



ERIC-PCR fingerprint profiling and genetic diversity of coagulase negative *Staphylococcus* in Malaysia

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ABSTRACT

Aims: The coagulase-negative staphylococci (CoNS) are a group of *Staphylococcus* that is gaining clinical significance as major agents of nosocomial infections, especially amongst neonates and immuno-compromised patients. The identification of CoNS remains problematic, and there has been little information on their molecular genotyping. The overall aim of this study was to evaluate Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) as a rapid and cost-effective tool for the genotyping of CoNS isolates from within a hospital setting.

Methodology and results: A total of 200 isolates of CoNS were collected from Hospital Tuanku Ampuan Rahimah, Klang, Malaysia and identified via *sodA* gene sequence analysis. Genetic diversity among the isolates was evaluated using the ERIC-PCR. The most frequently isolated species was *S. epidermidis* (37%) followed by *S. haemolyticus* (30%), *S. hominis* (18%) and *S. capitis* (8.5%). ERIC-PCR was found to be efficient for the differentiation of *S. hominis* isolates with a discriminatory index (*DI*) of 0.949 and satisfactory for *S. epidermidis* isolates at *DI* of 0.808. Poor discriminatory power was observed in *S. haemolyticus* (0.377) and *S. capitis* (0.111). The majority of the *S. haemolyticus* and *S. capitis* isolates were found to be genetically homogenous which imply that the source of these infections are due to hospital-derived contaminants. In contrast, the *S. epidermidis* and *S. hominis* strains displayed high genetic diversity suggesting the presence of different endemic strains and inflow of exogenous strains brought in by non-local residents.

Conclusion, significance and impact of study: ERIC-PCR is a useful tool to differentiate and track selected species of CoNS.

Keywords: Diversity, identification, fingerprinting, genotyping

INTRODUCTION

The CoNS are a group of *Staphylococcus* which are distinguished from the more virulent *S. aureus* by their inability to coagulate plasma. To date, more than 40 species of CoNS have been identified (Parte, 2014). CoNS were once considered to be non-pathogenic and have long been dismissed as culture contaminants since they are normal inhabitants of human skin and mucous membranes. The potential pathogenicity of CoNS in human was first reported in 1958, but it was only in the 1970s that these organisms gained prominence as agents of clinically significant infections, particularly in nosocomial infections among immune-compromised patients and premature newborns (Piette and Verschraegen, 2009). In the USA, data collected through the National Nosocomial Infections Surveillance (NNIS) system revealed that CoNS accounted for 36% of all bloodstream isolates in medical intensive care units

(Richards *et al.*, 1999a) and 38% from the paediatric intensive care units (Richards *et al.*, 1999b) making these organisms the most common cause of nosocomial bloodstream infections. In England and Wales, a study over a 10-year period reported that, at a prevalence of 28%, CoNS are the most frequent causative agents isolated from bacteraemia patients (Henderson *et al.*, 2010). In most cases, the infection by CoNS have been associated with medical devices such as prosthetic valves, cerebrospinal fluid shunts and intravascular, urinary and dialysis catheters, due to their ability to form a biofilm structure on the surfaces of these devices (Mack *et al.*, 2006).

The increasing importance of CoNS as opportunistic pathogens in hospitals has led to substantial interest in their molecular epidemiology. As CoNS are common skin commensals, an outbreak could be due to either an endemic strain, or a recently-introduced strain from a new patient. Distinguishing this is important, as each case

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requires a different management strategy. Molecular variability, in the form of genomic fingerprints may also be correlated with pathogenic profiles, providing a useful diagnostic tool for differentiating and tracking virulent strains. For genotyping purposes, pulse-field gel electrophoresis (PFGE) has always been considered as the gold standard. A number of PCR- fingerprinting techniques have also been employed for whole genome typing of bacterial species, including ERIC-PCR, Box-PCR and AFLP analysis. The PCR-based genotyping methods have the advantages for being simpler, fast and more cost effective as compared to PFGE and MLST, with good discriminatory power (Nath *et al.*, 2010; Yuan *et al.*, 2010).

MATERIALS AND METHODS

A total of 200 CoNS isolates were collected from the Pathology Lab of Hospital Tuanku Ampuan Rahimah, Klang, Malaysia in 2011. These isolates were recovered on Mannitol Salt Agar (MSA) from various clinical samples including 139 (69.5%) from blood specimen, 31 (15.5%) from the respiratory tract specimen, 21 (10.5%) from pus while the remaining 9 (4.5%) isolates were from urine and other body fluids.

Identification of the CoNS isolates

Identification of the 200 CoNS isolates was performed via both phenotypic and genotypic method. Biochemical identification to the species level was performed using the Microgen Staph ID (UK) kit as according to the manufacturer's instructions. A probability score of $\geq 80\%$ was considered a good identification. The identities of some of the strains with probability scores below 80% were further confirmed through analysis of the *sodA* gene sequence (Abdul-Aziz, 2015).

Amplification of the *sodA* gene sequence

Sequence-based identification was performed using the highly conserved sequence of the *sodA* gene. Genomic DNA of each of the isolates was extracted using a DNeasy Blood & Tissue Kit (Qiagen, USA). Degenerated primers d1 (5-CCITAYICITAYGAYGCIYTIGARCC-3) and d2 (5-ARRTARTAIGCRTGYTCCCAIACRTC-3) were used to amplify a fragment which represents approximately 83% of the *sodA* gene (Poyart *et al.*, 2001).

The PCR reaction mix was prepared using Gotaq Flexi kit (Promega) in a total volume of 50 μL containing : 1 \times amplification buffer, 1 μL of 1 μM of each deoxynucleoside triphosphate, 1.5 mM MgCl_2 solution 0.5 μM of each primer, 0.5 U of Go-Taq DNA polymerase (Promega), and 750 ng of genomic DNA. Amplification was performed in an Eppendorf Mastercycler with an initial denaturation cycle for 3 min at 95 $^\circ\text{C}$; followed by 30 cycles of the following: 60 sec of annealing at 37 $^\circ\text{C}$, 45 sec of elongation at 72 $^\circ\text{C}$, and 30 sec of denaturation at 95 $^\circ\text{C}$. DNA sequencing was performed by a third-party service provider. The DNA sequences of the *sodA* gene

were matched against sequences in the NCBI database using the BLAST function. The match with the highest percentage of similarity (with a minimum of 99% similarity and 99% coverage) was considered a good identification.

ERIC-PCR

ERIC-PCR fingerprinting for all the 200 clinical isolates was performed using the primers ERIC1: 5'-ATGTAAGCTCCTGGGGATTAC-3' and ERIC2: 5'-AAGTAAGTACTGGGGTGAGCG-3' (Versalovic *et al.*, 1991). The reference strains used in this experiment are as listed in Table 1. The PCR reaction mixture contains 1 \times Go-Taq Flexi amplification buffer, 750 ng of DNA as template, 1.5 μM of each primer, 2 μM concentration of each deoxynucleoside triphosphate, 0.5 U of Go-Taq DNA polymerase (Promega) and 3.0 mM MgCl_2 in a final volume of 50 μL . The cycling conditions begins with an initial denaturation step (95 $^\circ\text{C}$ for 7 min) followed by 30 cycles of denaturation (94 $^\circ\text{C}$ for 1 min), annealing (52 $^\circ\text{C}$ for 1 min), extension (65 $^\circ\text{C}$ for 8 min) and a final extension step (65 $^\circ\text{C}$ for 16 min). All PCR products were resolved by electrophoresis at room temperature on a 1.2% agarose at 80V for 80 min.

Table 1: Lists of reference strains used in ERIC-PCR genotyping.

<i>Staphylococcus</i> species	Reference strains
<i>S. capitis</i> subsp. <i>capitis</i>	ATCC 27840
<i>S. cohnii</i> subsp. <i>urealyticum</i>	ATCC 49330
<i>S. cohnii</i> subsp. <i>cohnii</i>	ATCC 29974
<i>S. epidermidis</i>	ATCC 12228
<i>S. epidermidis</i>	ATCC 35984
<i>S. haemolyticus</i>	ATCC 29970
<i>S. hominis</i> subsp. <i>hominis</i>	ATCC 27844
<i>S. hominis</i> subsp. <i>novobiosepticus</i>	ATCC 700236
<i>S. lugdenensis</i>	ATCC 700328
<i>S. saprophyticus</i>	ATCC 49907
<i>S. sciuri</i>	ATCC 29062

The gels images were analyzed using the open-source software GelAnalyzer2010a (www.gelanalyzer.com). Band sizing was performed using a 100 bp DNA ladder as the molecular size standard after correcting for *Rf* migration curves. For each gel, the ATCC reference strain for the species under investigation was included in at least one lane. For cluster analysis, the open-source software FREETREE (Hampl *et al.*, 2001) was used. A reference tree dendrogram was constructed using UPGMA method and visualized using TREEVIEW (Page, 1996). The isolates were clustered using a baseline of 80% similarity.

To evaluate if ERIC-PCR fingerprinting provides Sufficient discriminative between different genetics clones, the Discriminative Index (*DI*) was calculated by applying the Simpson's Index of Diversity equation as follows (Hunter and Gaston, 1988);

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^S n_j(n_j-1)$$

where N is the total number of isolates in the sample, S is the total number of types described and n_j is the number of isolates belonging to the j th type. A DI value of >0.90 is considered discriminative.

RESULTS

Distribution of the 200 clinical isolates of CoNS species

In total, 12 species and subspecies of CoNS were isolated as shown in Figure 1. The predominant species was *S. epidermidis* with 74 isolates (37%) followed by *S. haemolyticus* with 60 isolates (30%), *S. hominis* with 36 isolates (18%) and *S. capitis* with 17 isolates (8.5%). *Staphylococcus hominis* were further divided to two subspecies, 30 isolates of *S. hominis* subsp. *hominis* and six of *S. hominis* subsp. *novobiosepticus*. Of the 17 isolates of *S. capitis*, 14 were *S. capitis* subsp. *capitis* and three *S. capitis* subsp. *ureolyticus*. Other isolates include *S. lugdenensis*, *S. saprophyticus* subsp. *saprophyticus*, *S. sciuri* and *S. warneri*.

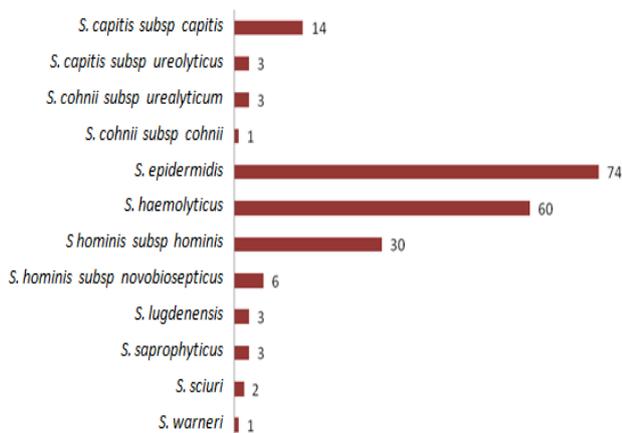


Figure 1: Distribution of clinical isolates of CoNS isolated from patients in Hospital Tuanku Ampuan Rahimah, Klang, Malaysia. Figure showing the distribution of CoNS clinical isolates as indicated by the number of isolates for each of the staphylococcal species.

ERIC-PCR Banding Patterns

Figure 2 shows a composite picture of representative banding patterns from the different species including the ATCC reference strains. The number of bands varied from a minimum of three to a maximum of 16 bands across the isolates. Common features include two bands of 510 and 570 bp in size which were predominant in the majority of the strains. Another predominant band of about 3000 bp was also observed in many of the species.

Comparatively, *S. haemolyticus* has the most complicated ERIC banding pattern, while *S. epidermidis* has the simplest.

ERIC-PCR fingerprints

The gel image of ERIC-PCR fingerprints from *S. capitis* is shown in Figure 3a along with the *S. capitis* subsp. *capitis* reference strain ATCC 27840. The presence of two intense bands with molecular sizes of 570 and 960 bp were observed in all the isolates. Also, with the exception of a strain in lane No. 7, all of the *S. capitis* isolates has an additional intensely-stained band with the size of 510 bp. Cluster analysis of the ERIC-PCR profile displayed in Figure 4 indicates that the *S. capitis* subsp. *capitis* ATCC 24780 strain is genetically distant to the local clinical isolates of similar species, with a low similarity of slightly more than 60% only. On the other hand, all the local isolates appear to be genetically similar to each other when clustered using a similarity value of 80%.

A subset of ERIC-PCR fingerprints from *S. epidermidis* isolates is shown in Figure 3b. The *S. epidermidis* isolates displayed considerable variability in their band patterns. A group of four bands with size of 570, 510, 410 and 280 bp is present in almost all isolates and serves as a distinctive feature for *S. epidermidis*. Cluster analysis resulted in 11 clusters at a similarity value of at least 80% (Figure 5). Cluster 1 comprises about 40% of the isolates and includes the ATCC 35984 strain. This cluster is characterised by the presence of five to six bands, very similar to the reference strain. Clusters II and III include isolates with seven to eight bands similar to another reference strain, ATCC 12228, while members of Clusters IV, V and XI lack the higher molecular size bands. In addition, cluster VI to X were distinguished by the presence of a variety of higher molecular size bands.

ERIC-PCR fingerprints from *S. haemolyticus* is shown in Figure 3c. The ATCC 29970 *S. haemolyticus* reference strain produced 13 bands that can be clearly scored. Three bands with sizes of 3050, 1140 and 720 bp were intensely stained and present in the majority of the isolates with two other bands of approximately 280 and 220 bp. Most of the variations among the *S. haemolyticus* isolates were attributable to bands of higher molecular size. Despite the seemingly complicated patterns, only four clusters can be identified when the minimum similarity level of 80% was applied. The cluster analysis of *S. haemolyticus* (Figure 6) also indicates that the majority of the isolates were genetically similar at $\geq 80\%$ similarity level. Hence, the majority of the *S. haemolyticus* isolates appears to be clonal and are probably the result of hospital-derived contaminants which caused opportunistic infections in the patients.

The *S. hominis* subsp. *novobiosepticus* ATCC 700236 strain produced ten bands while *S. hominis* subsp. *hominis* ATCC 27844 strain displayed only nine bands (Figure 3d) as the 2230 bp band was not present. Both the 2830 and 400 bp bands were present in all isolates of *S. hominis*. The number of total bands observed in each

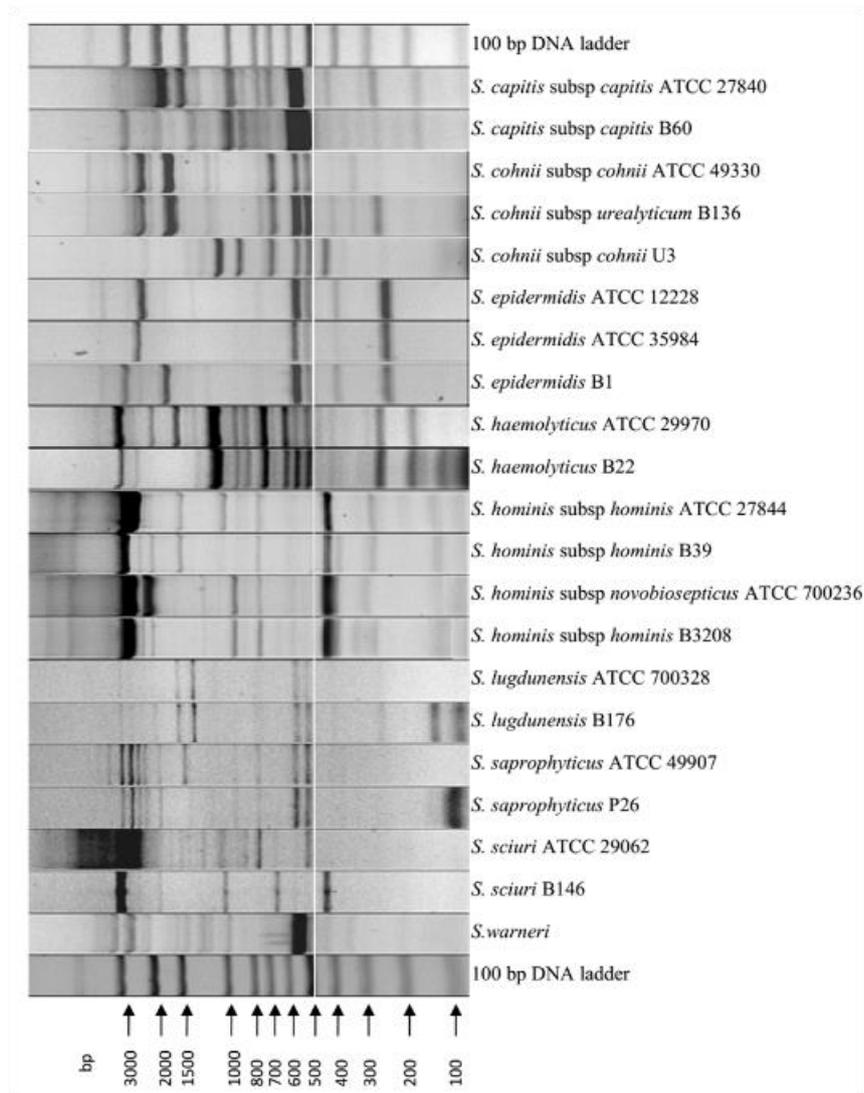


Figure 2: Composite gel image of ERIC-PCR patterns for selected species of CoNS. Gel images of representative ERIC-PCR banding patterns from the clinical isolates and reference strains of CoNS. Each species have a characteristic ERIC-PCR pattern that can be used to distinguish it from other species in this study. The vertical line indicates the position of the 500 bp fragment.

isolates ranges between three to eleven bands. Although the number is small, the variation amongst isolates is surprisingly high where most of the variations were attributable to the higher molecular size bands. Cluster analysis of the ERIC-PCR patterns resulted in ten clusters at a similarity cut-off level of 80%, as displayed in Figure 7. If a less stringent criterion of 75% similarity is applied, clusters IA, IB and IC will be merged as one major cluster and clusters IIA and IIB can be merged as a single cluster II. Both these clusters I and II will account for >50% of the isolates. The remaining five clusters are relatively minor consisting of four members at most. Both the ATCC reference strains are included in cluster IV. Thus the *S.*

hominis isolate displayed a considerable amount of variation at the genetic level.

ERIC-PCR as a genotyping tool for CoNS

An index of greater than 0.90 would be considered necessary if the typing results are to be interpreted with confidence (Hunter and Gaston, 1988). The *DI* for each of the staphylococcal species were 0.111, 0.808, 0.377 and 0.949 respectively for *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. hominis* indicating that ERIC-PCR have low discriminative power (<0.9) among the isolates except in *S. hominis*. The *DI* for other isolates were not

determined due to small sample size.

The ERIC patterns appear to be sufficiently distinctive amongst the 9 species studied and can be used as a supplementary diagnostics tool at the local level. However, there is not enough difference to distinguish between the subspecies. In all species, the ERIC patterns of the local isolates are different from their ATCC counterparts. Thus, geographically, the CoNS appear to display a high amount of genetic diversity. While this essentially precludes the use of ERIC banding patterns as a universal tool for diagnosis purposes, the method is still highly useful in epidemiology studies and also for the preliminary identification of local isolates.

DISCUSSION

The prevalence of *S. epidermidis*, *S. haemolyticus* and *S. hominis* in hospitals and clinical settings have long been recognized. The finding in the present study is in agreement to other studies on clinical CoNS which documented similar results (Kleeman *et al.*, 1993). The same distribution pattern of CoNS was observed in other studies including in hospitals in Germany (Gatermann *et al.*, 2007), Norway (Klingenberg *et al.*, 2007) and Istanbul, Turkey (Koksal *et al.*, 2009). The predominance of *S. epidermidis* in human infections has been linked to its overrepresentation in skin flora, resistance to antibiotics

and the ability to adhere to medical devices (Otto, 2009). *S. haemolyticus* is the second most common CoNS isolated from blood cultures especially in neonatal ICUs (von Eiff *et al.*, 2005). For other species of CoNS isolated in this study, their ability to cause various types of infections has also been reported in other studies.

Fingerprinting methods based on DNA sequence polymorphisms, such as Multi-loci Sequence Typing (MLST) are emerging as the methods of choice for genotyping. MLST and other sequence-based methods however can be costly to small laboratories. In addition, some studies have reported concerns over the discriminatory capacity of MLST for *S. epidermidis* (Monk and Archer, 2007; Thomas *et al.*, 2007). This finding was supported by another study which revealed that MLST failed to discriminate among *S. epidermidis* isolates to the same extent as PFGE (Klingenberg *et al.*, 2007). Fragment-based typing methods such as Pulse-Field Gel Electrophoresis (PFGE) of genomic restriction fragments are by far the most commonly used method for genotyping clinical strains. PFGE however is technically and resource demanding; requiring a high level of skill, expensive equipments and software. While the discriminative power is high, the above considerations and other limitations i.e. the inability to separate fragments that are too large or small limits the widespread adoption of PFGE. Alternative methods, such as using

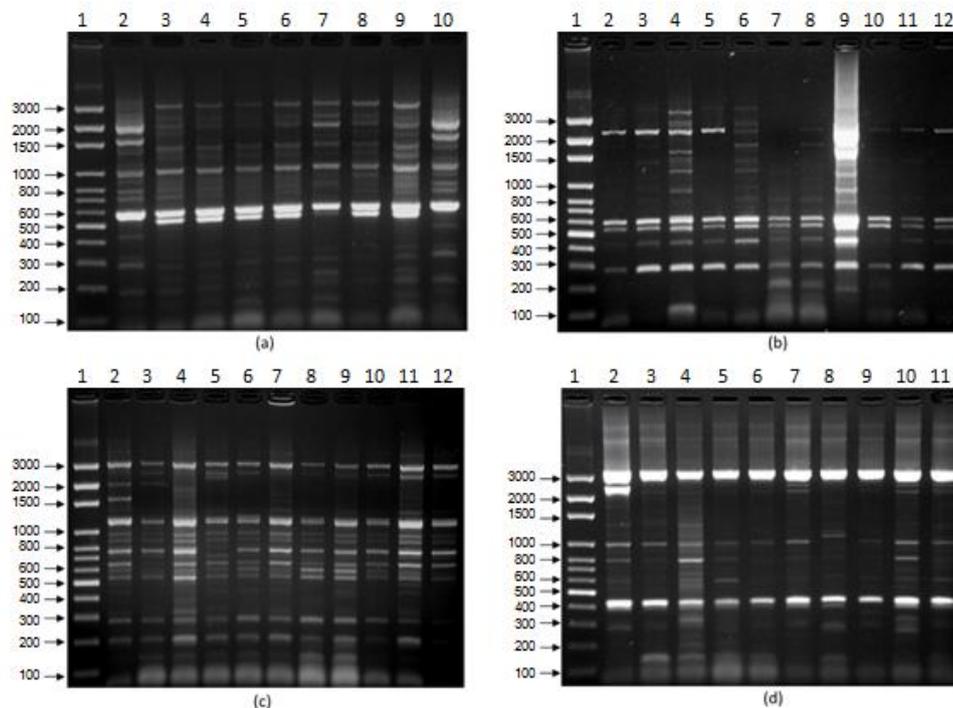


Figure 3: ERIC-PCR fingerprints of CoNS. Agarose gel images of ERIC-PCR fingerprints for the four most frequently isolated species. The first lane of each gel is loaded with a 100bp DNA ladder. ATCC type strains are included as comparison (a) *S. capitis* isolates. Lanes 2 & 10: *S. capitis* subsp. *capitis* ATCC 27840; (b) *S. epidermidis* isolates. Lane 2: *S. epidermidis* ATCC 35984, lane 3: *S. epidermidis* ATCC 12228; (c) *S. haemolyticus* isolates. Lane 2: *S. haemolyticus* ATCC 29970; (d) *S. hominis* isolates. Lane 2: *S. hominis* subsp *novobiosepticus* ATCC 700236.

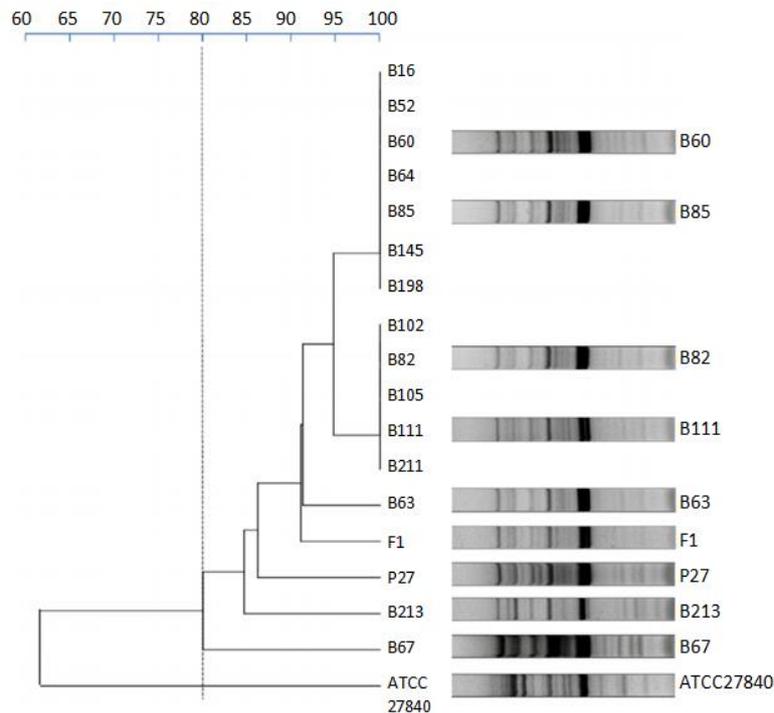


Figure 4: Dendrogram generated from the ERIC-PCR fingerprints profiles of 17 clinical strains of *S. capitis* including the reference strain *S. capitis* subsp. *capitis* ATCC 27840. Cluster analysis was performed using the open-source software FREETREE while similarity matrix was calculated using the Dice index. The scale bar at the top of the dendrogram indicates similarity while the dotted line indicates the cut-off value of 80% similarity applied to separate the clusters.

multiple markers are laborious in nature (Jaradat *et al.*, 2016). PCR-based methods, on the other hand are easy to perform and can be completed in a short period of time with lower labour costs. A number of repetitive sequence PCR has been developed for molecular typing and fingerprinting e.g. the ERIC-PCR, Box-PCR and AFLP analysis. The DNA fragments of ERIC-PCR banding profiles have been used to develop strain-specific probes or PCR primers for specific detection of bacteria in both clinical or environmental samples (Giesendorf *et al.*, 1993; Duan *et al.*, 2009) indicating that these amplified fragments are specific and could be a useful tool for fingerprinting studies. ERIC-PCR was also found useful in assessing the genetic diversity of isolates from different geographic locations (Macedo *et al.*, 2011; Ye *et al.*, 2012; Bilung *et al.*, 2018). The simple and rapid nature of the method provides a pragmatic option for small epidemiology studies e.g. a localised hospital outbreak. The discriminative power of these band-based methods however, has not been comprehensively evaluated. In *Staphylococcus*, the first attempt to identify staphylococcal species via ERIC-PCR was investigated in different strains of *S. epidermidis* and the high degree of similarity in their banding patterns suggests that the method could be a useful tool for rapid identification of the bacteria at species level (Wieser and Busse, 2000).

In this study, the applicability of ERIC-PCR as a molecular typing tool for CoNS was evaluated. With the number of isolates collected, large scale cluster analysis

was performed using *S. epidermidis* (74 strains) and *S. haemolyticus* (60 strains). Similar analysis were also carried out with isolates of *S. capitis* (17 isolates) and *S. hominis* (36 isolates), both of which comprises two subspecies. This is by far the largest scale analysis performed on clinical isolates of CoNS using ERIC-PCR fingerprinting. The results showed that ERIC-PCR does not provide enough discriminative power for *S. capitis* and *S. haemolyticus* but may however be suitable for distinguishing different genotypes of *S. hominis* and *S. epidermidis*.

For *S. capitis*, although the number of bands is sufficient for a robust analysis and the pattern is clear, ERIC-PCR fingerprints were not able to distinguish between the two subspecies at the 80% similarity cutoff point. All 17 isolates of *S. capitis* are considered as clonal. In addition, there is no indication of strong genetic relatedness amongst the subspecies. This is in contrast to a study on 60 isolates of *S. capitis* whereby a clear separation between the *S. capitis* subsp. *capitis* cluster from the *S. capitis* subsp. *ureolyticus* isolates using a combination of PFGE fingerprints and several phenotypic markers (Cui *et al.*, 2013). Similarly, the majority of the *S. haemolyticus* isolates were also found to be clonal. One important factor that should be considered is the possibility that the majority of the isolates were derived from only a few clones. This is likely in a hospital setting which is already contaminated by a pathogenic strain thus explaining the genetic similarity amongst the *S. capitis*

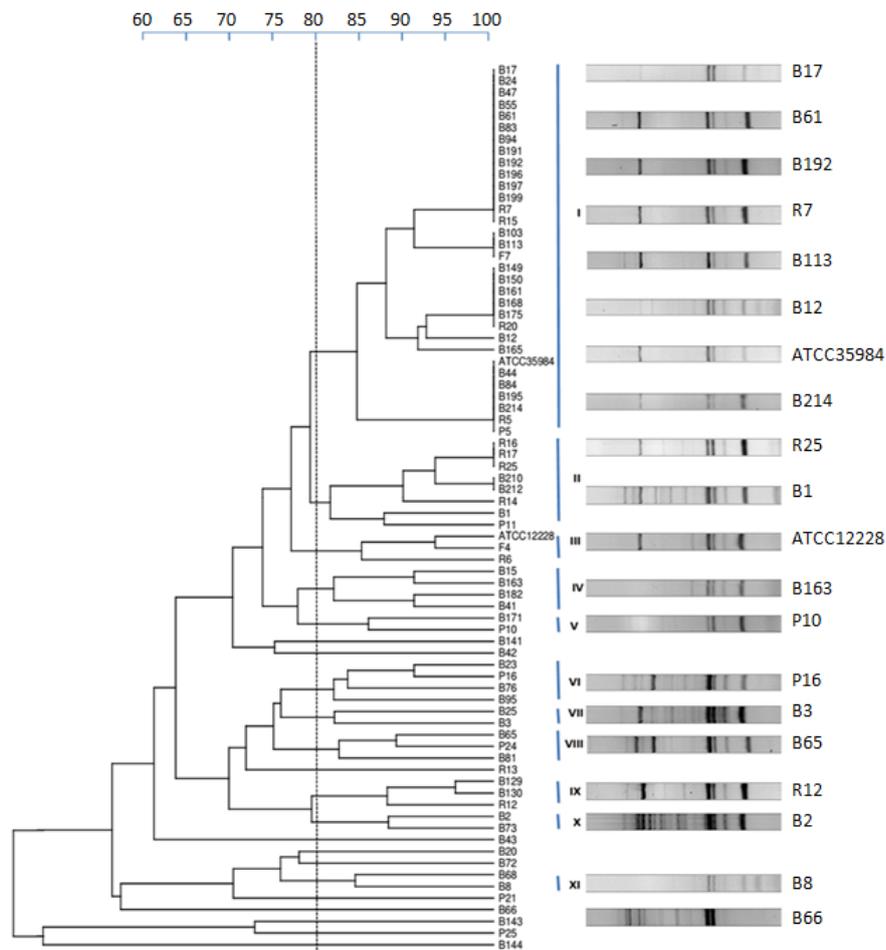


Figure 5: Dendrogram generated from the ERIC-PCR fingerprints profiles of 74 clinical strains of *S. epidermidis* including the reference strains ATCC 35984 and ATCC 12228. The scale bar at the top of the dendrogram indicates similarity while the dotted line indicates the cut-off value of 80% similarity applied to separate the clusters

and *S. haemolyticus* isolates respectively. Isolates of *S. epidermidis* fall in between the two extremes. While some of the isolates were likely to be clonal (Cluster I) and probably derived from a hospital-acquired infectious strain, a good portion of these isolates were however likely to be genetically different and reflect the true variation amongst local isolates. The *S. epidermidis* isolates show substantially higher genetic variability, as compared to *S. capitis*. *Staphylococcus epidermidis* is a common skin commensal and is carried by the majority of the population. Thus, the various clusters may represent true clonal variations of this species and are specific to the patient from which the isolate was obtained. The hospital from which these strains were collected acts as a referral centre and take in patients referred from smaller, rural hospitals. Thus, the clonal variation may also reflect differences in the locality from which the samples were derived. On the other hand, members of Cluster I may be derivatives of the same clone which infected multiple patients when they were hospitalised.

In contrast, *S. hominis* isolates displayed a wide degree of genetic diversity. The six species of *S. hominis* subsp. *novobiosepticus* were separated into four different clusters; isolates B40 and B45 were included in Cluster IB, isolates B134 and B128 were grouped in Cluster III while two more isolates of B6 and B48 were in their own separate clusters. On the other hand, the 30 isolates *S. hominis* subsp. *hominis* were divided into ten different clusters. Such diversity could be due to the presence of many different local strains, or the inflow of strains brought in by non-local residents. In addition, heterogeneity of PCR conditions cannot be ruled out, especially when interpreting faint and polymorphic bands. One suggestion to overcome this limitation is to use more stringent conditions i.e. higher annealing temperatures and the design of more specific primers. The inclusion of an internal standard that is amplified together with the ERIC bands will also help. Further improvement to the analysis pipeline for example using densitometry measurements instead of gel images, and the use of more informative indices e.g. the Pearson index, will also

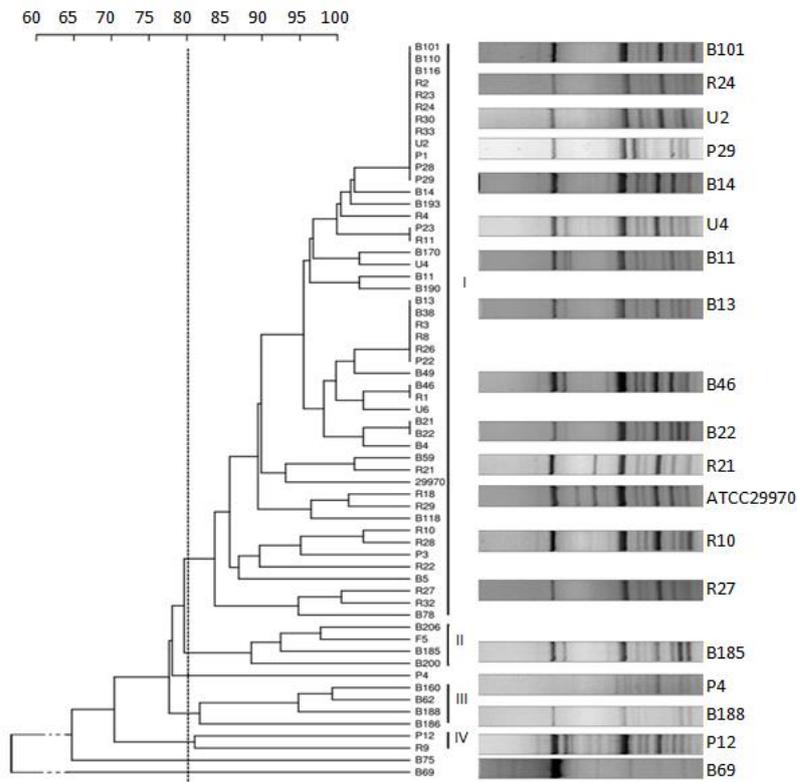


Figure 6: Dendrogram generated from the ERIC-PCR fingerprints profiles of 60 strains of *S. haemolyticus*, including the reference strain ATCC 29970. The scale bar at the top of the dendrogram indicates similarity while the dotted line indicates the cut-off value of 80% similarity applied to separate the clusters.

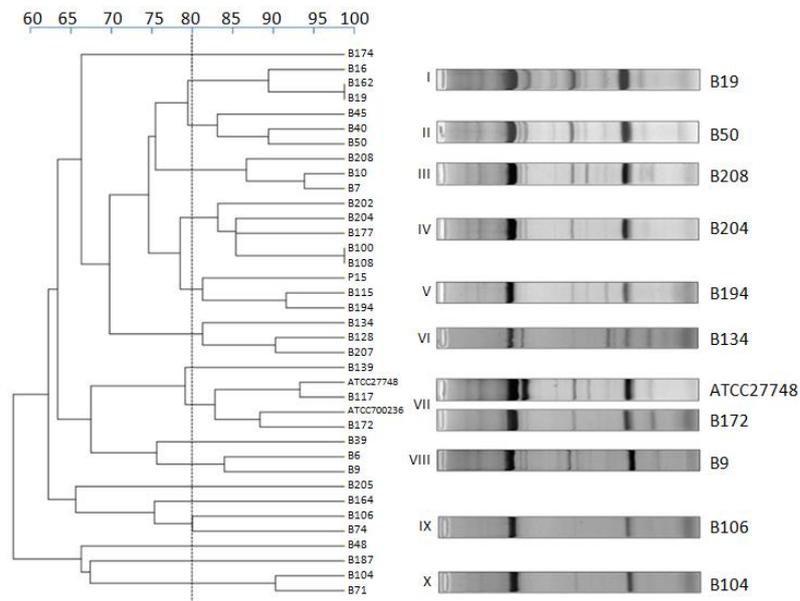


Figure 7: Dendrogram generated from the ERIC-PCR fingerprints profiles of *S. hominis* isolates including the reference strains *S. hominis* subsp. *hominis* ATCC 27844 and *S. hominis* subsp. *novobiosepticus* ATCC 700236. The scale bar at the top of the dendrogram indicates similarity while the dotted line indicates the cutoff value of 80% similarity applied to separate the clusters.

help to improve the reproducibility of the method. This will however increase both the cost and complexity of the method.

Although it does not appear to be fully discriminative when used for molecular typing, ERIC-PCR patterns can be useful as supplementary diagnostic aids for the differentiation of the subset of species. Each of the 12 species and subspecies in this study has a unique signature pattern that can be used for identification. Similar findings were observed in another study where different strains of *S. epidermidis* were found to display high degree of similarity (Wieser and Busse 2000).

CONCLUSION

CoNS species isolated from a hospital setting display varying levels of genetic diversity, possibly indicative of their respective source of origin. ERIC-PCR is a useful tool to differentiate and track selected species of CoNS.

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