Instability of characteristics amongst coagulase-negative staphylococci causing endocarditis

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Summary. Variation in typing of clinically significant isolates of coagulase-negative staphylococci (CNS) was determined by five typing methods with 143 isolates obtained from 19 patients over periods from 2 days to 1 year. In only one case did all isolates give exactly the same typing pattern by all five tests. No single method, or simple combination, provided a ready means of confirming the relatedness of separate isolates. The most frequently useful tests were antibiotic susceptibility and extrachromosomal DNA banding patterns. However, the results of biotyping, serotyping and phage typing were also helpful in showing the relationship between different isolates from a given patient. In most cases a core pattern varying by the gain or loss of a small number of features, characterised a given patient's isolates. In two cases, apparently radical changes in the infecting organism were observed, and confirmed by restriction endonuclease analysis. Care should be taken when successive isolates of CNS show distinct typing differences in deciding their clinical relevance.

Introduction

Coagulase-negative staphylococci (CNS) are only rarely pathogenic in man although they are widely distributed. However, they are responsible for many cases of prosthetic valve endocarditis (PVE)¹ and for a small percentage of cases of native valve endocarditis (NVE).² The species most often involved is *Staphylococcus epidermidis*. CNS are the organisms most frequently recovered from blood cultures, and although the vast majority of isolations represent contamination, clinically important infections occur and represent a major cause of septicaemia.³ Even after exclusion of the more obviously contaminated samples, a small number of potentially clinically relevant isolates remain.⁴ Individual strains of CNS differ considerably, and, except for infrequent instances of nosocomial outbreaks, clinically relevant strains are usually not closely related to each other.⁵ A frequently used criterion for the presumption of clinical importance is the repeated isolation of the same strain from the patient from successive samples over time or from different sites.³ The means used to establish the identity of different isolates are clearly crucial to this concept. Unfortunately, no single method is entirely satisfactory and many are insufficiently

developed or poorly standardised for CNS.^{6,7} Biotyping can be reliably performed, but is not sufficiently discriminatory;⁶ antibiotic resistance profiles can change quite rapidly and are influenced by the patient's treatment and previous history;^{5,6} phage typing can be helpful, but no universally recognised battery of phages allowing the typing of the majority of samples exists at present,⁶ serotyping suffers from the lack of available defined antisera; and plasmid profile analysis^{5,8,9} and restriction endonuclease patterns¹⁰ may be too costly and too technically complex for routine analysis; however, facilities should be available in specialised hospital centres.

In the present paper we describe the variations observed amongst successive isolates of CNS from 19 patients with PVE or NVE as determined by the five methods mentioned above. The CNS were in all cases shown to be clinically relevant, either by isolation from the infected valve, by histological evidence of CNS infection of the replaced valve or surrounding tissues, or by resolution of the clinical state on elimination of the CNS infection. A total of 143 isolates was obtained over periods from a few days to more than a year. Considerable variation in typing characteristics was observed frequently, even when the period studied was only a few days.

Materials and methods

Patients

Typing was performed on CNS isolates from successive specimens taken from 19 patients with endocarditis diagnosed at the Cardiological Hospital, Lyon, France since 1980, from whom at least two positive isolates were obtained. Eleven of these patients form part of a cohort previously studied⁵ to determine whether typing a typical CNS isolate could help to predict its clinical relevance; the other patients in that cohort were eliminated from the present study because the successive different isolates were no longer available. Eight further patients seen at the hospital since termination of the previous study complete the present group which is made up of 12 cases of PVE and seven cases of NVE. In 17 cases, the infecting organism was identified as *S. epidermidis*; one was *S. capitis* (No. 2), according to the classification of Schleifer¹¹ and using the API Staph gallery (API-System, Montalieu-Vercieu, France) and one was identified as *S. lugdunensis* (No. 7), a recently recognised CNS species.¹² The outcome of the infection varied from rapid resolution of the infection with antibiotic therapy (usually a β -lactam or vancomycin combined with an aminoglycoside, rifampicin, pefloxacin or fosfomycin) in 10 cases, through persistence of the infection until the valve was replaced (11–33 days) in four patients (Nos. 11–14) with one death (No. 12), to persistence of the infection despite valve replacement (74–346 days) in five patients (Nos. 15–19) with four deaths. The survivor from this last group (No. 19) recovered after receiving a heart transplant. The infections due to *S. capitis* and to *S. lugdunensis* occurred in patients in the rapid recovery group. A total of 143 isolates was obtained; 126 from blood cultures (2–13 per patient), 12 from prosthetic or native valve specimens from eight patients (Nos. 10, 11, 13–16, 18 and 19), four from wounds considered to be the portal of entry of the organism (Nos. 11 and 18), and one from an episode of sepsis occurring 18 months previously (No. 15).

Cultures

Isolates growing in both aerobic and anaerobic blood cultures and fulfilling other criteria as not being contaminants,⁴ or those obtained from specimens of the infected valves, surrounding tissues or the presumed portal of entry, were picked from agar plates, grown in brain-heart infusion broth, and stored at -20°C .

Biotyping

Biotypes were determined with API-Staph kits (API System) following the manufacturer's recommendations; the 19 biochemical reactions were used to generate a seven digit biotype code.

Phage typing

A battery of eight phages isolated in our laboratory was used to establish susceptibility profiles as previously described.¹³

Serotyping

The agglutinability of formalin-treated *S. epidermidis* by two antisera (186 and 260), which recognise distinct antigenic determinants, was determined as previously described.¹⁴

Antibiotic susceptibility testing

Sensitivity to a panel of antibiotics was determined by the agar diffusion method¹⁵ on commercially prepared Mueller-Hinton medium with antimicrobial disks (Bio-Mérieux, Marcy l'Etoile, France) containing: penicillin G 6 μg , oxacillin, 5 μg , gentamicin 15 μg , streptomycin, 10 IU, neomycin, 30 μg , chloramphenicol, 30 μg , tetracycline, 30 IU, minocycline, 30 IU, erythromycin, 15 IU, lincomycin, 15 μg , pristinamycin, 15 μg , co-trimoxazole, 1.25 + 23.75 μg , pefloxacin, 5 μg , rifampicin, 30 μg , novobiocin, 30 μg , fusidic acid, 10 μg , fosfomycin, 50 μg and vancomycin 30 μg . Incubation for 18–24 h was at 35°C except for oxacillin tests which were performed at 30°C . The isolates were tested for their antibiotic susceptibility on the same day as the determination of their extrachromosomal DNA pattern.

Extrachromosomal DNA banding

Extrachromosomal DNA was extracted from CNS cultures by the rapid boiling technique¹⁶ modified as described previously.⁵ Briefly, the staphylococci were exposed to lysostaphin at 37°C for 30 min, then lysed by boiling for 65 s. Plasmids from *Escherichia coli* strain V517 were used as molecular size standards.¹⁷ In the case of strains isolated from patient No. 4, plasmids were identified by hybridisation with probes specific for *ermC* and *tetK* genes, responsible for resistance to macrolide¹⁸ and to tetracycline¹⁹ respectively. The *tetK* probe was obtained by cloning a 870-bp *HincII* fragment of plasmid pT181 into the *HincII* site of pUC8.²⁰

The fragments of the *tetK* and *ermC* genes used as probes were radiolabelled by nick translation with a commercial kit (BRL, Cergy-Pontoise, France), and hybridised to the extrachromosomal DNA on Nytran sheets (Schleicher and Schuell, Dassel, Germany) as described by Maniatis.²¹

Restriction endonuclease analysis (REA)

In certain cases, the restriction enzyme pattern of chromosomal DNA from successive isolates was compared. CNS were grown overnight at 35°C in glycerol broth and harvested by centrifugation. The DNA was recovered by the simultaneous action of lysozyme (Sigma Chemical Co., OSI-France) and TBL₁ achromopeptidase (AMI-Labo, Lyon, France) as previously described.¹⁰ This DNA was exhaustively digested with *EcoRI* restriction enzyme (Boehringer Mannheim, Meylan, France) under conditions recommended by the manufacturer.

Results

The clinical significance of the CNS isolates from the 19 patients in this study is certain, since strains were grown from the replaced prosthetic or native valve in eight cases, and histological or clinical evidence of CNS infection was clear in the others. Nevertheless, for 18 of the 19 cases, successive isolates showed a certain degree of variability in typing by one or more of the tests employed, even over short periods. Generally the variants from an individual patient bore a clear resemblance to each other, but in two cases there was sufficient difference to suspect that the isolates derived from different parental clones (Nos. 15 and 16).

Variations were seen in all the tests used (tables I and II). In only four of 17 cases of *S. epidermidis* infection did all the isolates retain exactly the same biotype. The variations observed concerned characteristics not considered crucial for species identification—e.g., utilisation of mannose.

We used two sera that recognise independent antigenic determinants, giving four possible combinations. For 16 of the 17 cases, at least one isolate gave a positive reaction with one or both sera; formalinised bacteria from patient No. 4 agglutinated spontaneously in the absence of antiserum. Isolates from six patients consistently retained the same serotype, whereas variants were observed in the 10 other cases.

With the battery of eight phages, typable isolates were obtained from only seven of the 19 patients. In these seven patients, isolates from two retained the same phage susceptibility profile throughout the study period, whereas some isolates from the five other patients had different patterns, usually the development of resistance to lysis by any of our phages.

Profiles of antibiotic resistance were somewhat more stable, being conserved unchanged in 10 of 19 cases, and showing only minor modifications in less than one third of the isolates in six other cases. However, major changes involving a large proportion of isolates were observed in three cases (Nos. 13, 15 and 16) (table II). Emergence of resistant strains was observed only during the course of antibiotic treatment, and strains resistant to rifampicin and pefloxacin were isolated (Nos. 15 and 16). Generally, however, the antibiotic profile of strains isolated from a given patient were quite distinctive for that patient and tended to change rather little between isolates.

Similar results were obtained by analysis of the size patterns of extrachromosomal DNA. The patterns tended to be quite distinctive for isolates

from a given patient, and in nine of 19 cases were preserved unchanged in all isolates obtained. In a further eight cases, only minor modifications—usually the loss of a band—were observed (fig. 1). REA of chromosomal DNA from strains from case No. 13 indicated a very close relationship despite the variation in extrachromosomal DNA banding (results not shown). Major changes were seen in two cases (Nos. 15 and 16; see figs. 2 and 3). There is clearly a close relationship between modifications in the extrachromosomal banding patterns and the corresponding antibiotic profiles. However, case Nos. 4 and 5 showed minor variations in their extrachromosomal DNA profiles while conserving their antibiotic resistance, and No. 17 showed some variation in antibiotic susceptibility without any corresponding change in extrachromosomal DNA banding. The 2·8-Mda plasmid from case No. 15 hybridised with the *tetK* probe and the 1·7-Mda band with the *erm C* probe. It is probable that certain closely correlated bands represent different forms of DNA derived from the same plasmid; for example the weak band at c.1·7 Mda in fig. 1 might well represent a relaxed circular or linear form of the 1·2-Mda supercoiled form, and similarly for the 2·8- and 4·8-Mda bands. Confirmation could be achieved by nicking the major forms and observing the new migration patterns.

It should be noted that the isolates obtained from the infected valves or perivalvular tissue were also subject to variation. In three cases, multiple isolations were made from valvular tissue and, in one of these (No. 15), strains varying in phage type, serotype and antibiotic-resistance pattern were obtained (table II). However, these different isolates were identical by REA of chromosomal DNA (data not shown). Of five other single isolates from the infected valve, three showed loss of an extrachromosomal DNA band (case Nos. 10, 11 and 14), one had an altered serotype (case No. 16) and one an altered biotype (case No. 19) compared with at least one blood-culture isolate from the same patient. The isolates from wounds considered to be the portal of entry of the infection (one suppurating catheter scar, and three from a cholecystectomy wound) closely resembled blood-culture isolates from the same patients (case Nos. 11 and 18).

Complete stability of the results in all five testing procedures was observed in only one case (No. 8) in five separate isolates made over a 2-day period. In nine cases, stability of the results in two of the five methods was observed. These were most often their antibiotic profile and the extrachromosomal DNA pattern (seven cases; Nos. 1, 3, 6, 7, 9, 12 and 18). Isolates from five cases showed stability of

Table 1. Characterisation of multiple isolates of CNS from patients with endocarditis

Patient no.	Type of endocarditis	Outcome	Site of isolation				Serotype					Antibiotic susceptibility patterns	Extrachromosomal DNA patterns		
			Number of isolates	BC	E	P	Biotype	186+260			Phage type*				
								(n)	(n)	(n)				(n)	
1	PVE	Rapid cure	8	8			6606133 6604113	(7) (1)	0	8	0	(8)	Unchanged	Unchanged	
2	NVE	"	8	8			6102102 6100100	(5) (2)		ND		0	(8)	Loss of resistance to FOS: 1 isolate	Loss of a band: 3 isolates
3	NVE	"	3	3			6304113 6600012 6700012	(1) (1) (1)	1	1	1	0	(3)	Unchanged	Unchanged
4	PVE	"	8	8			6706013 6602113	(1) (8)	Spontaneous agglutination			0	(8)	Unchanged	See fig. 1
5	NVE	"	6	6			6606111 6706111	(5) (1)	1	0	0	all phages (1)	Unchanged	Loss of a band:	
6	PVE	"	3	3			6602113 6602112	(2) (1)	0	3	0	0	(3)	Unchanged	Unchanged
7	NVE	"	11	11			6712150 6716150	(6) (3)		ND		0	(11)	Unchanged	Unchanged
8	NVE	"	5	5			6716110 6700113	(2) (5)	0	5	0	392	(5)	Unchanged	Unchanged
9	NVE	"	6	6			6600112 6600113	(4) (1)	0	1	3	392	(1)	Unchanged	Unchanged
10	PVE	"	8	7	1		6706113 6706113	(1) (6)	0	0	8	0	(8)	Loss of resistance to SXT: 1 isolate	Loss of 1 band: 1 isolate
11	PVE	Persistence of infection	5	3	1	1	6606113 6703113	(2) (1)	0	0	4	550	(1)	Loss of resistance to SXT: 1 isolate	Loss of 1 band: 3 isolates
12	PVE	until valve replacement	2	2			6602112 6606113	(1) (1)	1	0	0	0	(2)	Unchanged	Unchanged
13	PVE	"	12	10	2				See table II					Loss of band: 6 isolates	
14	PVE	"	5	4	1		6200113 6204113	(4) (1)	0	5	0	0	(5)	Loss of resistance to GM: 1 isolate to OX and SXT: 1 isolate	Loss of 2 bands: 1 isolate
15	PVE	Persistence	10	7	3				See table II					See fig. 2	
16	PVE	of infection	6	5	1		6606113	(9)	2	4	0	0	(11)	Loss of resistance to C: 3 isolates	See fig. 3
17	PVE	despite valve	11	11			6606112 6604113	(2) (10)	11	7	0	0	(18)	Unchanged	Unchanged
18	PVE	replacement	18	13	2	3	6606113 6704113	(4) (4)							
19	PVE	"	8	7	1		6706113 6602113	(3) (3)	0	0	8	63/138	(1)	Loss of resistance to S: 1 isolate	Loss of band: 3 isolates
							6604113 6702112	(1) (1)				245/336			

BC, blood culture; E, endocardium; P, portal of entry; *Susceptibility to phages 41, 63, 118, 138, 245, 336, 392 and 550 (13); 0 = resistant to all 8 phages. PVE, prosthetic valve endocarditis; NVE, native valve endocarditis; FOS, fosfomycin; SXT, trimethoprim-sulfamethoxazole; GM, gentamicin; OX, oxacillin; C, chloramphenicol; S, streptomycin.

Table II. Examples of the variation in REA characteristics of clinically significant *S. epidermidis* isolates from three patients with PVE during periods of 13, 94 and 96 days

Isolate no.	Day of sampling	Site	Biotype	Phage type	Serotype		Antibiotic resistance pattern																REA patterns
					186	260	PG	OX	GM	S	N	C	TE	E	L	SXT	PF	RA	FO	S			
Patient 13																							
				41/63/118																			
1	1	BC	6706112	138/245/336	+	+	R	R	R			R							R		A		
2	1	BC	6706112	„	+	+	R	R	R			R	...	R	R				R		A		
3	2	BC	6706112	„	+	+	R	R	R			R	...	R	R				R		A		
4	2	BC	6706112	„	+	+	R	R	R			R	...	R	R				R		A		
5	2	BC	6706112	„	+	+	R	R	R			R							R		A		
6	6†	BC	6706112	„	+	+	R	R	R	R	...	R	...	R	R				R		A		
7	9	BC	6706112	„	+	—	R	R	R			R							R		A		
8	9	BC	6706112	„	+	—	R	R	R			R							R		A		
9	9	BC	6706112	„	+	—	R	R	R			R							R		A		
10	9	BC	6706112	„	+	+	R	R	R			R	...	R	R				R		A		
11	13	PV	6706112	„	+	+	R	R	R			R							R		A		
12	13	PV	6706112	„	+	+	R	R	R			R							R		A		
Patient 15																							
13	§	BC	6606112	0	—	+	R	R	R	...	R					R							
14	1	BC	6706112	0	—	—	R	R	R	R	R	...	R	...	R	R				R	B		
15	3	BC	6702112	0	—	—	R	...	R	R	R	...	R	R	R						B		
16	49	BC	6702112	0	—	—	R	R	R	R	R	...	R								B		
17	51	PV	6706112	(63/245/336)	—	+	R	R	R	R	R	...	R								B		
18	51	PV	6706112	63/138/245/336	—	—	R	R	R	R	R	...	R								B		
19	51	PV	6706112	(63/245/336)	—	+	R	R	R	R	R	...	R					R	...		B		
20	94	BC	6706112	„	—	—	R	R	R	R	R	...	R	R	R	R			R	...	B		
21	94	BC	6706112	63/138/245/336	—	+	R	R	R	R	R	...	...	R	R						B		
22	96	BC	6702112	0	—	—	R	R	R	...	R			R	R						B		
Patient 16																							
23	1†	BC	6704112	0	—	—	R	R	R			R	R			R	...	R	...		C		
24	4	BC	6704112	0	—	—	R	R	R			R	R	R	R	R		R	...		C		
25	5	BC	6704112	0	—	—	R	R	R			R					R	...	R	...	C		
26	68	BC	6200112	0	—	+	R	R	R	...	R				R	R	R	R	...		D		
27	72	BC	6200113	0	—	—	R	R	R	...	R				R	R	R	R	...		D		
28	94	PV	6700113	0	+	—	R	R	R	...	R				R	R	R	R	R		D		

PG, penicillin G; OX, oxacillin; GM, gentamicin; S, streptomycin; N, neomycin; C, chloramphenicol; TE, tetracycline; E, erythromycin; L, lincomycin; SXT, trimethoprim-sulphamethoxazole; PF, pefloxacin; RA, rifampicin; FOS, fosfomycin; R, resistant.

REA, restriction endonuclease analysis (restriction enzyme patterns A, B, C and D were clearly distinct, but comprised too many bands for listing—see fig. 3).

BC strains isolated from blood culture; PV, strains isolated from prosthetic valves.

0, Resistant to all eight phages; (), weak reactions.

†First day of antibiotic treatment.

§Strains responsible for septicaemia, 18 months before endocarditis.

the results in only one method and two cases showed the conservation of a distinctive underlying pattern even though no single character remained perfectly stable. In two cases (Nos. 15 and 16), the characteristics of certain isolates varied to such an extent that it cannot be affirmed that they were derived from the same ancestral clone. In order to clarify this point, the chromosomal DNA of isolates from these cases was digested with *EcoRI* restriction

endonuclease, and the size of fragments obtained was examined after electrophoresis in agarose gel. For case No. 15, similarity in the REA pattern of the strain obtained from a past episode of sepsis indicated that this isolate was closely related to the strains responsible for the episode of endocarditis (results not shown). For case No. 16 there is a noticeable difference in the banding pattern of isolates obtained early in the clinical course and

Fig. 1. Agarose gel electrophoresis of extrachromosomal DNA from *S. epidermidis* isolates from patient No. 4. *E. coli* V517 provides size reference fragments of 1.4, 1.8, 2.0, 2.6, 3.4, and 4.8 Mda. BC, strain isolated from blood culture. The first two strains were isolated 97 and 30 days before hospitalisation. The BC isolates taken at day 1 are presented in chronological order. Chr, chromosome band.

those obtained later (fig. 3); this change correlates with other major changes in the typing characteristics of isolates from this patient. No correlation between the time over which the isolates were obtained (2–346 days) and the variability of the different typing characteristics could be demonstrated.

The various isolates from patient No. 15 have been re-tested for their biotypes, phage types, antibiotic profiles and extra-chromosomal DNA patterns on three occasions over a period of 1 year of storage at -20°C . The phage, antibiotic-resistance and extrachromosomal DNA profiles had excellent stability on repeated testing. However, variation in the biotypes concerning features which were unstable in repeated isolates from the patient was observed frequently. Strains from patient No. 13 have also been re-typed after storage for 6 months, and found to give the same patterns as reported here.

Discussion

The difficulty in determining the clinical relevance of an isolate of CNS from a patient in the face of the universal presence of these bacteria is of considerable importance, given their major pathological role in PVE. The usual approach to this problem is to require repeated isolation from independent specimens from the patient, and that the isolates should share identical typing characteristics. It may be considered unlikely, given the intrinsic variability of these bacteria, that separate contamination by identical strains would occur—except possibly in the rare cases of nosocomial outbreaks. In this study we have examined the stability of typing characteristics of successive isolates of CNS of demonstrated clinical significance from patients with prosthetic or native valve endocarditis over periods ranging from 2 days to almost a year. Five typing methods were employed: biotyping, serotyping, phage typing, antibiotic susceptibility and extrachromosomal DNA banding. When necessary these results were confirmed by REA of the chromosomal DNA. In 18 of the 19 cases studied, some degree of variation in typing characteristics was observed among successive

Fig. 2. Agarose gel electrophoresis of extrachromosomal DNA from *S. epidermidis* isolates from patient No. 15. * Strain isolated during a period of sepsis 18 months previously. PV, strain isolated from replaced prosthetic valve. (See also caption to fig. 1).

specimens, and in several cases variants were isolated directly from the affected valve itself or from peripheral tissue.

No one test or any simple combination of those we have analysed here can provide unequivocal evidence of the essential identity, and thus the clinical relevance of isolates from a given patient; the most frequently helpful were antibiotic susceptibility and extrachromosomal DNA pattern. However, different isolates from the same patient do tend to retain a core pattern of characteristics, and to vary by simple loss or gain of a small number of features. Typing patterns of CNS isolates from different patients differed much more widely than did those of different isolates from the same patient. As described previously,⁵ no particular characteristics were found to be associated with pathological importance in these CNS.

In two cases we observed changes in the typing characteristics of successive isolates that appeared more radical than the usual simple variations on a conserved core theme. In one of these cases (No. 15), REA of the chromosomal DNAs of the distinctive isolates indicated that the isolates

showing clear differences in their extrachromosomal DNA patterns were closely similar in their chromosomal DNAs, even in a strain responsible for a previous episode of sepsis. This possibly reflects a change in the plasmid population within a conserved parenteral strain of CNS. In another case (No. 16) changes in the extrachromosomal DNA banding patterns occurring during the endocarditis-related infection were paralleled by clear changes in the REA of the chromosomal DNA (fig. 3). This strongly suggests that replacement of one infecting strain by another had occurred.

The present findings confirm and amplify other reports^{22,23} in which some variation was described in typing characteristics between CNS isolates from a smaller number of patients involved in outbreaks of prosthetic valve endocarditis. In our study, variations were also observed between isolates from the same patient. Thus, it appears that clinically relevant CNS isolates from the same patient can present significant differences in typing characteristics, even over relatively short periods. In view of these findings, caution should be exercised in rejecting the clinical relevance of CNS

Fig. 3. (a) Agarose gel electrophoresis of extrachromosomal DNA from *S. epidermidis* isolates from patient No. 16. (b) Agarose gel electrophoresis of restriction endonuclease (*EcoRI*) digests of chromosomal DNA of the same isolates. * Phage λ DNA digested with *Hind* III used as size markers; kb = kilobase pairs. (See also captions to figs. 1 and 2.)

even when successive isolates show distinct typing differences. Unfortunately, no simple recipe can be derived from our observations to decide whether any given degree of variation would indicate the appearance of a new strain. The conservation of a core of similar characteristics which vary essentially by the acquisition or loss of a small number of simple features may well indicate a continuity

between the different isolates. Weighing the significance of the variations requires the judgement of the experienced microbiologist.

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