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CHARACTERISATION OF THE *STAPHYLOCOCCUS SCIURI* GROUP BY MATRIX-ASSISTED LASER DESORPTION/IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

Staphylococcus aureus and coagulase-negative staphylococci (CNS) are recognised as important nosocomial pathogens. Laboratory identification and susceptibility testing remain important tools in the epidemiology and control of staphylococcal infection. Multi-drug resistance, particularly methicillin resistance is an escalating problem. The mechanism of resistance among S.aureus (MRSA) and CNS is encoded by the mecA gene, and has been found to be ubiquitous among the primitive animal CNS - *Staphylococcus sciuri*. It has been hypothesised that *S.sciuri* is a natural reservoir of methicillin resistance genes. Until recently *S.sciuri* has seldom been recovered from clinical material and rarely implicated in human infections. Over the past three years at Ashford Public Health Laboratory, 160 isolates of *S.sciuri* identified using the API32 Staph (Biomerieux) have been recovered from clinical samples, 30% of which expressed methicillin resistance. Seventeen ATCC strains of the *S.sciuri* group, comprising of *S.sciuri* subsp. *sciuri*, *S.sciuri* subsp. *carnaticus*, *S.sciuri* subsp. *rodentium*, *S.lentus* and *S.vitulinus* were analysed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and a database created. The aim of this study is to identify and compare clinical and non-clinical isolates using MALDI-TOF-MS. The results will be used to assess any correlation between subspecies of *S.sciuri*, and whether there is a link between expressed methicillin resistance in clinical isolates and assumed exposure to antibiotic pressure by comparing hospital acquired isolates with the community acquired isolates.

Introduction

Staphylococcus sciuri is a coagulase-negative novobiocin resistant staphylococcus primarily of animal origin, first described by Kloos *et al* in 1976. Over the last two decades this staphylococcus has seldom been recovered from humans and and other primates (Hajek V and Balusek J, 1985). It has been suggested that *S.sciuri* isolates recovered from humans may be a consequence of them having pets or recent contact with farm animals (Kloos WE, Schleifer, RF Smith RF, 1976).

However, in recent years there has been a greater incidence reported in clinical material, such as boils (Adegoke GO, 1986), wound infections (Udo EE *et al*, 1995, Kalowole DO, Shittu AO, 1997, Marsou *et al*, 1999, Aires De Sousa *et al* 2000), blood cultures (Udo EE *et al*, 1995, Marsou *et al*, 1999), CAPD-associated peritonitis (Marsou *et al*, 1999, Lang S *et al*, 1999, Wallet F *et al*, 2001), intravenous catheters (Horii T *et al*, 2001) and endocarditis (Hendin G and Widerstrom M, 1998).

The most significant aspect about this organism is the presence of a *mec*A gene, which in *S. aureus* and coagulase-negative staphylococci encodes for methicillin resistance (Chambers HF, 1997) which is a major nosocomial problem on a global scale.

The *mec*A gene is found uniformly distributed among S.sciuri (Couto *et al*, 1996, Wu *et al*, 1996, Wu *et al*, 1998). However, the mecA gene along with the transcriptional repressor gene mecI found in the chromosomal DNA of this organism may not have initially been involved in antibiotic resistance, but instead encoding for a protein as a normal function of cell wall biosynthesis (Couto *et al*, 1996, Wu *et al*, 1996).

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Expressed methicillin resistance among strains of *S.sciuri* subsp. *rodentium* has been observed in recent years. These isolates were shown to have more than one copy of the *mec*A gene i.e. a *mec*A gene similar to that of an MRSA called "MRSA *mec*A" and another called "sciuri *mec*A". In contrast strains of *S.sciuri* subsp. *carnaticus* and *S.sciuri* subsp. sciuri were susceptible to methicillin and carried only a single copy of the *mec*A gene - "sciuri *mec*A" (Couto et al, 1996, Wu *et al*, 1996, Couto *et al*, 2000).

These strains of *S.sciuri* subsp. *rodentium* were recovered from humans and animals that had been exposed to an antibiotic-rich environment. Resistance to methicillin seen in these strains of *S.sciuri* may have evolved due to point mutations in the mecA sequence reducing the affinity of an active site for methicillin, which may have been driven by increased antibiotic pressures (Couto *et al*, 1996).

In a recent study carried out by the same workers, strains of *S.sciuri* that possess the MRSA *mec*A gene, were recovered from humans that had not been exposed to an antibiotic rich environment thus indicating there is no correlation between carriage of *S.sciuri* with the MRSA *mec*A gene and antibiotic consumption (Couto *et al*, 2000).

Recent experimental evidence has shown that the *mec*A gene of *S.sciuri*, which has an as yet undefined domestic function, can be recruited to become a resistance determinant under conditions of antibiotic selection. The actual process that makes the silent "sciuri *mec*A" homologue of the methicillin susceptible *S.sciuri* strain become methicillin resistant determinant appears to be the replacement of a single nucleotide within the promoter sequence. This results in an increase in the rate of transcription of the gene into a penicillin binding like-protein, that closely resembles PBP2A of the *S.aureus mec*A determinant (Wu *et al*, 2001).

All methicillin-resistant strains of *S.aureus* are clonal descendants from a few ancestral strains that acquired the *mec*A gene. The *mec*A containing DNA is not readily self-transmissible, but some horizontal transfer must have occurred to have reached coagulase-negative staphylococci as well as *S.aureus* (Hiramatsu K *et al*, 1999). Recent evidence suggests that *in-vivo* horizontal transfer of *mec*A DNA from a *mec*A$^+$ *S.epidermidis* to a *mec*A- *S.aureus* did occur in a patient during treatment with flucloxacillin (Weilders CLC, *et al* 2001).

This supports the hypothesis that *S.sciuri* may serve as a reservoir of the *mec*A gene and may have been the evolutionary precursor of the structural gene PBP2a and the mechanism of methicillin resistance seen in *S.aureus* and coagulase-negative staphylococci (Couto *et al*, 1996).

An attempt was made to investigate whether expressed methicillin resistance is confined to a particular sub species of *S.sciuri* and subsequently a source of *mec*A by comparison of community and hospital acquired isolates. Conventional biochemical identification systems such as API (Biomeriuex), although able to confirm their identity, did not offer further speciation of the *S.sciuri* group. Molecular methods such as 16S rRNA and 16S-23S rDNA ITS-PCR although well established taxonomic tools, do require some degree of expertise and can be time consuming. Speciation of the *S.sciuri* group was achieved by performing matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS).

MALDI TOF MS is a rapid method of analyzing the cell surface components of microorganisms. It is these surface components which generate ions which produce a reproducible mass spectrum or fingerprint within a few minutes and is species, and in some case strain specific (Claydon M A, *et al* 1996). MALDI TOF MS has recently enabled the rapid discrimination between methicillin-sensitive

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and methicillin-resistant *S.aureus* (Edwards-Jones V, *et al* 2000) and would serve as a key epidemiological tool. It is highly likely that the distinguishing factor between these strains is the presence of a cell-surface moiety that is linked with the production of PBP2a.

Method

Bacterial cultures

Sixteen strains of the *S.sciuri* group were selected for analysis by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI TOF MS). These comprised: four strains of *S.sciuri* subsp. *sciuri* (ATCC 29059, ATCC 29061, ATCC 29062^T, DM93), three strains of *S.sciuri* subsp. *carnaticus* (ATCC 700058T, ATCC 700059, ATCC 700060), three strains *S.sciuri* subsp. *rodentiium* (ATCC 700061T, ATCC 700062, ATCC 700063), three strains of of *S.lentus* (ATCC 29070T, K-2, K-15) and three strains of *S.vitulinus* (ATCC 51145^T, ATCC 51162, ATCC 51163). The above were obtained from the North Carolina State University, Raleigh, North Carolina, USA. and are used to build a database. In addition a number of clinical isolates were analysed and compared to this database. All isolates were stored at -80°C using Cryobeads (Prolab diagnostics) and were sub-cultured on Columbia blood agar (CPHL), incubated at 37°C overnight prior to analysis.

Preparation of the bacteria for analysis by MALDI-TOF MS

Using a sterile disposable plastic 1uL loop a sweep of pure colonies was taken and applied to the wells of the target plate, to give a uniform surface coating. 12 replicates were used for each strain. Each sample well was overlain with 1uL of a freshly prepared matrix solution, 5-chloro-2-mercaptobenzothiazole (CMBT) at 3.0 mg/mL used for Gram positive organisms. The matrix was dissolved in acetonitrile:methanol:water (1:1:1) with 0.1% formic acid and 0.01M 18-crown-6 ether. A seven peptide mixture of known molecular weight standards, was pipetted onto the lock mass wells of the target plate. These peptides included bradykinin 1pmol/uL, angiotensin 1 1pmol/uL, Glu-fibrinopeptide B 1pmol/uL, renin substrate tetra deca peptide 1pmol/uL, ACTH (18-39 clip) 1pmol/uL, bovine insulin 2pmol/uL and ubiquitin 10pmol/uL. The target plate was then allowed to dry in air.

MALDI-TOF MS analysis was carried out using the M@LDI™ Linear time-of-flight mass spectrometer (Waters Corporation). A nitrogen laser giving a 337nm output of 3ns-pulse width was used and the laser fluence was set just above the threshold for ion production. The mass spectrometer was used in the positive ion detection mode using an acceleration voltage of +15kV. The data acquisition mass range was from *m/z* 500 to 10000 Da. The lock mass calibration was determined from the renin substrate ion at 1760 Da. For maximum throughput of samples the bacterial mass fingerprints were acquired automatically using the MAXspec real-time data selection algorithm to optimise the bacterial fingerprint signal in the mass range 600 to 3000 Da.

MicrobeLynx data analysis

For each strain, twelve replicates were compared for reproducibility using the root mean square (RMS) value. This value is obtained by comparing each replicate in turn with the average of the other 11 replicates, as shown in **Figure 1a and 1b**. An RMS rejection value of 3 was used to identify outliers significant at the 0.1% level. The acceptable spectra were then combined to give a representative spectral pattern for each organism, **Figure 2**, from which a database was prepared. Dendrograms were produced by an algorithm, where the proximity of one organism to another was generated using a spectral RMS calculation. Each node of the dendrogram is singly linked and organisms are grouped together using a simple average proximity.

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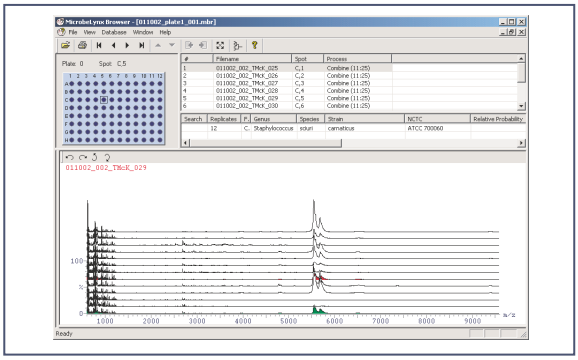

Figure 1a. MicrobeLynx Browser showing reproducibility of replicate spectra

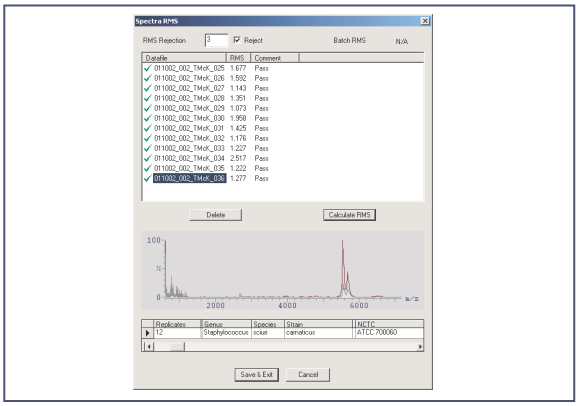

Figure 1b. Comparison of the RMS values for 12 replicate spectra from Staphylococcus scuiri sub species carnaticus ATCC 700060

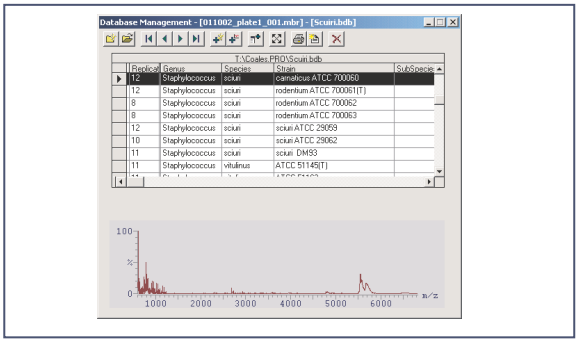

Figure 2. Average mass spectrum from database entry for Staphylococcus scuiri sub species carnaticus ATCC 700060

Results

A database was generated from the organisms listed in **Table 1**.

Genus	species	Sub species	strain
Staphylococcus	*sciuri*	*sciuri*	ATCC 29059
Staphylococcus	*sciuri*	*sciuri*	ATCC 29061
Staphylococcus	*sciuri*	*sciuri*	ATCC 29062^{T}
Staphylococcus	*sciuri*	*sciuri*	DM93
Staphylococcus	*sciuri*	*carnaticus*	ATCC 700058^{T}
Staphylococcus	*sciuri*	*carnaticus*	ATCC 700059
Staphylococcus	*sciuri*	*carnaticus*	ATCC 700060
Staphylococcus	*sciuri*	*rodentium*	ATCC 700061^{T}
Staphylococcus	*sciuri*	*rodentium*	ATCC 700062
Staphylococcus	*sciuri*	*rodentium*	ATCC 700063
Staphylococcus	*lentus*		ATCC 29070^{T}
Staphylococcus	*lentus*		K-2
Staphylococcus	*lentus*		K-15
Staphylococcus	*vitulinus*		ATCC 51145^{T}
Staphylococcus	*vitulinus*		ATCC 51162
Staphylococcus	*vitulinus*		ATCC 51163

Table 1.

These averaged spectra in the database were clustered together and displayed as a dendrogram. The dendrogram, **Figure 3**, shows the relationship between the different species and sub species of *Staphylococcus scuiri* group and infers the level of discrimination possible using MALDI TOF MS. These strains fall into four main groups with *Staphylococcus lentus* being the most distantly related strain. *Staphylococcus vitulinus* and *Staphylococcus scuiri* sub species scuiri are clearly separated from each other and from the other more closely related *Staphylococcus scuiri* sub species *carnaticus* and *Staphylococcus scuiri* sub species *rodentium*.

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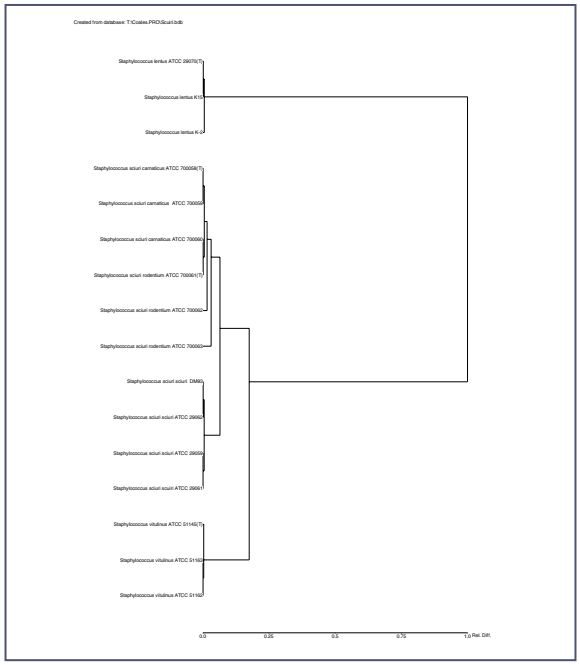

Figure 3. Dendrogram of the mass spectral fingerprints generated by MALDI TOF MS of the 16 strains of the Staphylococcus scuiri group

An initial study of 8 clinical isolates, which had previously been biochemically typed as *Staphylococcus scuiri* group only, was undertaken. Each of these isolates were searched against this Scuiri type-strain database and against a commercial database of ~700 entries generated by Manchester Metropolitan University, UK in collaboration with the NCTC, CPHL, London UK.

The MicrobeLynx results browser for these 8 clinical isolates is shown in **Figure 4**. The result for isolate SS1 highlighted and shows that the top eight hits are all *Staphylococcus scuiri*. More specifically there is a 97% probability that the best match is a *Staphylococcus scuiri* sub species rodentium.

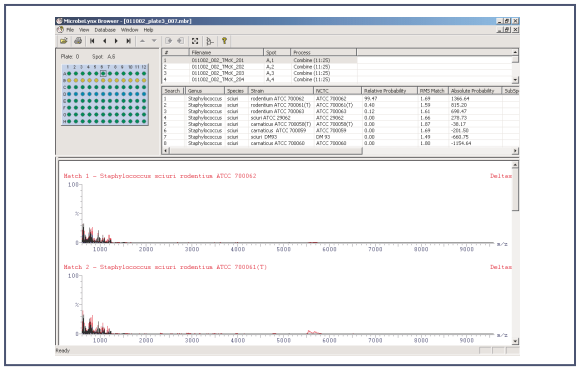

Figure 4. Results Browser for 8 clinical isolates showing the 2 best matches in the database

These eight clinical strains were clustered along with the scuiri database indicating their relationships to the database entries. This dendrogram is shown in **Figure 5** and shows the SS1 isolate clusters close to the *Staphylococcus scuiri* sub species *rodentium*, SS3 close to *Staphylococcus scuiri* sub species *carnaticus* and the other six isolates in the *Staphylococcus scuiri* sub species *scuiri* group.

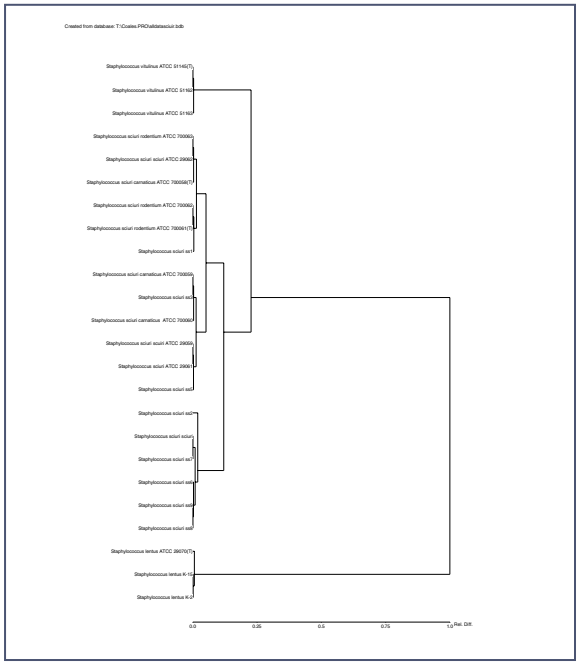

Figure 5. Dendrogram of the mass spectral fingerprints generated by MALDI TOF MS of the 16 strains of the Staphylococcus scuiri group clustered with 8 clinical isolates

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Conclusion

- A database was generated from sixteen different species and sub species of *Staphylococcus scuiri* group
- The relationship between the group members could be inferred by the generation of a dendrogram, produced by comparison of their mass spectral fingerprints.
- Four main groups could be discriminated using MALDI TOF MS with *Staphylococcus lentus* being the most distantly related strain.
- *Staphylococcus vitulinus* and *Staphylococcus scuiri* sub species *scuiri* are clearly separated from each other and from the other more closely related *Staphylococcus scuiri* sub species *carnaticus* and *Staphylococcus scuiri* sub species *rodentium*.
- The 8 clinical isolates were all identified as *Staphylococcus scuiri*.
- Further work is required to determine the level of discrimination possible by MALDI TOF MS

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