HOSPITAL INFECTION

Epidemiological study of an outbreak of infection with *Staphylococcus aureus* resistant to lincosamides and streptogramin A in a French hospital

C. ARPIN, I. LAGRANGE, J. P. GACHIE*, C. BEBEAR† and C. QUENTIN

Laboratoire de Microbiologie, Université de Bordeaux II, 146 rue Léo Saignat, *Laboratoire d'Hygiène Hospitalière and †Laboratoire de Bactériologie, Hôpital Pellegrin, Place Amélie Raba Léon, 33076 Bordeaux Cedex, France

A significant increase in the incidence of isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), that were also resistant to lincosamides and streptogramin A (LS_A-MRSA), was observed in a French university hospital. Twenty-seven isolates from the outbreak were characterised, including 17 isolates from a plastic surgery ward and six control strains of MRSA. The strains were examined by antibiotyping and biotyping, and by three molecular methods: plasmid analysis, ribotyping and insertion sequence (IS) typing with IS256 sequence as a probe. Antibiotyping (five antibiotypes) was discriminatory because of the uncommon resistance phenotype of the epidemic strain. Biotyping (three biotypes), DNA plasmid analysis (four profiles) and ribotyping (two profiles) were poorly sensitive, in contrast to IS-typing (12 profiles). By the latter method, a coefficient of similarity (percentage similarity) compared to the predominant IS profile was calculated. Strains with a coefficient of similarity $\geq 82\%$ were considered as highly related to the epidemic strain, while those with a coefficient of similarity $\leq 40\%$ were regarded as distant. Results obtained with the five markers confirmed that an outbreak of hospital infection had occurred in the plastic surgery ward, with spread of the epidemic strain throughout the hospital.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunist pathogen that causes frequent outbreaks of infection in hospitals. MRSA strains are phenotypically similar and appear to belong to an evolutionarily restricted subpopulation of *S. aureus* [1]. Most MRSA strains worldwide have gradually become resistant to additional antibiotics, such as aminoglycosides, macrolides, lincosamides, tetracyclines and, more recently, fluoroquinolones [2, 3]. As a result, discrimination between endemic and epidemic situations by antibiotyping is often difficult. At present, resistance to lincosamides and to factor A of streptogramin (LS_A phenotype) is uncommon among MRSA isolates and although the mechanism of resistance is incompletely understood, it appears to be chromosomally mediated [4]. A significant increase in isolates of this kind in the Pellegrin Hospital, Bordeaux, France, especially in a plastic surgery ward, strongly suggested an outbreak of hospital infection.

Epidemiological investigations of MRSA have been

based on non-molecular markers such as antibiotyping [5], lysotyping [6] and serotyping [7]. The two latter techniques are subject to considerable variations, are labour intensive, have limited availability, lack standardisation and are generally limited to reference laboratories. The development of DNA-based techniques has provided clinical laboratories with the means to characterise isolates at the molecular level [8, 9].

The value of antibiotyping, biotyping and three molecular methods (plasmid profile, ribotyping and insertion sequence [IS] typing with an IS256 sequence as a probe) were examined in the investigation of a presumed outbreak of hospital infection.

Materials and methods

Epidemiological investigation and MRSA strains

The Pellegrin Hospital, Bordeaux, is a 1500-bed university-affiliated hospital, including mainly surgical (c. 950 beds) and intensive care (c. 200 beds) units. Between May 1991 and May 1992, 38 isolates of MRSA that were also resistant to lincomycin, clindamycin and pristinamycin IIA, were obtained from 27 patients. Of these isolates, 28 were from wound

Received 30 May 1995; revised version accepted 31 July 1995.

Corresponding author: Dr C. Arpin.

samples of 17 patients in a plastic surgery unit. This unit is in a separate building and is composed of a 26-bed ward, a four-bed day care unit, a consulting office, an operating theatre suite and a recovery room (three intensive care beds).

For the study, isolates were divided into three groups: 17 isolates with the *LS_A*-MRSA phenotype from patients in the suspected outbreak ward (group I; only the first isolate from each patient was studied); 16 isolates of MRSA from other units of the hospital during the same period, including 10 isolates with the *LS_A* phenotype, and six random isolates of MRSA without this phenotype (group II); four strains (group III) obtained from one patient in the plastic surgery unit over an 18-month period.

S. aureus isolates were identified by a tube test for free coagulase and a slide test for clumping factor. They were cultivated in Brian Heart Infusion (BHI; Difco) Broth and Agar.

Escherichia coli strains and plasmids

Two *Escherichia coli* strains were used. *E. coli* HB101 harboured plasmid pKK3535, a pBR322 derivative plasmid containing the *rrnB* ribosomal operon of *E. coli* [10]. *E. coli* TG1 contained plasmid pISM1001 (a gift of F. C. Minion) which consists of an *E. coli* pKS plasmid carrying *S. aureus* Tn4001 [11]. Tn4001 is a small (4.7 kb) composite class I transposable element which encodes the bifunctional enzyme AAC(6')-APH(2'') specifying kanamycin-gentamicin-tobramycin resistance and which is flanked by the 1.3-kb insertion element IS256 [12].

The *E. coli* strains were grown in Luria Broth (LB, GibcoBRL) at 37°C with aeration.

Antibiotic susceptibility testing

Susceptibility to 32 antimicrobial agents was determined by a standard disk diffusion method [13]. Commercially available disks (Diagnostic Pasteur) loaded with the following antibiotics were used: penicillin G 6 µg, oxacillin 5 µg, streptomycin 10 I.U., spectinomycin 100 µg, kanamycin 30 I.U., neomycin 30 I.U., gentamicin 15 µg, netilmicin 30 µg, tobramycin 10 µg, amikacin 30 µg, erythromycin 15 I.U., spiramycin 100 µg, lincomycin 15 µg, clindamycin 2 I.U., pristinamycin 15 µg, ofloxacin 5 µg, tetracycline 30 I.U., minocycline 30 I.U., sulphonamides 200 µg, trimethoprim 5 µg, co-trimoxazole (trimethoprim:sulphonamides, 1.25: 23.75 µg), fosfomycin 50 µg, vancomycin 30 µg, teicoplanin 30 µg, fusidic acid 10 µg, rifampicin 30 µg, chloramphenicol 30 µg, bacitracin 130 µg, nitrofurantoin 300 µg and nitroloxline 20 µg. Additional disks containing either 20 µg of pristinamycin IIA or 40 µg of pristinamycin IB were prepared in our laboratory.

Biotyping

The *S. aureus* isolates were biotyped with the API Staph system (bioMérieux) according to the manufacturer's recommendations.

Plasmid and chromosomal DNA isolation and analysis

Plasmid DNA from *E. coli* was isolated and purified by caesium chloride-ethidium bromide gradient centrifugation [14]. Plasmid DNA from MRSA strains was extracted by a modification of the Birnboim and Doly procedure [15] in which lysostaphin (Sigma), 1 mg/ml, was substituted for lysozyme. Total cellular DNA of MRSA isolates was purified as described previously [16]. DNA samples were digested with the restriction endonucleases *Eco*RI and *Hind*III as recommended by the manufacturer (GibcoBRL). Electrophoresis of plasmid and total digested DNA was performed on agarose (0.8% w/v gels) in Tris-borate or Tris-acetate buffer (pH 8.3) as described by Sambrook *et al.* [14]. The λ DNA phage digested by *Pst*I or *Hind*III was used as a size marker.

Blotting and hybridisation

DNA was transferred from agarose gels to Hybond-N nylon filters (Schleicher and Schuell) by a vacuum blotting system (Appligene). The blotted DNA was fixed by UV irradiation at 305 nm for 5 min. Purified plasmid DNA (pKK3535 and pISM1001) was labelled with [α^{32} P] dCTP (110 TBq/mmol; Dupont NEN Research products) by the nick-translation labelling system (Amersham International) according to the manufacturer's instructions. The pre-hybridisation, hybridisation and washing steps were performed under stringent conditions as described previously [17]. The blots were exposed to Fuji RX films at -70°C with intensifying screens for various lengths of time.

Comparison of hybridisation patterns

The percentage similarity between pairs of hybridisation patterns was calculated from the formula [18]:

$$\%S = \frac{N \times 2}{T} \times 100$$

where N is the number of matching bands and T is the total number of bands. A dendrogram was constructed by computer by the unweighted pair group method with averages.

Results

Epidemiological data

The age of the 17 patients in the plastic surgery unit ranged from 20 to 64 years (mean, 37 years). Thirteen were male and four were female. The temporal

Fig 1. Temporal relationship between the 17 patients with LS_A-MRSA strains in the plastic surgery ward. Symbols: — period during which patient was in hospital; ↓ isolation of an LS_A-MRSA strain.

relationship between the patients of this ward shows the presence of at least one infected patient during the epidemic peak, i.e., from June 1991 to January 1992 (Fig. 1). The patients carrying isolates assigned to group II were geographically distant from the plastic surgery ward and no epidemiological link could be demonstrated between them.

Antibiotic susceptibility testing

All MRSA isolates were resistant to penicillin G and oxacillin. The phenotype of resistance to aminoglycosides was presumptively due to the production of at least two modifying enzymes including AAC(6')-APH(2'') [19]. All isolates were resistant to tetracyclines, quinolones and fosfomycin. Three phenotypes of resistance to macrolides-lincosamides and streptogramins were observed: resistance to lincomycin and pristinamycin IIA, and resistance to macrolides and pristinamycin IB either constitutively or inducibly expressed [20]. In addition, six isolates of group II were resistant to spectinomycin and co-trimoxazole; among them, one was also resistant to chloramphenicol. High or low level of rifampicin resistance was another characteristic. Accordingly, five antibiotypes (A1–A5) were defined (Table 1). All isolates of group I and 10 isolates of group II (strains with the LS_A phenotype) exhibited the same antibiotype. The six other isolates of group II displayed four different patterns of susceptibility.

Biotyping

All isolates were identified by the API system as *S.*

aureus. Three biotypes were recognised: those that produced arginine dihydrolase but not nitrate reductase; those that produced nitrate reductase but not arginine dihydrolase; and those that produced both. All isolates of group I and 10 isolates of group II with the LS_A phenotype belonged to the first biotype (B1). The six other isolates of group II were of two remaining biotypes (Table 1).

Plasmid profile

No plasmid was found in the isolates with the LS_A phenotype despite repeated attempts. In contrast, the six isolates of group II that did not exhibit the LS_A phenotype showed three distinct plasmid profiles. The approximate sizes of the bands of unrestricted plasmids are indicated in Table 2 and the distribution of these profiles among the isolates is shown in Table 1. For the four isolates with the antibiotic patterns designated A2 and A4, plasmid profiles P1 (four bands) and P3 (five bands) were observed. The two isolates with the antibiotypes A3 and A5 both showed plasmid profile P2 (two bands).

Hybridisation patterns obtained with probe pKK3535 (ribotyping)

With plasmid pKK3535 as probe, only two hybridisation patterns were obtained when the DNA of group I and II strains was digested with *Hind*III. No hybridisation was observed when the same DNA was probed with plasmid pBR322 (data not shown). The hybridisation patterns designated H1 (eight bands) and H2 (nine bands) were reproducible (Fig. 2); they differed only by

Table 1. Characteristics of MRSA isolates

Isolate no.	Patient no.	Source	Ward	Date	Antibiotype	Biotype	Plasmid	Ribotype	IS-type	Similarity coefficient
Group I										
1118	1	Drainage fluid	Plastic surgery	27/07/91	A: LS ₄ RA	B1	P0	H1	E1	100
1150	2	Wound	Plastic surgery	22/08/91	A1: LS ₄ RA	B1	P0	H1	E2	95
1178	3	Wound	Plastic surgery	03/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
1196	4	Wound	Plastic surgery	12/09/91	A1: LS ₄ RA	B1	P0	H1	E3	90
2000	5	Wound	Plastic surgery	17/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2020	6	Wound	Plastic surgery	24/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2025	7	Wound	Plastic surgery	28/09/91	A1: LS ₄ RA	B1	P0	H1	E4	95
2029	8	Wound	Plastic surgery	30/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2039	9	Wound	Plastic surgery	01/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2052	10	Wound	Plastic surgery	04/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2067	11	Wound	Plastic surgery	07/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2069	12	Wound	Plastic surgery	08/10/91	A1: LS ₄ RA	B1	P0	H1	E5	82
2071	13	Wound	Plastic surgery	16/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2096	14	Wound	Plastic surgery	04/11/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2101	15	Wound	Plastic surgery	08/11/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2124	16	Wound	Plastic surgery	26/11/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2328	17	Wound	Plastic surgery	23/03/92	A1: LS ₄ RA	B1	P0	H1	E2	95
Group II										
1024	18	Catheter	Orthopaedic surgery	31/05/91	A1: LS ₄ RA	B1	P0	H1	E1	100
1111	19	Wound	Neurosurgery 2	24/07/91	A2: MLS _{B₆} RA SP FA	B2	P1	H2	E12	35
1134	20	Gastric fluid	Gynaecology-obstetrics	05/08/91	A1: LS ₄ RA	B1	P0	H1	E2	95
1138	21	Placenta	Gynaecology-obstetrics	07/08/91	A1: LS ₄ RA	B1	P0	H1	E6	95
1155	22	Blood	Neurosurgery 2	26/08/91	A2: MLS _{B₆} RA SP FA	B2	P1	H2	E8	40
1175	23	Wound	Neurosurgery 1	09/09/91	A3: MLS _{B₆} (RA) SP SXT	B3	P2	H2	E9	35
2014	24	Catheter	Burn unit	20/09/91	A4: MLS _{B₆} (RA) SP SXT C	B3	P3	H2	E10	40
2023	25	Wound	Gynaecology-obstetrics	28/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2028	26	Ascites	Paediatric ICU	28/09/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2068	27	Catheter	Burn unit	07/10/91	A4: MLS _{B₆} (RA) SP SXT C	B3	P3	H2	E10	40
2078	28	Wound	Burn unit	19/10/91	A5: MLS _{B₆} (RA) SP SXT	B3	P2	H2	E11	40
2084	29	Wound	Urology	18/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2086	30	Wound	Medicine	18/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2089	31	Wound	Nephrology	19/10/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2105	32	Wound	Gynaecology-obstetrics	08/11/91	A1: LS ₄ RA	B1	P0	H1	E1	100
2280	33	Wound	Medicine	28/02/92	A1: LS ₄ RA	B1	P0	H1	E7	89
Group III										
2198	15	Wound	Plastic surgery	20/01/92	A1: LS ₄ RA	B1	P0	H1	E1	100
2382	15	Wound	Plastic surgery	12/05/92	A1: LS ₄ RA	B1	P0	H1	E1	100
2375	15	Wound	Plastic surgery	09/02/93	A1: LS ₄ RA	B1	P0	H1	E1	100
13	15	Wound	Plastic surgery	05/06/93	A1: LS ₄ RA	B1	P0	H1	E1	100

ICU, intensive care unit; LS₄, lincomycin and streptogramin A; MLS_{B₆} and MLS_{B₆}*, respectively, macrolides-lincosamides and streptogramin B, constitutively and inducibly expressed; RA and (RA), high and (low) level of resistance to rifampicin; SP, spectinomycin; FA, fusidic acid; SXT, co-trimoxazole; C, chloramphenicol.

Table 2. Analysis of DNA plasmid patterns of MRSA

Plasmid pattern	Size of DNA bands* (kb)
P0	No plasmid
P1	30, 35, 2.8, 2.5
P2	30, 35
P3	5.5, 2.9, 2.6, 1.3, 1.1

*Bands of unrestricted plasmids.

Fig. 2. Profiles H1 and H2 obtained by Southern blot analysis of *Hind*III-digested total DNA detected in the 32 MRSA strains with the α^{32} P-labelled pKK3535 plasmid as a probe. Numbers indicate the sizes of DNA fragments obtained in kb.

a single band of *c.* 3.9 kb which was present in the profile H2. All 17 isolates of group I and group II isolates with the LS_A phenotype gave the profile H1. The six other isolates of group II gave the profile H2 (Table 1).

Hybridisation patterns obtained with probe pISM1001 (IS-typing)

The DNA of the 33 isolates of groups I and II hybridised with the pISM1001 probe but not with the plasmid pKS which lacks the Tn4001 transposon (data not shown). Twelve different patterns consisting of 9–16 fragments were observed when the DNA was hydrolysed by *Eco*RI (profiles E) (Fig. 3A and B). At least two unrelated hybridisation experiments were done to ensure that these patterns were reproducible. Among group I isolates, five profiles were obtained (E1–E5). In group II, nine hybridisation patterns were found (E1, E2, E6–E12). Profile E1 was predominant in the two groups. The distribution of the E profiles and their percentage similarity (%S) based on the Dice index [18] was calculated for each MRSA isolate and compared with the profile E1 (Table 1). These percentages varied from 82 to 100% for LS_A-MRSA isolates and 35 to 40% for non-LS_A-MRSA isolates. Furthermore, when the overall similarity was calculated between each pair of MRSA isolates, the values ranged from 17 to 100%. The corresponding dendrogram shows that the isolates were divided in two groups (Fig. 4): the LS_A-resistant strains that exhibited a hybridisation pattern either identical to or highly related to E1, and the six remaining strains of group II with a very

Fig. 3. Profiles obtained by **a**, Southern blot analysis of *Eco*RI-digested total DNA detected in the 32 MRSA strains with the α^{32} P-labelled pISM1001 plasmid as a probe; **b**, schematic representation. Lanes 1–12 correspond respectively to profiles E1–E12.

Fig. 4. Dendrogram of percentage similarity based on computer-assisted comparison of DNA fingerprints obtained by the IS-type method for **a**, LSA-MRSA and **b**, non-LSA-MRSA strains.

different pattern. In the latter group, two strains respectively with the profiles E8 (1155) and E12 (1111) exhibited a highly different hybridisation pattern (coefficient of similarity: 17%) but were identical by their antibiotype (A2), biotype (B2), plasmid profile (P1) and ribotype (H2).

Genetic stability of *Tn4001*

The high degree of restriction fragment length polymorphism obtained with the pISM1001 probe indicated that the *Tn4001* transposon and the IS256 element, or both, were present in numerous copies in the chromosomal DNA of the MRSA isolates studied. To investigate the frequency of genetic re-arrangement in the DNA, four isolates collected from the same patient during a 1.5-year period were analysed (strains of group III, Table 1). No change in the profile E1 was observed (data not shown). The same result was obtained with two isolates from another patient belonging to group II. Moreover, one isolate (2101, group I) was subcultivated 20 times without any change in its E1 profile (data not shown).

Discussion

The 17 LSA-MRSA isolates from the plastic surgery unit were found to be identical or very similar by all five epidemiological markers, suggesting transmission of a single strain responsible for an outbreak of hospital infection. The primary means of cross-transmission of MRSA is on the hands of colonised staff [21], and this is likely to be the principal source of transmission, as most of the newly infected cases had an overlapping stay in the ward. Moreover, the simple re-enforcement of conventional hygiene measures was sufficient to control the epidemic situation. The 10 isolates of the control group II

gave the same results with the epidemiological typing methods used. This suggests the spread of the epidemic strain throughout the hospital, without direct transfer, as reported for other MRSA outbreaks [22]. The six isolates of group II that lacked the LSA phenotype were completely different by all markers.

Among conventional typing methods, the antimicrobial susceptibility profile is attractive as it is readily available, easy to perform and relatively inexpensive. However, it has limited sensitivity for organisms such as MRSA which are often multiresistant. Also, the antibiotic susceptibility pattern may be unstable owing to the selective pressure in the hospital. In the present study, antibiotyping worked very well because the epidemic strain harboured an uncommon antibiotic resistance phenotype. The presence of strains carrying an unusual combination of drug resistances is a good indication of the dissemination of an epidemic strain, but additional typing methods are required for a proper epidemiological assessment.

Biotyping is also simple to perform when commercial kits are available, as for staphylococci. However, these systems are designed for taxonomic rather than epidemiological purposes and often lack sensitivity, particularly for *S. aureus*. Furthermore, the biotype may be influenced by various technical and environmental factors [23, 24]. In this study, only three distinct biotypes were found (Table 1) but these results correlated well with the other markers.

Plasmid DNA analysis has been usefully applied in a number of epidemiological studies [5, 9, 23, 24], but the profile is markedly influenced by the plasmid extraction procedure, which may convert one molecular form of the plasmid to another. The discriminatory power of this method increases with the number of plasmids and the differences in their molecular sizes; strains may lose or acquire antibiotic resistance or cryptic plasmids, and existing plasmids can undergo molecular re-arrangements. Several reports indicate that MRSA isolates often carry few, if any, plasmids [25] perhaps related to the chromosomal location of resistance determinants in MRSA [26]. The study strains exhibited four plasmid profiles, but no plasmid was detected in the LSA isolates, so that this method was undiscriminatory.

Ribotyping has been proposed as a useful molecular marker for MRSA [27]. However, in general, MRSA typing with a rDNA probe shows relative genetic homogeneity, reinforcing the hypothesis that many MRSA isolates may have evolved recently from a single ancestral strain [1, 28]. In the present study, the two profiles that were found shared a high degree of homology, although isolates with the non-LSA phenotype were very different by other markers. Ribotyping did not adequately discriminate among the MRSA isolates examined.

Plasmids containing the genetic mobile element IS256 can be used as an epidemiological tool for typing gentamicin-resistant staphylococci with a good discriminatory power [28–30]. With a similar probe, 12 different hybridisation patterns were obtained. Among the LS_A strains, profile E1 was predominant (17 of 28 strains) and the six remaining patterns were very similar to E1. The six non-LS_A strains gave five hybridisation patterns that were very different from E1. As an IS element has inherently the potential to cause genetic re-arrangements [31], the stability of this marker was tested. No change in the profile of a strain was observed after a 1.5-year period in the same patient and of another strain after 20 in-vitro subcultures. These results demonstrate that under the conditions of this study, the mobile element IS256 is relatively stable during a considerable number of in-vitro and in-vivo generations. These results are in agreement with those of Dyke *et al.* [32] who found no evidence for mobility of IS256 after a total of 126 generations. However, considering that IS256 is able to transpose at different sites of the chromosomal DNA, strains with a similarity coefficient $\geq 82\%$ were regarded as related to the epidemic strain, and those with a coefficient $\leq 40\%$ as unrelated.

In a recent study [33], the strengths and weaknesses of 12 currently available typing systems were assessed for their ability to discriminate among MRSA and methicillin-susceptible *S. aureus*. No typing method prevailed clearly among others. Insertion sequence typing, with IS275/431 sequences as probes, was relatively effective with MRSA strains, but ineffective with methicillin-sensitive isolates which rarely carry these sequences [34]. In other studies, pulsed-field gel electrophoresis appears as the 'gold standard' test for typing *S. aureus* strains [35], even when compared to the arbitrarily primed PCR [36]. Ultimately, a combination of two methods might be most efficacious, the choice depending on the resources of each laboratory and the level of expertise of the personnel in particular techniques.

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