Genetic analysis of methicillin-resistant *Staphylococcus aureus* from a Nigerian hospital

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Summary. Methicillin-resistant strains of *Staphylococcus aureus* isolated during 1985 and 1987 at a Nigerian hospital were compared by resistance profiles, plasmid analysis, and pulsed-field gel electrophoresis of chromosomal DNA. The results indicated that the isolates from the two periods were unrelated with regard to all three aspects. None of the isolates was similar to the classical MRSA nor to the epidemic MRSA of Australia or the UK. The MRSA isolated in 1985 had a similar plasmid to MRSA isolates from Singapore, but differed from them when compared by pulsed-field gel electrophoresis.

Introduction

Multi-resistant *Staphylococcus aureus* continue to be a major cause of serious infections in man, both in hospitals and in the community.¹ Since they were first reported in the UK in 1961,^{2–4} methicillin-resistant *S. aureus* (MRSA) have been found in many countries, including Britain,⁵ France,⁶ USA,⁷ Ireland^{8,9} and Australia.¹⁰ MRSA are important clinically because their resistance to methicillin confers cross-resistance to other β -lactam antibiotics, including cephalosporins. In addition, MRSA are frequently resistant to most other antimicrobial agents except vancomycin.¹¹

MRSA isolated in the UK, Europe and Australia until the early 1970s shared common characteristics.^{12–15} Typically, they had high-level chromosomal resistance to streptomycin and inducible resistance to erythromycin. They contained two plasmids: one of c. 30 kb, encoding penicillinase and resistance to heavy metals, and one of 4.4 kb, encoding tetracycline resistance.¹⁵ These isolates have been described as the “classic” MRSA.¹⁵ Genetic studies on MRSA isolates from Eastern Australian hospitals in the 1980s (EA MRSA) revealed differences from the classic MRSA.^{13–15} EA MRSA typically have chromosomal determinants for penicillinase and resistance to heavy metals, tetracycline, minocycline, streptomycin (low-level) together with inducible resistance to erythromycin and lincomycin. They usually carry three plasmids: a cryptic plasmid (c. 1.8 kb), a chloramphenicol-resistance plasmid (c. 4.5 kb) and a c. 28-kb incompatibility group 1 plasmid, that encodes resistance to nucleic acid binding compounds and, sometimes, gentamicin, kanamycin and trimethoprim, together with penicillinase production.¹⁵ MRSA

resembling the EA MRSA have also been reported in some London hospitals.¹⁶ The similarity of the London and EA MRSA has been confirmed by examining the chromosomal DNA of the isolates with an M13 repeat probe¹⁷ and by pulsed-field gel electrophoresis.¹⁸

Despite the widespread incidence of MRSA in many parts of the world, they have been reported only recently in Nigerian hospitals.^{19,20} In a study of antibiotic resistance in *S. aureus* isolated at the Lagos University Teaching Hospital (LUTH) in 1985, < 2% of the isolates were methicillin-resistant (Udo and Grubb, unpublished results). By 1987, however, 50% of the *S. aureus* isolates at this hospital were methicillin-resistant.¹⁹ Seven of the 42 MRSA then collected were resistant to multiple antibiotics and were described as epidemic strains.¹⁹ This paper reports the genetic study of these seven isolates in comparison to one organism isolated at the same hospital in 1985. The isolates were also compared with MRSA from other parts of the world.

Materials and methods

Bacteria and plasmids

The isolates and reference strains studied are listed in the table.

Media and reagents

Brain Heart Infusion Broth (BHIB), Brain Heart Infusion Agar (BHIA), Trypticase Soy Broth (TSB) and Mueller Hinton Agar (MHA) were purchased from Gibco Diagnostics (Madison, WI, USA). Lysostaphin, ribonuclease A and proteinase K were purchased from Sigma. *Sma*I restriction endonuclease

duction. All strains were maintained by twice-weekly subculture on to Columbia blood agar and Skirrow's agar.

Growth of bacteria in broth cultures. Surface bacterial growth on a Columbia blood agar plate was harvested with a sterile swab and suspended in 5 ml of sterile saline 0.9% to form a concentrated suspension. A 1-ml inoculum was added to Brain Heart Infusion Broth (BHIB, Oxoid) supplemented with fetal calf serum (Flow Laboratories, Irvine) 8%, and vancomycin (AddaTab, Mast Laboratories, Bootle) 6.7 mg/L. The cultures were grown as shallow layers, 2–3 ml deep, in petri dishes (Bibby Sterilin, Stone) with the side walls perforated just below the lid, to promote gas exchange during incubation.

The cultures were incubated at 37°C for 72 h under micro-aerophilic conditions. The purity of each culture was checked by Gram's stain and urease activity. Bacteria were harvested by centrifugation at 2500 *g* for 10 min, and washed three times in saline 0.9%.

Preparation of radiolabelled bacteria. Bacteria were grown in supplemented BHIB, containing ³H adenine 10 µl/10 ml of broth. Uptake of radiolabel was satisfactory after incubation for 72 h. The bacteria were harvested by centrifugation at 750 *g* for 15 min, washed three times with phosphate-buffered saline (PBS), pH 7, and suspended in saline 0.9% to give an OD₆₁₀ of 0.13, which had been demonstrated previously to be equivalent to a viable count of 1 × 10⁷ cfu/ml.

Opsonisation of bacteria

A single pool of normal human serum (NHS) was derived from the blood of *c.* 20 healthy laboratory workers, and stored in small quantities at –70°C until use. NHS was assayed for anti-*H. pylori* surface antigen and anti-urease specific antibody by ELISA^{9,10} at the Public Health Laboratory, Preston. *H. pylori* antibody titres varied between 2000 and 4000, and urease antibody titres between 2000 and 8000.

NHS was diluted in Hanks's Balanced Salts Solution 10% supplemented with gelatin 0.1% (GHBSS) to obtain various serum concentrations. Complement activity was destroyed by heating NHS to 56°C for 30 min and the alternative pathway activity was destroyed by heating NHS to 50°C for 30 min.¹¹ Components of the classical pathway were blocked by treating NHS with ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetra-acetic acid (final concentration 10 mM) in the presence of equimolar MgCl₂ (EGTA-MgCl₂).¹²

H. pylori-specific antibody was removed by triple absorption against a pool of three strains of *H. pylori* at 0°C for 15 min. Titres of *H. pylori* antibody fell by between four- and eight-fold and anti-urease antibody titres by between eight- and 32-fold. Specific antibody and complement were removed by sequential heating and absorption. In some experiments, serum absorbed with *H. pylori* was further treated by triple absorption

(0°C for 15 min) with *Staphylococcus aureus* (Cowan 1 strain) as a source of protein A. Protein A binds to the Fc region, inactivating immunoglobulin irrespective of antigenic specificity.¹³

Suspensions of *H. pylori* in saline 0.9% were adjusted to either an OD₆₁₀ of 0.13 (viable count 1 × 10⁷ cfu) for the radiolabelled bacterial experiments, or OD₆₁₀ of 0.3 (viable count 2 × 10⁷ cfu) for the assessment of PMNL chemiluminescence. Equal quantities of bacterial suspension and NHS diluted in GHBSS were mixed and incubated at 37°C for 15 min in a rotary incubator. The bacteria were sedimented by centrifugation at 2500 *g* for 15 min and resuspended in their original volume in GHBSS. Opsonised bacteria were used immediately.

Preparation of PMNL

PMNL were freshly prepared for each experiment, by a method based on that of Böyum.¹⁴ Sixty ml of heparinised blood was taken from a series of healthy volunteers and mixed with 20 ml of dextran 150 in saline 0.9% for 30 min to sediment erythrocytes. Ten-ml volumes of leucocyte-rich supernate were decanted into conical centrifuge tubes containing 3–5 ml of Leucocyte Separation Medium (Flow Laboratories) and centrifuged at 750 *g* for 30 min. The PMNL-rich layer was then resuspended in NH₄Cl 0.87% to lyse any remaining red blood cells. PMNL were washed in GHBSS and resuspended to a concentration of 2 × 10⁷ PMNL/ml. In all experiments PMNL were used within 3 h of separation.

Measurement of phagocytosis by uptake of radiolabelled bacteria

The method was based on that previously described by Verhoef *et al.*¹⁵ in which each experimental limb consisted of a set of four reaction mixtures. Two of the four contained 0.1 ml of opsonised bacteria in GHBSS and an equal volume of PMNL. The other two contained 0.1 ml of bacteria with an equal volume of GHBSS but no PMNL. These acted as controls, allowing an estimate to be made of any non-specific binding of bacteria to the walls of the reaction vessels. Each experimental limb was performed in duplicate. After incubation at 37°C for 15 min, one vial containing PMNL (washed cells) and one containing GHBSS alone (control) were centrifuged at 750 *g* for 5 min to pellet the PMNL; unphagocytosed bacteria remained in suspension and were discarded with the supernate. Pellets were washed three times in ice-cold PBS to remove extracellular radiolabel, and then scintillation fluid (Canberra Packard, Caversham) was added to lyse all the remaining cells. The remaining two vials had scintillant added immediately after incubation to produce an estimate of the total radio-label present (total count).

The radioactivity of each sample was measured in an "Ultrabeta" scintillation counter (LKB, Milton

each dilution was spread on to selective media, as in mixed-culture transfer.

In both mixed-culture and conjugation experiments, controls consisting of donor and recipient only were used. Transfer was considered to have occurred when growth was obtained on selective plates from the donor plus recipient mixtures and not on selective media plated with either organism alone.

Plasmid isolation

Plasmid extracts suitable for restriction endonuclease analysis were isolated by the cetyltrimethylammonium bromide method.²⁵ Horizontal agarose gel electrophoresis and restriction enzyme analysis were performed as described previously.²³

Pulsed-field gel electrophoresis

Electrophoresis of DNA was performed by the contour-clamped homogeneous electric field (CHEF) method. Cells were treated as reported previously,¹⁸ though with some modifications. They were grown overnight in 5 ml of BHIB at 35°C, washed twice in 10 ml of 50 mM EDTA, pH 8.0, and resuspended in 6 ml of EC buffer—6 mM Tris; 1 M NaCl; 100 mM EDTA; Brij 58 (Sigma) 0.5%, sodium deoxycholate 0.2%, sodium lauroylsarcosine 0.5%, pH 7.5.²⁶ The cell density was then adjusted to $c. 3 \times 10^8$ cfu/ml. Equal volumes of this suspension and agarose (Sigma type 1, A-6013) 1% w/v were mixed and aspirated into glass capillary tubes (diameter, 1.6 mm). After these plugs had solidified, $c. 1$ cm was expelled into 200 μ l of lysing solution (lysostaphin 200 mg/L in 50 mM EDTA) and incubated at 37°C for at least 4 h. The plugs were then held overnight at 50°C in 200 μ l of EST buffer (0.5 M EDTA, 5 mM Tris, sodium lauroylsarcosine, 1%, pH 7.5) containing proteinase K, 20 g/L, then washed three times for 2 h in 50 mM EDTA at room temperature and stored at 4°C in 50 mM EDTA until used. Before use, the plugs were digested with *Sma*I restriction endonuclease, according to the supplier's instructions, and inserted into wells of an agarose (Sigma Type 1, A-6013) 1% gel. Electrophoresis was performed with a CHEF-DR II system (BioRad Laboratories, Richmond CA, USA) at 4°C for 42 h in half-strength TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) at 120 V. The pulse times were 10 s initial and 80 s final.

Results

Characteristics of the isolates

The seven isolates obtained in 1987—WBG7208, WBG7209, WBG7210, WBG7211, WBG7212, WBG7213 and WBG7214—all belonged to phage group III.¹⁹ They all produced penicillinase and were

resistant to methicillin, kanamycin, neomycin, streptomycin, tetracycline and cadmium. They harboured a single plasmid of $c. 30$ kb (fig. 1).

Strain WBG4763, isolated in 1985, had the phage pattern 53/85, harboured a single plasmid of $c. 30$ kb, produced penicillinase and was resistant to methicillin, gentamicin, kanamycin, neomycin, streptomycin, tetracycline, minocycline, cadmium, mercury, phenyl mercuric acetate and the nucleic acid binding compounds, propamidine isethionate and ethidium bromide. All the isolates were susceptible to mupirocin and vancomycin.

Plasmid analysis

During the curing experiments, only cadmium resistance was lost from the seven 1987 isolates. Its loss correlated with the disappearance of the 30-kb plasmid. Of 168 colonies of the 1985 isolate, WBG4763, screened for loss of resistance, one lost methicillin resistance and four lost resistance to cadmium, mercury, phenyl mercuric acetate, propamidine isethionate and ethidium bromide. Loss of resistance to the last four compounds corresponded to the disappearance of the 30-kb plasmid, but the loss of methicillin resistance appeared to be independent of plasmid loss. To test further whether methicillin resistance was associated with the plasmid, extrachromosomal DNA was isolated from strain WBG4763 and its methicillin-susceptible derivative (WBG6198) and digested with *Eco*RI restriction endonuclease. The fragments thereby generated were identical (not shown), con-

Fig. 1. Plasmid content of the isolates obtained in 1987. Lane 1, WBG4483, contains plasmid mol. wt markers; 2, WBG7208; 3, WBG7209; 4, WBG7210; 5, WBG7211; 6, WBG7212; 7, WBG7213; 8, WBG7214.

Fig. 2. *Eco*RI restriction enzyme analysis of plasmid pWBG663 from the Nigerian isolate WBG4763 with plasmids from Singaporean MRSA. Lane 1, pWBG663; 2, pWBG278; 3, pWBG281.

firming that the methicillin-resistance determinant was not located on the plasmid.

To study the isolates further, their plasmids were transferred to WBG1876 by mixed-culture transfer. Only cadmium resistance was transferred from WBG7208 to WBG7214. Their transcipts had acquired the 30-kb plasmid. With isolate WBG4763, penicillinase production and resistance to cadmium, mercury, phenylmercuric acetate, propamidine isethionate and ethidium bromide were transferred together with the 30-kb plasmid, designated pWBG663. Methicillin resistance was not transferred. None of the isolates transferred any of their resistance determinants to WBG541 by conjugation.

Fig. 3. *Sma*I restriction enzyme analysis of chromosomal DNA from the isolates. Lane 1, WBG7208; 2, WBG7209; 3, WBG7210; 4, WBG7211; 5, WBG7212; 6, WBG7213; 7, WBG7214; 8, WBG1437 (classic MRSA).

Plasmid pWBG663 carried resistances identical to those of the plasmids found in MRSA isolates WBG1983 and WBG1997, which were obtained in Singaporean hospitals.²⁷ *Eco*RI restriction digests of the plasmid pWBG663 and two plasmids, pWBG278 and pWBG281, from WBG1983 and WBG1997, respectively, revealed that they were closely related (fig. 2).

MICs of streptomycin

The MICs of streptomycin for all the LUTH isolates, including WBG4763, were > 2000 mg/L.

Analysis of chromosomal DNA

*Sma*I digests of the chromosomal DNA of all the isolates were analysed by pulsed-field gel electrophoresis (figs. 3 and 4). The 1987 isolates WBG7208 to WBG7214 inclusive had similar *Sma*I restriction patterns (fig. 3) and were different from the classic MRSA (WBG1437), WBG4763 and its methicillin-susceptible derivative WBG6198 (fig. 4) and from the EA MRSA (WBG525) (not shown).

As restriction enzyme analysis had suggested that plasmid pWBG663 from strain WBG4763 was related to the two plasmids from the Singaporean MRSA, the chromosomes of the two Singaporean MRSA were compared with that of WBG4763 and its methicillin-sensitive derivative, WBG6198. No relatedness was demonstrated (fig. 4).

Derivative WBG6198 lacked a 210-kb *Sma*I frag-

Fig. 4. *Sma*I restriction enzyme analysis of isolates and their derivatives. Lane 1, lambda concatemers; 2, WBG4763; 3, WBG6198; 4, WBG1983; 5, WBG1997; 6, WBG7208. Note that WBG6198 has lost a 210-kb band and gained a 170-kb band, as compared to WBG4763.

ment present in WBG4763 but had a 170-kb fragment absent from WBG4763 (fig. 4). As this was the only difference observed between the two organisms, it follows that the appearance of the 170-kb fragment in WBG6198 resulted from the loss of 40 kb of DNA from the 210-kb fragment in WBG4763. This loss corresponded to the loss of methicillin resistance.

Discussion

Until 1987, there were no reports of MRSA from Nigeria, nor any indication that this organism was being sought by clinical laboratories.²⁸⁻³¹ Consequently, it is difficult to ascertain whether the subsequent reports of MRSA reflect its recent introduction, or recent awareness of the importance of testing for methicillin resistance. A study of 53 drug-resistant *S. aureus* from LUTH in 1985 detected only one MRSA isolate (Udo and Grubb, unpublished results). A survey 2 years later at the same hospital reported that 50% of *S. aureus* isolates were MRSA.¹⁹ Although the 1987 survey was carried out only on patients of high risk of colonisation and infection,¹⁹ it nevertheless indicated an alarming increase in the incidence of MRSA. The 1985 MRSA isolate, WBG4763, had similar chromosomally-mediated resistances to the 1987 isolates, differing only in being

resistant to minocycline and gentamicin and in carrying a different 30-kb plasmid.

None of the Nigerian isolates resembled classic MRSA,¹⁵ although they shared this organism's high-level resistance to streptomycin,³² they differed in being susceptible to erythromycin and in having chromosomal resistance determinants to penicillin, kanamycin and neomycin. Moreover, they lacked the plasmids coding resistance to tetracycline and heavy metals. All had chromosomal tetracycline resistance which, although not typical of classic MRSA, has been reported for a few European isolates.^{33,34} The LUTH isolates were also quite different from the EA MRSA of Australia and the EMRSA of the UK in that they did not have chromosomal resistance to cadmium, mercury, phenylmercuric acetate, erythromycin and lincomycin nor, except for strain WBG4763, to tetracycline and minocycline. Also, they did not have the cryptic plasmid, the chloramphenicol-resistance plasmid or the nucleic acid binding compound-trimethoprim-gentamicin-resistance plasmid typical of EA MRSA.¹⁵ Isolate WBG4763 did have a resistance profile and a nucleic acid binding compound resistance plasmid similar to some Singaporean MRSA strains. However, WBG4763 was found to differ from these Singaporean isolates when its DNA was examined by pulsed-field gel electrophoresis. It was also different from the later LUTH isolates, which closely resembled one another.

Loss of methicillin resistance from isolate WBG4763 corresponded to the loss of *c.* 40 kb of DNA (fig. 4). This figure is high compared with the values reported by others.^{35,36} Also, these reports found other resistance phenotypes, such as resistance to cadmium, mercury and tetracycline, to be lost together with methicillin resistance whereas no other resistance phenotype was lost with methicillin resistance from WBG4763.

The LUTH isolates were resistant to kanamycin, neomycin and streptomycin. It will be interesting to determine whether these resistances are encoded by a transposon similar to Tn3854, already described from a Nigerian *S. aureus* isolate.³⁷ As mixed-culture transfer and curing experiments have demonstrated that WBG4763 has both chromosomal and plasmid-borne penicillinase determinants, it will be interesting to determine whether there is a duplication of the penicillinase determinant due to a penicillinase transposon.

This report is the first genetic analysis of Nigerian MRSA and indicates that a strain of MRSA was dominant in a Nigerian hospital and that this strain was different from other MRSA studied by the same methods.¹⁵ Whether this strain occurs in other Nigerian hospitals is not yet known.

We thank Professor V. O. Rotimi for providing the isolates. This work was supported by a National Health and Medical Research grant to W.B.G.

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