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 [1]. Methicillin-resistant *S. pseudintermedius* (MRSP) are believed to arise from clonal selection after routine antibiotic use, and have increasingly been reported worldwide [2]. Moreover, *S. pseudintermedius* can transiently colonize humans, such as veterinarians and others who are closely associated with dogs [1, 3], and can occasionally cause nosocomial infections, such as sinusitis, soft tissue infection and endocarditis in humans [4–6]. 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 [7–9]. 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 dogs with MRSP or methicillin-sensitive *S. pseudintermedius* (MSSP) and canine mortality and bacterial virulence [10]. *S. pseudintermedius* isolates from both healthy and clinically affected individuals harbour a variety of specific virulence genes, including leukocidin genes (*lukS*-I and *lukF*-I), the exfoliative gene (*siet*), exfoliative toxins of *S. pseudintermedius* (*expA* and *expB*) and the *Streptococcus intermedius* enterotoxin gene (*si-ent*) [11–16]. In addition, a variety of other staphylococcal enterotoxin (SE) genes exist, which vary between bacterial species and strains [17]. To date, fewer types and less variety have been described amongst *S. pseudintermedius* SE genes than have been found in *Staphylococcus aureus* [18]. Furthermore, knowledge about SEs in *S. pseudintermedius* that may relate to pathogenicity is still limited; the SE gene family products

### Abstract

**Purpose.** This study aimed to detect and identify staphylococcal enterotoxin (SE) genes in methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) strains from different sources, and to investigate the relationship between their sequence types (STs) and SE gene patterns.

**Methodology.** The profiles of 17 SE genes in 93 MRSP strains isolated from dogs (*n*=43), humans (*n*=18) and the environment (*n*=32) were detected by PCR. Multilocus sequence typing (MLST), SCCmec typing and pulsed-field gel electrophoresis (PFGE) were used to analyse the clonal relatedness between the molecular type and SE gene profiles.

**Results/Key findings.** The human MRSP strains harboured the greatest number of SE genes (12/17; *sea, sec, seq, sei, sek, sel, sem, sen, sea, sep, seq* and *tst-1*) compared to those from dogs (5/17; *sec, sel, sem, seq* and *tst-1*) and environmental sources (3/17; *sec, seq* and *tst-1*). Using MLST and PFGE, different SE gene profiles were found within the same clonal type.

**Conclusion.** We show that isolates of MRSP vary in their virulence gene profiles, depending on the source from which they have been isolated. This insight should encourage the development of appropriate monitoring and mitigation strategies to reduce the transmission of MRSP in veterinary hospitals and households.
produced by human isolates of *S. pseudintermedius*, known as superantigens (e.g. SEA, SEB, SEC and TSST-1) [19], are involved in food poisoning and anaphylactic shock associated with sepsicaemia, but little else is known.

Most studies to date have focused on the genetic characterization and antibiotic resistance profiles (antibiogram) of MRSP, but there have been few studies on the molecular epidemiology and ecology of MRSP from dogs, their owners and household or animal hospital environments. A previous study demonstrated that MRSP, but there have been few studies on the molecular epidemiology and ecology of MRSP from dogs, their owners and household or animal hospital environments. A previous study 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 [20, 21]. 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 [3, 6, 22]. *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 fingerprints and staphylococcal cassette chromosome mec (SCCmec) types of MRSP from different host or environmental origins were analysed, together with their SE profiles.

**METHODS**

**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 the 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 [21]. 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. The methods and criteria for environment sample collection were similar to those previously described [9]. The isolates were classified into their species by biochemical tests [23] and multiplex PCR for the *nuc* gene [24]. The methicillin resistance trait was classified by the oxacillin disk diffusion method [25] and *mecA* detection [26].

**DNA extraction**

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

**Detection of SE genes**

A total of 17 SE genes (*sea, seb, sec, sed, sei, sej, sek, sel, sem, sen, sep, seq, ser and tst-1*) were detected by multiplex PCR [27], and single PCR was used for *sea* with some modifications [28]. The 50 µl PCR mixture contained 1× reaction buffer, 200 µM dNTP, 1.5 mM MgCl₂, 2.5 U of Taq polymerase (Promega), 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) 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 pyrosequencing 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 the positive genes are KP659467 (*sea*), KP659468 (*sec*), KP659469 (*sei*), KP659470 (*seb*), KP659471 (*sem*), KP659472 (*seq*), KP659473 (*sei*), KP659474 (*ser*), LC020109 (*sea*), LC020110 (*seq*), LC020111 (*ser*), LC020112 (*sec*), LC020113 (*ser*), LC020114 (*seq*), LC020115 (*sea*), LC029795 (*sed*), LC209796 (*sei*) and LC209797 (*ser*). Laboratory *S. aureus* strain 77.3, which was 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 the Supplementary Material (Table S1, available in the online version of this article).

**SCCmec typing and multilocus sequence typing (MLST)**

The isolates from the environment with no SCCmec type and sequence type (ST) were subjected to SCCmec typing by the PCR method [29, 30]. The nontypeable SCCmec isolates were further identified using long-range PCR and restriction enzyme analysis with BSU-36I (New England Biolabs) for *v*SCCmec57395 [31]. MLST using the scheme for seven genes was performed as previously described [32]. The sequences of the housekeeping genes were analysed and used to define the ST in the *S. pseudintermedius* MLST database (https://pubmlst.org/spseudintermedius/).

**Pulsed-field gel electrophoresis (PFGE)**

A total of 18 MRSP ST45-4 V SCCmec57395 were randomly selected for PFGE analysis using restriction enzyme Cfr91 (Thermo Fisher Scientific). 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 MRSP ST182-V isolates that were shared by dogs (\(n=3\)) and the environment (\(n=4\)) were included in the PFGE analysis.

PFGE was performed following the previously published protocol from the Centers for Disease Control and Prevention [33, 34], with the minor modification of adding lysostaphin (1 mg ml\(^{-1}\)) (Sigma-Aldrich) for the lysis of cells in agarose plugs. Separation of the restricted DNA fragments was achieved using 6 V cm\(^{-1}\) 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) [20]. The genetic relatedness of the strains was analysed by dendrogram construction using unweighted pair group with mathematical average (UPGMA) in the GeneDirectory software (InGenius3 Syngene) and setting a position tolerance of 1.5 %.

### Table 1. Information on the *S. pseudintermedius* isolates and their SE gene profiles

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<th>SE genes profile</th>
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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 five isolates. For higher frequencies of occurrence, the χ²-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).

RESULTS
SE gene detection
Of the 17 SE genes, 12 (sea, sec, seq, sei, sek, sel, sem, sen, seo, seq, 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 1). 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 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 11 SE genes detected in human MRSP were statistically significantly more common there than they were in 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 strains from all three sources, while sek, sel and sem were found in MRSP from dogs and humans, but not in those from the environment.

Clonal relatedness between molecular type and SE gene profiles
To analyse the role of the host and source, the clonal relatedness of 46 MRSPs from 6 STs that harboured different SE gene profiles was examined. MRSP ST45-ψ SCCmec57395 was the only ST shared amongst the three isolation sources. Cluster analysis by PFGE showed 17 distinct electrophoretic types (A–Q) that were mostly in concordance with their ST and SCCmec type (Fig. 1). Interestingly, five MRSP isolates from the environment that belonged to ST45-ψ SCCmec57395 with few or no SE genes were distinguishable from the ST45-ψ SCCmec57395 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, the MRSP ST 182 V shared by isolates from dogs and the environment were grouped in the same PFGE group (group Q), and their SE gene profiles were quite similar.

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 clonal 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 of the MRSP their genotypic characterization corresponded to their pathogenic potential in relation to their recovery from lesions. Even though this study only evaluated a limited number of MRSP isolates (n=93), all had a potential connection between humans (owner or veterinarian) and dogs through their proximity and co-exposure within the hospital.

The SE gene profiles of the human MRSP strains showed a higher variation in frequency and type compared with previous studies on canine S. pseudintermedius [12, 35, 36]. With respect to the variation in the SE profiles, geographical and strain differences were probably 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). The high frequency and types of SE genes in the MRSP isolated from humans were possibly the result of their horizontal transmission via mobile genetic elements (MGEs) from other bacteria, especially S. aureus and coagulase-negative staphylococci (CoNS) [37]. S. pseudintermedius and/or MRSP may be either transient or persistent in the human nasal cavity, and therefore co-colonization and gene transfer may occur in this niche [38]. The presence of MGEs, e.g. S. aureus pathogenicity islands (SaPIs) encoding superantigen, has been shown to be involved in intra- and interspecies transfer in S. aureus [39–41]. This phenomenon could mimic that associated with human S. epidermidis strain FR909, which acquired the S. epidermidis pathogenicity islands (SePi) containing sec and sel from S. aureus [42]. The process of SE acquisition in this SePI is still poorly understood and the transfer is assumed to have been via a bacteriophage [42]. Certain genes, such as sea, sek and seq, are simultaneously located in the same S. aureus bacteriophages, ΦSa3ms and ΦSa3mW [19]. In our study, sea, sek and seq, and sek, seq and tst-1, were detected in human isolates, and these might also 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 [43]. 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 [43]. This study also detected some SE genes that are regarded as non-mobile genes on genomic islands, e.g. sei, seq and sem [14], as a previous study had [19]. The presence of SEs and their roles in other staphylococci are not known, since they have
been studied less, and their horizontal transfer has not been proved [42]. The properties of SE genes and MGEs in *S. pseudintermedius* from different hosts still need to be investigated.

In this study, *seq* was the SE gene that was found most often from the three sources. This gene encodes a non-emetic toxin and is located on a bacteriophage or a staphylococcal pathogenic island (SaPI) [19]. The expression of *seq* is found in every *S. aureus* growth phase and might be associated with the bacteriophage life cycle [44]. However, the biological activity of the *seq* of *S. pseudintermedius* is still not known. On the other hand, enterotoxin type C, and variant canine (*sec*canine) in particular, is believed to be a common specific SE gene of *S. pseudintermedius* isolates from dogs, especially those from pyoderma lesions [12]. In contrast, this study found a low prevalence of *sec* (16.3 %), represented by a single canine isolate (1.1 %; 1/43) derived from the perineum (i.e. not recovered from a lesion). This low prevalence may due to differences in geographical area and/or anatomical site, and in our study isolates with the *sec* gene were not necessarily specific to a canine source. The *sec* gene has also been detected in canine and human strains of *S. intermedius* and *S. aureus* with 95–97 % sequence homology, and it has been divided into at least four subtypes based on the nucleotide sequence: *sec*canine sequence and *sec*1–3 variant sequences [45]. We found one different variant of *sec* in human strain VB16, whereas the other sources were identical for *sec*canine (Fig. 2). The association of this finding with *S. pseudintermedius* adaptation in the human host needs to be clarified in future studies.

**Fig. 1.** Dendrogram of 46 MRSP strains isolated from dogs, humans and the environment showing the PFGE cluster, ST, SCCmec type and SE gene profiles. For PFGE, the cluster was grouped using UPGMA with a similarity coefficient with a position tolerance of 1.5 % and ≥80 % cut-off. D, H and E represent dogs, humans and the environment, respectively. * and †, isolates derived from the same household.
In contrast to the case of *S. aureus*, the roles of SEs in *S. pseudintermedius* have not been elucidated. Although there have been many studies on SEs and superantigens, it is not clear why *S. aureus* possesses a large number and diversity of SEs [46]. The presence of SE genes is suggested to be related to immune evasion, as they function as an immunomodulator [47], but without a high fitness cost to the bacteria, because most are encoded on mobile genetic elements that do not persist in the bacteria [46]. These genes are expressed in different growth phases of bacteria, with and without the regulation of the enterotoxin gene cluster (egt) operon and agr system [17, 44]. 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 [17]. In our study, the expression of SE genes was not studied.

MRSP ST45-ψ SCCmeC57395 has been shown to be the most frequent clonal type in Thailand [21], and this was found in humans, dogs, and the hospital environment. Thus, we analysed this major type, together with the other five minor STs that were shared between dogs and humans, or dogs and the environment. The canine isolates appeared to be more diverse (ST) than the human isolates, with human-associated MRSP lineages being dispersed in five STs and five PFGE types. This is consistent with PFGE grouping having greater discriminatory power than ST grouping in MLST analysis [48]. MRSP clonal types ST45, 112, 178, 181, which were all isolated from the nares of veterinarians, contained more virulence genes than the other sources, except for strains AQ25 and AQ30 from ST183, which were isolated from owners. Thus, veterinarians appear to have an increased risk of carrying highly pathogenic MRSP. The canine-associated MRSP lineages and those isolated from the environment had few or no SE genes. It is speculated that the sources of isolates might influence the findings.

In this study, the exact origin of all environmental isolates could not be established. It can be assumed that these groups of isolates may be contaminants from dogs (ST182-PFGE type Q) rather than from humans, 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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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The collection of isolates from dogs and humans was previously approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC) and the Ethical Review Committee for Research Involving Human research Subjects, Health Science Group, Chulalongkorn University (08/1/54).

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