Isolation and molecular characterization of multiresistant \textit{Staphylococcus sciuri} and \textit{Staphylococcus haemolyticus} associated with skin and soft-tissue infections

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The isolation, molecular identification and genotyping of multiresistant \textit{Staphylococcus sciuri} and \textit{Staphylococcus haemolyticus} from skin and soft-tissue infections are reported. Accurate and full identification of three coagulase-negative staphylococcal isolates was achieved using PCR, while the API STAPH method failed to identify an isolate of \textit{S. haemolyticus} fully. The PCR assay, which detects polymorphism in the 16S–23S rRNA spacer region, is shown to be potentially useful for rapid and accurate identification of coagulase-negative staphylococci. Identical PFGE type and antibiotic-resistance profiles of two methicillin-resistant \textit{S. haemolyticus} isolates in this study suggest the existence of a multiresistant community clone.

\textbf{INTRODUCTION}

Skin and soft-tissue infections (SSTIs) are among the most common infectious diseases and are a frequent cause of visits to health-care providers. They cover a wide clinical spectrum, from superficial, localized and sometimes self-limiting, to deep, rapidly spreading and potentially life-threatening. These infections may be due to a variety of infectious agents including viruses, mycobacteria, other bacteria or fungi. Early diagnosis and treatment of infections caused by bacteria remain a major clinical challenge (Alam et al., 2002). Most bacteria have multiple routes of resistance to any drug and, once resistant, can rapidly produce vast numbers of resistant progeny (Livermore, 2003). The widespread antibiotic resistance in bacteria found in SSTIs compounds treatment management by health-care practitioners, for example, resulting in prolonged hospital stay, increased trauma care and treatment costs (Bowler et al., 2001).

Among the members of the genus \textit{Staphylococcus} that are widely distributed in nature, some species are important human pathogens in SSTIs, causing substantial rates of morbidity and mortality (Engemann et al., 2003; Isaacs, 2003). \textit{Staphylococcus aureus}, a coagulase-positive staphylococcal species, is the main aetiological agent and most frequently isolated micro-organism in various SSTIs (Bowler et al., 2001; Charalambous et al., 2003). Coagulase-negative staphylococci (CNS) have long been regarded as harmless skin commensals and dismissed as culture contaminants. However, the incidence of CNS reported as causative agents of nosocomial infections has risen substantially with the increasing use of prosthetic devices and other invasive technologies (Peters, 1988; Huebner & Goldmann, 1999; Petinaki et al., 2001). Although \textit{Staphylococcus epidermidis} accounts for the majority of CNS infections, other species have also been identified in association with human infections (Buttery et al., 1997; Sandoe et al., 1999; Wallet et al., 2000). In addition, cases of multi-drug-resistant CNS species in human infection have been reported (Petinaki et al., 2001; Stepanovic et al., 2002; Basaglia et al., 2003). Limited treatment options and prolonged course of infection due to these CNS species could have severe consequences for patients. Full and accurate identification of CNS isolates in clinical samples is therefore of great importance for epidemiological purposes and infection control measures (Varaldo & Biavasco, 1997; Basaglia et al., 2003).

We present a detailed microbiological and molecular study on multiresistant \textit{Staphylococcus sciuri} and \textit{Staphylococcus haemolyticus} associated with SSTIs in three health institutions in Nigeria. A PCR-based genetic method assisted in prompt and accurate identification of the CNS isolates.
METHODS

Isolates. Patient 1: a wound swab was taken from a 6-year-old girl who was injured on the right side of her head. She was hospitalized at the Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria, and had been discharged from the health facility. The specimen was taken on 1 January 2001, when she reported for wound dressing at the outpatient department. The cause of the injury was unknown and further clinical information could not be obtained. She had been on antibiotic medication of ampiclox (a combination of ampicillin and cloxacillin) and gentamicin for 1 week. Patient 2: a 35-year-old woman registered for wound care at the Sabo Health Centre, a local health institution in Ile-Ife, Nigeria, on 12 December 2000. The wound type was a furuncle located at the left armpit of the patient. Patient 3: a 32-year-old man with a localized skin infection on his left hand. He reported for wound dressing at a local hospital in Ipitumodu (20 km from Ile-Ife), Nigeria, on 8 January 2001. Based on our enquiries, patients 2 and 3 were local residents of the community, had not been hospitalized prior to sample collection and were not on antibiotic medication at the time of sample collection.

The wound types were assessed as infected due to the presence of purulent discharge according to Cutting & Harding (1994). Wound samples were taken before wound cleansing and dressing performed by a nurse. Care was taken to avoid contamination by the normal skin flora.

Phenotypic identification. Wound swabs were immediately streaked on blood agar and mannitol/salt agar (MSA) and incubated at 37 °C for 24 and 48 h, respectively. Haemolysis on blood agar and growth and fermentation of mannitol on MSA were examined and noted. Two or three well-separated colonies from each medium were picked and subcultured onto nutrient agar for further characterization. Preliminary identification of isolates resembling staphylococci was performed on the identification of isolates resembling staphylococci was performed on the basis of colonial morphology and cultural characteristics on blood agar and MSA, Gram reaction (Gram-positive cocci in clumps), catalase, coagulase and DNase tests (Isenberg, 1992). CNS isolates were identified by API STAPH according to the manufacturer’s instructions (bioMérieux).

Nasal isolates used in PFGE were obtained from samples from medical personnel and students in a Nigerian hospital. Nasal swabs were streaked on MSA and incubated at 37 °C for 48 h. Staphylococci were identified as above.

Antibiotic sensitivity test. Susceptibility testing was performed on isolates using the agar disc-diffusion technique. The antibiotics included penicillin (10 U), ampicillin (10 μg), oxacillin (1 μg), streptomycin (30 μg), tetracycline (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), mupirocin (200 μg), novobiocin (30 μg), fusidic acid (10 μg), trimethoprim/sulfamethoxazole (25 μg), teicoplanin (30 μg), vancomycin (30 μg) and cefuroxime (30 μg). Performance of susceptibility testing and interpretation followed the recommendations given by the NCCLS (2000). Isolates with zone diameters ≤17 mm to novobiocin and ≤18 mm to fusidic acid (antibiotics not included in the NCCLS guidelines) were adjudged resistant. Isolates were screened for intermediate resistance to vancomycin on in-house-prepared brain heart infusion agar (Oxoid) containing 5 μg vancomycin ml⁻¹ (Sigma). An aliquot of 10 μl of 0·5 McFarland culture were spotted on the plates and incubated at 37 °C for 48 h.

Molecular confirmation and identification of CNS species. A multiplex PCR assay to detect the nuc and mecA genes (Bignardi et al., 1996; MacKenzie et al., 2002) was conducted to confirm phenotypic identification of the isolates as coagulase-negative and methicillin-resistant, respectively. Molecular identification of CNS isolates to the species level was performed by a simple and rapid PCR-based method, rRNA spacer-length polymorphism analysis (Couto et al., 2001; Lee & Park, 2001), using primers published previously by Jensen et al. (1993). Three to five colonies were resuspended in NET buffer (10 mM Tris/ HCl, 1 mM EDTA, 10 mM NaCl) containing 10 U a-chymopapetidase (Sigma) and incubated at 50 °C for 10–15 min. Two microlitres of extracted DNA was added to 12·5 μl Reddy-Load (Abgene) PCR mix (0·2 mM dNTPs, 3 mM MgCl₂, 0·625 U Taq polymerase, 20 mM Trizma base, 50 mM KCl) and 25 pmol primers G1 (5’-GAAATCG TAACAAGG) and L1 (5’-GAAAGCATCCAGCAGTG) (MWG-Biotech). Tissue-culture-grade water (Sigma) was added to give a final volume of 25 μl. Cycling conditions consisted of 34 cycles of 95 °C, 55 °C and 72 °C for 1 min each, followed by a final cycle of 72 °C for 7 min. Six microlitres of PCR product was loaded into 1·5 % Mastel GP agarose (Mast Diagnostics). Electrophoresis was performed in 0·5 × TBE buffer (pH 8) at 100 V for 10·5 h. Patterns of PCR products from clinical isolates were compared with those for reference strains.

PFGE typing. Genetic relatedness and characterization of isolates using PFGE typing of Smal (Invitrogen)-digested DNA was carried out by a modification of the protocol described previously by Bannerman et al. (1995). A colony was inoculated into brain heart infusion broth and incubated overnight at 37 °C without agitation. The pellet from 0·4 ml of this culture was washed in 0·8 ml NET buffer, mixed with 500 μg lysozyme (Sigma) and 30 U Lysostaphin (Sigma) and an equal volume of 2 % SeaPlaque agarose (Flowgen) at 50 °C was added. The cell/agarose suspension was loaded into block moulds (Bio-Rad) and allowed to solidify at 4 °C. Cells were lysed by incubation at 37 °C overnight in lysis buffer (6 mM Trizma base, 100 mM EDTA, 1 M NaCl, 0·5 % Brij 58, 0·2 % sodium deoxycholate, 0·5 % lauroyl sarcosine). This was followed by a second overnight incubation at 50 °C in 1 ml proteolysis buffer (1 % lauroyl sarcosine, 75 μg proteinase K in 0·5 M EDTA). The blocks were washed three times for 10 min each at room temperature in TE buffer (10 mM Trizma base, 1 mM EDTA). One quarter of each agarose block was digested with 30 U Smal for 3 h according to the manufacturer’s instructions and loaded into the wells of 1 % PFGE-certified agarose gel (Bio-Rad). Electrophoresis was performed in 0·5 × TBE buffer (pH 8) (Invitrogen) by the contour-clamped homogeneous electric field method with a CHEF system (Bio-Rad). Fragments were separated with a linear ramped pulse time of 6·8–63·8 s over a period of 23 h and gels were stained with 1 μg ethidium bromide ml⁻¹ (Sigma) solution for 30 min, visualized under UV and photographed.

RESULTS

Cultures obtained from wound swabs of the purulent SSTIs in this study yielded small, white, convex colonies with no haemolysis on blood agar for patient 1 and β-haemolytic, white, convex colonies for patients 2 and 3. Yellow colonies similar to those of S. aureus were observed in each wound specimen plated on MSA. Staphylococci were presumptively identified based on colonial morphology and cultural characteristics on blood and MSA, Gram reaction (coccii in clumps), catalase production and coagulase test. The isolates were coagulase-negative by the tube test using rabbit plasma after 4 and 24 h. The staphylococcal isolate from patient 1 (isolate THT) produced a weak-positive DNase result, while isolates from patients 2 and 3 were DNase-negative (isolates 14 and 34). The absence of the S. aureus-specific nuc (thermonuclease) gene supported the identification of these isolates as CNS (Fig. 1).

Isolate THT was identified as S. sciuri (95·8 %) with biocode 633580170 by the API STAPH method. Isolate 14 was identified as S. haemolyticus (biocode 6632051; 93·3 %). However, isolate 34 could be identified only to the genus level (biocode
6636051; 51.1%). rRNA spacer-length polymorphism analysis confirmed the API STAPH identification for isolates THT and 14 (Fig. 2). Isolate 34 was identified as *S. haemolyticus* based on the similarity of the DNA fingerprint pattern to that of *S. haemolyticus* NCTC 11042T, though it differed from the pattern of the reference strain by one band. PFGE patterns for isolates 14 and 34 were identical (pulsotype B; Fig. 3).

Susceptibility testing was performed on these isolates. *S. sciuri* (isolate THT) was resistant to penicillin, ampicillin, methicillin, fusidic acid, streptomycin, tetracycline, cefuroxime, trimethoprim/sulfamethoxazole and novobiocin, but sensitive to gentamicin, mupirocin, ciprofloxacin, teicoplanin and vancomycin. No growth was observed on the vancomycin screen plates after incubation for 48 h. The two *S. haemolyticus* isolates exhibited similar sensitivity patterns, with resistance to penicillin, ampicillin, methicillin, streptomycin, gentamicin, tetracycline and ciprofloxacin and sensitivity to fusidic acid, mupirocin, novobiocin, trimethoprim/sulfamethoxazole and cefuroxime. Pheno-

typic resistance to methicillin by the three CNS isolates was confirmed by detection of *mecA* using PCR. By the agar disc-diffusion technique, both *S. haemolyticus* isolates were sensitive to vancomycin and teicoplanin. However, confluent growth was observed on the vancomycin screen plates after 48 h incubation, indicating that these two isolates were intermittently resistant to vancomycin.

### DISCUSSION

Identification of the pathogen causing disease and understanding its resistance pattern has proved to be helpful in the selection of empirical antimicrobial therapy and in infection control measures in health institutions. Identification of staphylococci in many clinical microbiology laboratories is often limited to *S. aureus*, while non-*S. aureus* isolates are simply reported as CNS. A large number of CNS strains recovered from clinical samples have become a serious problem as they express methicillin resistance, which involves all β-lactam antibiotics and leads to significant limitation of therapeutic options (Bogado et al., 2001). Particular species of CNS are also associated with distinct types of infections and patterns of antimicrobial susceptibility (Kloos & Bannerman, 1994). Therefore, species identification of CNS is increasingly of clinical and epidemiological interest to clinicians. In this study, we char-

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Fig. 1. PCR detection of *mecA*, *nuc* and *mupA*. Lanes: 1, water control; 2, *mecA*-nuc-mupA-positive control; 3, *mecA*-negative, *nuc*-positive, *mupA*-negative control; 4, isolate 14 (*S. haemolyticus*); 5, isolate 34 (*S. haemolyticus*); 6, isolate THT (*S. sciuri*). Sizes of PCR products are indicated in bp.

Fig. 2. rRNA spacer length polymorphism. Lanes: 1, isolate THT (*S. sciuri*); 2, *S. aureus* (epidemic MRSA type 15); 3, *S. aureus* (epidemic MRSA type 16); 4, *S. sciuri* reference strain NCTC 12103T; 5, isolate 14 (*S. haemolyticus*); 6, *S. haemolyticus* reference strain NCTC 11042T; 7, isolate 34 (*S. haemolyticus*).

Fig. 3. PFGE profiles. Lanes: 1, *S. aureus* NCTC 8325; 2, isolate 14 (*S. haemolyticus*); 3 and 4, *S. haemolyticus* (medical personnel nasal isolates); 5, isolate 34 (*S. haemolyticus*).
acterized CNS isolates from SSTIs of three patients in Ile-Ife and Ipetumodu, Nigeria, using phenotypic and genotypic methods.

There have been few reports on the isolation of S. sciuri, principally found in animal species, in clinical specimens from SSTIs (Kolawole & Shittu, 1997; Marsou et al., 1999; Stepanovic et al., 2002). The isolation of S. sciuri from clinical specimens has generated a lot of interest in the potential of this micro-organism to cause infection in humans. However, from the clinical standpoint, most isolates recovered from humans have not been considered important and, at present, only five cases of S. sciuri infection have been established (Hedin & Widerstrom, 1998; Wallet et al., 2000; Horii et al., 2001; Stepanovic et al., 2002; Tsakris et al., 2002). The fact that the wound type was assessed as infected due to the presence of purulent discharge, according to Cutting & Harding (1994), and S. sciuri was isolated and subsequently identified by phenotypic and genotypic methods suggests that this organism can cause SSTIs. In addition, the strain, like those reported previously by Stepanovic et al. (2002) and Tsakris et al. (2002) in clinical infections, was multiresistant. The isolation of multiresistant S. sciuri from clinical sources is important not only because of its serious impact on the course of infection, but because this species is a potential source of genes encoding resistance to antibiotics for other staphylococci pathogenic for man (Wu et al., 2001). The clinical relevance of S. sciuri is still controversial, but it may be an important opportunistic pathogen in human infection.

S. haemolyticus has been associated with septicemia in neonates and various infections in individuals with compromised host defences and implanted foreign bodies (Peters, 1988; Mehta & Kumari, 1997). The emergence of strains with decreased susceptibility to vancomycin and especially teicoplanin has also been reported in health institutions (Sloos et al., 1998; Tabe et al., 1998). It is highly prevalent in the hospital environment, with a tendency to develop resistance to multiple antibiotics (Froggatt et al., 1989; Birnbaum et al., 1991; Perdreau-Remington et al., 1995). Such multiresistant S. haemolyticus strains have been described frequently causing colonization and infection in hospitalized patients (Mehta & Kumari, 1997). The vast majority of community-acquired SSTIs are caused by Gram-positive cocci or are polymicrobial in nature. Multidrug-resistant Gram-positive cocci are rarely associated with community-acquired SSTIs, but are frequently found in hospital-acquired SSTIs (Hau, 2002). Multiresistant S. haemolyticus is an infrequently reported staphylococcal species in community-acquired SSTIs. The two multiresistant strains were isolated from patients with SSTIs who had no record of hospitalization. The patients received treatment in two local health institutions located 20 km from each other. PFGE analysis revealed that the strains were of the same pulstype. In a separate study conducted in Ile-Ife, a methicillin-resistant S. haemolyticus isolate, recovered from a nasal swab from a member of the medical personnel, was noted with an identical PFGE type to that of isolates 14 and 34 in this study (A. Shittu and others, unpublished results). This suggests that there could be a multiresistant local clone in the community. Multidrug-resistant CNS could be underreported, remain largely unknown or uncharacterized because many clinical microbiology laboratories do not identify CNS to the species level. Moreover, the majority of patients with superficial infections are treated in outpatient departments, which are not guided by microbiological analysis. The community could therefore serve as a reservoir of multidrug-resistant CNS. More clinical and microbiological studies are needed in order to understand the epidemiology of S. haemolyticus in community-acquired SSTIs.

In many clinical laboratories, CNS are not identified to species level and, in most cases, identification of clinically significant CNS is carried out through conventional methods and commercial identification kits. However, correct identification of all clinical isolates of CNS is not easy, because the biochemical traits of the species are similar and many clinical isolates show intermediate traits (Kawamura et al., 1998). rRNA spacer length polymorphism analysis is a recognized method for both typing and identification of various bacterial species including the staphylococci and has been applied successfully to the identification of CNS to the species level (Couto et al., 2001; Lee & Park, 2001). This simple, rapid and cost effective assay might provide a potential tool, especially in hospital laboratories, for the full and accurate identification of CNS and thus help to understand better the epidemiology of CNS. Recent reports of misidentification of CNS species by API STAPH (Yasuda et al., 2002; Basaglia et al., 2003) and the inconclusive identification of one of the isolates in this study (isolate 34) underline the importance of molecular methods in the accurate identification and characterization of CNS.

In conclusion, multiresistant CNS strains could be important agents in human infections, especially SSTIs. The emergence of molecular methods for identification of bacterial pathogens is a welcome development that is expected to complement phenotypic identification and assist in the full identification of aetiological agents of infection for epidemiological purposes.

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