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Phage typing of coagulase-negative staphylococci and micrococci

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SUMMARY

A total of 3217 strains of coagulase-negative staphylococci and micrococci were tested for susceptibility to a collection of phages isolated from coagulase-negative cocci; it was concluded that a useful typing scheme could be developed. Of the strains of Baird-Parker's biotype 1, 72% were lysed by one or more phages, although rather a large proportion of strains are lysed by many phages to give a complex typing pattern.

Normal persons commonly yield 10 or more distinguishable strains of coagulase-negative cocci in cultures from the nose and the skin.

INTRODUCTION

During the last few years the coagulase-negative staphylococci (*Staphylococcus epidermidis* or *S. albus*) and micrococci have been the subject of several studies since it has become obvious that they can, in some circumstances, produce disease. In two conditions – endocarditis and bacteraemia originating in Spitz–Holter valves – it would be of particular value to have methods for type identification to enable studies of the origin of the infection to be pursued. We therefore set out to accumulate a collection of phages and to investigate their use for type identification. Shortly after we began work we found that Professor Winkler and Drs Verhoef and van Boven of Utrecht were similarly engaged and we have exchanged phages with them; some results from their studies have recently been published (Verhoef, van Boven & Winkler, 1971*a*, *b*, 1972).

MATERIALS AND METHODS

A total of 3217 strains of coagulase-negative, Gram-positive cocci were tested in our studies; 2413 were isolated from the nose and skin of hospital staff and patients and 804 from 'clinical' specimens. When allowance was made for repeat isolations from single individuals of strains having the same phage pattern and biotype (see below) a total of 1517 'independent' strains were available for analysis, and it is to these that all figures given in this paper refer.

All the strains were classified according to Baird-Parker's (1963) scheme, with the exception that, as suggested recently by Baird-Parker (1972, modifying Baird-

Table 1. *Biotypes of strains examined*

Biotype (Baird-Parker, 1965, modified 1972)	Subgroup (Baird-Parker, 1963)	Acid: Glucose Anaer.	Acid: Glucose Aer.	Acid: Mannitol Anaer.	Acid: Mannitol Aer.	Acid: Lactose	Production of: Phosphatase	Production of: Acetoin	No. of strains	
1	SII	+	+	−	−	(+)	+	+	832	972
	SV	+	+	−	−	+	−	+	140	
2	SIII	+	+	−	−	+	+	−	15	
3	SIV	+	+	−	−	−	−	+	38	
4	SVI	+	+	−	+	+	−	(+)	72	
*a**	(SI)*	+	+	+	+	+	+	+	39	
	M1	−	+	−	−	−	−	+	22	
	M2	−	+	−	−	+	−	+	43	
	M3	−	+	−	+	(+)	−	+	9	
	M4	−	+	−	+	+	−	+	10	
	M5	−	+	−	(+)	(+)	−	−	4	
	M6	−	+	−	+	+	+	−	5	
	M7	−	−	−	−	−	−	−	20	
	M unclass.	−	.	.	.	Various	.	.	187	
Unclassifiable		−	.	.	.	Various	.	.	81	
Total									1517	

* Strains with these characters are not included in Baird-Parker's (1972) classification and do not fall properly into his (1963) classification since his subgroup I strains were coagulase-positive.

(+) Variable reactions.

Table 2. *Characters of the typing phages*

Phage	Source	Propagating strain	Lytic pattern at 100 × RTD on the set of propagating strains																	
			15	27	RG	28	37	155	165	28A	A6C	A9C	71	275	48	82	456	157A	471A	459	275A
15	WFI	89	++	++	.	.	±	+	.	.	++	++	++	+	+	++	++	++	++	++	++
27	WFI	186	.	++	.	.	.	.	.	.	++	.	++	.	++	.	++	+	.	++	++
RG	Colindale	RG	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
28	WFI	170	.	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
37	WFI	192	.	.	.	.	++	++	.	.	.	.	.	.	.	.	.	.	.	.	.
155	WFI	296	++	++	.	.	±	++	.	.	++	++	++	++	++	±	++	++	++	++	++
165	WFI	8	.	.	.	.	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.
28A	WFI	SN14	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
A6C	WFI	Deal 3	.	++	.	.	.	.	.	.	++	++	±	±	±	±	++	.	++	++	±
A9C	WFI	Howe 4	.	.	.	.	.	.	.	.	.	++	.	.	.	.	.	.	.	.	.
71*	Utrecht	240	+	++	±	.	±	++	+	±	++	++	++	++	++	±	++	+	++	++	++
275*	Utrecht	98	+	++	±	.	±	.	.	±	++	++	++	++	+	±	++	++	++	++	++
48*	Utrecht	81	.	+	.	.	.	.	.	.	.	.	++	.	++	±	.	+	.	.	++
82*	Utrecht	87	.	.	.	.	.	.	.	.	.	.	.	.	.	++	±	.	.	.	.
456	Utrecht	76	.	++	.	.	.	.	.	.	++	.	++	.	.	.	++	.	++	++	±
157A†*	Utrecht	382	.	.	.	.	.	.	.	.	.	.	±	.	.	.	.	++	.	.	±
471A*	Utrecht	330	.	++	±	.	±	.	.	.	++	±	.	+	.	±	++	.	++	++	±
459*	Utrecht	407	++	++	±	.	+	+	.	.	++	++	++	++	++	++	++	++	++	++	++
275A*	Utrecht	380	++	++	.	.	±	.	.	.	++	++	++	++	+	±	++	++	++	++	++

WFI = Wright–Fleming Institute. RTD = routine test dilution. ++ = >50 plaques – confluent lysis. + = 20–50 plaques. ± = <20 plaques.

* Strains included in the provisional typing set described by Verhoef *et al.* (1971 *b*).

† Isolated from a biotype 4 strain.

Parker 1965), the term 'biotype' is used in place of 'subgroup' and biotype 1 is taken to include the strains previously classified as subgroup II and subgroup V. The tests used in our work are set out in Table 1. The methods used were substantially those of Baird-Parker but only a proportion of the strains were tested for fermentation of maltose. Tests for lactose fermentation and for phosphatase and acetoin production were performed as described by Baird-Parker (1963). Mannitol and glucose fermentation were examined by a modified method in media composed of (g/100 ml.): $NH_4H_2PO_4$ 0·1, KCl 0·02, $MgSO_4.7H_2O$ 0·02, yeast extract 1·0, tryptone 0·5, bromo-cresol purple 0·004, and agar 0·2. After sterilization, mannitol or glucose was added to a final concentration of 1·0%. In use, 10 ml. of the medium was inoculated with 0·5 ml. of an overnight broth culture and incubated for up to 5 days.

We observed 39 strains that were physiologically very similar to *Staphylococcus aureus* in that they fermented mannitol anaerobically, fermented lactose, and produced acetoin and phosphatase, but they all failed to produce a coagulase for human plasma and were insusceptible to the standard set of *S. aureus* typing phages; these are indicated in Table 1 as biotype *a* and are the same as the strains referred to by Corse & Williams (1968) as *S. albus*.

Phages were isolated by 'cross-culture', spotting the supernatant fluid from 6 hr. cultures onto a plate spread with a culture of similar age. After overnight incubation plaques were picked and the phages purified by subculture; one plaque was suspended in 0·5 ml. broth, which was immediately spotted on a plate flooded with a 6 hr. culture of the susceptible strain. After two single-plaque subcultures the phages were propagated in infusion broth (Southern Group Laboratories) containing 0·04% $CaCl_2$, filtered through Millipore (HAWP) membranes having a 0·45 μm. pore size, and preserved at +4°C.

The solid medium used for titration of phages and phage typing was formulated for us by Oxoid Laboratories. It contained 1·25% Peptone L 37 (Oxoid), 0·75% Lab–Lemco powder, 0·7% Agar Number 1 (Oxoid) and 0·04% calcium chloride.

All strains were tested with phages used at a titre of 100 × RTD (see Blair & Williams, 1961) and almost all untypable strains were retested with undiluted phage filtrates (mostly 1000 or 10,000 × RTD). The term 'typable' is used for strains showing lysis with any phage used at 100 × RTD or stronger.

A total of 13 phages were isolated and tested, and a further 14 were received from Professor Winkler and his colleagues. After testing 2894 strains with this set of 27 phages, the results were analysed and eight phages found to be redundant; these were discarded from the typing set.

The results in this paper are based on the set of 19 phages listed and characterized in Table 2.

RESULTS

Of the total collection of 1517 strains, 591 (39%) showed strong (++) lysis by one or more of the phages used at 100 × RTD, and a further 154 (10%) showed weak lysis. Of the untypable strains, 110 (7% of the total) were lysed when the stronger phage filtrates were used giving a total of 855 (56%) of typable strains.

Table 3. *Phage sensitivity of strains of different biotypes*

	No. of strains tested	% 'typable'*	No. 'typable' Short patterns†	No. 'typable' 'Long' patterns†
Staphylococcus				
Biotype 1	972	72	480	222
Biotype 2	15	27	4	0
Biotype 3	38	50	18	1
Biotype 4	72	45	31	1
Biotype *a*	39	56	19	3
Total 'staphylococcus'	1136	68	552	227
Micrococcus				
Biotype M 1	22	14	3	0
Biotype M 2	43	9	3	1
Biotype M 3	9	22	2	0
Biotype M 4–6	19	–	0	0
Biotype M 7	20	15	3	0
Unclassified micrococci	187	19	32	3
Total 'micrococcus'	300	16	43	4
Unclassifiable	81	36	26	3

* Typable = any pattern at RTD × 100 or stronger.
† 'Long pattern' = reactions with six or more phages (see text).

Table 4. *Number of reactions in lytic patterns*

No. of + + reactions	No. of strains	% of total strains giving + + reactions
1	239	40
2	57	10
3	34	6
4	34	6
5	17	3
6	15	3
7–9	93	16
10–12	84	14
13 or more	18	3
Weak reactions only	264*	
No reaction	662	
Total	1517	

NOTE. The 'long' pattern ordinarily comprises 6–13 lytic reactions, but not all of them are classed as + +.

* 154 gave weak (+) reactions with phage at RTD × 100 and 110 were typable with phage used at a higher concentration.

Table 5. *Phage patterns represented by ten or more cultures*

Lytic pattern	Total	Staphylococcus biotype			Micrococci	Unclassified
		1	2, 3, 4	*a*		
'Long' (i.e. reactions with 6 or more phages)	234	222	2	3	4	3
71	20	14	0	3	0	3
82	23	18	2	2	0	1
82 + several weak reactions	54	52	0	0	0	2
37	56	8	20	1	21	6
275A	19	15	1	1	2	0
157A	43	38	1	2	0	2
456; 456/459; 459	28	27	1	0	0	0
RG	24	14	2	0	5	3
28	12	12	0	0	0	0
A6C/A9C	96	87	2	1	4	2
15	10	5	0	3	1	1

The proportion of typable strains varied substantially between the different biotypes (Table 3); 72% of strains of biotype 1 were sensitive compared with 44% of the strains in biotypes 2, 3 and 4 and 16% of the micrococci. About half (56%) the strains in the *S. aureus*-like biotype *a* were sensitive. These differences probably reflect the fact that most of the phages were isolated from strains of biotype 1.

Most of the strains of staphylococci and micrococci were lysed by several different phages to give pattern reactions, as in the *S. aureus* typing system. A rather serious drawback, at present, is the fact that some 36% of the strains that are lysed by any phage are lysed by six or more phages and 17% were lysed by ten or more (Table 4). Not all these 'long patterns' are identical but the differences among them do not seem to be consistent enough to permit their subdivision into distinguishable patterns. The 'long' patterns were proportionately more common among strains of biotype 1 than among the relatively few typable strains of other biotypes (Table 3).

Table 5 sets out the distribution over the biotypes of strains belonging to phage 'types' represented by ten or more strains, and also the strains giving the 'long' pattern. All but one of the types were seen in strains of more than one biotype, although with the exception of lysis with phage 37, which was seen rather frequently among the micrococci, there was no segregation of particular patterns into particular biotypes.

Reproducibility

We typed a large number of strains received from Dr R. J. Holt, who had isolated them from patients with chronic bacteraemia complicating Spitz-Holter valves. Like Verhoef *et al.* (1972), who also examined some of these strains, we obtained either identical typing patterns from all the strains isolated from individual patients, or in some cases two or more sets of identical patterns.

We also examined the reproducibility of repeat tests on single strains examined

Table 6. *Percentage distribution of various phage patterns among staphylococci and micrococci from various sources*

Phage pattern	Source of strains: Carrier sites	Wounds	Blood culture	Urine
'Long'	16	15	26	4
71 + or − other reactions	3	1	.	.
82 + or − other reactions	6	6	3	3
37	3	11	5	6
275A	1	.	.	.
157A	3	.	2	.
456; 459; 456/459	2	1	7	1
RG	2	1	3	.
28	.	.	1	2
A6C/A9C	6	8	9	6
15	1	.	.	.
Others	13	14	6	5
N.T.	43	40	35	75
Total no. of strains	1132	84	86	107

N.T. = not typable.

on one day and after an interval and our results conformed with those of Verhoef *et al.* (1972); duplicate tests on the same day gave loss or gain of only one + + reaction in one of 17 tests, and in duplicate tests on separate days at 3 months' interval 2 of 10 strains showed loss or gain of 1 + + reaction and 1 strain 4 + + differences. In these tests the strains examined all showed at least 7 + + reactions.

Types in relation to source

Table 6 summarizes the distribution of the commoner types in carrier sites and pathological lesions. There are no striking differences between the strains from carrier sites and those from wounds or blood cultures; a large proportion of the strains from urine proved to be untypable.

Numbers of distinguishable types on carriers

A number of members of the laboratory staff had swabs taken from the nose and from six skin sites on two or more occasions. The swabs were inoculated on nutrient agar plates and incubated overnight. Up to five colonies of each distinguishable colony type were chosen at random and subcultured for phage-typing and biotyping. Strains were regarded as different if they were of a different biotype (even if, as rarely occurred, they had the same phage pattern) or if, regardless of biotype, the phage pattern differed significantly; for this purpose differences in typing patterns were interpreted in the way used in *S. aureus* typing (Blair & Williams, 1961). For this analysis, all untypable cultures were counted as one strain.

Table 7. *Numbers of distinguishable strains in cultures from nose and skin*

No. of distinguishable strains	No. of cultures contributing colonies																							
	13	15	11	8	8	11	7	5	7	9	13	6	3	3	7	4	2	1	0	2	0	0	3	1	2
11	.	.	.	.	.	.	.	.	.	.	1	1	1	.	.	1	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.	1	.	.
9	.	.	.	.	.	.	.	.	.	1	2	1	.	1	2	1	.	.	.	.	.	.	.	.	2
8	.	.	.	.	.	.	.	.	.	2	2	.	.	.	1	1	.	.	.	.	.	.	1	.	.
7	.	.	.	.	1	1	3	1	1	.	3	.	.	1	1	.	1	.	.	.	.	.	1	.	.
6	.	.	2	2	.	2	.	.	1	2	2	2	.	.	.	.	1	1	.	1	.	.	.	.	.
5	.	3	.	1	4	1	2	2	2	1	1	1	1	.	2	.	.	.	.	1	.	.	.	1	.
4	4	5	4	1	2	2	2	.	2	2	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.
3	4	4	1	3	1	2	.	.	1	1	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.
2	3	2	3	.	.	2	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1	2	1	1	1	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28

No. of colonies tested from each sample

The numbers of colonies examined from the different subjects varied between 2 and 125. Table 7 illustrates the results from 90 swab cultures, from each of which between 4 and 28 colonies were tested; the numerals in the body of the figure are the number of cultures yielding the indicated number of distinguishable types. It will be seen that when more than 10 different colonies were tested, about 7–11 strains could generally be distinguished in each culture. There were also five subjects who, from a total of 16 swabs, each yielded more than 90 colonies; they had between 25 and 45 distinguishable strains.

The same study showed that individuals commonly harbour staphylococci having the same phage pattern over periods at least up to 16 weeks; in 12 of the 19 persons in whom prolonged carriage was observed, the strain concerned belonged to biotype 1 and showed the 'long' phage pattern.

DISCUSSION

The preliminary studies reported here, in confirmation of the work reported by Verhoef *et al.* (1971*a*, *b*, 1972), demonstrate the practicability of bacteriophage type-identification of the coagulase-negative staphylococci. Most of the strains for which some pathogenic role is postulated fall into Baird-Parker's biotype 1 (previously known as Subgroups II and V) and in our experience isolation of phages for strains of biotype 1 has been easier than for other biotypes or for micrococci. We have not found any advantage from the use of Mitomycin C (Verhoef *et al.* 1971*a*) as an inducing agent for the isolation of phages.

We have included 19 phages in our provisional typing set; this includes 8 of the 18 phages in the set proposed by Verhoef *et al.* (1971*b*), one other phage received from these workers but not included in their set, 10 phages isolated by us, and 1 phage from a culture collection. This set was selected from a larger collection after testing nearly 3000 strains of staphylococci and micrococci. With it useful lytic reactions can be obtained with about 72% of biotype-1 *S. epidermidis*. The main practical difficulty has been the rather large proportion of strains that are very sensitive to the phages and are lysed by ten or more different phages.

The reproducibility of the reactions, both in repeated tests of single isolates, and in repeated isolates from infected patients, seems to be quite adequate, although there is certainly variability among the strains with the 'long' typing pattern; distinct types within this group have not yet been recognized but may well exist.

Normal persons evidently carry quite large numbers of distinguishable strains of staphylococci on their body. If any attempt is to be made to seek the origins of post-operative wound infections by pre-operative examinations, it will therefore be necessary to type a considerable number of isolates.

There is not, from our work, any clear indication that particular phage patterns within biotype 1 characterize strains having special pathogenic properties, but our experience is not yet sufficient for a clear statement on this point.

Since coagulase-negative staphylococci are commonly resistant to several antibiotics, we wondered whether resistance could be conveyed from them to

coagulase-positive *S. aureus* by transduction. So far we have been quite unsuccessful in demonstrating this.

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