skin lesions including crusts, pustules, wounds, and abscesses (n=35). The environmental isolates were collected from the floor or equipment from 6 units of a veterinary teaching hospital, including dermatology, gynaecology, surgery, emergency and intensive care; general and preventive medicine; and special clinic as previously described (Fungwithaya et

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al., 2017). The isolates from carriage sites in dogs, humans, and the environment were identified to the species level by biochemical tests (Chanchaithong and Prapasarakul, 2011), and multiplex PCR (Sasaki et al., 2010). For clinical isolates from dogs, all were screened and identified using the Vitek2 (bioMérieux, France), and their identity were confirmed by the previous multiplex PCR (Sasaki et al., 2010).

2. DNA extraction

The bacteria were grown on tryptic soy agar with 5% sheep blood at 37° C for 24 h. The DNA was extracted using a commercial kit (Promega, USA), modified by adding lysostaphin (1 mg/ml) and lysozyme (10 mg/ml) to assist in breaking the bacterial cell wall. The quality and quantity of the DNA template was checked using a Colibri spectrophotometer (Titertek-Berthold, Germany). DNA at a final concentration of 10-100 ng/µl was used as the template in mPCR development and for detecting the prevalence of *sps* genes amongst the isolates.

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3. Multiplex PCR development

A set of mPCR reactions was developed as a rapid means for detecting the 18 sps genes, following described procedures (Edwards and Gibbs, 1994; Henegariu et al., 1997). The sequence of *S. pseudintermedius* ED99 (accession number: CP002478.1) from NCBI's Genbank was used as the prototype template for targeting the 18 *sps* genes (Bannoehr et al., 2011). The thermonuclease gene (*nuc*) specific to *S. pseudintermedius* was used as the internal control in all mPCR sets (Sasaki et al., 2010). Primer pairs that were unique, specific, of appropriate amplicon sizes, and selected to avoid variable

repeat motifs, were designed using NCBI's Primer BLAST and Primer3 software (Table 1). Uniplex PCR reactions were first performed on the reference and representative strains to test the primer specificity. Initially the components of the uniplex PCR reactions were adjusted and the annealing temperature (Ta) varied from 49-58° C until an optimal Ta was reached which could amplify a single amplicon of the expected size without producing non-specific bands. The uniplex reaction comprised a final volume of 50 µl, containing 1x reaction buffer (Promega, USA), 200 µM dNTP, 1.5 mM MgCl2, 0.2 µM of forward and reverse primers, 2.5 U of Taq polymerase (Promega, USA), and 1 µl of DNA template at a final concentration of 10-100 ng/µl. The thermal cycling for the uniplex PCRs included an initial step of 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 4 min, and 72 °C for 5 min. The mPCRs were divided into 4 sets each comprising 4-5 primer pairs for the various *sps* genes, and primers specific for the *nuc* gene as the internal control (**Table 4**). The mPCR reaction sets 1, 3, and 4 consisted of the same reaction as in the uniplex PCRs. The concentration of the primers *spsH* in mPCR set 2 was increased from 0.2 to 0.4 µM. The thermal cycling for the mPCRs included an initial step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 4 min, and 72 °C for 5 min. The PCR products were separated by electrophoresis on a 2% agarose gel stained using RedSafe (iNtRON Biotechnology, Korea). The positive products produced in mPCR using three representative *S. pseudintermedius* strains in our laboratory were sent for commercial sequencing and analyzed for similarity using nucleotide BLAST at

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NCBI. All PCR products with unexpected sizes were sequenced and analyzed by BLAST, and aligned nucleotide sequences by MUSCLE (www.ebi.ac.uk).

**Table 4.** Oligonucleotide primers, product size, and set of a multiplex PCR developed in the study

| Gene | Primer (5'-3') | Amplicon size (bp) | PCR set |
|---|---|---|---|
| *spsA* | CGAATGGTAAGGCGAGTGGA | 407 | 1 |
| | ACCACCACGACCTAACAACG | | |
| *spsB* | AAGGGACGAACTCATATGTTAAAAA | 733 | 1 |
| | TGCGTCGTCGTGTAAAGAAC | | |
| *spsC* | AACTGAAACGCCCGTAGAAG | 619 | 1 |
| | ATCCGCTTTCGTTTCATTTG | | |
| *spsE* | CGCATCGAAATGGACGTGG | 212 | 1 |
| | TTATGTTGTGTTGGCGGCAC | | |
| *spsF* | ATACAGATGGCGCGGTTTCA | 429 | 2 |
| | TGCTCGTTACTGCTGGGTTT | | |
| *spsG* | TGCTGTTAAGACTGCGCCAA | 219 | 2 |
| | GCTACAAATCAAGTGGCGGC | | |
| *spsH* | GAACCCGAGTGATGAGGTCC | 379 | 2 |
| | ACCGTTATCAGTGGCAGGTG | | |
| *spsI* | CGACATTGCAGGCGAATCTT | 561 | 2 |
| | GATGCTTCATTTCCGTCGGC | | |
| *spsJ* | ATGGCTCTTCCAACCACGAG | 296 | 2 |
| | TCTGAAGCTTCCTTGCCCTG | | |

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| Gene | Primer (5'-3') | Amplicon size (bp) | PCR set |
|---|---|---|---|
| *spsD* | TGGTGTAAAAAGCCCTTCAGGTA | 528 | 3 |
| | TTCCCTTCCCCACTTGCATTA | | |
| *spsK* | AGACCCAAGTAGCGCACAAA | 459 | 3 |
| | GCGCTTACTTCAGGTGCTTG | | |
| *spsL* | AACTCCAAAGGCCGAAGAAT | 201 | 3 |
| | CCAGCAACAAGAAGGAGAGG | | |
| *spsM* | AAGCATGGCCGAGTGATGTT | 306 | 3 |
| | CATAAGCACCTGGACCCACT | | |
| *spsN* | GGCGTCATCGCTTCAACATC | 284 | 4 |
| | GCGCAAGTTGCTTCTGTGAA | | |
| *spsO* | ACGTCACCTAGTGCTGTTGATT | 696 | 4 |
| | TGCAACTGGCCGTTACAATAA | | |
| *spsP* | CAAAGCTGAAGCGAAAGCAGA | 366 | 4 |
| | GCGATACTTACGCCACCCG | | |
| *spsQ* | CAGACAAAGGTATGGACAAAGCG | 217 | 4 |
| | ATTTCGTGGTTTGCTTTAGCTT | | |
| *spsR* | CACTGTAATTTGCACGCTGCT | 408 | 4 |
| | ACGAATCATATGCCGCAGAA | | |
| *nuc* | CGGCGACATGTTTCACGAAC | 135 | 1,2,3,4 |
| | TGTCACCGTCGACAGCTTTG | | |

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4. Sensitivity, specificity, and application of the multiplex PCRs

To evaluate the sensitivity of each set of novel mPCRs, the lowest level of detection of genomic DNA was determined by serial dilution (from 10 ng/µl-1 fg/µl) (Hwang et al., 2007). In addition, the primer specificity was evaluated by PCR amplification of DNA from the reference strain *S. pseudintermedius* ED99, *Staphylococcus intermedius* group (SIG) members Staphylococcus intermedius CVMP 0309 and *Staphylococcus delphini* CVMP 0109, the closely related canine pathogen *Staphylococcus schleiferi* subsp. coagulans AI42, as well as *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The four sets of mPCRs were applied to detect the presence of genes encoding CWA proteins in the 135 *S. pseudintermedius* isolates. The mPCRs were performed in duplicate at different times. Where the mPCRs indicated a lack of genes this was confirmed by use of the corresponding uniplex PCR.

5. Statistical analysis

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The occurrence of *sps* distributed among each source of isolates was compared by using the $\chi^2$-test. Differences were considered statistically significant at $p < 0.05$. The data analysis was performed using SPSS version 22 software (IBM, USA).

**Results**

1. Multiplex PCR

The use of 4-5 primers in each mPCR set, including targeting an internal control gene, to amplify sps from DNA extracted from *S. pseudintermedius* strain ED99 and three other representative strains, gave no primer complementary and it was easy to distinguish each gene within the same set (**Figure 3**). DNA sequencing of the representative amplicons showed high similarity (97-100%) to those of *S. pseudintermedius* ED99 (**Table 5**). There were no discrepancies in results between uniplex and mPCRs. However, *spsR* amplification generated an unexpected product size in the three human isolates VA77, AP49 and AQ50, which produced amplicons of 260 bp instead of 408 bp. Their sequences were about 98% identical to *spsR* of ED99. When their sequences were aligned with reference (ED99) and representative sequence (VA8), there was a 150-bp deletion (**Figure 4**) that corresponded with a 50-amino acid deletion at the same site in their products (**Figure 5**). The sequences were further analyzed for protein function in the Conserved Domain Database (CDD) of NCBI and Prosite (http://prosite.expasy.org). The entire SpsR of ED99 was annotated as a putative cell wall-associated of gram positive bacteria or a hypothetical protein, and the deleted part did not contained any predicted function.

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2. Sensitivity and specificity

For sensitivity testing, the lowest level of detection for all primers in the 4 mPCR sets was 1 pg/µl (**Figure 6**). For specificity testing, non-specific and faint bands with unexpected product sizes were observed in non-*S.*

*pseudintermedius* strains. However, there were some fragments with clear and exact product size produced in the mPCRs. The identity of their DNA was confirmed in uniplex PCRs and they were positive for *spsE* and *spsF* produced from *S. intermedius*, and *spsP* produced from *S. delphini*, respectively. The non-*S. pseudintermedius* strains were negative for the *nuc* gene (**Figure 7**).



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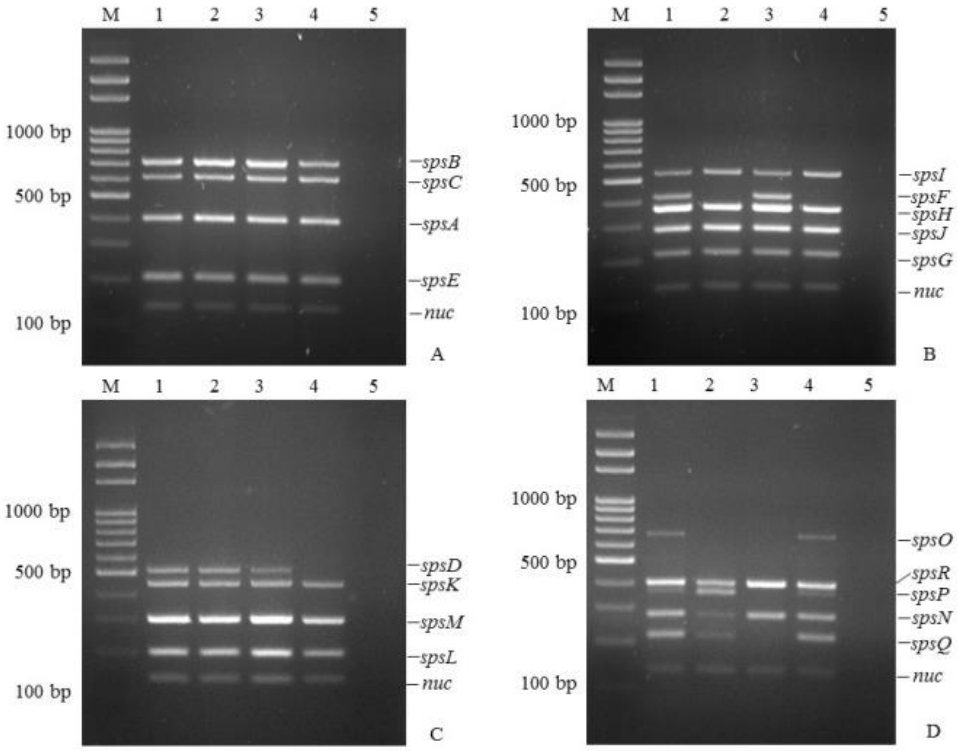


**Figure 3.** Multiplex PCR panels for detection of genes encoding cell wall-associated proteins in *S. pseudintermedius*. Lane 1: DNA of *S. pseudintermedius* ED99, Lane 2: DNA of *S. pseudintermedius* MN1, Lane 3: DNA of *S. pseudintermedius* G5, Lane 4: DNA of *S. pseudintermedius* VA8, Lane 5: DNase free water (negative control), Lane M: 100 bp DNA ladder marker**;** A) Set 1 comprise of *spsA* (407 bp), *spsB* (733 bp), *spsC* (619 bp), and *spsE* (212 bp); B) Set 2 comprise of *spsF* (429 bp), *spsG* (219 bp), *spsH* (379 bp), *spsI* (561 bp), and *spsJ* (296 bp); C) Set 3 comprise of *spsD* (528 bp), *spsK* (459 bp), *spsL* (201 bp), and *spsM* (306 bp); D) Set 4 comprise of *spsN* (284 bp), *spsO* (696 bp), *spsP* (366 bp), *spsQ* (217 bp), and *spsR* (408 bp); *nuc* (135 bp): internal control

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**Table 5.** Percentage similarity of partial sequence of genes encoding cell wall-associated proteins derived from representative *S. pseudintermedius* isolates in this study

| Strain | Source | Gene | % similarity to *S. pseudintermedius* ED99 |
|---|---|---|---|
| MN1 | Dog | *spsA* | 99 % |
| | | *spsB* | 99 % |
| | | *spsC* | 98 % |
| | | *spsD* | 99 % |
| | | *spsE* | 98 % |
| | | *spsG* | 99 % |
| | | *spsH* | 98 % |
| | | *spsI* | 99 % |
| | | *spsJ* | 100 % |
| | | *spsK* | 99 % |
| | | *spsL* | 100 % |
| | | *spsM* | 99 % |
| | | *spsN* | 98 % |
| | | *spsP* | 99 % |
| | | *spsQ* | 99 % |
| | | *spsR* | 99 % |
| G5 | Dog | *spsF* | 99 % |
| | | *nuc* | 97 % |
| VA8 | Veterinarian | *spsO* | 99 % |
| | | *spsR* | 98 % |

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**Figure 4.** Partial alignments of spsR sequences of the three human *S. pseudintermedius* isolates VA77, AP49, and AQ50 compared with representative strain VA8 and reference strain ED99. The multiple sequence alignment was performed using MUSCLE (http://www.ebi.ac.uk). Dashes (-) represent gap in the sequence and stars (*) represent consensus sequence

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VA77      ------------------YESYAAETIQNNTSSSETNQNSDQAPLDHY-----------      30
AP49      ------------------YESYAAETIQNNTSPSETNQNSDQTPLDHY-----------      30
AQ50      ------------------YESYAAETIQNNTSPSETNQNSDQTPLDHY-----------      30
ED99      MKKTISVLGLGLLATFFVSNESYAAETIQNNTSSSETNQNSDQTPLDHYIRKADGTLVEP      60
VA8       --------------------TNHAAETIQNNTSSSETNQNSDQTPLDHYIRKADGTLVEP      40
                             .:********** *********:*****

VA77      ----------------------------------------IRKADGTLVEPNVYPHKDYVE      51
AP49      ----------------------------------------IRKADGTLVEPNVYPHKDYVE      51
AQ50      ----------------------------------------IRKADGTLVEPNVYPHKDYVE      51
ED99      NVYPHKDYVENEGPLPEFKFQVDSKKDSSDPNQAPLDHYIRKADGTLVEPNVYPHKDYVE      120
VA8       NVYPHKDYVENEGPLPEFKFQVDSKKDSSDSNQAPLDHYIRKADGTLVEPNVYPHKDYVE      100
                                                 **********************

VA77      NEGPLPEFKLMYADKQNHHDQQSKNNKDKQRANYS-------------------------      86
AP49      NEGPLPEFKFMYADKQNHHDQQSKNNKDKQRANYS-------------------------      86
AQ50      NEGPLPEFKFMYADKQNHHDQQSKNNKDKQRANYS-------------------------      86
ED99      NEGPLPEFKFMYADKQNHHDQQSKNNKDKQRANYSDKKHNDQPGHPKAVTPAVQHDKAVT      180
VA8       NEGPLPEFKFMYADKQNHHDQQGKNNKDKQRAYS-------------------------      134
          *********:************.*********

VA77      ---------------------------------------                          86
AP49      ---------------------------------------                          86
AQ50      ---------------------------------------                          86
ED99      SNATVKALPNTGESDKTTQLPIVLSLLSVGILVLLKLRK                          219
VA8       ---------------------------------------                          134
```

**Figure 5.** Amino acid alignment of SpsR of three human *S. pseudintermedius* isolates (bold letters), VA77, AP49, and AQ50 compared with representative strain VA8 and reference strain ED99. The alignments were performed by using Clustal Omega (www.ebi.ac.uk). Dashes (-) represent gap in the sequence, Symbol *, :, . represent consensus sequence

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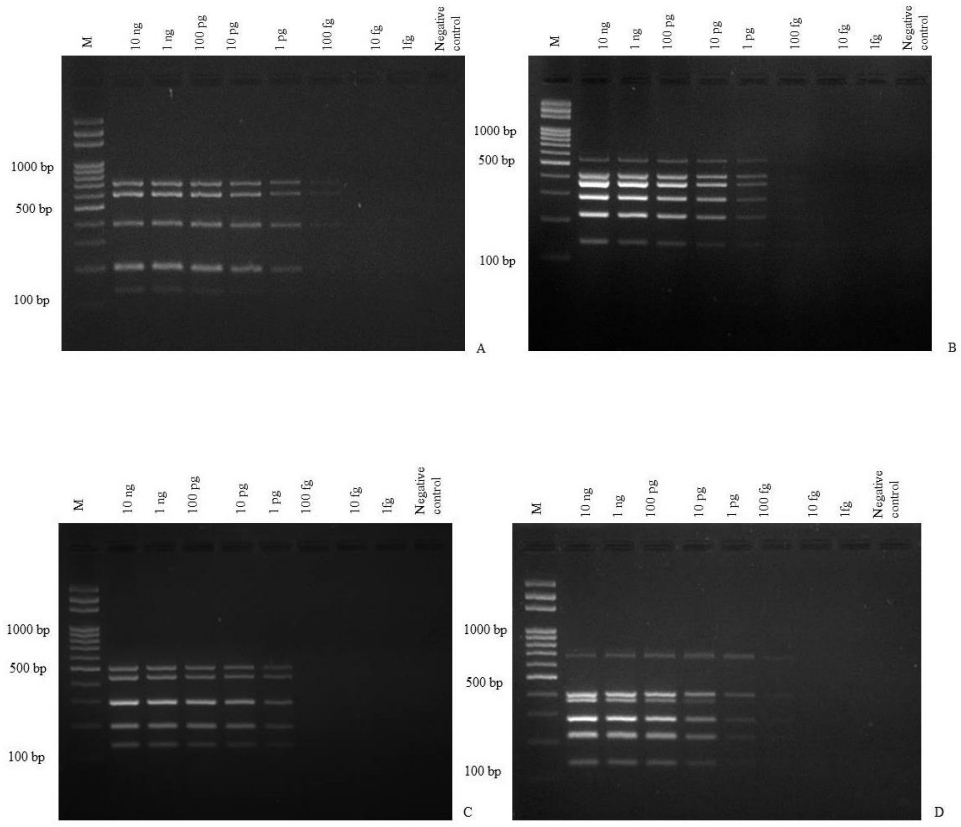


**Figure 6.** Sensitivity of 4 sets of new mPCRs**.** Tenfold serial dilution of DNA from *S. pseudintermedius* ED99 amplified by the new developed multiplex PCR. Set 1 (A) comprise of *spsA* (407 bp), *spsB* (733 bp), *spsC* (619 bp), *spsE* (212 bp), and *nuc* (135 bp); Set 2 (B) comprise of *spsF* (429 bp), *spsG* (219 bp), *spsH* (379 bp), *spsI* (561 bp), *spsJ* (296 bp), and *nuc* (135 bp); Set 3 (C) comprise of *spsD* (528 bp), *spsK* (459 bp), *spsL* (201 bp), *spsM* (306 bp), and *nuc* (135 bp); Set 4 (D) comprise of *spsN* (284 bp), *spsO* (696 bp), *spsP* (366 bp), *spsQ* (217 bp), *spsR* (408 bp), and *nuc* (135 bp), M: 100 bp ladder marker, Negative control: DNase free water

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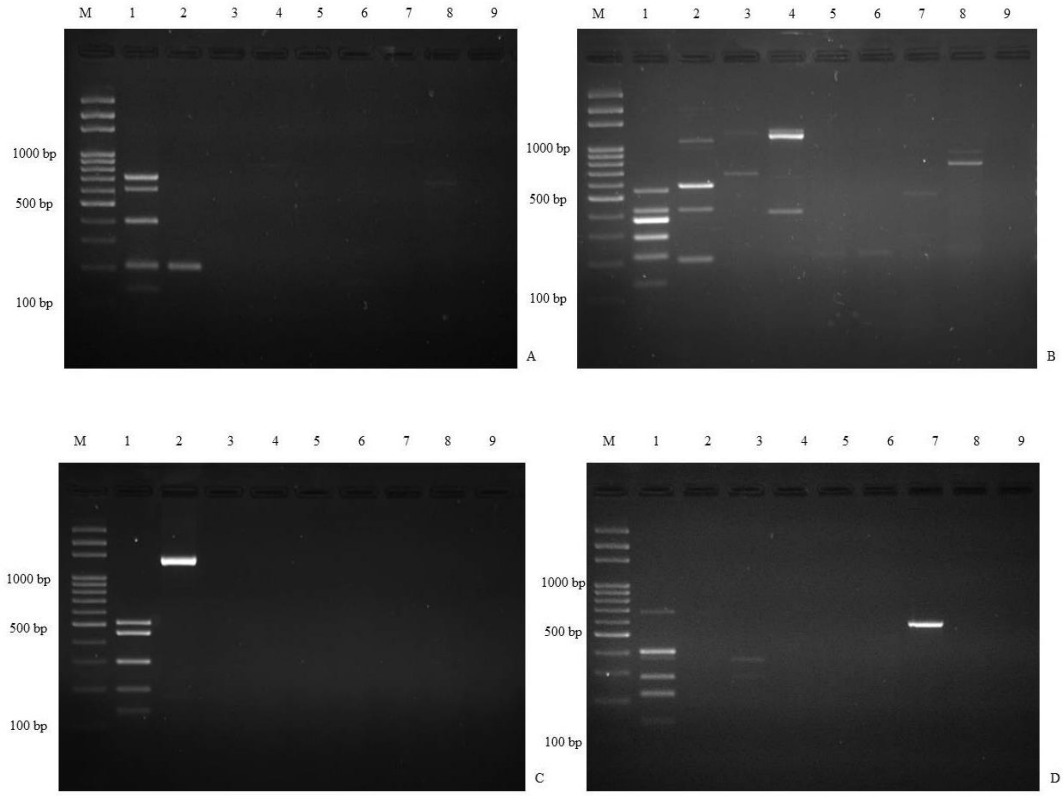


**Figure 7.** Specificity testing of the new developed multiplex PCR (mPCR) set 1 (A), set 2 (B), set 3 (C), and set 4 (D), respectively. The DNA of *S. pseudintermedius* ED99 (Lane 1), *S. intermedius* CVMP0309 (Lane 2), *S. delphini* CVMP0109 (Lane 3), *S. schleiferi* subsp. coagulans (Lane 4), *S. aureus* ATCC 25923 (Lane 5), *S. aureus* ATCC 29213 (Lane 6), *E. coli* ATCC 25922 (Lane 7), and *P. aeruginosa* ATCC 27853 (Lane 8) were amplified by mPCRs. Lane M: 100 bp ladder marker, Lane 9: DNase-free water.

3. Gene encoding CWA proteins of *S. pseudintermedius* isolates

A total of 23 *sps* gene profiles each consisting of 12 to 18 genes were found in the *S. pseudintermedius* isolates. The profile patterns of isolates from the different sources were diverse, with some specific patterns such as for environmental isolates containing a profile of all 18 *sps* genes (**Figure 8**). Isolates harboring 14 genes (lacking *spsD, spsF, spsP* and *spsQ*), were the most prevalent (22.2 %, 30/135), especially from the environment (n=17) and canine isolates (infected site: n=5; carriage site: n=7). The next most common profile comprised of 13 genes, without *spsD, spsF, spsO, spsP* and *spsQ* (19.3%, 26/135), particularly from dogs at both infected (n=7) and carriage sites (n=6), and from humans (n=9). The third most common profile was 15 genes, without *spsD, spsF*, and *spsO* (11.9%, 15/135), which was predominant in the isolates from infected (n=7) and carriage sites (n=2) in dogs.

Six genes were variably found, being *spsD* (23.0 %, 31/135), *spsF* (17.8%, 24/135), *spsI* (81.5%, 110/135), *spsO* (40.0 %, 54/135), *spsP* (40.0%, 54/135), and *spsQ* (40.0%, 54/135). The pair *spsP* and *spsQ* were always present together. The prevalence of these variably present genes among *S. pseudintermedius* isolates derived from dogs, humans and the environment was not statistically significant ($p < 0.05$), except for *spsP* and *spsQ* ($p = 0.048$) from carriage sites and infected sites of dogs, as showed in grey boxes in **Table 6**.

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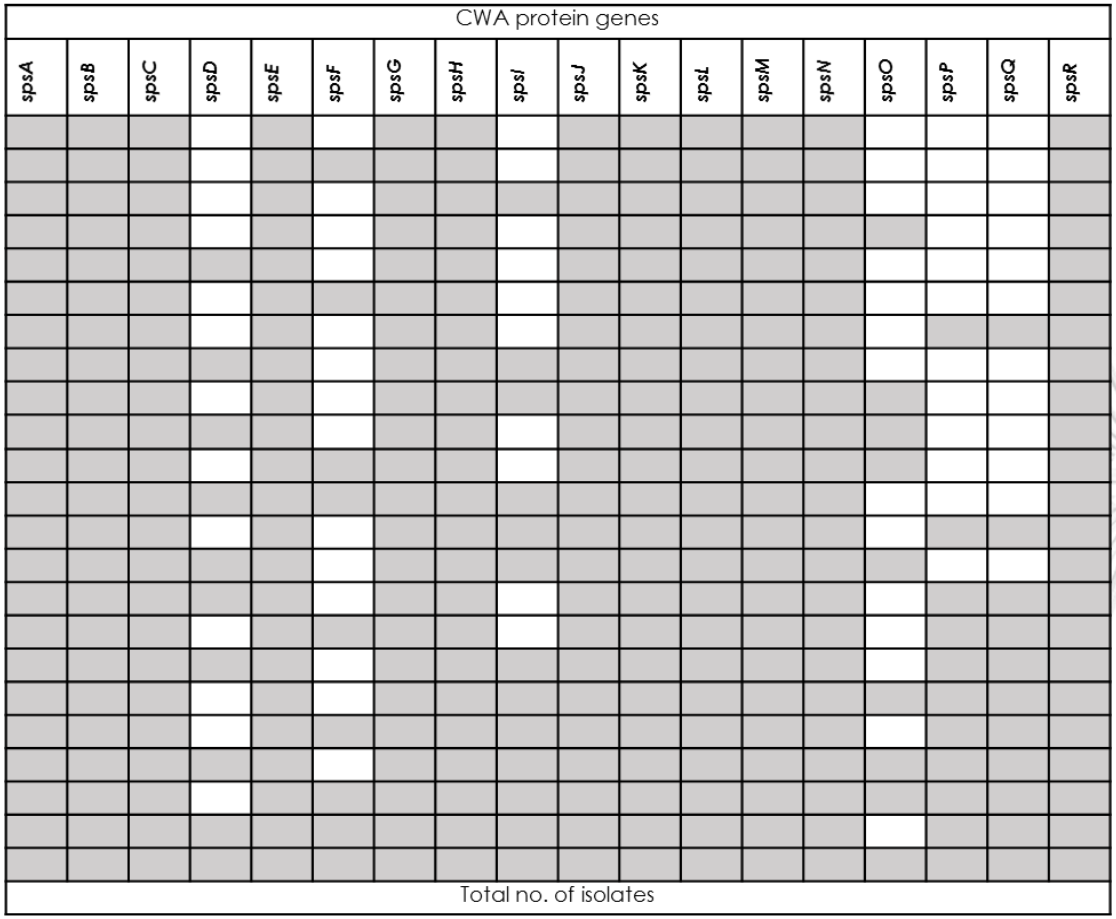


| No. of isolates | | | |
|---|---|---|---|
| Dog | Human | Environment | Total |
| 4 | 0 | 0 | 4 |
| 2 | 1 | 0 | 3 |
| 13 | 9 | 4 | 26 |
| 1 | 2 | 0 | 3 |
| 1 | 0 | 1 | 2 |
| 2 | 0 | 0 | 2 |
| 1 | 1 | 0 | 2 |
| 3 | 0 | 2 | 5 |
| 12 | 1 | 17 | 30 |
| 1 | 0 | 0 | 1 |
| 2 | 0 | 0 | 2 |
| 2 | 0 | 0 | 2 |
| 9 | 3 | 3 | 15 |
| 1 | 0 | 0 | 1 |
| 0 | 1 | 0 | 1 |
| 2 | 1 | 4 | 7 |
| 4 | 2 | 2 | 8 |
| 6 | 1 | 0 | 7 |
| 0 | 1 | 1 | 2 |
| 4 | 2 | 0 | 6 |
| 0 | 0 | 1 | 1 |
| 0 | 0 | 2 | 2 |
| 0 | 0 | 3 | 3 |
| 70 | 25 | 40 | 135 |

**Figure 8.** *S. pseudintermedius* surface (*sps*) genes profiles of *S. pseudintermedius* isolated from dogs, humans and the environment detected in this study. The white and grey boxes represent absence and presence of the gene, respectively.

**Table 6.** Percentage and numbers in parentheses of variable genes detected among *S. pseudintermedius* isolated from dogs, humans and the environment

| Genes | Dogs | | | Humans | Environment |
|---|---|---|---|---|---|
| | Carriage (n=35) | Clinical (n=35) | Total (n=70) | (n=25) | (n=40) |
| *spsD* | 28.6 (10) | 17.1 (6) | 24.3 (16) | 20.0 (5) | 25.0 (10) |
| *spsF* | 14.3 (5) | 14.3 (5) | 14.3 (10) | 12.0 (3) | 27.5 (11) |
| *spsI* | 80.0 (28) | 80.0 (28) | 80.0 (56) | 76.0 (19) | 87.5 (35) |
| *spsO* | 42.9 (15) | 34.3 (12) | 38.6 (27) | 24.0 (6) | 52.5 (21) |
| *spsP* | 25.7 (9) | 48.6 (17)* | 37.1 (26) | 48.0 (12) | 40.0 (16) |
| *spsQ* | 25.7 (9) | 48.6 (17)* | 37.1 (26) | 48.0 (12) | 40.0 (16) |

***** Statistically significant by $\chi^2$-test ($p$ <0.05) between carriage and clinical group of isolates

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## Discussion

In this study a rapid assay to detect 18 *sps* genes of *S. pseudintermedius* was successfully developed. This used 4 sets of mPCRs and had a high sensitivity. The mPCR method has many advantages over uniplex PCR, including being less time consuming, easy to perform, and more cost effective (Edwards and Gibbs, 1994). The 18 *sps* genes were selected for mPCR development based on their presence in the complete whole genome annotation of *S. pseudintermedius* ED99. This study examined the greatest number of putative *S. pseudintermedius* surface proteins analysed to date (Bannoehr et al., 2011). The mPCR assay could detect a very low concentration of DNA template (at least 1 pg/µl). In relation to specificity, it is important to be aware that certain genes were also found in members of the *S. intermedius* group, *S. delphini* and *S. intermedius* (Bannoehr et al., 2011), together with non-specific band products for certain non-*S. pseudintermedius* strains. However, these phenomena should not impact on detection since the process of isolation and identification must be performed prior to the mPCR, and all non-*S. pseudintermedius* strains were negative for the *nuc* gene, the internal control. The *nuc* gene is conserve and has a moderate diversity among members of the genus *Staphylococcus* that has proved useful for distinguishing and identifying coagulase positive *Staphylococcus* spp. (Sasaki et al., 2010). Moreover *S. intermedius* and *S. delphini* are mainly found in pigeons and are uncommon in dogs (Sasaki et al., 2010). Therefore, our mPCRs can be applied in research and clinical laboratory settings. Data about genes encoding CWA proteins of *S. pseudintermedius* may

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also support understanding of their role in pathogenesis and lead to new therapeutic strategies.

The distribution of the genes encoding CWA proteins was diverse, with various gene combinations generating 23 profiles from 135 *S. pseudintermedius* isolates. All isolates had at least 12 of the 18 genes, consisting of *spsA, spsB, spsC, spsE, spsG, spsH, spsJ, spsK, spsL, spsM, spsN,* and *spsR*. Previous reports have found different number of common genes, being 14/18 and 13/18, respectively (**Table 7**) (Bannoehr et al., 2011; Latronico et al., 2014). These 12 *sps* genes are suggested as being the essential genes representing a basic complement of cell wall-associated proteins. Overall there were three major gene profiles where each was detected in more than 10% of the population. In *S. aureus* differences in surface gene profiles also have been found, varying according to the strain, origin, sequence type, and detection tool used (Mirzaee et al., 2015). The variation in surface protein gene profiles might be a result of mobile genetic elements exchange in an ecological niche (Nienaber et al., 2011), for example *spsO* which is flanked by transposase genes was variably disseminated by horizontal gene transfer or deleted by recombination (Bannoehr et al., 2011). Regarding gene distribution, the six genes *spsD*, *spsF*, *spsI*, *spsO*, *spsP*, and *spsQ* were distributed at different but not statistically significantly different frequencies among isolates from dogs, humans and the environment. In addition, *spsF, spsO, spsP,* and *spsQ* also presented as variable genes in previous studies (Bannoehr et al., 2011; Latronico et al., 2014), with different prevalences (**Table 6**). Genes *spsP*, and *spsQ* were frequently found in isolates derived from infection sites of dogs, and this was consistent with

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previous findings (Garbacz et al., 2013). In addition, *spsP* and *spsQ* are tandemly arranged in the genome, which likely accounts for them being present together. *S. pseudintermedius* genes *spsP* and *spsQ* have been shown to have an orthologous conformation with *S. aureus spa*, encoding protein A (Bannoehr et al., 2011). It is speculated that the role of protein A in *S. pseudintermedius* infection maybe comparable to that of *S. aureus,* which involves evading the phagocytosis process by inactivating complement and blocking the Fc fragment of IgG (Peterson et al., 1977; Garbacz et al., 2013). Thus, strains containing *spsP* and *spsQ* may be better able to evade the immune response in canine skin infections. Not all clinical isolates harboured *spsP* and *spsQ*, however, and these isolates might have other virulent factors to assist them to maintain colonization, whilst skin defects also could facilitate infection (Peacock et al., 2002; Tristane et al., 2003; Nienaber et al., 2011).

No correlation was observed between *sps* gene profiles and host origin in this study. This confirms that no single gene or set of genes is responsible for host colonization or environment survival, as previously suggested in *S. aureus* models (Vancraeynest et al., 2004; Mirzaee et al., 2015). The diversity of genes encoding CWA proteins may not affect adherence to the host surface. Interestingly, the positive amplicons of *spsR* gene in three human isolates showed gene deletions that were similar to the sequence in *S. aureus* ST398 (Uhlemann et al., 2012). The variation and difference of surface protein genes between human and animal *S. aureus*, including deletions, insertions, and truncation or pseudogenes have been reported and suggested to differentially affect host-specific adaptation (McCarthy and Lindsay, 2010; Uhlemann et al.,

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2012). To demonstrate *S. pseudintermedius* adaptation to its host, other approaches such as adherence assays on host corneocytes and whole genome sequencing of additional isolates should be considered in future studies.

Since *S. pseudintermedius* ED99 was used as the only prototype available, there might be other missing surface protein genes that have never been assigned in *S. pseudintermedius,* and genes presenting polymorphic nucleotides may be a limitation in future studies.

In conclusion, a novel four panel mPCR set with satisfactory sensitivity and specificity was developed for rapid CWA protein gene detection in *S. pseudintermedius*. A total of 23 *sps* gene profiles were found in *S. pseudintermedius*, and these were not related to the origins of the isolates. The genes *spsP* and *spsQ* may be associated with pathogenicity of *S. pseudintermedius* in canine skin infection.

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**Table 7.** Prevalence of *sps* genes in *S. pseudintermedius*

| Gene | Current study (n=135) | The other studies | |
|---|---|---|---|
| | | Bannoehr et al., 2011 (n=20) | Latronico et al., 2014 (n=7) |
| *spsA* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsB* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsC* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsD*[a] | 23.0 (31/135) | 100 (20/20) | 71.4 (5/7) |
| *spsE* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsF*[a] | 17.7 (24/135) | 50.0 (10/20) | 71.4 (5/7) |
| *spsG* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsH* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsI*[a] | 81.5 (110/135) | 100 (20/20) | 100 (7/7) |
| *spsJ* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsK* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsL* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsM* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsN* | 100 (135/135) | 100 (20/20) | 100 (7/7) |
| *spsO*[a] | 40.0 (54/135) | 30.0 (6/20) | 28.6 (2/7) |
| *spsP*[a] | 40.0 (54/135) | 40.0 (8/20) | 57.1 (4/7) |
| *spsQ*[a] | 40.0 (54/135) | 60.0 (12/20) | 57.1 (4/7) |
| *spsR* | 100 (135/135) | 100 (20/20) | 100 (7/7) |

[a] Variable genes are highlighted in grey colour.

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## PART 2.2: ADHESION ABILITY OF MRSP TO CANINE AND HUMAN CORNEOCYTES

**TITLE:** Investigating the ability of methicillin-resistant *Staphylococcus pseudintermedius* isolates from different sources to adhere to canine and human corneocytes

### Introduction

This study part aimed to determine the adherence ability of five MRSP derived from different sources, and to look for association between the presence of genes for cell wall-associated (CWA) proteins and adherence. We focused on MRSP strains that commonly found on canine skin after treatment with antibiotics, and are emerging pathogens in human patients (Chanchaithong et al., 2014). MRSP colonize humans who have contact with dogs, and they can contaminate the environment in veterinary hospitals and households (van Duijkeren et al., 2011). The initial step in colonization and infection is adhesion to skin cells (corneocytes), which may be influenced by bacterial CWA proteins, encoded by *S. pseudintermedius* surface protein genes (*sps*) (Latronico et al., 2014). The ability of *S. pseudintermedius* to adhere both to canine and human corneocytes has been used as evidence for zoonotic potential (Woolley et al., 2008). Sequence type (ST) 71, a major European clone, has high adherence ability (Latronico et al., 2014); however, the adherence ability of individual strains may relate to their source and place of isolation. Thus, to estimate their pathogenic potential, their ability to adhere to human and canine corneocytes needs to be assessed. Moreover, the association between adherence and genes encoding CWA proteins is still not clear.

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## Materials and Methods

1. Bacterial isolates

Five MRSP isolates were selected from their sources, ST and *sps* gene profiles (**Table 8**). All of them were obtained from the culture collection at the Department of Microbiology, Veterinary sciences, Chulalongkorn University. They included two each from dogs (AP65-ST45 and MIC460-ST733) and the environment (SW1F4O1-ST45 and SW1F4O2-ST433), and one from a human (VB88-ST45). Their sequence type (ST) in multilocus sequence typing (MLST) and their profile of genes encoding CWA proteins (*sps*) previously had been established (Chanchaithong et al., 2014; Phumthanakorn et al., 2017).

**Table 8.** Information on MRSP strains used in this study

| Strain | Origin | Site | ST | SCC*mec* | No. of *sps* (18) and absent genes (*spsA-spsR*) |
|---|---|---|---|---|---|
| AP65 | Dog | Perineum | 45 | ΨSCC*mec*$_{57395}$ | 14 except *spsD, spsF, spsP,* and *spsQ* |
| VB88 | Human | Nasal cavity | 45 | ΨSCC*mec*$_{57395}$ | 14 except *spsD, spsF, spsP,* and *spsQ* |
| SW1F4O1 | Environment | Floor of dermatology unit | 45 | ΨSCC*mec*$_{57395}$ | 14 except *spsD, spsF, spsP,* and *spsQ* |
| SW1F4O2 | Environment | Floor of dermatology unit | 433 | Non-typeable | 18 |
| MIC460 | Dog | Pyoderma | 733 | V | 12 except *spsD, spsI, spsF, spsO, spsP,* and *spsQ* |

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2. Ethics approval

In this study, the corneocytes collection in dog and human were approved by Chulalongkorn University Animal Care and Use Protocol (CU-ACUP) no.1731009 (dated March 7, 2017) and the Research Ethics Review Committee for Research Involving Human Research Participants, Health Science Group, Chulalongkorn University no. 225.1/59 (dated February 9, 2017). The consent from and questionnaire were sent to dog's owner and volunteer before sampling.

3. Corneocytes collection

The corneocytes were collected from the abdominal area of five dogs, and from the medial biceps area of five volunteer humans who had little contact with dogs, as previously described (Saijonmaa-Koulumies and Lloyd, 2002; Latronico et al., 2014). All dogs and humans were clinically healthy and did not have a history of skin disease. Debris and normal microbiota were removed from the sampling sites by applying five successive adhesive tape strips (Scotch tape, 3M, USA), and then 22 mm medical adhesive discs (D-Squame, CuDerm Corporation, Dallas, USA) were placed on the skin to collect corneocytes. A total number of 24 discs were collected per individual dog and human at a single time and disc with influence corneocytes were selected for doing the experiment.

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4. Adherence assay

To test the reproducibility, the pilot study was done by randomized single bacterial strain and tested with one individual dog, and human corneocytes. The pilot assay was performed until no statistical difference

significant was found. The adherence assay was performed in duplicate with some minor modifications from previous protocols (Latronico et al., 2014). The MRSP isolates were grown in Brain Heart Infusion (BHI) broth and 25 ml of the culture at mid-exponential phase (14-16 h) was centrifuged at 1500 *g*, 4 ℃ for 5 min. The bacterial pellet was thrice washed with sterile phosphate buffered saline (PBS) and centrifuged at 800 *g*, 4 ℃, 10 min. The corneocyte discs were incubated with 500 µl of bacterial suspension for 45 min, and one disc from each individual was incubated with PBS as a negative control. After incubation, the discs were washed with PBS, stained with 0.5% crystal violet, and washed with water for 20 s of each steps. Ten fields (1000x) of confluent corneocytes per disc were examined for bacterial adhesion counts (BAC).

5. Statistical analysis

The Mann-Whitney *U* test was used to compare the adherence ability to canine and human corneocytes. Kruskal-Wallis test was used to find the difference between strains. The statistical analysis was performed using SPSS ver. 22 software (IBM, Armonk, USA).

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**Results**

1. Adhesion ability of 5 MRSP isolates

The three strains in ST45, the major clone in Thailand, from all sources adhered to both canine and human corneocytes significantly more than the two strains in ST733 and ST433 ($p<0.001$) (**Figure 9**). The isolates SW1F4O1-ST45 from the environment and VB88-ST45 from a human showed the greatest adhered to canine and human corneocytes, respectively (**Figure 9**).

Interestingly, AP65-ST45 and MIC460-ST733 from dogs showed significantly better adherence to human corneocytes than to canine corneocytes ($p$=0.007 and $p$<0.001), respectively. In addition, VB88-ST45 from a human showed significantly greater adhesion to human than to canine cells ($p$<0.001). The environmental isolates, SW1F4O1-ST45 and SW1F4O2-ST433 had no difference significant in adherence to both corneocytes.

2. Association with *sps* gene profiles

In this study, no positive association was found between adherence and the *sps* gene profiles, as SW1F4O2- ST433 with the highest number of sps (18/18) had the least adherence to both canine and human corneocytes (**Figure 9** and **Figure 10**). The clinical isolate MIC460-ST 733 harbouring the lowest number of *sps* gene (12/18) also showed the higher adhesion ability to both canine and human corneoctyes than SW1F4O2- ST433.

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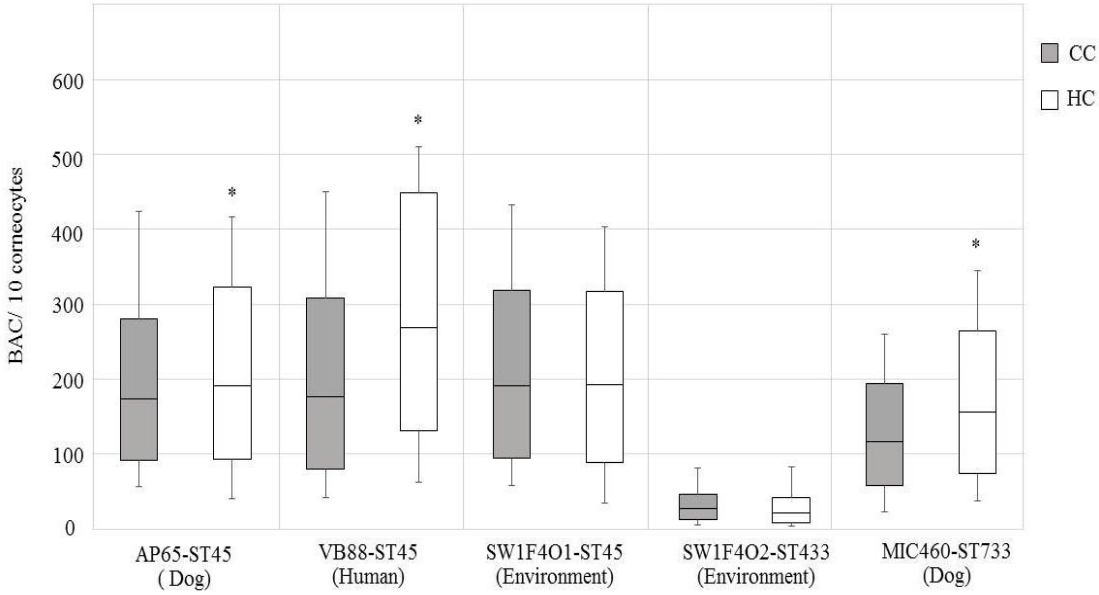


**Figure 9.** Boxplots represent bacterial adhesion counts (BAC) of 5 MRSP isolates to canine corneocytes (CC) and human corneocytes (HC) in this study. The top and bottom of the box represent lower quartile (Q1) and upper quartile (Q3). The line in the box is the median and the whisker is range of the data. *, statistically significant difference at *p*-value < 0.05

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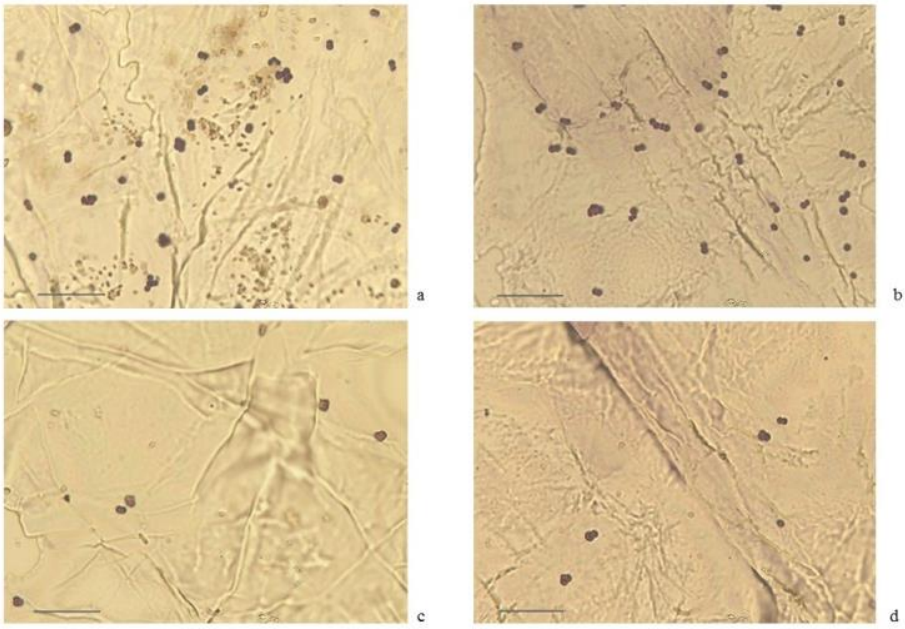


**Figure 10.** Illustration the adhesion of MRSP to canine and human corneocytes. (a) SW1F4O1-ST45 adhered to canine corneocytes, (b) VB88-ST45 adhered to human corneocytes, (c) and (d) SW1F4O2-ST433 adhered to canine and human coneocytes, respectively. Bar = 10 μm

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**Discussion**

The adherence assay is a reproducible and rapid method for quantitative measurement of staphylococci adherence to corneocytes (Lu and McEwan, 2007; Moodley et al., 2012). Nevertheless, it is difficult to perform this assay with a high number of isolates because it is required a large number of host corneocytes. The results showed that strains from the major Thai clone, ST45, had the greatest potential to adhere to canine and human corneocytes, reflecting the situation with the major European type, ST71. This suggests that strains in this ST are better adapted to attach to canine and human skin than are strains in other STs (Latronico et al., 2014). The three strains that showed greater adhesion to human than to canine corneocytes might have a greater ability to adapt to human corneocytes *in vivo* than other strains (Latronico et al., 2014). Interestingly, in this study no association was found between *sps* profiles and BAC. Bacterial colonization is a multifactorial process, therefore products encoded by other gene types may be require for host binding, as described in *S. aureus* (Fleury et al., 2017). A combination of *in vivo* experiments, whole genome comparisons and gene expression analysis using canine and human MRSP should provide more understanding of basic bacterial host adaptation (Moodley et al., 2012; Uhlemann et al., 2012).

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In summary, MRSP ST45 isolates from a dog, a human, and the environment adhered to both canine and human corneocytes. The clone type of MRSP may influence the initial stage of colonization and infection, but without a direct relationship to surface protein gene profiles.

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# CHAPTER III

## General Discussion

To investigate the distribution of virulence factors associated with bacterial adaptation and survival in different hosts, the detection of staphylococcal enterotoxin genes (SEs) and CWA protein genes in *S. pseudintermedius* were performed. This study was divided into 2 parts composed of detection of SE genes in MRSP isolates and CWA protein genes in isolates from dogs, humans, and environments. Moreover, the adhesion ability to canine and human corneocytes were determined.

The main finding of the first part was the higher detection of SE genes in MRSP isolated from humans than dogs and environmental isolates. The isolates in the same clone contained the different types and number of SE genes when were collected from different hosts and sources. The high prevalence of SE genes in the MRSP isolated from humans could be due to their horizontal transmission of mobile genetic elements (MGEs) from other commensal *Staphylococcus* sp. such as *S. aureus* and *S. epidermidis* in human nasal mucosal niche. The human isolates derived from veterinarians and dog's owners who could act as a transient or persistent carriers. The evidence of SE genes associated with horizontal gene transfer was previously reported in *S. epidermidis* pathogenicity islands (SePI) containing *sec* and *sel* from *S. aureus.* This finding also supported the increased risk of carrying highly pathogenic MRSP in human that might refer to ability of MRSP adaptation in human host. Nevertheless, SE

gene expression and their location in the genome are required in the further study.

The second part of this study was previously named as adhesin genes detection. However the function and structure of all *S. pseudintermedius* surface protein genes (*sps*) are not well elucidated. Therefore, it should be named as cell-wall associated protein genes or CWA protein genes instead of adhesins. The novel multiplex PCR (mPCRs) was constructed followed the principle guideline. The sensitivity and sensitivity were satisfied. The results revealed the diverse of 23 *sps* profiles compose of 12 common genes and 6 variable genes. There was no host specific profile in dogs, humans, and environmental isolates suggested as 12 genes were the essential genes representing a basic complement of cell wall-associated proteins. The higher prevalence of *spsP* and *spsQ* genes were found in canine clinical isolates with statistically significance. *spsP* and *spsQ* encoding Spa A protein may be associated with the pathogenicity of *S. pseudintermedius* in canine dermatitis. Interestingly, the 150- bp deletion in *spsR* of 3 human isolates were observed. This situation may be associated with different host colonization as well as the report in *S. aureus* ST398 (Uhlemann et al., 2012). However, it should be noted that this situation possibly found in the other *sps* genes and isolates. The further comprehensive investigation on whole genome sequencing base will be useful.

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Furthermore, the 5 representative MRSP strains were selected according to their *sps* profiles for adhesion ability assay. The bacterial adhesion to dermal cells is the adherence assay using corneocytes. Interestingly, the most common Thai clone type, ST45, had the greater ability to adhere to canine and human

corneocytes than others. The adhesion ability was not related with sources of isolate and *sps* profiles. The relationship with ST and the adhesion ability are still unknown (Latronico et al., 2014). The other gene types may be require for host cell binding and colonization. The further study of *sps* gene including expression, structure and function, and adhesion ability to other skin cell types should be elucidated. It will increase our understanding of pathogenesis leading to better management of *S. pseudintermedius* infection and control.

The relation of SE genes and CWA protein genes were attempted to describe in *S. aureus* (Peacock et al., 2002; Nienaber et al., 2011). Peacock and colleges demonstrated the combination of 7 virulence-associated factors in *S. aureus* isolates from invasive disease, including fibronectin binding protein A (*fnbA*), collagen adhesion protein (*cna*), serine-aspartate repeat proteins E (*sdrE*), intercellular adhesion protein (*ica*), exfoliative toxins (*eta*), and staphylococcal enterotoxin J (*sej*). Moreover, the relationship of 3 adhesins and 5 SE genes composed of clumping factor B (*clfB*), *cna*, and extracellular adherence protein (*map/eap*), and SE genes: *sea, sed, see, sei,* and *tst* were significantly higher in a group of *S. aureus* CC30 causing infective endocarditis than soft tissue infection isolates (Nienaber et al., 2011). Although the particular association of adhesins and SE genes were unknown, they were suggested to play a roles in pathogenesis and could be the representative biomarker genes for detection and prediction the severity of disease (Peacock et al., 2002; Nienaber et al., 2011). For this study, the isolates used in both parts of study were included and analyzed (**Table 9**). The association of specific SE genes and CWA protein genes among dogs, humans, and environmental sources were not observed. For example, strain AK9,

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AQ25, and G1TB1 collected from dog, human, and environment that contained single *seq* and different of *sps* genes combination of 15, 16, and 14 genes, consequently. Strain AP64, VB98, and P2F3B3 from dog, human, and environment had similar *sps* profiles (13/15 genes) but different in number and SE genes combination of 0, 9, and 0 gene, respectively. In addition, the adhesion ability was not related with virulence profiles as showed in 3 strains, AP65, VB88 ,and SW1F4O1 belonging to ST45 contained similar of *sps* profile but different SE genes. They had quite similar adhesion ability to canine and human corneocytes. The highly diversity of SE genes distributed in human isolates was effect this relation.

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**Table 9.** SE gene and CWA protein gene profiles of representative isolates used in study part 1 and 2

| Strain | Source | No. and type of SE genes | No. and type of CWA protein genes | Adhesion ability testing |
|---|---|---|---|---|
| **AP65** | Dog | 2: *seq, tst-1* | 14, except *spsD,* | Yes |
| **VB88** | Human | 8: *sea, sec, seg, sei, sek, sem, sen, seo* | *spsF, spsP,* and *spsQ* | Yes |
| **SW1F4O1** | Environment | none | | Yes |
| **AK9** | Dog | 1: *seq* | 15, except *spsD, spsF, spsO* | No |
| **AQ25** | Human | 1: *seq* | 16, except *spsD,* and *spsO* | No |
| **G1TB1** | Environment | 1: *seq* | 14, except *spsD, spsF, spsP,* and *spsQ* | No |
| **AP64** | Dog | none | 13, except *spsD,* | No |
| **VB98** | Human | 9: *sea, sec, seg, sei, sek, sem, sen, seo, tst-1* | *spsF, spsO, spsP,* and *spsQ* | No |
| **P2F3B3** | Environment | none | | No |

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This study had several limitation. The isolates used in each study were previously derived from different time period and places therefore the relation of isolates and sources were linked by STs. In general, it was difficult to derive *S. pseudintermedius* isolates from dog, human, and environment at the same time of collection. In addition, the isolates from human infection that possible be a good representative of host adaptation was not available.

In conclusion, SE genes associated with MGEs and CWA protein genes might play a role in different host colonization and adaptation of *S. pseudintermedius* especially in human. The presence of these virulence factors may reflect the pathogenicity of *S. pseudintermedius* from different hosts. This study provides the primary data of SE and CWA protein genes distributing among *S. pseudintermedius* isolates from dogs, humans, and environment. Further study such as whole genome sequencing analysis, virulence gene expression and surface protein binding assay is likely to expand the better understanding of its pathogenesis, adaptation, and evolution

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APPENDIX

## แบบฟอร์มการขออนุญาตเก็บตัวอย่างในสุนัขและคนที่ใช้ในวิทยานิพนธ์

**ใบอนุญาตการใช้ร่างกายสัตว์เพื่องานวิจัย**

งานวิจัยเรื่อง การเปรียบเทียบความสามารถในการเกาะติดเซลล์ corneocytes จากสุนัขและคน ของเชื้อ *Staphylococcus pseudintermedius* ที่แยกได้จาก สุนัข คน และสิ่งแวดล้อม

โดย นางสาวนทิตา ภูมิธนากรณ์ 5675309331

ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์

จุฬาลงกรณ์มหาวิทยาลัย

ข้าพเจ้า.....

ที่อยู่.....

.....

.....เบอร์โทรศัพท์.....

ขอลงนามในใบยินยอมการใช้ร่างกายสัตว์เพื่อการวิจัยทางสัตวแพทย์ เพื่อแสดงว่าข้าพเจ้าอนุญาตให้เก็บตัวอย่างจากผิวหนัง ของร่างกายสุนัข ชื่อ..... เพื่อให้นิสิตได้ใช้ศึกษาในงานวิจัย ตามที่เห็นสมควร

โดยทั้งนี้ข้าพเจ้าเข้าใจดีว่าคณาจารย์และนิสิตจะปฏิบัติตามจรรยาบรรณของสัตวแพทย์ในการทำวิจัย

ลงนาม .....

(.....)

เจ้าของสัตว์

วันที่.....

Chulalongkorn University

**หนังสือแสดงความยินยอมเข้าร่วมการวิจัย**

ทำที่.....

วันที่.....เดือน.....พ.ศ. .....

**เลขที่** ประชากรตัวอย่างหรือผู้มีส่วนร่วมในการวิจัย.....

ข้าพเจ้า ซึ่งได้ลงนามท้ายหนังสือนี้ ขอแสดงความยินยอมเข้าร่วมโครงการวิจัย

ชื่อโครงการวิจัย การตรวจหาและการกระจายตัวของกลุ่มยีนเอนเทอโรทอกซินและแอดฮีซินของเชื้อสแตฟฟิโลคอคคัส ซูดอินเทอร์มีเดียส ที่แยกได้จากสุนัข คน และสิ่งแวดล้อม

ชื่อผู้วิจัย นางสาวนทิตา ภูมิธนากรณ์ นิสิตป.เอก ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ที่อยู่ที่ติดต่อ .....

.....

..... โทรศัพท์ .....

ข้าพเจ้า **ได้รับทราบ**รายละเอียดเกี่ยวกับที่มาและวัตถุประสงค์ในการทำวิจัย รายละเอียดขั้นตอนต่างๆ ที่จะต้องปฏิบัติหรือได้รับการปฏิบัติ ความเสี่ยง/อันตราย และประโยชน์ซึ่งจะเกิดขึ้นจากการวิจัยเรื่องนี้ โดยได้อ่านรายละเอียดในเอกสารชี้แจงผู้เข้าร่วมการวิจัยโดยตลอด และ**ได้รับคำอธิบาย**จากผู้วิจัย **จนเข้าใจเป็นอย่างดี**แล้ว

ข้าพเจ้าจึง**สมัครใจ**เข้าร่วมในโครงการวิจัยนี้ ตามที่ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย โดยข้าพเจ้ายินยอมให้เก็บตัวอย่างจาก **ผิวหนังบริเวณแขนด้านใน** ด้วย **แผ่นกาวสำหรับเก็บตัวอย่างผิวหนังทางการแพทย์ขนาด** 22 **มิลลิเมตร** จำนวน 20 ชิ้น โดยเมื่อเสร็จสิ้นการวิจัยแล้วข้อมูลที่เกี่ยวข้องกับผู้มีส่วนร่วมในการวิจัยจะถูกทำลายอย่างเหมาะสม

ข้าพเจ้าได้รับคำรับรองว่า ผู้วิจัยจะปฏิบัติต่อข้าพเจ้าตามข้อมูลที่ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย และข้อมูลใดๆ ที่เกี่ยวข้องกับข้าพเจ้า ผู้วิจัยจะ**เก็บรักษาเป็นความลับ** โดยจะนำเสนอข้อมูลการวิจัยเป็นภาพรวมเท่านั้น ไม่มีข้อมูลใดในการรายงานที่จะนำไปสู่การระบุตัวข้าพเจ้า

**หากข้าพเจ้าไม่ได้รับการปฏิบัติตรงตามที่ได้ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย** ข้าพเจ้าสามารถร้องเรียนได้ที่คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย 254 อาคารจามจุรี 1 ชั้น 2 ถนนพญาไท เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์/โทรสาร 0-2218-3202

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ข้าพเจ้าได้ลงลายมือชื่อไว้เป็นสำคัญต่อหน้าพยาน ทั้งนี้ข้าพเจ้าได้รับสำเนาเอกสารชี้แจงผู้เข้าร่วมการวิจัย และสำเนาหนังสือแสดงความยินยอมไว้แล้ว

| | |
|---|---|
| ลงชื่อ..... | ลงชื่อ..... |
| (นางสาวนทิตา ภูมิธนากรณ์) | (.....) |
| ผู้วิจัยหลัก | ผู้มีส่วนร่วมในการวิจัย |
| ลงชื่อ..... | ลงชื่อ..... |
| (รศ.น.สพ.ดร. ณวีร์ ประภัสระกูล) | (.....) |
| อาจารย์ที่ปรึกษาวิทยานิพนธ์ | พยาน |

# VITA

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ACADEMIC PUBLICATIONS:

1. Phumthanakorn N, Chanchaithong P and Prapasarakul N. 2017. Development of a set of multiplex PCRs for detection of genes encoding cell wall-associated proteins in Staphylococcus pseudintermedius isolates from dogs, humans and the environment. Journal of Microbiological Methods 142: 90-95.

2. Phumthanakorn N, Fungwithaya P, Chanchaithong P, Prapasarakul N. 2018. Enterotoxin gene profile of methicillin-resistant Staphylococcus pseudintermedius isolates from dogs, humans, and the environment. Journal of Medical Microbiology 67: 866-873.

ACADEMIC PRESENTATIONS:

1. Poster presentation: Distribution and frequency of staphylococcal enterotoxins in Staphylococcus pseudintermedius isolated from dogs, humans, and environment, 14th Chulalongkorn University Veterinary Conference (CUVC 2015)

2. Oral presentation: Enterotoxin gene profiles of Staphylococcus pseudintermedius from canine origin in Thailand, VPAT Regional Veterinary Congress (VRVC 2015)

3. Poster presentation: Diversion of enterotoxin gene profiles in a homologous clone of methicillin-resistant Staphylococcus pseudintermedius from dogs, humans, and environment, 15th Chulalongkorn University Veterinary Conference (CUVC 2016)

4. Oral presentation: Development of a multiplex PCR for adhesin genes detectionof Staphylococcus pseudintermedius isolated from carriage and infected sites of dogs, 16th Chulalongkorn University Veterinary Conference (CUVC 2017)

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