

Molecular Epidemiology of *Staphylococcus haemolyticus* Strains Isolated in an Albanian Hospital

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A recent outbreak of erythroderma in young children in an Albanian hospital was investigated. The etiology was not established, but *Staphylococcus haemolyticus* was frequently isolated from the affected children and from staff working in the same unit. Possible relationships among the isolates were investigated by using classical techniques (biotype, antimicrobial susceptibility, and extrachromosomal DNA pattern) and by restriction endonuclease analysis (REA) of total DNA. Control isolates of proven pathogenicity from hospitalized patients in Lyon, France were subjected to the same procedures. Distinct REA patterns were obtained after digestion with two enzymes in 7 of 10 isolates from five affected children. Six distinct patterns were observed in nine isolates from six staff members; two REA patterns from patient isolates and two from staff members were identical, and these were distinguishable by the other markers examined. Only two different REA patterns were found in the pathogenic control isolates despite the use of a third additional enzyme. Again, the isolates with the same REA patterns could be distinguished by their plasmid profile or antimicrobial resistance profile. REA of total DNA used in combination with other markers indicated that the Albanian isolates differed considerably, whereas the French pathogenic isolates showed little variability.

Staphylococcus haemolyticus is a normal member of the human bacterial flora and accounts for some 10 to 20% of clinical coagulase-negative staphylococcal isolates (13). Clinically significant infections of the urinary tract may occur (3, 13, 15, 16), and wounds, indwelling catheters, or continuous peritoneal dialysis (12) may be sites of infection, sometimes resulting in bacteremia (9-11), particularly in immunocompromised patients in a hospital setting. Many *S. haemolyticus* strains are multiply resistant to a variety of antibiotics (9-11, 19, 20), and, together with *Staphylococcus epidermidis*, have been considered to constitute an important reservoir of resistance genes (2) which may be transferred to other coagulase-negative staphylococci (17).

In the course of our investigations of a recent outbreak of acute desquamative erythroderma in a pediatric service in a hospital in Tirana (Albania), we observed a high frequency of isolations of *S. haemolyticus* from the affected children and from staff working in the same unit. Although none of the isolates were demonstrably pathogenic and the microbial etiology of the erythroderma was not established, we studied their relatedness by comparing restriction endonuclease profiles of their total DNA as well as their biotypes, antimicrobial resistance patterns, and extrachromosomal DNA profiles as epidemiological markers (6, 8, 18). The results were compared with those obtained with *S. haemolyticus* strains considered to be responsible for clinical infections in the Louis Pradel hospital in Lyon (France). The restriction enzyme analysis (REA) of total DNA proved to be a reliable epidemiological marker and was more stable in vitro and in vivo than the other three markers examined.

MATERIALS AND METHODS

Albanian isolates (Table 1). Ten isolates of *S. haemolyticus* were obtained from the skin, nose, or pharynx of five children aged under 3 months with manifest erythroderma whose etiology was not determined, and nine more isolates were obtained from the skin or nose of six members of the staff working in the children's unit. Swab samples were plated on horse blood agar, and isolates were identified by using the Staph ID gallery (API-bioMérieux, Marcy l'Etoile, France).

Control isolates (Table 2). Pathogenic *S. haemolyticus* isolates were obtained from three successive blood cultures from patient 1 (in intensive care after a trauma), five blood cultures from patient 2 (after cardiac surgery), two urine cultures from patient 3 (with a urinary tract infection), and two blood cultures from patient 4 (after a heart transplant).

Biotyping. Biotypes were determined by using the Staph ID gallery in accordance with the manufacturer's instructions, and the 32 biochemical reactions were used to generate a 9-digit biotype code (5).

Antimicrobial susceptibility testing. Antibiograms were determined on Mueller-Hinton medium by using impregnated discs (bioMérieux). The cultures were incubated for 18 to 24 h at 35°C for penicillin G, gentamicin, streptomycin, tetracycline, chloramphenicol, erythromycin, lincomycin, pristinamycin, trimethoprim-sulfamethoxazole, fosfomicin, and rifampin and at 30°C for oxacillin. Results were expressed as susceptible, intermediate, or resistant according to the criteria adopted by the Comité Français de l'antibiogramme (1).

Extrachromosomal DNA banding. Extrachromosomal DNA was extracted from the *S. haemolyticus* cultures by the rapid technique of Holmes and Quigley (14), modified as described previously (6). Briefly, the staphylococci were

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TABLE 1. Epidemiological markers of *S. haemolyticus* isolates from Albanian pediatric patients and hospital staff

Reference no.	Source ^a	Sample origin	Biotype no.	Antimicrobial resistance ^b										Plasmid profile	REA pattern	
				PEN	OXA	GEN	TET	CMP	ERY	SXT	FOS	STR	RFA			
141	AA	skin	553610043	R										R	O	I
142	AA	pharynx	551610043					R		R	R	R			A	II
143	AA	nose	551610043							R	R	R			O	II
144	AA	nose	553610043	R							R	R			O	I
145	AA	nose	553612043	R			R			R	R	R			B	III
3	CO	pustule	552610043	R	R		R	R	R	R		R			C	IV
6	AB	skin	453210043	R	R	R	R	R	R	R	R	R			D	V
8	AC	skin	553610043	R	R					R	R	R			O	VI
11	AD	skin	553610043	R	R	R	R	R	R	R		R			D	VII
12	AD	skin	553610043	R	R	R	R	R	R	R		R	R		E	VII
13	VD	skin	557610043	R	R	R	R		R	R		R			F	VIII
14	EP	nose	553610043	R	R		R	R	R	R	R	R			G	IX
146	EP	nose	553610043	R	R		R	R		R		R			H	IX
147	EP	nose	557610043	R			R					R			I	X
15	SM	skin	553610043	R	R		R		R	R	R	R			J	XI
16	SH	skin	551610043	R					R	R		R			K	IX
17	SK	skin	553610043	R	R		R		R	R		R			J	I
18	SK	nose	553610043	R		R		R	R	R	R	R			L	I
19	BB	nose	557612043	R	R	R	R		R	R	R	R			M	VII

^a Sources AA through AD are patients; sources VD through BB are staff members.

^b PEN, penicillin G; OXA, oxacillin; GEN, gentamicin; TET, tetracycline; CMP, chloramphenicol; ERY, erythromycin; SXT, trimethoprim-sulfamethoxazole; FOS, fosfomicin; STR, streptomycin; RFA, rifampin; R, resistant.

exposed to lysostaphin at 37°C for 30 min and then lysed by boiling for 65 s. Plasmids from the supercoiled DNA ladder (GIBCO-Bethesda Research Laboratories [BRL], Cergy-Pontoise, France) were used as molecular size standards.

Total DNA REA. Isolates of *S. haemolyticus* were grown on brain-heart agar plates (Diagnostics Pasteur, Marnes-la-Coquette, France) for 18 h at 37°C. The bacteria were resuspended in 10 ml of 0.9% NaCl and adjusted to an optical density of 1.5 at 540 nm. The cells were harvested by centrifugation, resuspended in 0.5 ml of Tris-EDTA buffer (50 mM each), pH 8.0, and lysed as described by Bialkowska-Hobrzanska et al. (4). DNA was extracted three times with phenol-chloroform (1:1 [vol/vol]) and precipitated overnight in two volumes of ethanol containing 1 µg of glycogen. The pellets were washed in 70% ethanol.

DNA from the Albanian isolates was cleaved with *EcoRI* (18) and then with *SalI* in the few instances in which the first

patterns were very similar. Control DNAs from French pathogenic isolates were all digested with *EcoRI*, *PstI*, and *PvuII*, as their patterns were frequently similar.

Electrophoresis. Samples were electrophoresed at 1.5 V/cm for 18 h in horizontal agarose (0.5 or 0.85% in 0.089 M Tris borate-boric acid buffer, pH 8.0, containing 0.002 M EDTA) slab gels at room temperature. The patterns obtained were compared visually, and particular attention was paid to the plasmid bands, particularly where these were not restricted.

RESULTS

Albanian isolates (Table 1). The majority of the 19 isolates were resistant to a wide range of antimicrobial agents; all were resistant to streptomycin, and 17 were resistant to penicillin G. Resistance to trimethoprim-sulfamethoxazole

TABLE 2. Epidemiological markers of *S. haemolyticus* isolates from French hospital patients^a

Reference no.	Patient no.	Sample origin	Biotype no.	Antimicrobial resistance										Plasmid profile	REA pattern
				PEN	OXA	GEN	TET	CMP	ERY	SXT	FOS	STR	RFA		
20	1	BC	557610043	R	R	R			R	R		R	R	1	A
21	1	BC	557610043	R	R	R			R	R		R	R	1	A
24	1	BC	557610043	R	R	R			R	R		R	R	1	A
1	2	BC	557610043	R	R	R	R			R	R	R	R	2	A
2	2	BC	557610043	R	R	R	R			R	R	R	R	2	A
3	2	BC	557610043	R		R	R			R	R	R	R	2	A
5	2	BC	557610043	R	R	R	R			R	R	R	R	2	A
6	2	BC	557610043	R	R	R	R			R	R	R	R	3	A
97	3	urine	556620043	R	R	R	R		R					4	B
98	3	urine	457620043	R	R	R	R		R					4	B
38	4	BC	557610043	R	R	R			R	R	R	R		5	A
39	4	BC	457610043	R	R	R			R	R	R	R		5	A

^a BC, blood culture; see Table 1 for rest of abbreviations.

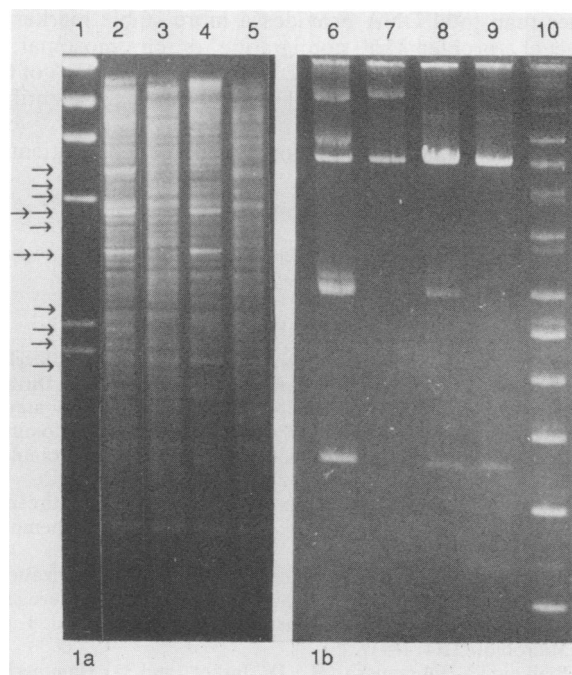


FIG. 1. Total DNA REAs and plasmid profiles of some Albanian isolates. (a) REA profiles. Lanes: 1, phage λ DNA digested with *Hind*III (23,700 to 1,980 bp) 2 to 5, DNA from specimens 6, 8, 12, and 146 digested with *Eco*RI. (b) Plasmid profiles. Lanes: 6 to 9; extrachromosomal DNA from specimens 6, 8, 12, and 146; 10, supercoiled DNA ladder-BRL (16,200 to 2,067 bp). \rightarrow , differences between patterns; $\rightarrow\rightarrow$, possible plasmid position.

(16), tetracycline (12), oxacillin (11), erythromycin (10), and fosfomycin (10) was also frequently observed. On the other hand, all isolates were susceptible to lincomycin, and 18 were resistant to rifampin. No two isolates from patients or staff showed identical resistance patterns. Extrachromosomal DNA bands were present in 15 of 19 isolates. Identical patterns were present in two pairs of isolates; samples 6 and 11 contained three apparently identical bands and showed similar resistance profiles, differing only in their resistance to fosfomycin. Samples 15 and 17 both contained a single small band but also differed in fosfomycin resistance. These two pairs of samples could also be distinguished by their REA profiles. Similarly, the four plasmid-free specimens (141, 143, 144, and 8) possess different antimicrobial resistance profiles, although, in this case, two of them share identical REA profiles (141 and 144).

The REA patterns of 10 isolates from five children and of 9 isolates from six staff members from the Albanian hospital showed considerable variation and yielded 11 distinct banding patterns (Fig. 1). Seven different patterns (I to VII) were distinguished in the children's isolates. Each child showed a distinct pattern, and child AA harbored three distinct variants, none of which were shared by any other child. The six staff isolate patterns were likewise distinct from each other, and two patterns from staff member EP were identified. Pattern I was found four times in two specimens from child AA and in two specimens from staff member SK. These isolates shared the same biotype but differed in antimicrobial susceptibility and plasmid content. Two other REA patterns, II (twice) and III (once), were seen in other samples from child AA. Pattern VII was found in the two isolates from

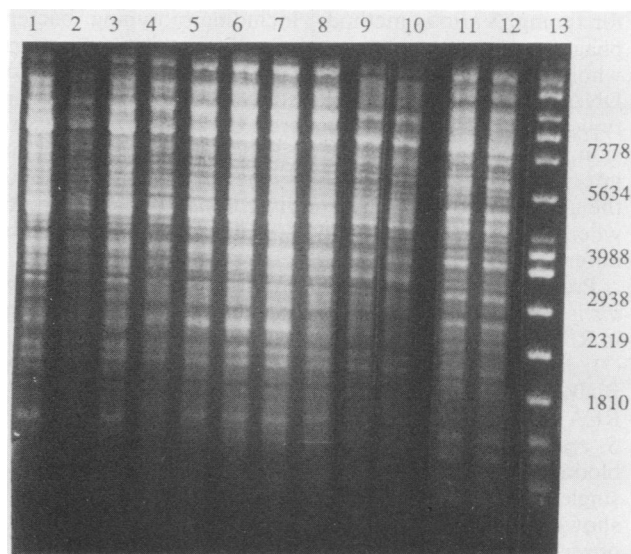


FIG. 2. Patient REAs. Total DNA was digested by *Eco*RI. Lanes: 1 to 12, samples 20, 21, 24, 1, 2, 3, 5, 6, 38, 39, 97, and 98; 13, base pair markers (Appligene).

child AD and in the isolate from staff member BB, and pattern IX was present in two of three isolates from staff member EP and in the isolate from staff member SH. In these cases, different biotypes were associated with the individual donors. Pattern VII isolates differed in susceptibility to chloramphenicol, rifampin, and fosfomycin and had completely different plasmid profiles. Pattern IX isolates differed in resistance to oxacillin, tetracycline, chloramphenicol, erythromycin, and fosfomycin, and again, all had quite distinct plasmid profiles. Variations in biotype occurred independently of the other markers; the most common biotype (553610043) was observed in conjunction with five different REA patterns (I, VI, VII, IX, and XI) and was not correlated with any particular antibiotic or extrachromosomal banding pattern.

Control isolates (Table 2). As a control in the procedures, isolates from clinically recognized infections at the Louis Pradel hospital were typed by the same techniques. Only two REA patterns were found (Fig. 2): type A from patients 1, 2, and 4, and type B from patient 3. The same biotype was found in 9 of the 10 type A isolates, while the two type B isolates differed. All isolates showed wide antimicrobial resistance but were uniformly susceptible to chloramphenicol. Extrachromosomal banding distinguished between isolates from the four patients, the only internal variation being the loss of a band in one isolate from patient 2. Little variation in antimicrobial resistance among samples from the same patient was observed: samples 3 and 6 from patient 2 lost resistance to oxacillin and tetracycline, respectively. The samples from patient 3 with REA pattern B were also distinguished from all other isolates by their susceptibility to trimethoprim-sulfamethoxazole and streptomycin and by discrete differences in biotype.

DISCUSSION

The characterization of strains of coagulase-negative staphylococci for epidemiological studies is a difficult problem because of the instability of many of the properties used

for typing. Various methods, including biotyping, bacteriophage typing, serotyping, antimicrobial resistance patterns, whole-cell protein analysis, plasmid DNA analysis, and total DNA REA have been employed. Methods based on phenotypic characteristics are hindered by variability in expression, and plasmids may be lost or gained over short time intervals. REA of total DNA has the advantage of examining the genotype of the organism but can be difficult in practice when a large number of bands must be examined for subtle differences.

Previous studies have shown that pathogenic *S. epidermidis* isolates from different patients tend to vary considerably in REA pattern (4, 18), whereas *Staphylococcus lugdunensis* (7) or *S. haemolyticus* (4) isolates show less variability. Bialkowska-Hobrzanska et al. (4) detected seven different REA patterns in 11 blood cultures from eight neonates with *S. epidermidis* infections, whereas all of a small number of blood isolates of *S. haemolyticus* in the same series gave a single pattern. Mucocutaneous isolates of *S. epidermidis* showed patterns both identical to and different from those observed in the blood cultures from the same patients.

In the present study, we have compared the REA patterns of nonpathogenic *S. haemolyticus* isolates from an Albanian hospital with those of pathological isolates from patients in the Louis Pradel Hospital. Wide variation in REA pattern was observed in the Albanian isolates. Identical patterns were observed in only three pairs of subjects, and differences in antimicrobial susceptibility and plasmid profile suggest that these were recent divergences from common ancestors. Individual patients sometimes yielded more than one distinct REA pattern, and isolates with the same pattern frequently differed by other criteria. In contrast, the pathogenic French isolates were much more homogeneous; 10 isolates from three patients had identical REA profiles, though minor variation in plasmid profile (patient 2), biotype (patient 4), and antimicrobial resistance (patient 2) could be seen. Two samples from a fourth patient (patient 3) had a distinct REA pattern. There was no obvious epidemiological link between these patients, their hospitalization being in different units and at different dates. In the cases in which identical REA patterns were associated with variation in other markers, notably in pathogenic isolates, antimicrobial resistance profiles and plasmid patterns provide more sensitive markers for epidemiological tracing. The similarity of REA patterns from the pathogenic isolates might suggest that pathogenic strains of *S. haemolyticus* collected from patients in different units at different times show less variability than do apparently nonpathogenic isolates taken at one time from patients in a single service. However, in the absence of studies of pathogenic strains isolated from other hospitals, we cannot at present give a particular clinical significance to strains showing these REA patterns. Such a homogeneity has been shown among pathogenic strains of *S. haemolyticus* isolated by blood culture and which were identical to mucocutaneous strains from the same patients (4).

Classical markers such as biotype or antimicrobial resistance have proved unsatisfactory for epidemiological studies of *S. epidermidis* because of their instability (8, 18), and in the present study, similar problems were encountered with *S. haemolyticus*. The biotype is for this reason an unsatisfactory epidemiological marker; however, reproducible major changes in antimicrobial resistance in the presence of stability of REA, or even plasmid pattern (control isolates), can shed light on the recent evolution of the strains.

It has been suggested that the REA of chromosomal DNA

rather than total DNA provides a more stable marker (4); however, problems of comigration of chromosomal and plasmid-derived bands can occur. In this study, REA of total DNA has proved most useful in both cases for identifying related isolates despite minor phenotypic variations, even in clinically relevant isolates from a single patient (patient 2).

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