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An epidemic methicillin-resistant strain of *Staphylococcus aureus* in Spain

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SUMMARY

During 1990, a strain of methicillin-resistant *Staphylococcus aureus* became epidemic in Spain and spread in a manner analogous to that of EMRSA-1 in England. Isolates of this strain produced little protein A and were resistant to a number of antibiotics including ciprofloxacin. Beta-lactamase production was encoded by a *c.* 39 kb plasmid, which also conferred resistance to mercury, cadmium, ethidium bromide and propamidine isethionate.

Investigation showed that two variants, separable by supplementary and Fisk phage typing, were circulating. The B variant appeared to spread more readily than the A variant.

The opportunity was taken to compare the discriminatory power of traditional typing methods with molecular techniques. The discriminatory power of the molecular techniques used only reached the same level as the traditional methods when double enzyme digestion of total cellular DNA by *Eco*R I and *Cla* I was performed.

INTRODUCTION

The epidemic potential of particular strains of *Staphylococcus aureus* is well known. A classic example is the pandemic of infection caused by strain 80/81 in the late 1950s and early 1960s [1]. More recent reports of epidemic strains have focused on methicillin-resistant *S. aureus* (MRSA) [2,3]. Kerr and co-workers [4] in a study of MRSA from England and Wales used a conservative definition of 'epidemic' to describe 14 such strains affecting more than one hospital and also defined ten distinct strains causing epidemics in single hospitals.

Though epidemic methicillin-resistant strains have been isolated from a number of countries around the world [5], they had not been considered a problem in Spain. Indeed, only 3 of 195 *S. aureus* isolates from 74 Spanish hospitals collected in 1986 were methicillin-resistant [6]. Late in 1989 an increase in the number of MRSA isolates referred to the Laboratorio de Referencia de Estafilococos was

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noted from several hospitals and from different cities. Amongst these isolates were many of indistinguishable phage type 29/77/84/932. This paper describes the laboratory characterization of selected isolates of this phage type and their separation into distinct variants. The opportunity was taken to investigate the discriminatory power of the traditional typing methods based on phage and antibiotic susceptibility characters compared with molecular techniques including restriction fragment length polymorphisms and probing of Southern blots.

MATERIALS AND METHODS

Selection of strains

Several collections of MRSA from Spain from 1979 until mid 1990 were available to us. Within these collections isolates from 10 hospitals in three cities in Spain which had the phage type 29/77/84/932 at 100 × RTD could be identified. Late in 1990, an infected patient returned from Spain to the United Kingdom and local spread was noticed. A set of 20 isolates, two, when available, from each outbreak was selected by the Madrid Laboratory for further study. All were isolated in 1989 and 1990. The provenance of these isolates is given in Table 1. The selected isolates and any derivatives were stored in glycerol nutrient broth (60% glycerol/40% Oxoid nutrient broth No. 2) at −20 °C. Appropriate control strains were incorporated into all tests. Control strains are shown in Table 2.

Bacteriophage (phage) studies

Phage typing. The method of Blair and Williams [15] was used. Isolates were re-tested at RTD and 100 × RTD against phages of the current international set [16], at 100 × RTD against four experimental phages, 88A, 90, 83C and 932, useful for dividing phage group III strains and at RTD against 10 supplementary phages [17].

Fisk typing [18]. Mitomycin C induction was used [19]. Induced phage from each isolate was spotted onto all the isolates, and onto 36 propagating or test strains for *S. aureus* phages and the host range of selected induced phages was determined [17]. Phage-sensitive control strains were 57 and RN 450. In some experiments, supernatants of young broth cultures were examined for phage. Lysogens were constructed and tested by the method of Vickery and colleagues [20].

Serotyping of phage

Standard anti-phage antisera used in the initial determination of serotypes of *S. aureus* phages were available [21]. Phages were diluted to give semiconfluent lysis on PS 47 and mixed with a 1/100 dilution of antiserum A, B, F or with normal rabbit serum. Two 20 μl drops of the mixture were spotted immediately onto staphylococcal typing agar [15] seeded with PS 47. After 1 h incubation at 37 °C, two further 20 μl drops were spotted onto the same plate. The drops were allowed to dry and the plate incubated overnight at 30 °C.

Enterotoxin production

Isolates were tested for the production of enterotoxins A, B, C and D and for toxic shock syndrome toxin 1 (TSST-1) by reverse passive latex agglutination using commercially available kits (Oxoid products TD900 and TD940 re-

Table 1. *Selected isolates of phage type 29/77/84/932.*

Isolate	Year	Source	Town/hospital†	Variant‡
207	1989	—*	Madrid, 5	A
208	1989	Wound	Seville, 1	B
209	1989	Wound	Seville, 1	B
210	1990	Wound	Seville, 1	B
211	1990	Wound	Seville, 1	B
212	1990	—	Madrid, 3	A
213	1990	—	Madrid, 3	A
214	1990	Skin	Madrid, 6	A
215	1990	Nasal exudate	Madrid, 6	A
216	1990	Wound	Madrid, 2	A
217	1990	Pleural exudate	Madrid, 2	A
218	1990	—	Madrid, 8	A
219	1990	—	Madrid, 8	A
221	1990	—	Madrid, 1	B
222	1990	Osteomyelitis	Barcelona	B
223	1990	Wound	Madrid, 2	A
224	1990	Wound	London, UK	B
225	1990	Wound	London, UK	B
226	1990	Wound	Madrid, 9	A
227	1990	Urine	Madrid, 9	A

* — information not available in patient's notes.

† Spanish hospitals coded according to the convention used by the Laboratorio de Referencia de Estafilococos.

‡ See text.

spectively). Controls were EMRSA-1 for enterotoxin A (SEA) and toxin supplied by the manufacturer for TSST-1. Six isolates, selected to give a wide range of source hospitals, were tested for production of enterotoxins A, B, C, D, E and TSST-1 by the double gel diffusion technique of Simkovicova and Gilbert [22] in the Food Hygiene Reference Laboratory.

Biotyping

Isolates were examined for

(i) Haemolysis on 5% (v/v) horse, human and sheep blood agar in Oxoid Colombia base (CM331). The sheep blood was supplemented with magnesium ions to a final concentration of 10 mM.

(ii) Hydrolysis of Tweens 20, 40, 60 and 80 to 1% (v/v) final concentration in Sierra's base [23].

(iii) Lipolysis on egg yolk medium (Oxoid CM3 agar with 15% Oxoid SR47 egg yolk).

(iv) Caseinase on milk agar [12·5% (v/v) sterile skimmed milk in nutrient agar base].

(v) Lactose fermentation on McConkey (Oxoid CM7) and CLED (Difco 0971) agars.

(vi) Tellurite reaction on Baird-Parker medium (Oxoid CM2756, SR 054C).

(vii) Gelatinase production using conventional gelatin stabs [23].

(viii) Urease production using conventional urea slopes [23].

Plate tests were inoculated with 10 μl (10^6–10^7 c.f.u.) of an overnight broth

Table 2. *Control strains*

Strain	Synonym	Control characters	Reference
NCTC 6571	Oxford	Susceptible to antimicrobial agents, but Cd^r	Phillips, et al, 1991 [7]
NCTC 7121	Wood 46	Protein A negative	Burnet, 1929 [8]
NCTC 8319	PS 3A	Representative of phage group II	
NCTC 8325	PS 47	Susceptible to phages of lytic groups I and III	
8325-4	RN 450	Delysogenized variant of NCTC 8325. β-haemolysin producer	Novick, 1967 [9]
NCTC 8363	PS 52A	Representative of phage group I	
NCTC 8509	PS 6	Representative of phage group III	
NCTC 9789	PS 80	Positive for protein A. Hg^r, Cd^r	
NCTC 10442	ST60/13136	Original MRSA	Jevons, 1961 [10]
NCTC 10530	*S. simulans*	Strongly proteolytic	Kloos and Schleifer, 1975 [11]
NCTC 10971	PS 95	Representative of miscellaneous phage group	
NCTC 11940	EMRSA-1	Produces urease, SEA. Weak protein A producer. Gentamicin sensitive	Marples, et al, 1986 [12]
NCTC 12232	EMRSA-2	β-lactamase positive, Hg^r, Cd^r, $Etbr^r$, $propamidine^r$ Inducible macrolide resistance	Richardson and Marples, 1988 [13]
NCTC 12233	EMRSA-2	β-lactamase negative derivative of NCTC 12232. Hg^s, Cd^s, $Etbr^s$, $propamidine^s$	
NCTC 50192	39R 861 *E. coli* K12	Molecular-weight markers	
NCTC 50193	V 517 *E. coli* K12	Molecular-weight markers	
—	57	Susceptible to phages of lytic groups I and III. β-haemolysin producer	Winkler, et al, 1965 [14]

culture using a rod inoculator; urea slopes and gelatin stab with 100–200 μl of an overnight broth culture using a Pasteur pipette to ensure that inoculum was delivered to the bottom of the tube. Tests were incubated at 37 °C. Haemolysin type, lactose fermentation, growth on tellurite and egg yolk reaction were read after 1, 2, 3 and 5 days. Tween hydrolysis, caseinase, gelatinase and urease activity were read on days 1, 2 and 5. Results for all tests were recorded in an empirical scale of: −, negative; ±, weak positive; +, positive and + + or + + +, strong positive. Control strains were: RN 450, 57, NCTC 10530 and EMRSA-1.

Protein A production

A rapid, semi quantitative, dot blot method was used [24]. Control strains were Wood 46, EMRSA-1 and PS 80. The test was developed until EMRSA-1 gave a definite purple/blue colour.

Antibiotic susceptibility testing

Disk susceptibility testing. The methods described in Richardson and co-workers [24] were used. Antibiotic disks and disk content were penicillin 1 i.u., streptomycin 10 μg, neomycin 10 μg, kanamycin 30 μg, gentamicin 10 μg, tobramycin 10 μg, spectinomycin 200 μg, tetracycline 10 μg, minocycline 30 μg, chloramphenicol 10 μg, erythromycin 10 μg, lincomycin 2 μg, fusidic acid 10 μg, vancomycin 30 μg, rifampicin 5 μg, mupirocin 5 μg, ciprofloxacin 5 μg, trimethoprim 2·5 μg and methicillin 10 μg.

Minimum inhibitory concentration studies. All 20 isolates were examined. Agar incorporation plates were made on the schedule described by Ericsson [25]. Methicillin and penicillin were tested in DST agar (Oxoid CM261) and nutrient agar. Both diluted (1/500 of an overnight broth culture) and undiluted cultures were used. Parallel series of plates were incubated at 30 and 37 °C. Plates were read after 24 and 48 h incubation. Other antibiotics were tested in DST agar using, except for erythromycin and lincomycin, a diluted broth culture. Plates were read after 24 h incubation at 37 °C. For streptomycin, spectinomycin and kanamycin concentrations of antibiotic up to 4 g/l were used. The control organism was the Oxford staphylococcus.

Erythromycin and lincomycin resistance

A 2 ml aliquot of an overnight broth culture was incubated with a final concentration of 0·1 mg/l erythromycin for 1 h at 37 °C. This was the induced culture. Both the induced and non-induced cultures were inoculated onto parallel series of plates containing either erythromycin, 0·125–200 mg/l; lincomycin, 0·125–200 mg/l or lincomycin, 0·125–200 mg/l and erythromycin, 0·1 mg/l in DST agar. Plates were read after overnight incubation at 37 °C. Control strains were the Oxford staphylococcus and EMRSA-2.

Resistogram typing

One per cent peptone water agar was used throughout. Incorporation plates containing mercuric chloride (Hg) 10 mg/l, cadmium sulphate (Cd) 10 mg/l, ethidium bromide (Etbr) 50 mg/l or propamidine isethionate (propamidine) 100 mg/l were made. These levels were chosen empirically to give a differentiation between known resistant and sensitive strains under the test conditions. Plates

were inoculated with 10 μl of a 1/500 dilution of an overnight broth culture using a rod inoculator, and incubated overnight at 37 °C. Presence or absence of growth as compared with a nutrient agar plate was recorded. Control organisms were: the Oxford staphylococcus, PS 80, EMRSA-2 and NCTC 12233.

Penicillinase production

Penicillinase was detected by a starch/iodine method [26]. Curing of penicillinase production was attempted for seven isolates. Two-hour, shaken broth cultures of the strains to be examined, were used to inoculate a small volume of fresh nutrient broth. This broth was then incubated at 43 °C for an extended period. The grown culture was then diluted so that a 100 μl volume would contain 100–200 c.f.u. Aliquots of 100 μl were plated onto starch agar and the plates incubated overnight at 37 °C. The plates were then developed and β-lactamase negative colonies selected. These colonies were also tested for resistance to antibiotics, Hg, Cd, ethidium bromide and propamidine. Their plasmid content was determined.

Plasmid preparation

A rapid method [27] was used. Plasmid preparations were analysed by agarose gel electrophoresis. Concentrations of 0·7 or 0·8% agarose were used in standard tris/borate buffer [28] at 35 V for 17–18 h. Plasmid bands were viewed by u.v. light after staining with ethidium bromide (7 mg/l) for 30 min. Control strains of *Escherichia coli* bearing plasmids of known size were obtained from the National Collection of Type Cultures.

DNA fingerprinting

Total cellular DNA isolation and analysis. Total cellular DNA was isolated by the guanidium thiocyanate method of Pitcher and co-workers [29] modified for *S. aureus* [30]. Single digestion of DNA was by the restriction endonucleases *Bgl* II, *Sal* I, *Eco*R I or *Hin*d III (Gibco-BRL) according to the manufacturers' instructions. Double digestions using *Eco*R I and *Cla* I or *Eco*R I and *Hin*d III were also performed. Electrophoresis of singly digested DNA was in 0·9% w/v agarose gels (Bio-Rad Ltd) in tris-borate buffer at 35 V constant voltage for 18–20 h at 4 °C. Doubly digested DNA was run in 1% agarose gels under the same conditions. Molecular-weight markers were either 1 kb DNA ladder (Gibco-BRL) or a biotinylated *Hin*d III digest of phage λ DNA (Bocheringer-Mannheim).

Blotting and hybridization (ribotyping). DNA was transferred from agarose gels to nylon filters (Hybond, Amersham International plc) by vacuum transfer (Vacugene Pharmacia) as instructed by the manufacturer. Biotinylated cDNA to *E. coli* 16S+23S r-RNA labelled by the method of Pitcher [29] was used as the probe. Hybridization and development of genes was carried out on the schedule given in Richardson and colleagues [30]. *Bgl* II and *Sal* I digests were not probed.

RESULTS

Phage studies

Twenty isolates with the phage type 29/77/84/932 at 100 × RTD were examined. These 20 isolates were indistinguishable when typed by the international phages, though two variants could be distinguished by the sup-

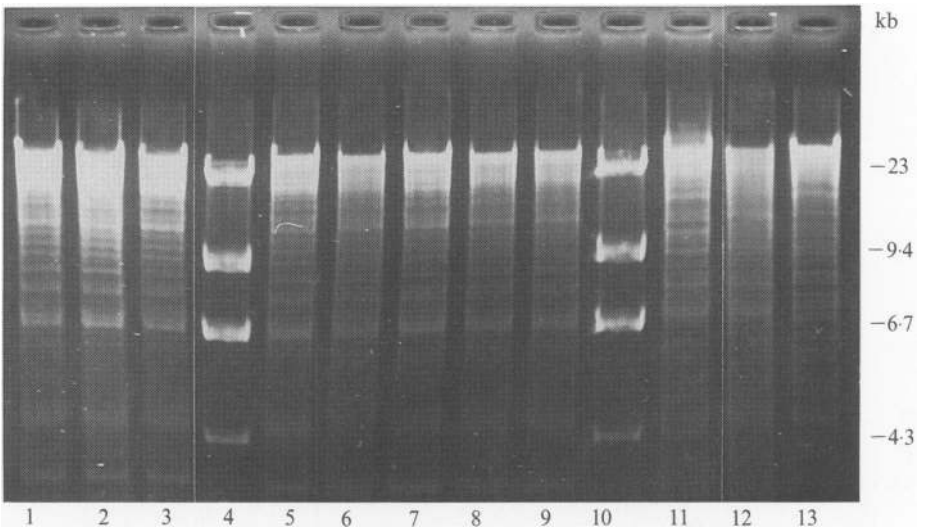

Fig. 1. Banding patterns of *Bgl* II digests. Lanes 1–3, 5, 8, 9, isolates from 4 Madrid hospitals (variant A); lanes 6, 7, isolates from the UK (variant B); lanes 11–13, propagating strains 52A, 6, 95; lanes 4, 10, λ DNA digested with *Hind* III.

plementary phages. Twelve isolates: 207, 212–219, 223, 226 and 227, non-typable by the supplementary phages, were assigned to variant A. Eight isolates 208–211, 221, 222, 224 and 225 lysed strongly by phage 618 of the supplementary set, were classified as variant B. Fisk typing confirmed these differences. Both variants carried phages belonging to lytic group III. Phage from variant A belonged to serogroup F, that from variant B was serum sensitive and, therefore, ungroupable by the standard antisera. Both titrated on PS 47 and RN 450 with an RTD of 1/500; lysogenization of RN 450 with either variant did not alter its phage type pattern nor β haemolysin production. Both phages lysed propagating strains 6, 7, 31, 42E, 47, 53, 83A, 88 and 90. In addition to these propagating strains, A variants also lysed propagating strains 42C, 54, 75 and 84 while B-variants lysed propagating strains 42B, 80, 81 and 95. Additionally, A-phage lysed B-variants and *vice versa*. Sufficient phage could be detected in the supernatant of young broth cultures to allow differentiation of A and B variants.

Biochemical and resistance characters

The 20 isolates tested were consistent in their biochemical and resistance characters. All produced enterotoxin A but were negative for other enterotoxins and for TSST-1. All were very poor producers of protein A; all hydrolysed Tweens 20, 40 and 60 but not Tween 80. All were weakly β-haemolytic, lipolytic and caseolytic, gelatinase-negative, formed black colonies of Baird-Parker medium and were urease positive. With one exception, isolate 219, all were lactose positive. This isolate was also ONPG negative. All were resistant to streptomycin (MIC > 4 g/l), spectinomycin (MIC > 4 g/l), kanamycin (median MIC 2 g/l, range 1–2 g/l), neomycin (median MIC 64 mg/l, range 32–64 mg/l), gentamicin (MIC 128 mg/l), tobramycin (MIC > 200 mg/l), tetracycline (MIC 64 mg/l), minocycline (MIC 2 mg/l), erythromycin (uninduced MIC > 200 mg/l), lincomycin (uninduced MIC > 200 mg/l) and ciprofloxacin (MIC 32 mg/l). Susceptibility to rifampicin was reduced (MIC 4 mg/l). They were uniformly susceptible to the

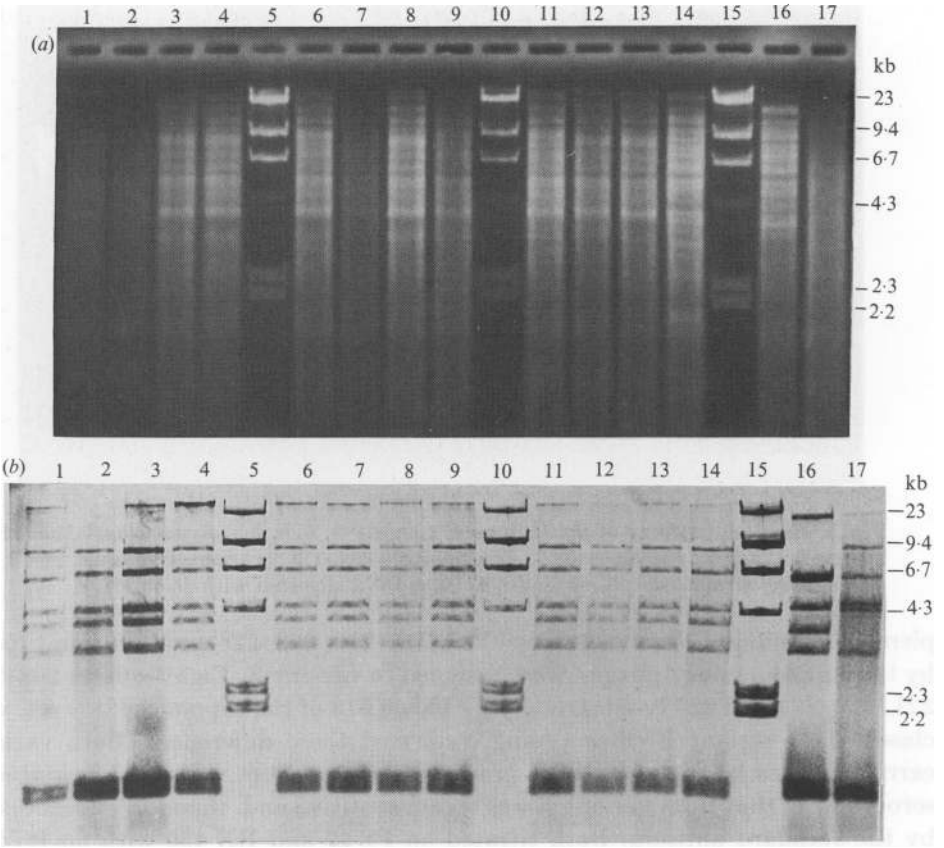

Fig. 2.(*a*) Banding patterns of *Eco*R I digests. Lanes 1–4, 6, 8, 12, 13, isolates from 4 hospitals in Madrid (variant A); lane 7, isolates from Barcelona (variant B); lanes 9, 11, isolates from the UK (variant B); lane 14, EMRSA-1; lanes 16, 17, propagating strains of *S. aureus* phages 52A and 3A; respectively, lanes 5, 10, 15, biotinylated *Hin*d III digests of λ DNA. (*b*) Hybridization with biotin-labelled cDNA to *E. coli* 16S+23S rRNA. Lanes as in (*a*).

other non-betalactam antibiotics tested. All isolates produced beta-lactamase which could be detected in the starch iodine test (median penicillin MIC 32 mg/l, nutrient agar at 30 °C) and were resistant to methicillin (MIC > 200 mg/l, nutrient agar at 30 °C). All the isolates were also resistant to Hg (10 mg/l), Cd (10 mg/l), ethidium bromide (50 mg/l) and propamidine (100 mg/l). All carried a *c.* 39 kb plasmid. Loss of this plasmid, at rates varying from 1·4–96 %, after growth at 43 °C, resulted in loss of beta-lactamase production with concomitant loss of resistance to Hg, Cd, ethidium bromide and propamidine. Interestingly, beta-lactamase-negative derivatives of variant A isolates showed a lesser degree of hetero-resistance to penicillin and methicillin than did derivatives of B variants.

Restriction endonuclease digestion

Digestion with Bgl II *or Sal* I. Digestion of total cellular DNA of the 20 isolates of *S. aureus* with either of these enzymes generated 40–60 fragments of DNA. Qualitative comparisons between isolates of variants A and B and between either variant and the control strains included indicated that while variants A and B

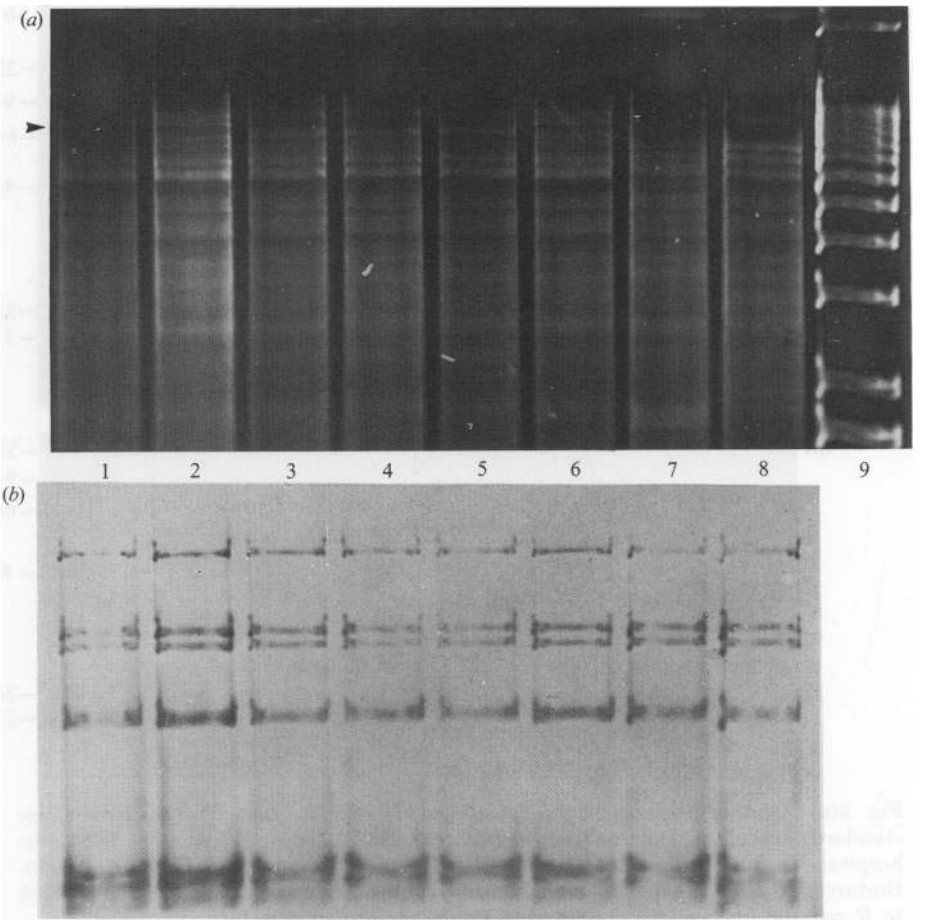

Fig. 3 (*a*) Banding patterns of *Eco*R I + *Cla* I digests. Lanes 1, 2, 6, isolates from Seville (variant B); lanes 3–5, isolates from 3 Madrid hospitals (variant A); lanes 7, 8, EMRSA-1 and EMRSA-2 respectively; lane 9, 1 kb ladder size standard. Arrow indicates the 8–12 kb region of variability between type A and type B variants. (*b*) Hybridization with biotin-labelled cDNA to *E. coli* 16S + 23S, rRNA. Lanes 1–8 as in (*a*).

gave indistinguishable RFLP patterns, this patterns could be distinguished from those given by the control strains (Fig. 1).

*Digestion with Hind III or Eco*R I. Many bands were produced following digestion with these enzymes. The overall impression given by the restriction patterns obtained with either enzyme was that, as with *Bgl* II or *Sal* I patterns, the fingerprint of variant A was indistinguishable from that of variant B, though both could be distinguished from those of the control strains (Fig. 2*a*). After probing, the distinctions between the control strain of EMRSA-1 and the test isolates was lost and variants A and B could not be distinguished; as expected, the two non-group III propagating strains (PS 52A and PS 3A) had distinct probe profiles. (Fig. 2*b*).

Double enzymic digestions. After digestion of total cellular DNA with *Eco*R I and *Cla* I, qualitative differences could be seen in the 8–12 kb region between isolates of variant A (lanes 3–5) and variant B (lanes 1, 2, 6) and between both variants

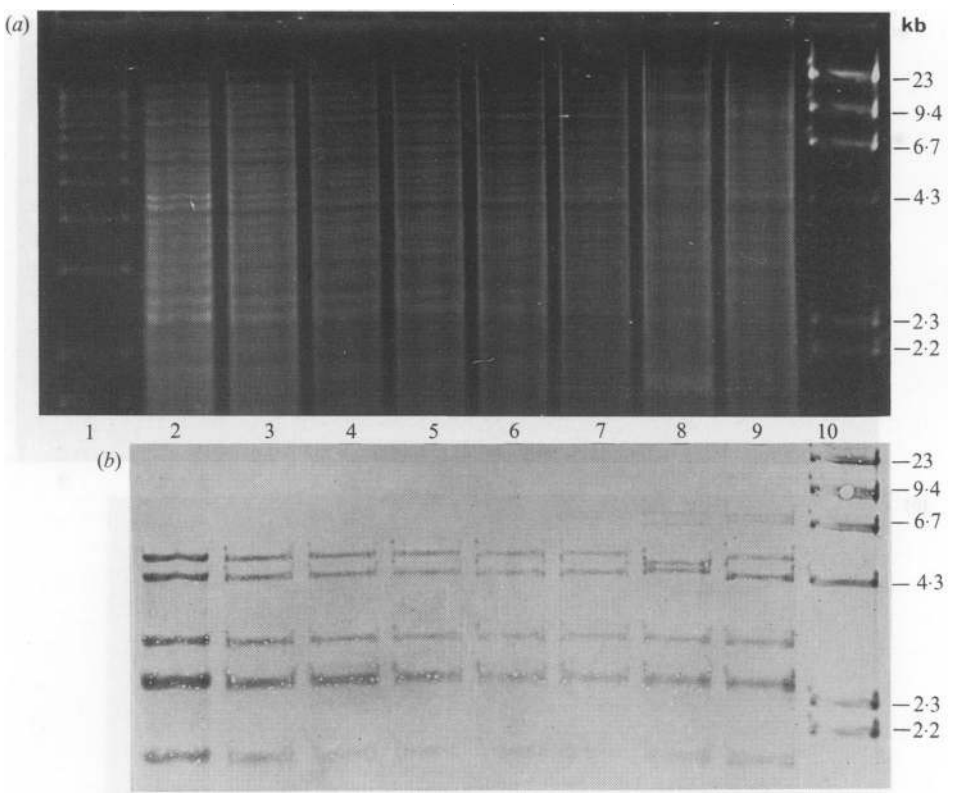

Fig. 4(*a*) Banding patterns of *Eco*R I+*Hin*d III digests. Lane 1, 1 kb ladder size standard; lanes 2, 3, isolates from Seville (variant B); lanes 4–7, isolates from four hospitals in Madrid (variant A); lane 8, EMRSA-1; lane 9, EMRSA-2; lane 10, Biotinylated *Hin*d III digest of λ DNA. (*b*) Hybridization with biotin-labelled cDNA to *E. coli* 16S+23S rRNA. Lanes as in (*a*).

and EMRSA-1 (lane 7) and EMRSA-2 (lane 8). EMRSA-1 and EMRSA-2 could also be distinguished from each other (Fig. 3*a*). After probing, all these distinctions were lost (Fig. 3*b*).

In contrast, when *Eco*R I and *Hin*d III were used, though EMRSA-1 and EMRSA-2 could be distinguished from each other and from the Spanish isolates, variants A and B were indistinguishable from each other (Fig. 4*a*). Interestingly, after probing, EMRSA-1 and EMRSA-2 remained distinct.

DISCUSSION

Initially, the aim of this study was to characterize isolates from different parts of Spain with the phage type 29/77/84/932 as a potential epidemic strain. At the beginning of this study, as all the selected isolates were indistinguishable, it was thought that one strain had become epidemic throughout Spain spreading in a manner analogous to EMRSA-1 in England or the Australian MRSA [2,3]. Molecular typing of selected isolates appeared to confirm this and it was only when Fisk phage typing was used that it became clear that two variants, possibly demonstrating divergent evolution, were circulating. It may be that the distinct

carried phages were acquired by the strain during passage through hospital personnel and patients. Variant B was found in Madrid and Barcelona as well as in Seville; the isolates found in the UK were unequivocally variant B. It is possible that this variant has the greater potential for spread.

Though it is possible to differentiate qualitatively between EMRSA-1 and EMRSA-2 by both phage and antibiogram typing [4] as well as by restriction fragment length polymorphisms [30], irrespective of enzymes or enzyme combinations used, this distinction was reduced when ribotying was performed. Similarly, though the DNA fingerprints of the variants of the Spanish strain were distinct from those of EMRSA-1 and EMRSA-2, it was only after the *Eco*R I/*Hin*d III double digestion that this distinction, clear by conventional methods, could be made by molecular methods. Additionally, only *Eco*R I/*Cla* I double digestion would distinguish between the Spanish A and B variants to a degree comparable to supplementary or Fisk phage typing though this distinction was lost after probing. It appears, therefore, that in this instance genotyping was less discriminatory than phenotyping.

The intention, when including molecular methods in this study was to determine whether they would improve discrimination when added to phenotypic typing. In the event, qualitative analysis of these methods indicated that, in the situation described here, they served to blur distinctions, clear when conventional methods were used. As the response in an epidemiological investigation must be rapid, this lack of extra discrimination coupled with the expense of genotyping it was thought that the application of, at least ribotying, would be in a research rather than a clinical situation.

It remains here to reiterate that in this paper we have defined one strain of MRSA in Spain; its initial spread throughout that country and importation into the UK. The strain is multiresistant including resistance to ciprofloxacin and reduced susceptibility to rifampicin, Tween 80 negative and a weak producer of protein A. Penicillinase and heavy metal resistances are carried on a large, curable plasmid of the classic type. It differs from EMRSA-1 where the penicillinase is chromosomally mediated and propamidine resistance is borne on a different plasmid [4]. It is similar to EMRSA-1 in the low production of protein A. These characters may assist in recognition of this strain in the laboratory. The two variants can readily be separated by the different carried prophages.

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