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Gentamicin-resistant staphylococci: genetics of an outbreak in a dermatology department

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SUMMARY

The genetics of gentamicin resistance in *Staphylococcus aureus* strains isolated during an outbreak of infection in a dermatology department have been studied. The predominant strain of *S. aureus* did not appear to possess a plasmid mediating gentamicin resistance though one isolate yielded a plasmid coding for penicillin and gentamicin. Three distinct plasmids were isolated from other phage types of *S. aureus* which appeared towards the end of the epidemic. There appeared to be a stepwise loss of gentamicin resistance in the predominant strain.

INTRODUCTION

There are several reports of infection due to gentamicin-resistant staphylococci associated with the use of topical gentamicin. In few, however, has the opportunity been taken to study the genetics of the staphylococci involved. This paper presents such a study.

Since preliminary phage typing showed that there was one predominant strain together with several others with different phage typing patterns, all resistant to gentamicin, the question resolved into one of the nature of the resistance: was a single plasmid involved which had spread between different staphylococci, was more than one plasmid involved, or had the topical use of gentamicin selected chromosomally resistant variants from the staphylococci usually present in skin lesions?

MATERIALS AND METHODS

The Department of Dermatology at the University of Munich consists of three separate outpatient clinics: (1) women and children, (2) men, (3) private patients and six inpatient wards. There are 180 beds for dermatology patients. Outpatients are seen in separate cubicles but some treatments such as dressing changes for

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leg-ulcer patients or phototherapy are done in treatment rooms where patients may come in contact with each other. The average hospital room has two or three beds but there are a few larger rooms. Topical treatment of inpatients is done in treatment rooms (one on each ward) where several patients may be present at the same time.

Methods for phage-typing, determination of minimal inhibitory concentrations (MIC) and plasmid transfer studies were those described by Naidoo & Noble, (1978, 1981). Loss of plasmid DNA was induced by culture in Oxoid nutrient broth containing ethidium bromide (3 mg/l) or sodium dodecyl sulphate (SDS) (2 mg/l).

Five *Staphylococcus aureus* recipient strains were used in transfer experiments. These were: 8325N, propagating strain for phage 47; NCTC 10039, propagating strain for phage 83A; 80CR5, a restriction-deficient mutant of propagating strain 80 (a gift from Dr Engel); B111, a wild type recipient isolated in the Institute of Dermatology of phage type 3A/53/85 and resistant to penicillin only; MI, a wild type isolated at the Institute of Dermatology of phage type 79 sensitive to all antibiotics. These strains were made resistant to rifampicin or streptomycin by serial subculture and were then designated 8325rif, 8325strep. etc.

Analysis of plasmid DNA. For analysis of plasmid DNA by restriction enzyme digestion the plasmid DNA was prepared by CsCl-ethidium bromide equilibrium density centrifugation by the method of Novick *et al.* (1979).

*Restriction Endonucleases. Hin*d III, *Eco*R1, *Bgl* II and *Pvu* II were from Uniscience Ltd, Cambridge, U.K. and were used according to suppliers instructions.

Electrophoresis. For whole plasmids, precipitated DNA was collected by centrifugation and dissolved in 100–150 μl TES (0·05 M-NaCl, 0·005 M-EDTA, 0·05 M-Tris, pH 8). Dye solution consisting of bromophenol blue (0·1 %) and glycerol (50 %) in water was added to the DNA and to the restriction enzyme digests prior to loading on gels. Electrophoresis was carried out in a vertical gel apparatus (Pharmacia Fine Chemicals) with 0·8–1·2 % agarose (Seakem, Marine Colloids Inc.) gels in Tris-borate buffer. Gels were run at 30 V overnight or until the dye reached the bottom. They were then stained in a solution of ethidium bromide (5 mg/l^{-1}) for 30 min and destained with distilled water. DNA bands were visualized with a long-wave UV light transilluminator (C-62, Ultra-Violet Products, Inc.) and photographed.

Standards for assessment of approximate molecular weight were 8325 containing the penicillinase plasmid pI 258 and tetracycline plasmid pT1044 and a *Hin*d III digest of phage λ DNA.

RESULTS

The epidemic

In July and August 1979 gentamicin-resistant *S. aureus* isolates were recovered from lesions of 30 patients at the Munich University Hospital dermatology department; 13 of these patients were attending outpatient departments (seven at the men's clinic, four at the women's clinic and two private patients) four were subsequently admitted to the wards; the remainder were distributed in five inpatient wards. All these patients proved to be infected with a strain of *S. aureus* phage type 77/84 but with a number of different sensitivity patterns in relation to penicillin, tetracycline, erythromycin and neomycin. During the next 4 months

Table 1. *Source, phage type and sensitivity pattern of gentamicin-resistant staphylococci*

Source	Number of isolates*	Phage type	Sensitivity patterns† P	T	N	E
OPD	9	77/84	R	R	R	R
	3	77/84	R	R	—	R
	1	77/84	—	R	—	R
	1	77/84	—	R	R	R
Ward 1	6	77/84	R	R	R	R
Ward 3	1	77/84	R	R	R	R
	1	77/84	R	R	—	—
Ward 4	4	77/84	R	R	R	R
	2	77/84	R	R	—	R
Ward 5	7	77/84	R	R	R	R
	1	77/84	R	R	—	—
Ward 9	3	77/84	R	R	R	R
	2	77/84	—	R	R	R
Ward 10	12	77/84	R	R	R	R
	1	77/84	—	R	R	R
OPD	1	85	R	R	R	—
Ward 1	1	85	—	R	R	R
Ward 5	1	85	R	R	R	—
	1	85	—	R	R	R
Ward 10	1	85	R	R	R	—
Ward 4	1	29/52	R	—	—	—
Ward 1	1	80	R	—	—	—
Not known	1	47/75/77	R	R	R	R
OPD	1	6/42E/54/75	R	—	R	—
Ward 5	1	29/52/79/95/6/42E/47/53/54/77/84	R	R	R	R

* Five patients yielded two isolates, only the first of which is included here.

† All isolates were resistant to gentamicin. P, Penicillin; T, tetracycline; N, neomycin; E, erythromycin; R, resistant; —, sensitive.

a further 16 patients, only one of whom was from an outpatient department, were infected with the 77/84 strain but two more patients were infected by new gentamicin-resistant strains, a phage type 85 and a type 29/52. In the last 4 months of observation a further eight patients acquired the 77/84 strain, four more acquired the type 85, two of whom were in different wards and one in outpatients, and one each yielded a phage type 80, a type 47/75/77, a type 6/42E/54/75 and a type 29/52/79/95/6/42E/47/53/54/77/84 (designated Gp I/III) (see Table 1).

Although initially topical gentamicin was used extensively in outpatients and in some wards, one ward in which it was not used nevertheless harboured 12 patients with gentamicin-resistant strains of three distinct phage types. Systematic searches for carriers of resistant staphylococci were not made and the epidemiology

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Table 2. *Loss of resistance after incubation in broth containing ethidium bromide or SDS*

Resistance tested	Strain number	Phage type	Number of colonies tested	Number sensitive to antibiotic
Gentamicin	A110	85	822	521
	A102	77/84	402	119
	A53	85	398	20
	A41	77/84	866	12
	A6	77/84	520	5
	A19	77/84	589	5
	A10	77/84	296	3
	A10I	77/84	1498	1
	A118	Gp I/III	1885	1
	A4	77/84	361	0
	A11	77/84	205	0
	A22	77/84	367	0
	A34	77/84	271	0
	A45	77/84	257	0
	A116	77/84	2788	0
	A104	29/52	1934	0
	A106	85	1717	0
	A109	85	727	0
	A114	80	1568	0
	A128	47/75/77	1152	0
Tetracycline	A102	77/84	402	1
	A41	77/84	538	0
	A110	85	369	0
	A118	Gp I/III	559	0
	A128	47/75/77	143	0
Neomycin	A41	77/84	538	0
	A102	77/84	402	0
	A110	85	369	0
	A118	Gp I/III	559	0
	A128	47/75/77	829	0
Penicillin	A104	29/52	399	0
	A114	80	435	0
	A118	Gp I/III	559	0

must therefore remain anecdotal. In one instance a staff member yielded the epidemic 77/84 strain on two occasions separated by nearly 3 months and was probably responsible for transmission of the strain to four patients on one ward. On four separate occasions admission to a ward of a patient known to have been infected in outpatients appeared to have initiated episodes of infection in other patients in the ward. A reduction in the use of topical gentamicin was co-incident with the end of the epidemic.

Aminoglycoside resistance

Strains were initially selected on the basis of resistance to gentamicin determined by disk diffusion techniques. All epidemic strains had MIC's of gentamicin over 32 μg/ml. On occasion MIC's of more than 128 μg/ml were recorded but this

Table 3. *Attempts to transfer gentamicin resistance from epidemic* S. aureus *to recipient strains in broth culture*

Donor	Phage type	Recipient			
		8325Nrif	10039rif	B111rif	80CR5rif
A6	77/84	0	c 1×10^{-8}	0	0
*A10	77/84	1×10^{-7}	c 1×10^{-8}	0	0
A37	77/84	1×10^{-8}	ND†	0	ND
A102	77/84	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}
A4, A41	77/84	0	0	0	0
‡A12, A33, A34, A36, A37, A43, A45, A47, A48, A49	77/84	ND	0	ND	ND
A4, A10, A19, A41, A102	77/84	0	0	0	ND
A4R, A41, A10R A10I, A19R, A19I	77/84	0	ND	0	0
A10, A6R, A6I A19R, A19I	77/84	0	0	0	0
§A41, A53, A102	77/84	ND	0	0	0
A19R, A19I A41, A6, A102	77/84	0	0	ND	ND
A118	Gp I/III	4×10^{-7}	1×10^{-7}	6×10^{-7}	4×10^{-7}
A118	Gp I/III	5×10^{-7}	8×10^{-7}	3×10^{-7}	1×10^{-7}
§A118	Gp I/III	0	0	0	0
A110	85	1×10^{-8}	0	1×10^{-8}	1×10^{-8}
A110	85	0	0	0	ND
A104	29/52	0	0	ND	ND
A107	6/42E/54/75	1×10^{-7}	ND	0	ND

* Strain designations appearing more than once represent separate isolates.
† ND = Not done.
‡ These crosses were also attempted on filters.
§ These crosses were also attempted in mice.

appeared unrelated to any other finding. All gentamicin-resistant isolates were uniformly resistant to kanamycin (30 μg disks) and tobramycin (10 μg disk) by disk test. Some difficulty was experienced when testing for resistance to amikacin (10 μg disk) using the disk test, some zone diameters falling within the range regarded as 'intermediate' (10–12 mm) though most would be recorded as sensitive. With one exception (strain A107, phage type 6/42E/47/75) streptomycin and neomycin resistance were coincident.

Loss of resistance

Loss of gentamicin resistance was accompanied by loss of resistance to kanamycin and tobramycin but not to neomycin or streptomycin. The variable resistance exhibited to penicillin, tetracycline, neomycin and erythromycin suggested that these resistances might be plasmid mediated and therefore readily lost. With the exception of strain A102, loss of penicillin but not gentamicin resistance was observed during storage (none of 269 isolates direct from the agar slopes was sensitive to gentamicin). In strain A102 there was linked loss of penicillin and gentamicin (129 isolates showed loss amongst 138 isolates directly from an agar

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Table 4. *Secondary transfer studies in broth cultures*

	Original host strain	First transfer strain	Second transfer strain	Frequency of second transfer†
A6	77/84	10039rif	B111strep	0
A10	77/84	8325rif	B111strep	0
A37	77/84	10039rif	B111strep	2×10^{-8}
A102	77/84	8325rif	B111strep	4×10^{-7}
A53	85	8325rif	B111strep	2×10^{-9}
A107	6/42E/54/75	8325rif	B111strep	4×10^{-7}
A110	85	B111rif	MIstrep	5×10^{-9}
A118*	Gp I/III	8325rif	B111strep	9×10^{-9}
A118*	Gp I/III	8325rif	B111strep	1×10^{-7}

* Separate isolates.

† Frequency expressed as transferant:recipient ratio.

slope). This plasmid later proved labile in strain 8325 recipients; 10 isolates of 140 tested had simultaneously lost penicillin and gentamicin resistance after storage on agar. Following exposure to ethidium bromide, gentamicin resistance proved to be very labile in one of the phage type 85 isolates (A110) and moderately so in another (A53) but not in A106 and A109. Amongst the isolates which typed 77/84, isolate A102 proved to be moderately labile and four other isolates A6, A10, A19 and A41 also yielded sensitive variants. Six other isolates represented by 4249 colonies examined failed to yield variants however (Table 2).

When the gentamicin MIC's were examined it was found that sensitive variants of the type 85 strain had MIC less than 1 μg/ml (parent isolates MIC > 32 μg/ml). Amongst the phage type 77/84 isolates however, two MIC ranges were found at 8 μg/ml and < 1 μg/ml (parent isolates > 32 μg/ml). Variants from A4, A10, A19 and A41 all gave a mixture of MIC values; the parent is referred to as R, the MIC 8 μg/ml as I and MIC < 1 μg/ml as S variants in the tables. As shown in Table 2, A10I yielded one isolate in 1498 tested which was reduced from 8 μg/ml to MIC < 1 μg/ml. All of 129 isolates tested from A102 which had lost resistance during storage on agar were reduced to MIC < 1 μg/ml.

Transfer of plasmid genes

Attempts to transfer gentamicin resistance from isolates of the phage type 77/84, 85, 6/42E/54/75 and Gp I/III strains were successful in mixed culture, but only at low frequencies (Table 3). In contrast to previous studies, experiments in which transfer was attempted on filters and on mouse skin were unsuccessful.

All recipients which became resistant to gentamicin were also resistant to tobramycin and kanamycin but, with the exception of recipients from type 6/42E/54/75 or Group I/III, were sensitive to neomycin and streptomycin; all showed MIC to gentamicin in the parent 32 μg/ml range, transfer of 8 μg MIC was not achieved. Recipients from A107 (6/42E/54/75) and A118 (Group I/III) showed intermediate resistance to neomycin. Further transfer experiments were carried out with isolates of 8325rif, 10039rif and B111rif which had acquired gentamicin resistance from wild strains. Table 4 shows that the resistances which had proved easiest to transfer from the original host were also those that were most mobile in the new host.

Table 5. *Sizes of DNA pieces (kilobases) produced by digestion of plasmids from* A102, A53, A118 *and* A107 *by restriction endonucleases Hind* III, *Eco* R1, *Bgl* II *and Pvu* III

	Hind III				*Eco* R1				*Bgl* II				*Pvu* II			
	A102	A53	A118	A107	A102	A53	A118	A107	A102	A53	A118	A107	A102	A53	A118	A107
	—	16	—	—	—	19·6	—	—	—	26	—	—	33·3	53	—	22
	—	12	—	—	18	18	17	21	—	—	19	—	—	—	17·5	—
	—	—	9·3	9·3	12	—	—	—	—	14	18	18	—	—	11·3	11·3
	8·5	—	9·2	9·2	—	9·2	9·2	9·2	9·0	—	—	17·5	—	—	9·7	9·7
	—	—	9·1	9·1	—	—	—	—	8·7	—	—	—	—	—	—	—
	5·7	—	5·7	5·7	—	7·0	7·0	—	6·0	6·6	—	—	—	—	9·0	—
	5·0	—	5·0	5·0	—	—	6·3	6·3	5·1	—	—	—	—	—	—	—
	4·4	4·3	4·5	4·5	—	—	—	—	—	—	—	—	—	—	—	4·0
	3·6	3·6	—	—	—	—	4·6	4·6	4·5	—	—	—	—	—	—	—
	—	3·5	—	—	—	—	—	4·5	3·7	3·8	3·8	3·8	—	—	—	—
	3·1	3·0	—	—	2·9	—	4·2	4·2	—	—	3·4	3·4	3·1	—	2·5	2·5
	—	—	—	—	—	—	—	—	—	—	2·7	3·0	—	—	—	—
	—	2·7	2·5	2·5	2·5	—	—	—	—	2·4	—	—	—	—	—	—
	2·4	2·3	2·4	2·4	—	—	2·1	—	—	—	1·9	1·9	—	—	—	—
	1·5	1·7	—	2·35	1·6	—	—	—	—	—	—	1·7	1·7	—	—	—
	1·4	1·3	—	1·7	—	—	—	—	1·1	—	1·5	1·5	—	—	1·3	1·3
	1·0	1·1	1·0	—	1·0	—	—	—	—	0·7	—	—	—	—	—	—
	—	1·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	0·8	0·8	0·8	0·8	—	—	—	—	—	—	—	—	—	—	—	—
	0·5	—	0·5	0·5	—	—	—	—	—	—	—	—	—	—	—	—
Total	37·9	53·3	49·0	53·1	38·0	53·8	50·4	49·8	38·1	53·5	50·3	50·8	38·1	53	51·3	50·8

Nature of resistance

Recipient isolates of 8325N which acquired resistance to gentamicin were examined by electrophoresis. No plasmids were detected in recipients from A6 or A10 but A102 exhibited a single large plasmid coding for both penicillin and gentamicin resistance; all these isolates were of the epidemic 77/84 strain. A recipient from A53 (type 85) had a 53kb plasmid and a recipient from A118 (Gp I/III) had a 50kb plasmid coding for gentamicin but not for other antibiotic resistances. A recipient from A107 (6/42E/54/75) carried a similar plasmid coding for gentamicin resistance and ethidium bromide resistance.

Endonuclease cleavage studies showed a close relationship between the plasmids from A107 and A118 but little similarity between these and A102 or A53 (Table 5).

DISCUSSION

Outbreaks of infection due to gentamicin-resistant *S. aureus* have been reported by several authors, in many cases the outbreak occurred in a dermatology unit and/or was associated with the use of topical gentamicin (Shanson, Kensit & Duke, 1976; Speller *et al.* 1976; Bint *et al.* 1977; Price, Brain & Dickson, 1980; Graham *et al.* 1980; Chattopadhyay & Teli, 1981; Schaefler *et al.* 1981).

In two further reports (Wyatt *et al.* 1977; Faden *et al.* 1979) a pattern similar to that of the epidemic reported here was seen, in that a single strain predominated but with other phage types also involved. Faden *et al.* remark that a common plasmid was isolated but do not give the evidence for this. Wyatt *et al.* were unable to demonstrate a plasmid (Wyatt, personal communication). Greenhood *et al.* (1979) reported infection with three distinct gentamicin-resistant staphylococci in about equal proportions in a time sequence and suggest that plasmid transfer may have been responsible but no genetic studies are reported.

In view of the evidence that gentamicin resistance can be transferred between strains of *S. aureus*, and between *S. epidermidis* or *S. hominis* and *S. aureus* (Naidoo & Noble, 1978, 1981; Jaffe *et al.* 1980) the simplest explanation for the appearance of resistance in several distinct strains would be transfer of a single plasmid. However, in the study reported here distinct plasmids were found in strains of type 6/42E/54/75 (A107), Gp I/III (A118) and 85 (A53) which appeared late in the epidemic.

The strain of type 77/84 presents several problems. No plasmids were found in recipients where only gentamicin transfer occurred but a 38kb plasmid which also coded for penicillinase production was found in A102 (see Dyke, Naidoo & Noble, 1983). It is tempting to speculate that this transferable resistance represents genetic material which can become integrated into the chromosome or which, in the one isolate, had recombined with a pre-existing penicillinase plasmid. There is evidence for the latter, but the former is based on negative evidence in that a plasmid cannot be isolated from the gentamicin-resistant recipients or the original strain.

A further peculiarity of the 77/84 strain was that under the influence of ethidium bromide the level of resistance dropped from an MIC of 32 μg/ml to either 8 μg/ml or less than 1 μg/ml. Where this phenomenon has been observed previously,

for example with tetracycline (Lacey, 1975), it has been due to the presence of a tetracycline resistance plasmid coding for high level resistance whilst the low level was coded for by chromosomal genes. In strain 77/84, except isolate A102, no plasmid was detected coding for either resistance level; where resistance was transferred, it was to an MIC of over 32 μg/ml. Further, the 8 μg/ml MIC isolates yielded a < 1 μg/ml MIC variant suggesting stepwise loss.

One possible explanation is that this phenomenon is related to the number of copies of the genes conferring gentamicin resistance in each bacterium. When present as a plasmid there are likely to be many copies and so the MIC will be 32 μg/ml or higher, loss of the plasmid will result in an MIC of 8 μg/ml if a copy or copies of the gentamicin resistance genes remain in the chromosome but to < 1 μg/ml if no copy is retained. It would also be possible for an MIC of 32 μg/ml to be due to several copies of the genes in the chromosome as perhaps in most examples of strain 77/84.

In contrast to our previous studies on gentamicin resistance (Naidoo & Noble, 1978, 1981) and to unpublished data (Naidoo, in preparation), resistance was not readily transferred from these strains to standard recipients. However, this is in agreement with the finding that at least three separate plasmids were involved together with a putative transposable element that had not spread during the epidemic. We have observed that, when transfer first occurs under experimental conditions, the recipients exhibit labile resistance, that is the resistance plasmids are easily lost from the cultures. Repeated subculture in the presence of antibiotic results in the resistance becoming more stable, perhaps by selection of stable clones. It seems possible that, in the epidemic described here, the free topical therapeutic use of gentamicin had selected stable resistant clones. The origin of the plasmids is not known, but there are similarities between these and other gentamicin resistance plasmids recovered from coagulase negative strains isolated in Britain or the U.S.A. (unpublished observations) and it may therefore be that the plasmids (or chromosomal genes) were acquired by *S. aureus* from *S. epidermidis* or *S. hominis* strains carried by the patients. If this were so, it would emphasize the role of the normal flora as a pool for resistance genes.

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