Differential growth of epidemic methicillin-resistant *Staphylococcus aureus* in vancomycin

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ABSTRACT

This study examines the ability of isolates representing the 17 epidemic methicillin-resistant strains of *Staphylococcus aureus* to grow in increasing levels of vancomycin. Only EMRSAs 1, 2, 8, 11, 12 and 15 showed any growth and were designated EMRSAs 1A, 2A, 8A, 11A, 12A and 15A. On population analysis, these strains all produced clones that grew on 32 μg/mL vancomycin, while EMRSA 12A and 15A grew at 128 μg/mL. This was associated with increased resistance to lysostaphin and teicoplanin, a loss of phage sensitivity and an increase in cell wall diameter. Typing by pulsed-field gel electrophoresis following *SmaI* digestion showed no change in EMRSA 8A and 15A, while the other EMRSAs all lost or gained at least one band. EMRSAs 1A, 2A and 15A became more resistant to methicillin, and EMRSAs 8A, 11A and 12A became less resistant to methicillin. These results suggest that more than one mechanism is responsible for this phenomenon.

KEY WORDS: Methicillin resistance.
Resistance factors.
Staphylococcus aureus.
Vancomycin.

Introduction

Staphylococcus aureus has long been recognised as the most common cause of significant infection, with a high mortality in hospitalised patients.[1] This has achieved even greater importance with the increasing resistance to the β-lactams and also the hetero-resistance to glycopeptides that has now been described.[2–4] In the UK, 17 epidemic methicillin-resistant *S. aureus* (EMRSA 1–17) strains have been documented.[5]

In a British hospital, EMRSA 15 was dominant between 1994 and 1998. In 42 cases of septicaemia, vancomycin monotherapy carried a mortality rate of 78%, while combination with rifampicin reduced this figure to 4%. All isolates were susceptible to vancomycin by conventional laboratory testing, but this was lost by passaging the strain in vancomycin, during which a minimal inhibitory concentration (MIC) of 8 μg/mL was achieved. This proved stable on subculture. The genetic fingerprint of the strain remained constant but changes to the phenotype included loss of phage sensitivity and a thickened cell wall.[6] These findings have also been observed in other vancomycin-resistant isolates.[2–4, 7–8]

This study aims to ascertain if other EMRSA strains can grow in increasing concentrations of vancomycin, and whether or not this is associated with consistent changes in phenotype. The importance of this is that clinical failures have been reported with vancomycin therapy alone,[9,10] and this may reflect antibiotic failure in the absence of antibiotic resistance defined by routine clinical testing. This may be a strain-specific phenomenon that correlates with, and perhaps is characterised by, an ability to grow in sequentially increasing concentrations of drug.

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Materials and methods

Induction of vancomycin resistance

The 17 parental strains (EMRSA 1–17) were inoculated in 10-mL nutrient broth (Oxoid, Basingstoke, UK) containing vancomycin at 1 μg/mL and incubated at 37°C with shaking. When the broth became turbid, 200 μL was used to inoculate another 10-mL broth containing 2 μg/mL vancomycin. This was repeated with increasing concentrations of vancomycin at 1 μg/mL intervals until no growth was apparent after five days' incubation. Isolates were subcultured on Mueller-Hinton agar for further characterisation.

Population analysis profiles of the vancomycin-resistant isolates

A population analysis profile of vancomycin-resistant isolates was determined by plating overnight cultures of the above in nutrient broth diluted to a turbidity of 0.5 (McFarland standard), equivalent to $\geq 10^{10}$ colony-forming units (cfu)/mL, on Mueller-Hinton agar plates. The agar contained antibiotic concentrations of vancomycin ranging from zero to 128 mg/mL. Plates were incubated at 37°C for 48 h. Three colonies of both the original isolate and the subsequent isolate with the highest resistance to vancomycin were typed by pulsed-field gel electrophoresis (PFGE) following *SmaI* digestion.[11]

Table 1. Details of the phenotype changes seen in the EMRSA strains.

EMRSA strain	Vancomycin MIC (μg/mL)	Teicoplanin MIC (μg/mL)	Methicillin MIC (μg/mL)	Lysostaphin MIC (μg/mL)	Cell wall diameter (nm)
1	2	0.5	32	0.39	18
1A	32	24	256	1.56	34
2	1	1	16	0.39	23
2 A	24	12	24	0.78	75
8	1	1	32	1.56	28
8 A	24	24	1	3.12	80
11	2	2	32	0.39	30
11 A	24	12	1	1.56	70
12	1	1	16	1.56	24
12 A	24	48	0.75	6.25	45
15	1	2	16	1.56	30
15 A	32	64	24	3.12	84

Phenotype changes in the vancomycin-resistant isolates

Antibiotic sensitivities (penicillin, rifampicin, chloramphenicol, gentamycin, erythromycin, clindamycin, ciprofloxacin, fusidic acid, trimethroprim, tetracycline and mupirocin) were determined on all vancomycin-resistant isolates by standard disc testing.[12] Methicillin, vancomycin and teicoplanin sensitivity tests were performed by E-test (AB Biodisk, Piscataway, NJ, USA) according to the manufacturer's instructions.

The MIC of lysostaphin was determined in Mueller-Hinton broth with lysostaphin (Sigma, Poole, Dorset) suspended in buffer (0.15 mol/L NaCl, 50 mmol/L Tris [pH 7.6]). The lysostaphin dilution ranged from 0.125 to 32 μg/mL, with an initial inoculum of 10^5 cfu and incubation at 37^{O}C for 48 h.[13]

Phage susceptibilities were determined by the method of Blair and Williams,[14] using the current set of international typing phages (PHLS Laboratory, South Manchester, UK). Cell wall changes were demonstrated by transmission electron microscopy, as previously described.[6]

Resistance to vancomycin, as measured by E-test, was repeated after 10 subcultures on antibiotic-free medium. This was then repeated after a further passage in 4 μg/mL vancomycin in Mueller-Hinton broth.

Results

Induction of vancomycin resistance

Isolates of EMRSA grew in maximum concentrations of vancomycin ranging from 6 to 103 μg/mL. Subculture of EMRSA 3, 4, 5, 6, 7, 9, 10, 13, 14, 16 and 17 demonstrated either no, or up to a four-fold, increase in the MIC to vancomycin and teicoplanin. This phenomenon disappeared on single subculture and there was no change in genotype on PFGE. The remaining isolates tolerated over 64 μg/mL vancomycin, and inocula from the antibiotic-resistant cultures were subcultured on Mueller-Hinton agar and described as EMRSA 1A, 2A, 8A, 11A, 12A and 15A, respectively.

Population analysis profiles of the vancomycin-resistant isolates

There was no evidence of a resistant population in the original strains and the profile of the antibiotic resistant isolates is illustrated in Figure 1. Typing by PFGE demonstrated no obvious change in the genotypes of EMRSA 8A and 15A, while the other EMRSA strains all showed changes that included the loss or gain of at least a single band. EMRSA 1A, 2A and 11A lost a band at 220 kbp and gained a band at 130 kbp. Isolate 12A gained a band at 146 kbp and showed a thickened band at 361 kbp (Fig. 2).

Antibiotic sensitivity test of the vancomycin-resistant isolates

The antibiotic sensitivity patterns of EMRSA 1A, 2A, 8A, 11A, 12A and 15A were identical to the progenitor strains, with the exception of the methicillin and glycopeptide

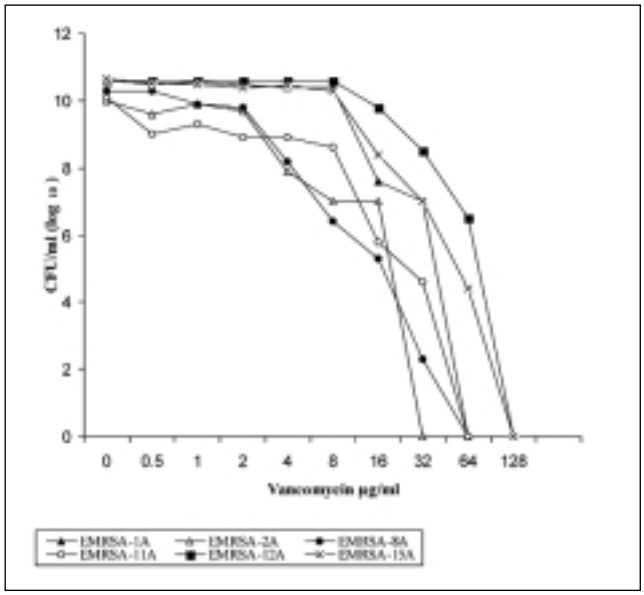

Fig. 1. Population analysis profiles of the vancomycin-tolerant isolates.

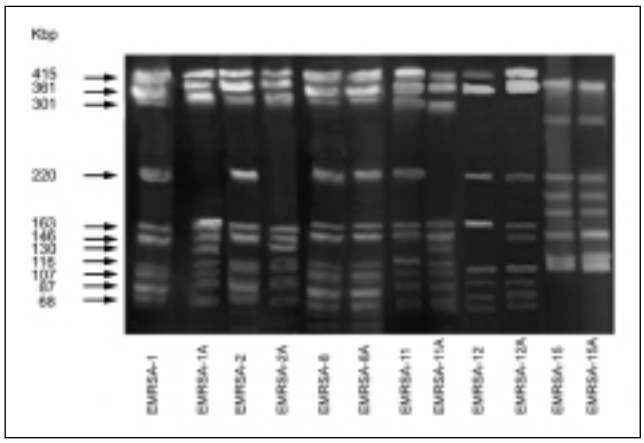

Fig. 2. Pulsed-field gel electrophoresis of EMRSA strains.

sensitivities (Table 1). Resistance to vancomycin was not induced alone but was shared with teicoplanin. The response to methicillin was variable, with EMRSA 1A, 2A and 15A becoming more resistant, and EMRSA 8A, 11A and 12A becoming sensitive.

These changes were mirrored by either a two- or four-fold increase in the MIC to lysostaphin. When the lysostaphin MIC was repeated for clones isolated from the subpopulation that showed the greatest vancomycin resistance in the population analysis profile, the values increased to 3.12 µg/mL (EMRSA 11A), 6.25 µg/mL (EMRSA 1A, 2A and 15A) and 12.5 µg/mL (EMRSA 8A and 12A).

All vancomycin-resistant subclones became untypable by the original phages. Transmission electron microscopy demonstrated increased extracellular material associated with the cell wall. Measurement of the diameter showed obvious increments (Table 1, Fig. 3).

When the E-test to vancomycin was repeated after 10 subcultures on antibiotic-free medium, results were 4 mg/mL (EMRSA 1, 2, 11), 8 µg/mL (EMRSA 8 and 12) and 16 mg/mL (EMRSA 15), but full resistance returned following subculture in vancomycin.

Discussion

This study demonstrated that representatives of the 17 type strains of EMRSA vary in their ability to grow in increasing concentrations of vancomycin. Since the original description of heteroresistance in Japanese isolates of MRSA,[2] similar strains have been described from all around the world.[4,8,15–17] However, there has been debate about how widespread these isolates are, even in Japan,[18] and whether or not the existence of this form of resistance alters the course of a clinical infection.[19,20]

In a previous study,[6] the treatment of a septicaemia with vancomycin alone was associated with a mortality rate of 78%. These infections were due to strains of EMRSA 15, which grew in the highest concentration of vancomycin and produced clones with the most stable glycopeptide resistance in the present study.

Currently, the dominant EMRSAs in the UK are types 15 and 16. In a recent survey of 26 hospitals, these strains were responsible for 95.6% of all bacteraemias, of which 60.2% were due to EMRSA 15 and 35.4% were due to EMRSA 16.[21] Screening 26,000 isolates from the UK failed to show heteroresistance,[22] although a heteroresistant strain belonging to EMRSA 15 has been reported from Bristol.[23]

The EMRSA 15 strain has also been reported in New Zealand, where it accounted for 48% of MRSA isolates during the first six months of 2000.[24] It has also caused outbreaks in Australia,[25] where treatment failure in a patient with infection due to a MRSA with reduced susceptibility to vancomycin has been described,[26] and a similar strain was isolated from 29 hospitals in Germany in 1998.[27]

The contrast in behaviour between the EMRSA 15 and EMRSA 16 isolates in this study was striking. The latter showed no evidence of an ability to grow in vancomycin, while the former and globally more dominant strain produced a stable vancomycin-resistant clone. In a clinical situation where glycopeptide therapy is dominant, this raises the question about whether or not outcome can be correlated with this phenomenon.

This may be especially important in the UK, where the two most common strains are so different and there has been debate about the value of controlling MRSA spread.28 In further support of this, strains that show progressive resistance to a glycopeptide during therapy have been reported in rats[10] and in humans,[29–31] and have been matched with treatment failures.[32–34]

When changes in phenotype have been examined in other strains of MRSA, there has been an increase in cell wall diameter, a loss of phage sensitivity, an increase in resistance to lysostaphin breakdown, and a reduction in the rate of growth. This was independent of whether the strain was produced in a laboratory[7,10,13,31] or occurred naturally.[2,4,6,8,15–17,29] All of the strains examined in the present study produced these changes.

The production of lysostaphin-resistant mutants by the growth of an oxacillin-resistant *S. aureus* in subinhibitory concentrations of lysostaphin has been linked to the loss of resistance to methicillin.[35] This is due to alterations in the activity of *femA* rather than changes in cross-bridge formation. With induction of vancomycin tolerance, all strains in the present study showed an augmented resistance to lysostaphin, while either becoming more or less resistant to methicillin.

Strains showing either a constant resistance to methicillin or hypersensitivity to methicillin have been reported when an isolate loses sensitivity to vancomycin.[2,4,6,8,10,13,15,17,29,31,36,37] This suggests that at least two separate pathways exist by which a strain can become resistant to vancomycin. This is further complicated by the ability of some strains to be resistant to teicoplanin and not vancomycin.[10,30]

A further assumption, as demonstrated by a constant PFGE fingerprint, is that the genotype is stable [2,4,6,15–17] In the present study, EMRSA 8A and 15A confirmed this, but the other isolates showed obvious changes. This is a finding that has been seen previously.[8] Three of the strains lost a band at 220 kbp and gained a band at 130 kbp, which suggests a degree of shared genotype change. Furthermore, strains lost their resistance to vancomycin on passage in antibiotic-free medium, which is a phenomenon that proved easily reversed and has been seen with other similar strains.[29, 37]

Overall, this study illustrated that individual EMRSA react differently when grown in vancomycin and this has raised

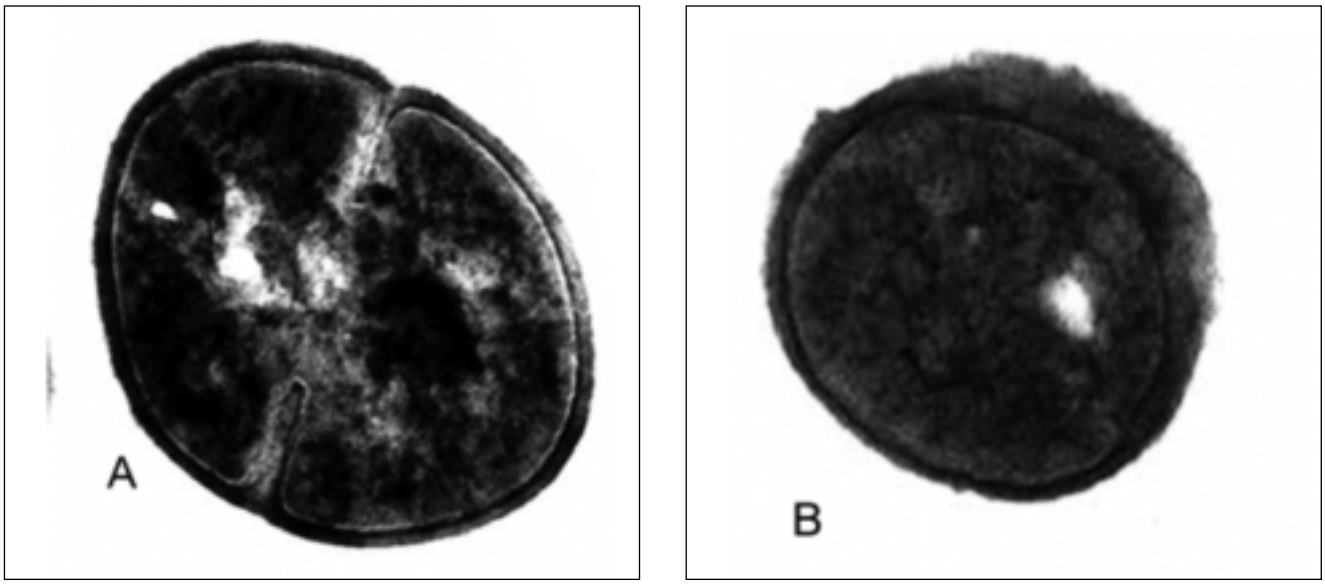

Fig. 3. Transmission electron microscopy of (A) EMRSA-2 vancomycin-sensitive strain and (B) EMRSA-2A vancomycin-resistant derivative showing a thicker cell wall.

the issue of whether or not this could act as a predictor of clinical response. This is important because it is now thought that the majority of MRSA worldwide are related to five clones.[38] □

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