DETERMINING THE CLONALITY AND SIGNIFICANCE OF COAGULASE–NEGATIVE STAPHYLOCOCCI ISOLATED FROM BLOOD CULTURES IN HUSM

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ABSTRACT

DETERMINING THE CLONALITY AND SIGNIFICANCE OF COAGULASE – NEGATIVE STAPHYLOCOCCI ISOLATED FROM BLOOD CULTURES IN HUSM

Coagulase-Negative Staphylococci (CoNS) is a group of microorganisms that are increasingly implicated as a cause of significant infection and has emerged as the most frequent cause of nosocomial bloodstream infection. CoNS species are normal skin flora and, it can be difficult to determine if CoNS species isolated from blood cultures reflect infection or microbial contamination. At the moment, pulsed-field gel electrophoresis (PFGE) is considered as a gold standard to diagnose the significance of CoNS isolated from blood cultures. In this study, significance of repeated CoNS isolated from blood cultures were evaluated by species identification, antibiogram and a molecular method, PFGE.

A total of 101 pairs of Coagulase-Negative Staphylococci were analyzed during this period of study. These isolates were identified to the species level by API Staph ID. Out of 101 pairs (, only 84 pairs (83.1%) of CoNs identified were similar species. These 84 pairs were further analyzed by pulsed-field gel electrophoresis (PFGE) to determine its clonality (genotypic). However only 33 pairs had eligible PFGE result for interpretation

In this study, *Staphylococcus epidermidis* was the predominant species isolated (52.5%), followed by *S.capitis* (10.4%) and *S.chromogenes* (7.9%). The percentage of methicillin-resistant CoNS was higher (68.68%) as compared to methicillin-sensitive CoNS. The

percentage of same phenotype with same antibiogram pattern (40.7%) was lower as compared to same phenotype and different antibiogram pattern (59.3%). There was a significant association between same phenotype and same antibiogram pattern.

Out of 33 pairs of CoNS analyzed, 87.9% (29 pairs) had indistinguishable patterns which denote the same bacterial strains. However the association between phenotypic and genotypic cannot be made because due to failure to maintain reproducible PFGE results, hence resulted in insignificant statistical findings. Overall laboratory concordance case was defined as a case which were concordant for the triple tests conducted: phenotypic, antibiogram pattern and genotypic. Out of 33 pairs of CoNS with PFGE results, 14 cases (42.4%) were concordant cases which represent laboratory true bacteraemia and 19 cases (57.6%) were regarded as contaminants.

In this study, there was significant association between same phenotype and antibiotic treatment. The percentage of those who had antibiotic treatment and same phenotype was higher (90.9%) as compared to those without antibiotic treatment (9.1%). There was significant association for leukocyte count, nosocomial infection and antibiotic treatment with antibiogram pattern. The association of laboratory concordance cases with clinical parameters showed significant association between laboratory concordance cases with blood pressure, leukocyte count and antibiotic treatment. All clinical parameters were not significantly associated with concordance genotype. The outcome of this study showed that genotype by pulsed-field gel electrophoresis (PFGE) failed to show any association with clinical bacteraemia by statistical calculation due to small sample size.

ABSTRAK

PENENTUAN KLONALITI DAN SIGNIFIKASI *COAGULASE-NEGATIVE STAPHYLOCOCCI* YANG DIISOLASI DARIPADA KULTUR DARAH DI HUSM

Coagulase-Negative Staphylococci (CoNS) adalah sekumpulan bakteria yang semakin meningkat sebagai penyebab jangkitan yang signifikan dan kini menjadi penyebab utama jangkitan darah nosokomial. CoNS adalah flora normal kulit dan ini menyebabkan sukar untuk ditentukan samada CoNS yang diisolasi daripada kultur darah merupakan penyebab jangkitan atau kontaminasi mikrobial. Sehingga kini, *pulsed-field gel electrophoresis* (PFGE) dianggap sebagai ujian utama untuk mendiagnosa signifikasi CoNS yang diisolasi dari kultur darah. Dalam kajian ini, signifikasi CoNS yang diisolasi dari sebanyak dua kali dari kultur darah telah dinilai menggunakan ujian pengenalan spesis, antibiogram dan kaedah molekular iaitu PFGE.

Sebanyak 101 pasang CoNS berjaya dianalisa sepanjang tempoh kajian ini. 101 pasang CoNS (202 isolat CoNS) ini dikenalpasti sehingga tahap spesis menggunakan ujian API Staph ID. Dari 101 pasang CoNS, hanya 84 pasang (83.1%) yang dikenalpasti mempunyai fenotipik yang sama (sama spesis). 84 pasang CoNS ini seterusnya dinilai dengan ujian PFGE untuk penentuan klonaliti (genotipik). Bagaimanapun hanya 33 pasang sahaja keputusan PFGE yang sesuai untuk ditafsirkan.

Kajian ini menunjukkan bahawa *Staphylocccus epidermidis* merupakan spesis yang paling banyak diisolasi (52.5%), diikuti *S.capitis* (10.4%) dan *S.chromogenes* (7.9%). Peratus

CoNS yang resistan terhadap metisilin adalah lebih tinggi (68.68%) berbanding peratus CoNS yang sensitif terhadap metisilin. Peratus fenotipik yang sama dengan corak antibiogram yang sama (40.7%) adalah lebih rendah berbanding fenotipik yang sama dengan corak antibiogram yang berbeza (59,3%). Bagaimanapun terdapat hubungkait yang signifikan antara fenotipik yang sama dan corak antibiogram yang sama.

Dari 33 pasang CoNS yang dianalisa dengan PFGE, 87.9% (29 pasang) mempunyai corak PFGE yang sama yang menunjukkan strain yang sama. Bagaimanapun hubungkait antara fenotipik dan genotipik yang sama tidak dapat dilakukan kerana kegagalan hasil ujian PFGE mengakibatkan penghasilan keputusan yang tidak signifikan secara statistik. Kes yang mempunyai keputusan yang sama bagi fenotipik, corak antibiogram dan genotipik didefinisikan sebagai kes keseluruhan keputusan makmal yang sama yang mewakili bakterimia benar makmal. Dari 33 pasang CoNS, 14 kes adalah bakterimia benar makmal (42.4%) manakala 19 kes (57.6%) adalah kes kontaminasi.

Dalam kajian ini, terdapat hubungkait signifikan antara fenotipik yang sama dan rawatan antibiotik. Peratus pesakit yang menerima rawatan antibiotik dan fenotipik yang sama adalah tinggi (90.9%) berbanding peratus pesakit yang tidak menerima rawatan antibiotik (9.1%). Terdapat hubungkait yang signifikan antara pengiraan leukosit, jangkitan nosokomial dan rawatan antibiotik dengan corak antibiogram. Hubungkait antara kes makmal yang sama (fenotipik sama dan corak antibiogram sama) dengan parameter klinikal menunjukkan hubungkait yang signifikan dengan tekanan darah, pengiraan leukosit, dan rawatan antibiotik.

Kesemua parameter klinikal adalah tidak mempunyai hubungkait yang signifikan dengan genotipik yang sama. Keputusan kajian ini menunjukkan bahawa genotipik menggunakan PFGE gagal menunjukkan hubungkait dengan bakterimia klinikal disebabkan oleh pengiraan statistic kerana pengurangan saiz sampel.

Chapter 1

INTRODUCTION

1.1 ABOUT COAGULASE-NEGATIVE STAPHYLOCOCCI

1.1.1 Description of the family Coagulase-Negative Staphylococci (CoNS)

Coagulase-Negative Staphylococci (CoNS) comprise an ever-expanding group of bacteria whose medical importance has emerged in the past decades. They now count among the most frequent of nosocomial pathogens featuring prominently among blood culture isolates, often in association with intravascular devices, and as a cause of infection of more deep-seated prosthetic implants (Finch, 2006). Clinically, infection may be silent, overt and occasionally fulminant, and this reflects the diverse pathogenic profile of this group of organisms (Finch, 2006). CoNS are also characterized by an unpredictable pattern of susceptibility to commonly used antibiotics. Multiple drug resistance is common and adds to the difficulties of treating these infections.

Taxonomically, CoNS, together with *Staphylococcus aureus*, are members of the family micrococcaceae. They are Gram-positive facultative anaerobes which appear in clusters, are non-motile, non-spore forming and catalase positive and in general do not produce the enzyme coagulase. A thin capsule may be detected in some strains. CoNS are divided into more than 44 species and more than a dozen subspecies, of which approximately half have been associated with humans (Kloos and Bannerman, 1994). The remainder are associated with domestic and other species of mammals. The relatedness of these species has been confirmed by guanine + cytosine ratios. DNA sequence homology of

>50% has been used to group the species, although a number of species are too distantly related to fit into this arrangement (Finch, 2006).

Coagulase production is generally absent among CoNS, although some strains of S. *intermedius* and *S.hyicus* are weak producers. Heat-stable thermonuclease is produced by *S.intermedius, S.hyicus, S.schleiferi* and some strains of *S.carnosus, S. epidermidis* and *S.simulans* and permits cleavage of nucleic acids.

1.1.2 Classification of the Staphylococci

CoNS have been divided into various species based on a variety of characteristics including colonial morphology, coagulase and phosphatase production, acid formation from maltose, sucrose, D-mannitol, D-trehalose and D-xylose as well as susceptibility to novobiocin using a 5-μg disc (Pfaller and Herwaldt, 1988). More extensive biochemical testing is necessary to speciate less common strains such as *S.warneri, S.capitis, S.simulans* and *S.hominis*, although little call is made for this outside reference or research laboratories (Kloos *et al.*, 1991). *Staphylococcus intermedius* is coagulase positive as are many strains of *S.hyicus*.

Table 1.1.1 Key identification characteristics for the most common genus *Staphylococcus*

Table 6.2 Laboratory characteristics of the genus *Staphylococcus*

	S. aureus	*S. intermedius*	*S. hyicus*	*S. chromogenes*	*S. epidermidis*	*S. capitis* subsp. *capitis*	*S. auricularis*	*S. saccharolyticus*	*S. haemolyticus*	*S. hominis* subsp. *hominis*	*S. warneri*	*S. simulans*	*S. saprophyticus* subsp. *saprophyticus*
Growth anaerobically	+	+	+	+	+	w	w	+[a]	+	w	+	+	w
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–
VP[e]	+	–	–	–	+	+	d	?	+	+	+	–	+
Coagulase[e]	+	+	d	–	–	–	–	–	–	–	–	–	–
Acid from													
Lactose	+	+	+	+	D		–	–	D	+	–	+	+
Maltose[e]	+	–	–	d	+	–	d	–	+	+	d	–	+
Mannitol	+	+	–	d	–	+	–	–	+[b]	–	d	+	+
Fructose	+	+	+	+	+	+	+	+	d	+	+	+	+
Sucrose	+	+	+	+	+	+	d	–	+	+	+	+	+
Trehalose[e]	+	+	+	+	–	–	+	–	+	+	+	+	+
Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–
Cellobiose	–	–	–	–	–	–	–	?	–	–	–	–	–
Raffinose	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	+	+	+	+	+	+	–	+	–	–	–	d	–
Phosphatase[e]	+	+	+	+	+	–	–	?	d	–	–	w	–
Nitrate	+	+	+	+	+	+	d	+	+	+	–	+	–
Arginine[e]	+	+	+	+	+	+	–	+	+	+[d]	+[d]	+	d
Urea	d	+	+	+	+	–	–	?	–	+	+	+	+
Protease	+	D	+	+	w	w	–	–	–	–	–	–	–
Novobiocin[e]	s	s	s	s	s	s	s	s	s	s	s	s	r

Table 6.2 (Continued)

	S. cohnii subsp. *cohnii*	*S. xylosus*	*S. caprae*	*S. carnosus*	*S. caseolyticus*	*S. arlettae*	*S. equorum*	*S. gallinarum*	*S. kloosii*	*S. lentus*	*S. sciuri*	*S. lugdunensis*	*S. schleiferi* subsp. *schleiferi*
Growth anaerobically	w	w	+	+	w	–	–	w	–	–	w	+	+
Oxidase	–	–	–	–	+	–	–	–	–	w	+	–	–
VP[b]	+	–	+	+	–	–	–	–	D	–	–	+	+
Coagulase[e]	–	–	–	–	–	–	–	–	–	–	–	–	–
Acid from													
Lactose	–	+	+	d	+	+	+	d	d	+	–	–	–
Maltose[e]	+	+	d	–	+	+	+	+	+	d	+	+	–
Mannitol	+	+	–	+	–	+	+	+	+	+	+	–	–
Fructose	+	+	–	+	+	+	+	+	+	+	+	+	–
Sucrose	–	+	–	–	d	+	+	+	–	+	+	+	–
Trehalose[e]	+	+	+	d	d	+	+	+	+	+	+	+	d
Xylose	–	+	–	–	–	+	+	+	–	–	–	–	–
Cellobiose	–	–	–	?	?	–	–	+	–	+	+	–	–
Raffinose	–	–	–	–	?	+	+	+	–	+	–	–	–
Mannose	–	+	+	+	–	d	+	+	–	+	d	+	+
Phosphatase[e]	+	+	+	?	?	+	+	+	+	+	+	–	+
Nitrate	–	+	+	+	+	–	+	+	–	+	+	+	+
Arginine[e]	–	–	+	?	?	–	–	?	–	?	?	–[c]	+
Urea	d	+	+	–	?	–	+	+	–	d	–	?	–
Protease	–	–	–	–	+	–	–	–	–	w	+	?	?
Novobiocin[e]	r	r	s	s	s	r	r	r	r	r	r	s	s

+, 85–100% strains are positive (all, most, many, usually); –, 0–15% strains positive (none, one, few, some); ?, not known or insufficient information; d, 16–84% strains positive (many, several, some); w, weak reaction or growth; D, different reactions given by lower taxa (genera, species, varieties); s, sensitive; r, resistant.

[a] No growth anaerobically.

[b] Usual reaction.

[c] Ornithine decarboxylated.

[d] Inferred reaction.

[e] These tests are usually sufficient to identify the species that may infect humans

Adapted from *Principles and Practice of Clinical Bacteriology, Second Edition*

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1.1.3 Natural habitat

The distribution and concentration of CoNS on the human skin and surface mucosa varies. The density of organisms ranges from 10^3 to 10^6 CFU/cm^2, with the lowest counts in the dry areas of the skin. They are particularly concentrated in the perineum, inguinal region, axillae and anterior nares. The distribution pattern is reflected in the relative frequency with which the various species are isolated from pathological specimens.

For example, *Staphylococcus capitis* subsp. *capitis* generally prefers the scalp and face. *S.epidermidis* is the most widely distributed species on the skin and achieves the highest concentrations in moist skin areas. *S.haemolyticus* and *S.hominis* are most numerous in the perineum, axillae and inguinal regions in association with apocrine glands. *S.auricularis* is largely confined to the external auditory meatus. *S.warneri* and *S.lugdunensis* are less frequent commensals than are other species, but when present they are widely distributed (Kloos and Bannerman, 1994)

The increasing importance of CoNS as human pathogens has been recognized over the past 25 years. Prior to this, *S.epidermidis* was an uncommon pathogen, although *S.saprophyticus* was recognized as an occasional cause of community-acquired urinary tract infection in sexually active young women. However, community-acquired CoNS infections are rare but may arise in patients who have been discharged from hospital following the insertion of prosthetic implants such as joints, heart valves and intracardiac patches. Infection by these organisms is generally a delayed expression of hospital-acquired infection. However, the recent growth in continuous ambulatory peritoneal dialysis

(CAPD), earlier discharge of patients from hospital and interest in home intravenous drug therapy suggest that CoNS infections will increase within the community.

1.2 COAGULASE-NEGATIVE STAPHYLOCOCCI IDENTIFICATION SCHEME

1.2.1 Historical perspectives

Staphylococcus aureus was first described by Rosenbech in 1884. Its pathogenic profile includes local invasion, systemic spread, toxin-mediated disease and increasing resistance to commonly used antibiotics (Finch, 2006). It was included among the group of pyogenic cocci, and hence, its former description, *S.pyogenes*. In contrast, CoNS were considered for many years to be non-pathogenic commensal organisms of the skin. *S.albus* was widely used to describe all CoNS as distinct from the colonial appearance of the golden pigmented *S.aureus*. The first widely accepted pathogenic role of CoNS was the association of *S.saprophyticus* (novobiocin resistant CoNS) with urinary tract infections in women (Pereira, 1962).

The widely held view that CoNS were largely commensals and indeed contaminants of clinical specimens frustrated recognition of the pathogenic potential of this group of organisms for many years. However, by the 1980s CoNS had clearly been identified with a wide variety of clinical problems such as bacteraemia, endocarditis of both prosthetic and native heart valves, septic arthritis, peritonitis complicating continuous ambulatory peritoneal dialysis (CAPD), mediastinitis, pacemaker associated infections, cerebrospinal fluid (CSF) shunt device infections, prosthetic joint and other orthopaedic device infections, osteomyelitis, urinary tract infection and prostatitis (Kloos and Bannerman, 1994). While *S.saprophyticus* was clearly associated with the urinary tract, the predominant

species among the remaining infections was *S.epidermidis.* However, many hospital diagnostic laboratories have used the species *S.epidermidis* description loosely to encompass all CoNS, further frustrating recognition of the diverse microbial nature of CONS infections. This has changed in recent years largely as a result of increased awareness of the importance of CoNS infections, together with the availability of commercial identification systems.

1.2.2 Conventional biochemical tests

Conventional methods for determining phenotypic characters that differentiate the new human. *S.lugdunensis* can be distinguished from all other CoNS species by its rapid positive ornithine decarboxylase activity. *S.schleifen* subsp.*schleiferi* can be distinguished from all other CoNS species by its positive thermonuclease and clumping factor activities. This subspecies can be separated from *S.schleiferi subsp. coagulans* by its negative tube coagulase test, positive clumping factor activity, and negative urease activity.

Several additional tests that might be useful in distinguishing *S.schleiferi* subsp. *schleiferi from S.lugdunensis. S.lugdunensis* is resistant to polymyxin B and bacitracin (10U) but *S.schleiferi* is susceptible to these antibiotics (Hebert, 1990). *S.capitis* subsp. *ureolyticus* can be differentiated from *S.capitis* subsp. *capitis* on the basis of its positive urease activity and acid production from maltose. This subspecies can be distinguished from the close relative *s.caprae* by its negative alkaline phosphatase activity, acid production from sucrose, and lack of acid production from trehalose and turanose. *S.cohnii* subsp. *urealyticum* can be differentiated from *S.cohnii* subsp. *cohnii* on the basis of its positive alkaline phosphatase, urease, 3-glucuronidase, and 3-galactosidase activities.

Although character analysis by many of the conventional methods requires 1 to 3 days before a final identification can be made, conventional methods are quite accurate and have served as a source of reference for studying the accuracy of rapid identification systems. A numerical code system for the reference identification of *Staphylococcus* species and subspecies based on the results of 18 primary conventional biochemical tests has been proposed by Rhoden and co-workers (Rhoden *et al.*, 1993).

The system, referred to as the Centers for Disease Control Micrococcaceae profile system, identified more than 95% of the 824 strains tested. Of the new species and subspecies, *s.lugdunensis* was well represented in this system (45 strains), but *S.schleiferi* (1 strain), *S.capitis* subsp. *ureolyticus* (9 strains), and *S.cohnii* subsp. *urealyticum* (10 strains) were underrepresented. *S.caprae* was represented by only two strains. Despite the small number of strains analyzed for certain species, the Micrococcaceae profile system approach appears to be a reasonable alternative for laboratories that require reference identification for members of the Micrococcaceae, including CoNS.

1.2.3 Commercial kits identification systems

To expedite the identification process for use in the clinical laboratory, several manufacturers have developed rapid species and subspecies identification kits or automated systems requiring only a few hours to 1 day for completing tests. The major companies marketing products for the identification of CoNS species and subspecies include the following: (i) bioMerieux Vitek, Inc., Hazelwood, Mo.,USA (API STAPH IDENT, STAPH Trac System, ID 32 STAPH, RAPiDEC STAPH System, and Gram Positive Identification Card for use with the automated Vitek and Vitek Jr.); (ii) Baxter Diagnostics Inc.,

MicroScan Division, West Sacramento, California, USA. (MicroScan Pos ID panel, Pos Combo Type 6 panel, MicroScan Rapid Pos ID panel, and Rapid Pos Combo Type 1 panel for use with the automated auto SCAN-W/A system); (iii) Becton Dickinson Microbiology Systems, Cockeysville, Md.,USA (Minitek Gram-Positive Set); (iv) Becton Dickinson Diagnostic Instrument Systems, Towson, Md., USA (Sceptor Staphylococcus MIC/ID Panel and Sceptor Gram Positive Breakpoint/ID Panel); (v) Biolog, Haywood, California, USA (GP MicroPlate test panel); and (vi) MIDI, Newark, Del.,USA (Microbial Identification System).

Identification of most human *Staphylococcus* species with the commercial systems can be made with an accuracy of 70 to >90% (Kloos and George, 1991). Identification of *Staphylococcus* species and sub- species has improved somewhat with the Baxter Diagnostics MicroScan Pos ID (West Sacramento, California, USA) and Rapid Pos ID (West Sacramento, California, USA) panel systems and the bioMerieux Vitek Gram Positive Identification Card (Hazelwood, Mo.,USA) (Bannerman *et al.*, 1993b) by increasing the data bases. It is expected that the reliability of these and other commercial systems will continue to increase as the result of growing data bases and the addition of more discriminating tests. The new *Staphylococcus* species and subspecies have been incorporated recently into several data bases. Accuracy in the identification of *S.lugdunensis* and *S.schleiferi* can be increased significantly by the addition of the ornithine decarboxylase test (now employed in the bioMerieux Vitek ID 32 STAPH) and the thermonuclease test (Remel Laboratories, Inc., Lenexa, Kansas.), respectively.

1.2.4 Molecular methods

1.2.4.1 Cellular fatty acid (CFA) analysis

Cellular fatty acid (CFA) analysis has received little attention as a method for strain delineation, perhaps because of the need to standardize substrates and growth conditions to obtain reproducible results. CFA analysis comparable to standard techniques (antibiogram, biotype, and plasmid profiles) for distinguishing between multiple CNS blood isolates (Kotilainen *et al.*, 1991). Their results showed that numerous morphologically identical isolates of a strain from a patient gave a correlation value of >95, while numerous nonidentical isolates of the same species from a patient gave a correlation of <95.

The study showed some strain discrimination; however, more studies are necessary to show the epidemiological usefulness of this method. CFA analysis is relatively inexpensive, simple, and quick, and a large number of isolates can be tested at one time. CFA analysis combined with numerical correlation analysis for subgrouping isolates may prove to be of some use in strain identification.

1.2.4.2 Pyrolysis-mass Spectrometry

Another method is application of pyrolysis-mass spectrometry for strain discrimination of CoNS has given encouraging results (Freeman *et al.*, 1991). In this technique, the organisms are pyrolyzed, the pyrolysates are examined by mass spectrometry, and the results are analyzed and compared mathematically to produce a dendrogram. Investigators have found pyrolysis-mass spectrometry to be comparable to typing schemes that incorporate antibiogram, biotype, and plasmid analysis (Freeman *et al.*,

1991). This technique is relatively, rapid, and reproducible. It is necessary to include epidemiologically unrelated control strains so that the significance of the different spectrograms of the strains can be estimated.

1.2.4.3 Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis is based on analysis of the electrophoretic profile of genetically controlled variants of metabolite enzymes (isoenzymes). These isoenzymes are distinguished on the basis of their movement in a starch or polyacrylamide gel and reaction with specific stains. The advantages of this technique are the ease in performance, the availability of reagents, a high degree of reproducibility, and the stability of the profiles. The disadvantage is the expense of the enzymes and staining reagents (Pfaller, 1992). Musser *et al.*, (1990) incorporated the technique successfully to study the genetic structure among strains of *S.aureus* that cause toxic shock syndrome. In early studies of CoNS isoenzymes for species identification, it was apparent that this method could distinguish strains of certain species (Myrvik *et al.*, 1989). Further analysis of the technique is necessary to determine its usefulness in strain identification of CoNS and the numbers and types of enzymes necessary to differentiate between strains.

1.2.4.4 Plasmid profiling

Plasmid profiling and the restriction endonuclease analysis of specific plasmids can serve as a valuable typing system, especially for those strains that carry multiple plasmids. Recent reviews have given the advantages and disadvantages of plasmid profiling (Pfaller and Herwaldt, 1988;Pfaller and Hollis, 1989). The CoNS that often carry multiple plasmids are *S.epidermidis, S.haemolyticus, S.hominis, S.capitis, S.warneri, S.saprophyticus,*

S.cohnii and *S.xylosus,* while *S.auricularis* and *S.lugdunensis* seldom have plasmids or have only one or two (Kloos, 1990). Restriction enzyme analysis of the plasmids may further extend the sensitivity of this technique and is particularly useful for differentiating plasmids of the same size.

However, some common plasmids are highly conserved and often have identical fragment patterns irrespective of the strain, for example, the small tetracycline plasmids and the small macrolide-lincosamide-streptogramin B (MLS) resistance plasmids. One major disadvantage of this technique is that plasmids are somewhat unstable elements, and the lack or addition of one plasmid may not truly designate a different strain. The technique is a relatively simple and inexpensive way to discriminate between strains and thereby, in combination with other strain typing methods, will be useful in epidemiology studies (Kloos and Lambe, 1991).

1.2.4.5 Whole-cell protein profiling

Whole-cell protein profiling employs gel electrophoresis of cellular proteins. The detection methods include using either Coomassie blue staining, 35S-methionine (radio-PAGE), or immunoblotting. SDS-PAGE with staining by Coomassie blue and radio- PAGE examine all major bacterial proteins, while immunoblotting examines surface-exposed antigens that are immunoreactive to antibodies. SDS-PAGE with Coomassie blue staining has produced distinct banding patterns for species of CoNS (Clink and Pennington, 1987).

Maggs and Pennington (1989) took the technique further to show that it could discriminate between clones of *S.capitis* inhabiting different regions of the skin of human subjects. Several studies have compared the detection methods. Thomson-Carter and

Pennington (1989) compared SDS-PAGE and immunoblotting. Both were reproducible; immunoblotting was more sensitive, and SDS-PAGE was easier to perform. Dryden *et al.* (1992) found that SDS-PAGE had a higher discriminatory power (69%) than immunoblotting (57%). Both techniques were technically demanding, and standardization of each step was necessary for reproducibility.

1.2.4.6 Chromosomal analysis

Chromosomal DNA analysis by restriction endonuclease fingerprinting has been used to type various pathogens, but little research has been done on CoNS. Bialkowaka-Hobrzanska *et al.* (1990) studied a total of 48 isolates of *S.epidermidis* and 19 isolates of *S.haemolyticus* for optimal conditions and reliable restriction endonuclease fingerprinting analysis. They investigated 12 restriction endonucleases and found *ClaI, PstI, BglII,* and *SacI* to be the most discriminatory. They concluded that restriction by *ClaI* was more discriminatory than plasmid profiling, and the results were stable. Wilton *et al.* (1992) screened 13 enzymes including *ClaI*; however, they found that *BclI* gave the most distinct banding patterns, with excellent reproducibility. This technique lacks standardization, and the banding patterns are often difficult to analyze because of the large number of fragments generated by the restriction enzymes and the single electrofield used to separate fragments.

Two new approaches to chromosomal analysis, ribotyping and field inversion gel electrophoresis or pulsed field gel electrophoresis (PFGE), have shown promise in the identification of different strains. Ribotyping incorporates the use of nucleic acid probes to highlight specific rDNA-containing bands upon restriction of the chromosomal genome. Several groups have begun to investigate the usefulness of this technique to further resolve

strain identification. De Buyser *et al.* (1989) used radiolabelled 16S rDNA from *Bacillus subtilis* and noted different rRNA gene restriction patterns between various species and strains of CoNS following *HindIII* and *EcoRI* cleavage of total DNA. Thomson-Carter *et al.* (1989) found similar results with their study of 22 strains of seven different species; however, they used 16S plus 23S rRNA from *E.coli* as a probe.

Bialkowaka-Hobrzanska *et al.* (1990) studied 78 strains and 15 species by comparing ribotyping, using the above probe, with *ClaI* cleavage and no probe. They found that the patterns given by ribotyping were easier to read, yet slightly less discriminatory. (Izard *et al.*, 1992) examined the intraspecific typing ability of ribotyping on 86 strains of *S.epidermidis*. Upon digestion with *EcoRI* and *HindIII*, they found 11 and 10 ribotypes, respectively. Discriminatory power varied from 14.3 to 15.1% with the use of one enzyme. When both enzymes were used, the discriminatory power was 31.6%. Field inversion gel electrophoresis and PFGE allow the use of restriction enzymes that infrequently cut chromosomal DNA and therefore separate large DNA fragments.

This in turn allows a better interpretation of the banding patterns. Goering and Duensing (1990) used field inversion gel electrophoresis to examine strains of methicillin-resistant *S.epidermidis*. They found that strain interrelationships could be established on the basis of *SmaI*-generated chromosomal restriction fragment length polymorphisms. Goering and Winters (1992) made their technique more rapid by preparing and analyzing the DNA in a total time of 2 days without a decrease in sensitivity or reproducibility. Preliminary studies on the use of PFGE for strain delineation of CoNS have shown promise. George and Kloos (1994) have incorporated PFGE in the study of *S.epidermidis, S.capitis* subsp. *capitis*, *S.capitis* subsp. *ureolyticus*, and *S.caprae* for strain identification and genome

sizing. Upon restriction of the DNA with *SmaI*, they found considerable conservation in fragment patterns for different strains of *S.capitis* subsp. *capitis*, *S.capitis* subsp. *ureolyticus*, and *S.caprae*.

On the other hand, *S.epidermidis* displayed a variety of different patterns. A small percentage of *S.epidermidis* strains demonstrated clonal variation in their fragment pattern, involving a size change in one or two of the bands. Bannerman *et al.* (1993a) found that strains of *S.caprae* isolated from goats produced fragment patterns different from those produced by strains of *S.caprae* isolated from humans. Further studies of different CoNS species and the use of different enzymes are needed to determine how well field inversion gel electrophoresis and PFGE will discriminate among strains of CoNS.

1.3 ANTIBIOTIC SUSCEPTIBILITY

The growing importance of CONS as a cause of human infection has been matched by an increasing problem of drug resistance. Multiply antibiotic-resistant strains of CoNS are now commonly recognized either as colonizers or as pathogens (Finch, 2006). Evidence for cross-infection between patients and staff is accumulating and CoNS, as part of the normal human flora, provide a reservoir in which antibiotic resistance can be spread to other CoNS and other potentially pathogenic bacteria. Cloxacillin is the drug of choice for CoNS bacteraemia. But the majority of CoNS are able to elaborate an inducible β-lactamase and hence are resistant to penicillin, while the production of a low-affinity penicillin-binding protein, PB2a, is responsible for resistance to methicillin and related anti-staphylococcal drugs (Chambers, 1987).

The *mec*A gene is common to all staphylococci exhibiting methicillin resistance and is expressed heterotypically among a variable minority of isolates within a bacterial population. Recognition is sometimes difficult to detect, as the more resistant subpopulation is in the minority and grows more slowly. More than 80% of hospital-acquired CoNS are methicillin resistant (Schulin and Voss, 2001) and of these, more than half will be resistant to other agents such as gentamicin, trimethoprim, erythromycin and clindamycin (Diekema *et al.*, 2001). Tetracycline, chloramphenicol and quinolone resistance is more variable. Methicillin resistant CoNS should be considered to be multi-resistant unless there is *in vitro* evidence to the contrary (Archer and Climo, 1994)

Vancomycin is currently the glycopeptide of choice for the treatment of most serious CoNS infections. Most CoNS remain susceptible to 4 mg/l of vancomycin or less, although occasional strains of *S.haemolyticus* are resistant (Srinivasan *et al.*, 2002). Vancomycin resistance among *S.epidermidis* isolates is even more uncommon, but concerns have been heightened following the detection of vancomycin-resistant *S.aureus*. Teicoplanin is being increasingly used as an alternative to vancomycin in the treatment of a wide range of staphylococcal infections. However, CoNS of reduced susceptibility (minimal inhibitory concentration > 8 mg/l) among *S.epidermidis* and *S.haemolyticus* are now recognized (Bannerman *et al.*, 1991). Resistance to *S.haemolyticus* can be readily reduced *in vitro* and among *S.epidermidis* by stepwise exposure (Biavasco *et al.*, 1991). However, such strains are currently uncommon.

Quinupristin–dalfopristin (von Eiff *et al.*, 2000) and linezolid (Henwood *et al.*, 2000) are two new agents licensed for the treatment of Gram-positive infections including those caused by susceptible CoNS. Experience to date remains limited but is encouraging

in the case of linezolid. However, despite *in vitro* susceptibility of many isolates, there is little evidence to suggest that the antibiotic treatment of medical device-associated infections has been improved. While *in vitro* susceptibility to various antibiotics can be demonstrated, biofilm-embedded microorganisms are less susceptible to the effects of antibiotic. Originally it was felt that the biofilm prevented drug penetration, but this is now known to be incorrect. Diffusion is often rapid, and concentrations are high in the case of vancomycin and rifampicin (Schulin and Voss, 2001). In vitro biofilm models of infection have shed some light on the activity of established drugs (Widmer *et al.*, 1992) and new agents such as quinupristin–dalfopristin (Gander and Finch, 2000) and linezolid (Gander *et al.*, 2002).

1.4 ISSUES ON CLINICAL SIGNIFICANCE OF COAGULASE-NEGATIVE STAPHYLOCOCCI

Coagulase-Negative Staphylococci (CoNS) are a group of micro-organisms that are increasingly implicated as a cause of significant infection (Gemmell, 1986;Weinstein *et al.*, 1997). CoNS are the main causal agents of bacteraemia in patients with indwelling medical devices eg. central and peripheral venous catheters, valvular prostheses, artificial heart valves, pace-makers and orthopaedic prostheses and other infections involving biofilm formation on implanted biomaterial (Huebner and Goldmann, 1999). CoNS species have emerged as the most frequent cause of nosocomial bloodstream infection, accounting for 27–32% and 50% of such infections among adult and paediatric patients, respectively (Bearson *et al.*, 2004).

Clinical criteria to predict whether CoNS isolated from blood cultures are associated with bloodstream infection are neither sensitive nor specific (Herwaldt *et al.*, 1996). The suggested microbiological criteria for bacteraemia include growth within 48 hours and multiple blood cultures positive for the same organism. In contrast, contamination is unlikely if the time to blood culture positivity is long, if polymicrobial growth of skin organism is observed, or if organisms grow while patient is receiving adequate antibiotic. Several studies, however, have shown that these criteria are of limited value (Krause *et al.*, 2003). These uncertainty regarding the significance of CoNS isolated from blood cultures usually resulted in over diagnosis and indirectly over used of anti-staphylococci especially vancomycin. Uncontrolled used of vancomycin may lead to development of resistant that will increased morbidity, mortality and total hospital cost.

Both phenotypic and genotypic characteristics of CoNS have been examined to differentiate infection-associated isolates from contaminants. Several indicators have been investigated in order to differentiate true bacteraemia from contamination, including number of positive blood cultures, species of CoNS and biotype, quantitative antimicrobial susceptibility testing and similarity in colony morphology and clonality (Garcia *et al.*, 2004). These variables have the following two problems: none has a high positive predictive value and they are useful only when two or more blood cultures in one series of such cultures are positive. This is because they are based on a demonstration that the strains isolated are identical and that the probability of contamination is very low.

Strain typing of CoNS by pulsed-field gel electrophoresis (PFGE) appears to have a higher positive predictive value than phenotypic characterisation by speciation, biotype and resistance profile. Quantitative antibiogram determination correlates well with CoNS

genetic relatedness assessed by PFGE, and could be rapid and cost-effective technique to aid in characterising CoNS blood isolates. PFGE is a widely used technique for analyzing a large amount of chromosomal DNA found in bacterial chromosomal fragments generated by endonuclease digestion. PFGE is considered as gold standard for subtyping of bacteria.

The advantage of PFGE is that it can profiles a large section of genome and the method is well established and reproducible. Periodic cross-sectional studies based on PFGE findings might be useful to estimate local contamination rates in an institution, which in turn can be used to improved the accuracy of the clinical diagnosis of bacteraemia (Senger *et al.*, 2007). The aim of study is to determine the significance and clonality of CoNS in multiple positive blood cultures by correlating the phenotypic (species identification and antibiotic pattern) the genotypic (pulsed-field gel electrophoresis) with clinical bacteraemia. The outcome of this study might be able to reduce the unnecessary used of antibiotic and to prevent or delay the emergence of vancomycin-resistant CoNS in the future in our local institution.

1.5 AIM OF THE STUDY

The aim of this study is to determine the significance and clonality of Coagulase-Negative staphylococci isolated from repeated blood culture in Hospital Universiti Sains Malaysia.

1.6 OBJECTIVES

1. To determine the predominant species of CoNS isolated from blood cultures from patients in Hospital Universiti Sains Malaysia.
2. To determine the clonality of CoNS isolated on multiple blood cultures by PFGE
3. To determine the association between phenotypic (API Staph ID and antibiogram pattern) and genotypic (PFGE) methods for species-level identification of clinical isolates of CoNS.
4. To determine the association between the clinical presentation and repeated isolation of CoNS as determined by phenotypic and genotypic.

1.7 HYPOTHESIS

Null Hypotheses

1. H_0 : There is no association between the phenotypic and genotypic methods for species-level identification of clinical isolates of CoNS.
2. H_0 : There is no association between the clinical presentation and repeated isolation of CoNS as determined by phenotypic and genotypic

Alternative Hypotheses

1. H_1 : There is association between the phenotypic and genotypic methods for species-level identification of clinical isolates of CoNS.
2. H_1 : There is association between the clinical presentation and repeated isolation of CoNS as determined by phenotypic and genotypic

Chapter 2

METHODS AND MATERIALS

2.1 SAMPLE SIZE

Sample size in this study was calculated using single proportion with power of 80% and confident interval of 95%.

Formula:

Sample size = $(z/\Delta)^2 \times P(1-P)$

$= (1.96 / 0.05)^2 \times (0.91 \times 0.09)$

= 126 pairs (CoNS isolated from blood culture)

P = Prevalence of Coagulase-Negative Staphylococci based on study done by Bearson *et al.* (2004) = 91%

Z = z distribution when $\alpha = 0.05 = 1.96$

2.2 STUDY DESIGN

This is a cross-sectional study (12 months duration) from June, 2007 till July, 2008. All isolates were identified as Coagulase-Negative Staphylococci (CoNS) based on the Gram stain (gram positive cocci), catalase positive and coagulase negative. Further species identification was done using API Staph ID (bioMerieux, Inc, Durham, USA) and clonality of repeated isolates were determined using pulsed-field gel electrophoresis

(PFGE). All procedures were done in Department of Medical Microbiology & Parasitology, School of Medical Sciences, USM.

2.2.1 Inclusion criteria

1. Clinical isolates - Repeated isolates of CoNS from blood culture samples were collected during this study.
2. The interval between the repeated isolates should not be more than 2 weeks.

2.2.2 Exclusion criteria

1. The interval between the repeated isolates exceeded more than 2 weeks.

2.3 SETTING

This study is conducted in Hospital Universiti Sains Malaysia (HUSM) and Medical Microbiology Laboratory, School of Medical Sciences. HUSM is an 800-bedded tertiary teaching hospital that is located in Kelantan, Malaysia.

2.4 SAMPLE COLLECTIONS

A total of 202 CoNS isolates were analyzed in this study. All isolates were collected from blood specimen which were sent to the Microbiology laboratory in Hospital Universiti Sains Malaysia during the study period. Subsequently these isolates were preserved in Microbank™ at -20°C.

2.5 SAMPLE STORAGE

All isolates that within the inclusion criteria were kept in the Microbank™ (Pro Lab Diagnostics, Ontario, USA) before the analysis.

2.6 IDENTIFICATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI BY CONVENTIONAL AND COMMERCIAL METHODS.

2.6.1 Catalase test

2.6.1.1 Principle

This test is used to differentiate between catalase producing bacteria, such as staphylococci, from non-catalase producing bacteria such as streptococci (Monica, 2000). Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer (Monica, 2000).

2.6.1.2 Procedure

One drop of hydrogen peroxide solution was put on a glass slide. By using a sterile wooden stick or a glass rod (*not* a nichrome wire loop), several colonies of the test organism were removed and immersed in the hydrogen peroxide solution. Care was taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur. Look for immediate bubbling.