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An alternative method of phage-typing *Staphylococcus aureus**

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INTRODUCTION

The present internationally agreed system for the phage-typing of *Staphylococcus aureus* (Blair & Williams, 1961; Report, 1967) is derived from the method of Wilson & Atkinson (1945). A plate of nutrient agar is flooded with a 6 hr. broth culture of the staphylococcus, and a drop of each of the basic set of 22 phages, diluted to the routine test dilution (RTD), is placed on the plate. The RTD is defined as that dilution of phage which produces near-confluent lysis on the propagating strain. If none of the phages produces lysis to the extent of 50 plaques or more, the culture is retyped on the following day with the same phages at 1000 times the routine test dilution (RTD × 1000). Williams & Rippon (1952) found that, when typing is carried out at RTD, less than 5 % of epidemiologically related sets of cultures showed differences of typing pattern of more than one strong phage reaction (i.e. no lysis of one culture and a reaction exceeding 50 plaques with another). No similar investigation of the variation in typing patterns with phages at RTD × 1000 has been reported, but there is evidence that reproducibility is relatively poor, either because of confusion between lysis and inhibition reactions or because of the frequency with which staphylococci are lysed by many phages at this dilution (Pöhn, 1957).

It seemed, therefore, that although the use of the phages at RTD × 1000 increased the proportion of typable staphylococci, it might add little information about the relation of one culture to another. An investigation was therefore carried out to see if, by making additions to the present basic set of phages, it would be possible to type an acceptably high percentage of staphylococci at RTD and so to remove the necessity to use phages at RTD × 1000. A large number of phages from the collection maintained in this laboratory were examined, and 20 phages were eventually chosen for use, together with the phages of the present basic set, for typing at RTD. A comparison was made of the reproducibility of the individual reactions and of the patterns produced by the basic-set phages and the extra phages.

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MATERIALS AND METHODS

Propagating strains

Freeze-dried preparations of the propagating strains for the phages were reconstituted in broth. Single colonies were picked and typed at RTD × 1000 with the present basic set of phages. RTD × 1000 was used since the original records indicated that typing of many of the propagating strains had been only with phages at that strength. Many of the propagating strains were untypable at RTD, and for these strains no useful comparison could be made of phage reactions except at RTD × 1000.

Typing method

Typing was performed by the method of Blair & Williams (1961). Dilutions of phage were placed in holes in a Perspex block and were applied to the surface of the agar plates with a multiple-loop applicator (Lidwell, 1959). Plastic Petri dishes were used containing a base layer of the following medium: 1 % Evans peptone, 0·5 % NaCl, 1·2 % shred agar. This was covered with a layer of a second medium: 2 % Difco no. 2 nutrient broth, 0·5 % NaCl, 1·2 % shred agar. Calcium chloride was added to a final concentration of 400 μg./ml.

Plates were read with a large hand-lens after overnight incubation at 30° C., and phage reactions were recorded as follows: + + = 50 plaques or more, + = 20–49 plaques, ± = 10–19 plaques, ± 1, ± 2, ± 3, . . ., ± 9 = 1, 2, 3, . . ., 9 plaques.

Phages

Phages had been stored either as broth suspensions at 4° C. or freeze-dried. Some of those stored as broth suspensions were of adequate titre for the experiment. Freeze-dried phages were reconstituted in broth. When necessary, phages were propagated by the soft-agar layer method originally described by Hershey, Kalmanson & Bronfenbrenner (1943) and later modified by Swanstrom & Adams (1951), and were titrated by placing 0·02 ml. drops of tenfold dilutions on a lawn of the appropriate indicator strain.

In all, 119 staphylococcal phages other than those of the present basic set were available in the laboratory; 27 of them had at some time been rejected from the basic set or from the set of additional phages used between 1953 and 1962; the rest had been sent to the Staphylococcus Reference Laboratory as potentially useful phages for typing staphylococci. Phage P3, which was received from Mrs H. Landau of Belfast after the investigation had begun, was included at a later stage.

After a preliminary study, 20 phages were chosen for a more detailed investigation. The lytic ranges of these phages were determined in the same manner, and with the same set of test staphylococci, as is recommended for the basic-set phages (Blair & Williams, 1961). With a high-titre phage preparation, the ratio was found of the dilution of phage which gave a standard amount of lysis of each test strain to the dilution which gave the same degree of lysis of the propagating

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strain. Phage suspensions were diluted from stock suspensions to RTD each week and stored at 4° C., and test dilutions were checked on the propagating strains before use.

For the purposes of the investigation, typability was taken as a phage reaction of + or more (20 plaques or more). When staphylococci were typed with the basic-set phages at RTD × 1000, no account was taken of inhibition reactions or of plaques in an area of inhibited growth (Blair & Williams, 1961).

Cultures of Staphylococcus aureus *typed*

Cultures of *Staph. aureus*, which had been received sequentially at the Staphylococcus Reference Laboratory, were used to test the usefulness of various phages for typing. Cultures from outside the British Isles, and from veterinary sources, were excluded. In all, 5000 cultures received during 1966 and 1967 were typed. The reproducibility of the typing results and of the ability of the different sets of phages to distinguish between staphylococcal cultures was investigated with 782 pairs of cultures which had been isolated from the same person in St Bartholomew's Hospital at an interval of 5–10 days. Antibiotic sensitivity tests had been carried out on these cultures.

RESULTS

Selection of the extra phages

It was impracticable to investigate more than a proportion of the 119 available phages, so a preliminary selection was made on the basis of the available records of the character and performance of the phages. For example, when several phages were known to have a similar host-specificity, only one was included. Phages of serological groups D, G and H were generally not chosen, because their lytic activity is usually too non-specific. If a phage had been exhaustively tested before and found to be of no value it was also discarded. Only one phage, KS6, splitting the 52, 52A, 80, 81 complex was included. A representative selection of phages found useful for typing bovine staphylococci (Davidson, 1961) was also included. Only one phage of lytic group II was added (phage 51), because on the whole those in the present basic set were considered to produce an adequate differentiation of the staphylococci in this group. No phages were used which lysed only coagulase-negative staphylococci, and highly specific phages were not tested.

Phages 7 and 3B had been discarded from the basic set in July 1966 (Report, 1967) after it had been found that they were the sole phage lysing a culture of *Staph. aureus* in only two out of 14,000 strains (E. H. Asheshov, personal communication). They were therefore not studied in the present investigation. Phage 1380, now designated phage 87 (Report, 1967), was not included, because a recent survey carried out in this laboratory had not revealed any organisms lysed by it. Phage D, isolated by Wallmark, was also not included because of its resemblance to phages 84 and 85. Fifty-eight phages were discarded in this manner, and the remaining sixty-one (Table 1) were used in the first experiment.

Three thousand sequential cultures submitted to the Staphylococcus Reference

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Laboratory for typing were first examined. The number typable by the basic set of phages at RTD was 2279 (75·9 %; see Table 2). When the untypable cultures were tested with the same phages at RTD × 1000, a further 411 were typed, bringing the percentage typed to 89·7. The addition of the 61 extra phages at RTD to the basic set at RTD resulted in the typing of 436 more cultures, a total percentage typed of 90·5; but 431 of these 436 cultures were lysed by only 20 extra phages. Thus the use of a combined set of 43 phages at RTD, which would have been accommodated easily in two standard typing blocks, resulted in the typing of 90·3 % of the cultures.

Table 1. *Set of* 61 *phages examined for their usefulness in typing cultures of* Staph. aureus *untypable with the basic set at RTD*

Phages with numbers allotted by the International Subcommittee			Other phages	
			Laboratory no.	Origin
29A*	47D*	77A*	UC 13, UC 14	
31*	51*	78*	UC 16, UC 20	Hill
31A*	52B*	86* (UC 18)	L 948, L 971	Leyton
31B*	57*	102†	B 33, C 33, D 33	Colquhoun
42B*	58*	103†	304, 377, 756, 950	Milch
42C*	62*	105†	SA 446, SA 313	Purandare
42F*	69*	107†	812	Rische
44*	70*	108†	Y 22, 777	Sharpe
44A*	73*	111†	Rosa, Israel C	Sompolinsky
47A*	75A*	190	A, B, C, E, KS 6	Wallmark
47B*	75B*	191		
47C*	76*	192		

* Previously in the basic set, or in the set of additional phages.
† 'Bovine' phages, see Davidson (1961).

Table 2. *Typing of* 3000 *cultures of* Staph. aureus *by the basic set of phages at RTD and at RTD* × 1000, *and by the basic set together with additional phages at RTD*

(A culture was considered typable if a phage produced 20 plaques or more.)

Typable by	No.	Percentage
Basic set at RTD	2279	75·9
Basic set at RTD + basic set at RTD × 1000	2690	89·7
Basic set at RTD + 61 extra phages at RTD	2715	90·5
Basic set at RTD + 20 extra phages at RTD	2710	90·3
Total number examined	3000	100

Characters of the extra phages

The origins of the 20 'useful' extra phages were found from the records (Table 3). They included phages 44, 47A and 69 previously in the original basic set, phages 31, 52B, 75A and 75B originally amongst the additional phages between 1953 and 1962 and phage 86 (originally UC 18). All the 'bovine' phages (Davidson, 1961) are excluded. Meyer (1967) found that only a very small additional number of human staphylococci were lysed by group IV phages other than 42D. An

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additional phage, P3, which was currently under investigation, was added to the 20 phages at this stage of the investigation.

The typing patterns of the propagating strains of these extra phages with the present basic set at RTD × 1000 were investigated (Table 4). These patterns were similar to those recorded when the staphylococci were first examined in the laboratory.

Table 3. *Origins of phages used in the extra typing set capable of typing cultures of* Staph. aureus *which were untypable by the basic set at RTD*

Phage	Lytic group	Serological group	Origin	Date	Ref.
190	Misc.	B	Wahl	1954	.
191	Misc. and I	B	Wahl	1954	.
192	III	A	Wahl	1955	.
C 33	Misc.	B	Colquhoun	1958	.
D 33	?	B	Colquhoun	1958	.
Rosa	Misc.	B	Sompolinsky	1960	.
SA 446	I	B	Purandare	1960	.
69	I	B	Hood	1951	2
L 971	III	?	Leyton	1953	.
KS 6	I	A	Wallmark	1961	3, 5
UC 13	III	B	Hill	1963	.
86 (UC 18)	III	B	Hill	1963	6
E	Misc.	B	Wallmark	1962	4
C	III	F	Wallmark	1962	4
75B	III	A	Hood	1951	.
31	I	B	Wilson & Atkinson	1943	1
44	I	B	Wilson & Atkinson	1943	1
52B	III	B	Heimer	1948	2
47A	Misc.	A	Wilson & Atkinson	1943	.
75A	III	A	Hood	1951	.
P 3	III	B	Landau	1967	.

Figures in italic indicate internationally agreed phage numbers.
References: (1) Wilson & Atkinson (1945); (2) Williams, Rippon & Dowsett (1953); (3) Comtois (1960); (4) Wallmark & Finland (1961); (5) Wallmark (1954); (6) Thomas, Hill, Culbertson & Altimeier (1960).

Finally the 20 extra phages and P3 were characterized by their lytic ranges (Table 5). These lytic ranges are those found by the author, and are based on two separate estimations for each phage. The lytic ranges generally resembled those found in the earlier records. The few major changes do not, however, invalidate the experiment. Where these changes have occurred it must be noted that the phage had probably undergone some change either by mutation, host-induced modification or contamination.

Assessment of redundancy of the phages in the new typing set

The extra phages had been used in the first part of the investigation as a secondary typing system for cultures of staphylococci untypable by the basic set of phages at RTD. They were now used together with the basic set at RTD to obtain an estimate of the frequency with which reactions with the new phages

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Table 4. *Typing patterns of the propagating strains of 20 extra phages with the basic set of phages at* $RTD \times 1000$

Phage	Propagating strain	Lysis by phage 29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	81	42D	187
190, 192	854	.	**++**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Cl	.	.
191	838	Non-typable																		
C 33, D 33	33	++	++	++	+	Cl	.	.	.	.	.	++	Cl	Cl	++	Cl	++	++	.	.
Rosa	Rosa	.	.	.	.	.	.	.	.	.	.	.	.	Cl	.	Cl	.	.	.	.
SA 446	446	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	Cl	++	.
69	8397	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.
L 971	8329	.	.	.	.	.	.	.	.	.	.	.	Cl	Cl	Cl	Cl	Cl	.	.	.
KS 6	KS 6	.	Cl	Cl	+	Cl	.	.	.	.	.	.	.	.	.	.	.	.	.	.
UC 13	UC 13	.	.	.	.	.	.	.	++	.	Cl	++	Cl	Cl	++	+	.	++	.	.
86	86	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	in	.	.	.
E	E	.	.	.	.	.	.	.	.	.	++	++	++	++	++	++	.	++	.	.
C	C	.	.	.	+	.	.	.	.	.	.	++	.	Cl	.	.	++	.	.	.
75B	9309	.	.	.	.	.	.	.	.	.	.	.	GR/Cl	.	.	Cl	.	in	.	.
31, 44	8508	Cl	Cl	Cl	Cl	Cl	.	.	.	±	.	*Cl*	Cl	*Cl*	Cl	++	*Cl*	Cl	*Cl*	.
52B	9303	.	+	.	.	.	.	.	+	.	*Cl*	++	Cl	Cl	*Cl*	.	*Cl*	++	+	.
47A	8317	+	.	.	.	±	.	.	.	.	.	.	.	.	.	.	.	.	.	.
75A	9307	.	±	.	.	.	.	.	.	.	.	.	.	.	.	++	.	.	.	.

± = less than 20 plaques; + = 20 to 49 plaques; + + = 50 plaques or more; Cl = confluent lysis; *Cl* = not quite confluent lysis; 0 = inhibition; in = weak inhibition; **+ +** = lysis in an area of inhibition; GR/Cl = confluent lysis with resistant growth.

Propagating strains with four-figure numbers indicates that they are lodged with the National Collection of Type Cultures.

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Table 5. *Lytic ranges of the 20 extra phages and phage* P3

	190	191	192	C 33	D 33	Rosa	SA 446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3
3A	.	.	.	2	2	.	.	.	.	.	.	.	.	.	.	2	1	1	.	.	2
3B	.	1	.	.	.	.	1	.	.	.	.	.	.	1	.	1	.	.	.	1	2
3C	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	2	.	1	1	1	0
6	4	4	4	.	3	4	4	3	5	4	4	5	3	5	5	0	.	4	1	5	5
7	4	4	3	.	3	4	3	3	4	3	4	5	3	5	5	.	.	5	.	3	> 5
29	4	.	.	.	.	4	.	3	.	4	.	1	.	0	.	4	.	.	.	.	.
29A	4	3	3	.	.	3	3	3	2	3	.	.	0	4	3	4	.	4	.	.	4
31/44	4	3	3	4	3	4	4	3	3	3	3	3	5	3	3	5	5	4	.	3	4
42B/47C	5	3	5	2	2	3	4	.	2	5	.	1	1	0	2	0	.	2	.	.	1
42C	4	4	.	4	.	4	4	.	2	.	3	4	.	4	2	.	.	0	.	3	4
42E	4	4	3	0	3	0	2	0	.	3	3	4	3	3	0	0	.	4	.	.	0
44A	4	4	3	.	.	0	4	3	3	3	.	.	2	0	0	0	1	2	3	0	0
47	2	0	.	.	3	3	3	1	2	2	4	.	2	3	5	3	1	5	1	4	> 5
47B	2	.	.	.	.	0	.	1	.	.	.	2	.	2	2	0	.	0	.	2	> 5
52	3	1	.	.	.	.	0	.	.	0	.	.	0	0	.	0	.	4	.	.	0
52A/79	3	5	.	3	2	0	2	.	.	.	.	1	1	0	.	0	.	4	.	.	0
53	0	.	.	.	3	4	.	.	2	.	4	0	1	4	5	.	.	0	.	5	5
54	4	1	3	.	3	4	2	2	5	3	4	1	2	4	5	1	.	3	1	5	> 5
71	.	2	.	1	.	.	.	1	.	.	.	0	.	.	.	3	.	1	.	.	.
75	0	.	.	.	.	3	3	2	4	.	4	.	2	4	.	.	.	3	.	5	.
77	0	.	.	.	.	4	.	2	.	.	4	4	.	4	2	.	.	4	.	.	.
80	2	.	5	.	.	.	5	.	.	5	.	.	.	.	.	.	.	2	.	.	.
2009	4	3	.	.	.	3	4	.	0	.	4	0	.	0	5	.	.	4	.	4	.
8719	3	2	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.
P.S.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Dilutions of the phage giving minimal + + reactions on the test strains are compared with the dilution giving approximately the same reaction on the propagating strain. (P.S.) Recorded as follows:

5 = a ' + + ' reaction in the same dilution as on the propagating strain.

4 = a ' + + ' reaction in a dilution 10–10^2 times more concentrated than that giving a + + on the propagating strain.

3 = a ' + + ' reaction in a dilution 10^3–10^4 times more concentrated than that giving a + + on the propagating strain.

2 = a ' + + ' reaction in a dilution 10^5 times more concentrated than that giving a + + on the propagating strain.

1 = very weak lysis.

In general, a grade 3, 4 or 5 reaction where none existed previously or the complete absence of such a reaction where one should exist is an indication of a change in the phage preparation.

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appeared in patterns with the old phages or with each other. A method of assessing redundancy of any of the phages was devised. One of two phages was considered redundant if the two phages always lysed the same culture of *Staph. aureus*. An ideal phage set is one in which there is as little redundancy as possible; when combinations of phage reactions occur, two phages must not invariably lyse the same culture of staphlococci. A further 2000 cultures of staphylococci were now typed by the basic set and the additional typing block of now 21 phages at RTD (after phage P3 had been added).

Table 6. *Numbers of cultures of staphylococci (out of* 2000) *lysed by the individual phages at different degrees of lysis*

Phage	+ +	+	Phage	+ +	+
29	361	30	190	397	44
52	476	44	191	127	94
52A	365	55	192	263	35
79	261	32	C 33	60	49
80	472	54	D 33	31	29
3A	38	1	Rosa	85	143
3C	59	3	SA 446	627	42
55	109	6	69	155	38
71	66	3	L 971	56	21
6	170	12	KS 6	106	20
42E	194	42	UC 13	117	52
47	229	18	86	194	64
53	225	37	E	34	17
54	225	25	C	264	62
75	307	32	75B	289	51
77	272	49	31	91	107
83A	171	28	44	121	53
84	355	41	52B	197	85
85	541	45	47A	2	4
81	419	30	75A	185	46
42D	22	9	P 3	200	29
187	4	0			

A modification of the sorting procedure designed by Davidson (1966) originally for computer analysis was now used. Patterns of lysis were coded and punched on standard 'Hollerith' punch cards on an International Computers and Tabulators (I.C.T.) Card Punch no. 129. The resultant holes were verified by a separate observer on an I.C.T. Verifier no. 129. Redundancy was detected by carrying out the following sorting procedure with an I.C.T. Sorter no. 302:

(1) Cultures were sorted into those lysed by each phage at RTD at two levels of lysis; + + (50 plaques or more) and + (20–49 plaques). The results at this stage are shown in Table 6.

(2) The cards sorted in (1) for each phage at the + + strength of reaction were then sorted for every other phage, again at the + + level of lysis.

(3) A similar sorting procedure to (2) was used but including all the + + and + reactions.

(4) A triangular matrix was constructed (Table 7) for the + + reactions, and a similar matrix (Table 8) for the combined + + and + reactions.

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Table 7. *Matrix showing how many cultures of* Staph. aureus *were lysed by two phages at the same time at the* + + *level*

	29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	83A	84	85	81	42D	187	190	191	192	C 33	D 33	Rosa	446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P3
29																																											
52	247																																										
52A	133	319																																									
79	126	151	150																																								
80	187	369	318	131																																							
3A	1	5	6	4	7																																						
3C	1	5	6	4	6	21																																					
55	9	10	6	4	11	18	48																																				
71	6	0	0	4	0	12	33	46																																			
6	34	27	35	56	28	10	8	6	1																																		
42E	53	69	78	81	71	12	10	9	2	102																																	
47	43	30	38	53	31	13	8	8	5	146	113																																
53	30	35	49	62	36	4	2	1	1	111	108	149																															
54	50	44	53	75	49	14	11	9	1	128	130	158	111																														
75	45	37	37	54	32	4	2	2	1	117	102	184	151	145																													
77	102	82	39	48	56	2	1	5	1	57	59	104	101	80	146																												
83A	23	36	47	50	38	11	9	8	1	71	80	83	92	79	70	46																											
84	15	17	24	30	17	1	0	0	0	30	37	69	82	48	119	122	58																										
85	52	44	52	63	46	11	9	9	1	90	81	130	128	116	182	125	99	308																									
81	96	195	173	91	248	13	11	13	8	100	135	108	77	112	92	67	65	21	58																								
42D	0	11	12	8	8	0	0	0	0	9	10	7	6	9	7	12	4	4	1	16																							
187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																						
190	232	261	162	116	216	12	11	14	1	28	67	35	24	46	40	86	32	14	55	145	6	0																					
191	39	61	74	70	64	11	10	10	1	26	47	29	15	42	28	16	37	13	36	65	3	0	80																				
192	92	109	100	66	140	5	4	5	1	59	81	77	45	97	81	42	30	23	65	165	7	0	105	35																			
C 33	25	36	40	33	41	7	6	6	2	23	34	26	19	29	20	10	19	6	14	52	4	0	38	38	28																		
D 33	7	13	13	16	14	3	3	3	0	14	17	18	17	23	15	11	7	6	18	20	0	0	12	15	16	16																	
Rosa	36	33	26	17	32	0	0	5	6	14	20	26	25	18	24	28	12	15	34	24	6	0	48	14	17	10	5																
SA 446	252	377	264	135	393	11	9	18	7	41	67	59	31	63	68	110	32	19	65	293	10	0	291	78	161	42	12	35															
69	120	105	35	30	76	0	0	5	0	14	24	18	17	18	14	66	13	14	12	46	5	0	138	15	22	9	3	21	134														
L 971	12	9	12	9	7	1	1	0	0	42	33	49	20	50	48	27	36	16	35	33	0	0	7	2	28	1	1	4	14	3													
KS 6	27	41	34	10	71	0	0	0	0	17	12	22	4	23	21	20	3	4	15	83	0	0	22	7	59	2	1	3	92	8	7												
UC 13	9	11	20	19	11	1	0	0	0	40	46	75	99	45	77	63	55	56	74	29	3	0	2	1	13	5	1	15	7	6	24	1											
86	60	63	68	58	62	5	5	7	0	35	52	51	64	53	49	58	91	50	105	62	7	0	61	44	16	27	15	16	70	36	15	3	45										
E	6	14	0	12	17	7	7	7	0	13	17	12	8	20	6	1	8	0	22	18	3	0	10	12	13	12	4	2	16	1	0	1	0	8									
C	41	57	63	55	53	10	7	7	4	102	121	125	146	130	143	98	97	76	176	106	19	0	60	38	63	23	17	26	71	25	25	6	77	66	9								
75B	43	49	51	62	46	7	5	5	1	109	97	172	153	139	245	135	76	107	212	100	4	0	49	41	78	30	16	22	77	13	51	20	77	56	10	149							
31	88	80	61	50	75	1	1	4	0	8	28	12	14	18	10	32	11	11	15	38	3	0	72	28	22	20	4	20	74	39	3	2	3	42	6	20	12						
44	77	100	74	53	97	4	4	8	0	24	47	26	34	38	25	44	26	18	32	51	4	0	88	31	40	18	8	27	93	57	8	6	13	35	13	40	31	46					
52B	79	126	121	84	117	5	5	5	0	49	73	56	72	59	54	41	76	24	49	94	6	0	85	42	60	34	5	22	111	26	33	8	47	64	7	90	76	46	53				
47A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
75A	31	30	29	37	25	7	5	4	0	80	75	115	97	109	174	93	56	64	109	70	3	0	34	28	60	20	15	12	50	11	42	12	42	35	6	110	177	9	21	44	0		
P 3	12	15	23	24	18	0	0	3	2	61	48	86	105	49	85	66	90	114	146	37	0	0	5	10	20	11	1	15	13	2	36	2	73	63	1	76	91	1	16	50	0	52	

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Table 8. *Matrix showing how many cultures of* Staph. aureus *were lysed by two phages at the same time at both + + and + levels*

	29	52	52A	79	80	3A	3C	55	71	6	42E	47	53	54	75	77	83A	84	85	81	42D	187	190	191	192	C 33	D 33	Rosa	446	69	L 971	KS 6	UC 13	86	E	C	75B	31	44	52B	47A	75A	P 3
29																																											
52	268																																										
52A	169	352																																									
79	147	183	180																																								
80	230	426	350	156																																							
3A	1	9	8	4	7																																						
3C	1	9	7	4	6	23																																					
55	9	13	7	4	11	21	50																																				
71	6	1	0	4	0	12	33	47																																			
6	49	43	57	68	39	10	9	7	1																																		
42E	81	89	103	101	89	12	11	7	2	137																																	
47	60	45	61	65	48	14	9	10	5	156	142																																
53	58	61	79	87	65	6	6	3	1	124	140	167																															
54	78	66	84	92	69	15	12	11	1	146	162	181	140																														
75	67	54	61	69	49	5	4	3	1	124	124	192	170	161																													
77	141	109	64	63	92	2	1	5	1	62	86	116	122	99	166																												
83A	53	71	80	72	71	13	11	9	1	78	106	93	109	100	82	64																											
84	40	29	41	43	32	3	1	1	0	40	59	95	103	76	153	158	73																										
85	79	69	83	91	65	18	10	9	1	106	124	155	161	142	216	154	124	336																									
81	111	221	191	107	269	13	11	14	8	123	167	134	102	139	108	83	85	39	86																								
42D	5	17	18	9	12	0	0	0	0	14	17	13	14	16	15	19	12	11	6	22																							
187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																						
190	243	280	181	136	249	12	11	15	1	41	89	50	43	68	56	111	62	33	83	161	11	0																					
191	99	121	115	103	111	11	10	13	1	42	64	38	27	60	48	45	59	31	61	91	5	0	142																				
192	108	127	130	87	162	6	4	7	1	81	127	101	75	124	109	58	47	45	94	194	13	0	118	71																			
C 33	54	70	69	53	74	10	9	11	3	37	58	41	38	49	33	28	35	17	33	73	5	0	70	58	44																		
D 33	45	33	36	31	34	8	7	7	1	28	48	34	36	42	27	17	29	17	36	38	2	0	33	27	30	36																	
Rosa	116	110	65	49	106	0	0	7	6	52	62	72	80	65	69	112	42	50	80	75	15	0	127	43	48	31	13																
SA 446	273	417	297	159	435	11	10	19	8	47	84	70	55	84	97	146	57	42	88	309	13	0	322	139	185	75	29	113															
69	149	133	62	50	126	1	1	5	0	21	39	23	28	30	21	95	29	21	30	57	7	0	167	43	43	29	10	92	166														
L 971	28	21	25	19	22	2	1	1	0	58	50	68	32	68	66	44	45	29	51	47	4	0	16	14	51	10	7	27	30	11													
KS 6	31	51	45	16	85	0	0	0	0	21	21	27	10	29	24	23	7	11	21	97	0	0	29	14	68	9	4	13	105	11	20												
UC 13	23	24	32	35	27	2	1	0	0	65	80	105	148	77	113	102	80	87	110	59	10	0	14	9	30	17	11	57	21	15	40	6											
86	117	115	88	84	108	5	5	9	0	40	67	58	77	66	58	90	104	61	118	83	8	0	115	90	36	41	20	63	120	69	18	9	75										
E	7	27	18	21	28	9	9	9	1	22	26	20	13	29	12	2	11	1	26	31	6	0	17	22	22	20	14	10	27	3	0	2	3	10									
C	66	86	94	88	79	10	9	9	5	116	155	143	183	156	167	135	116	120	230	132	26	0	86	51	86	43	38	76	101	45	51	14	120	79	22	1								
75B	66	77	87	77	73	13	11	13	4	129	131	195	175	164	264	155	91	139	249	138	10	0	65	62	107	50	34	72	111	21	70	27	118	66	22	188							
31	184	163	95	82	149	1	1	6	1	16	48	19	34	33	18	77	34	19	24	64	4	0	150	74	42	47	15	95	157	111	9	13	13	95	7	42	22						
44	119	139	97	73	136	5	5	9	0	40	67	41	59	55	38	85	55	29	53	72	8	0	123	61	62	40	17	76	130	99	20	12	28	60	19	65	46	110					
52B	109	209	197	112	199	5	5	5	0	60	96	68	92	77	65	58	101	35	69	152	13	0	111	71	89	56	20	61	188	54	40	24	67	78	13	110	98	75	81				
47A	1	4	3	0	3	3	3	2	0	3	3	3	2	3	1	0	3	0	3	4	0	0	3	2	0	3	1	3	4	1	1	0	0	1	2	3	3	1	1	1			
75A	53	50	61	50	44	13	10	9	0	104	113	141	121	142	205	117	76	94	145	101	11	0	57	53	92	37	32	42	76	20	63	20	77	49	16	140	212	14	37	55	3		
P 3	27	27	36	40	33	0	3	2	3	70	71	102	127	56	96	74	99	124	172	58	4	0	21	24	35	20	9	67	23	12	40	8	100	78	2	103	108	14	32	59	0	60	

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(5) A percentage matrix was then calculated from the results of the sorting procedure (2) as percentages of the numbers of cultures strongly lysed (+ +) by each phage (Table 6). The corresponding percentage matrix with the + + and + reactions combined was also calculated. Tables 9 and 10 show part of each of these matrices.

Table 9. *Part of the percentage matrix showing the results from Table* 7 *as a percentage of the total number of cultures lysed by each phage at the* + + *level.*

	29	52	52A	79	80	3A	3C	55	71	6
29	100	68	37	35	52	0	0	2	2	9
52	52	100	67	32	78	1	1	2	0	6
52A	36	87	100	41	87	2	2	2	0	10
79	48	58	58	100	50	2	2	2	2	21
80	40	78	68	28	100	1	2	2	0	6
3A	3	13	16	10	18	100	55	47	32	26
3C	2	8	10	7	10	36	100	81	56	14
55	8	9	6	4	10	17	44	100	42	6
71	9	0	0	6	0	18	50	65	100	1
6	20	16	20	33	16	6	5	4	1	100

Table 10. *Part of the percentage matrix showing the results from Table* 8 *as a percentage of the total number of cultures lysed by each phage at both the* + + *and* + *levels*

	29	52	52A	79	80	3A	3C	55	71	6
29	100	68	43	38	59	0	0	2	2	12
52	52	100	68	33	82	2	2	3	0	8
52A	40	84	100	43	83	2	2	2	0	14
79	50	63	62	100	53	1	1	1	1	23
80	44	81	67	30	100	1	1	2	0	7
3A	3	23	20	10	18	100	54	54	31	26
3C	2	15	11	6	10	37	100	81	52	15
55	8	11	6	3	10	18	43	100	41	6
71	9	1	0	5	0	17	48	68	100	1
6	27	24	31	37	21	5	5	4	1	100

The percentage matrices were now examined for each phage and any percentage of 50 or more was noted. Pairs of phages were identified which had 50–59, 60–69, 70–79 and 80 % or more similarities both ways. The lesser of the two percentages was taken as the 'both ways' answer. For example, the part of the + + percentage matrix shown in Table 9 shows that 67 % of the cultures of staphylococci lysed by phage 52 were also lysed by phage 52A, and 87 % of the cultures lysed by phage 52A were also lysed by phage 52. 67 % is therefore taken as the 'both ways' answer. Results were scored arbitrarily against each phage in the following manner: 80 % or more, 4; 70–79 %, 3; 60–69 %, 2; 50–59 %, 1. These scores were added separately for each phage both at the + + degree of lysis and for the combined + + and + degrees of lysis. The higher the score of a phage, the more redundant was that phage. The phage having the highest score was eliminated; in this experiment it was phage 47. The scores were reassessed in the absence of

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phage 47 and the highest scoring phage was again eliminated, and so on. The next two phages to go were phages 52 and 75B.

A stage was now reached when phage 80 would have been eliminated. Consideration of the + reactions as well as the + + indicated that phages 53 and 54 would have been eliminated at the same time. This therefore appeared to be a useful place at which to draw the line. Ideally, phages should have been eliminated until the scores were all zero, but at this time the number of typable cultures would have fallen to an unacceptable level. Only two cultures became untypable following the rejection of phages 47, 52 and 75B.

Reproducibility

A comparison was made of the reproducibility of the phage-typing patterns obtained at RTD with the basic-set phages, the extra phages, and the two sets of phages used in combination. A series of pairs of cultures isolated within a short period of time from the same site were used for this purpose. A similar comparison of the patterns of lysis by the extra set of phages at RTD and the basic-set phages at RTD × 1000 was made in similarly related pairs of cultures that were untypable with the basic set of phages at RTD.

A survey of staphylococci carried by the patients and staff of St Bartholomew's Hospital included 782 pairs of cultures isolated from the nose swabs of the same person within a period of 5–10 days. About a third of these cultures were previously included in the 2000 cultures used in the assessment of redundancy. The additional cultures were retyped both with the basic set and with the extra phages. Cultures untypable at RTD by the basic-set phages were also retyped by these phages at RTD × 1000.

Williams & Rippon (1952) considered that a difference of two strong reactions between the phage-typing patterns of staphylococci, i.e. one culture lysed strongly (+ +) by two phages which gave no lysis of the other culture, or strong lysis of each of two cultures by a phage which gave no lysis of the other culture, was a good indication that the cultures came from two distinct populations of bacteria. These criteria were applied to the pairs of cultures above, and a comparison was made of the proportion of pairs which would have been considered 'the same' or 'different' when they were typed in different ways.

The numbers of pairs of cultures of staphylococci showing various differences in the pattern of lysis by the two sets of phages at RTD are shown in Tables 11 and 12. The differences are arranged into a series of categories (1–6) of increasing magnitude; pairs in which there was no common reaction (category 7) or in which one or both of the cultures were untypable (categories 8 and 9) are shown separately. In each of the tables, the first column gives the number of pairs of cultures showing differences in each category, and the second column gives the percentage distribution of these differences. In the third column, the percentages are recalculated when only strictly comparable cultures are considered, that is to say, after the exclusion of categories 7, 8 and 9, as suggested by Williams & Rippon (1952). It is reasonable to assume in most cases that when two cultures isolated from the same site within a short interval of time have no common phage

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reactions, they are from different populations. When one or both of the cultures is untypable there is no way of assessing their relationship.

The percentages of presumably related pairs of cultures (column 3 in Tables 11 and 12) in which one culture is found to differ from the other by the two-strong differences rule were found for the two sets of phages at RTD. For the basic-set

Table 11. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the basic-set phages at RTD)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 652)*
1. None	177	22·6	27·2
2. Strength of reaction only	73	9·3	11·2
3. Loss or gain of + or ± reactions	182	23·2	27·9
4. Loss or gain of one + +, with or without change in + and ± reactions	125	16·0	19·2
5. Loss or gain of two + +, with or without change in + and ± reactions	51	6·5	7·8
6. Loss or gain of three + +, with or without change in + and ± reactions	44	5·6	6·7
7. No common reactions	26	3·4	.
8. One culture untypable	59	7·6	.
9. Both cultures untypable	45	5·8	.

* Excluding categories 7, 8 and 9.

Table 12. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the extra phages at RTD.)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 583)*
1. None	67	8·6	11·5
2. Strength of reaction only	71	9·1	12·2
3. Loss or gain of + or ± reactions	292	37·3	50·1
4. Loss or gain of one + +, with or without change in + and ± reactions	86	11·0	14·7
5. Loss or gain of two + +, with or without change in + and ± reactions	29	3·7	5·0
6. Loss or gain of three + +, with or without change in + and ± reactions	38	4·8	6·5
7. No common reactions	30	3·8	.
8. One culture untypable	53	6·8	.
9. Both cultures untypable	116	14·9	.

* Excluding categories 7, 8 and 9.

phages this percentage was 14·5 (7·8 + 6·7) and for the extra phage set it was 11·5 (5·0 + 6·5). If cultures of staphylococci with dissimilar antibiograms had been assumed to have come from different populations these percentages would

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have been 13·6 and 11·3 respectively. Consideration of the close epidemiological relationship between the pairs of cultures leads us to believe that the extra phage set shows a greater stability of pattern than the basic set. However, it must be remembered that the basic-set phages type more cultures of *Staph. aureus* than those of the extra phage set, this having been the original reason for choosing the phages for the basic set.

Eighty-seven pairs of cultures of staphylococci which were untypable at RTD by the basic set and were therefore retyped at RTD × 1000 were now used to compare the reproducibility of the patterns produced at this strength of phage with those of the extra phages at RTD (Table 13). Of the comparable pairs of cultures (Williams & Rippon, 1952) 18 of 47 typed by the basic set at RTD × 1000 showed loss or gain of two strong reactions while no pairs of cultures of a similar group (0 of 55) typed by the extra phages showed these differences.

Table 13. *Numbers of pairs of cultures of* Staph. aureus *untypable with the basic set at RTD differing in their pattern of phage lysis when typed either with the extra phages at RTD or with the basic set at RTD* × 1000.

(Eighty-seven pairs of cultures isolated from nose swabs of the same person at an interval of 5–10 days were considered.)

Category of the difference in phage pattern	Extra phages typed	Basic set at RTD × 1000, ignoring reactions less than + +
1. None	17	12
2. Strength of reaction only	10	—
3. Loss or gain of + or ± reactions	20	—
4. Loss or gain of one + +, with or without change in + and ± reactions	8	17
5. Loss or gain of two + +, with or without change in + and ± reactions	0	18
6. Loss or gain of three + +, with or without change in + and ± reactions	0	0
7. No common reactions	6	3
8. One culture untypable	7	13
9. Both cultures untypable	19	24

Reproducibility of the individual degrees of phage lysis by the extra phages was not as good as that of the basic-set phages. The changes in the strength of reaction were mainly in the weak reactions which would rarely have changed the reported phage-type (Tables 11 and 12, category 3).

Discrimination

With the enlarged typing set, how many differences should be 'allowed' before cultures of staphylococci were considered to have come from different populations of bacteria? The percentage of pairs of cultures (see Tables 11 and 12) differing by two or more + + reactions when typed by the basic set and the extra phage set, respectively 14·5 and 11·5, equal approximately the percentage of pairs (11·9) differing by three or more + + reactions when typed by the combined sets

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of phages (Table 14). It is therefore suggested tentatively that a 'three-strong differences' rule should be applied to patterns of lysis produced by the combined sets of phages much in the same way as the 'two-strong differences' rule suggested by Williams & Rippon (1952) is applied to the basic set alone at RTD.

Table 14. *Differences in the pattern of phage lysis of* 782 *pairs of cultures of* Staph. aureus *isolated from the nose swabs of the same person at an interval of* 5–10 *days*

(Phage-typing by the basic set and extra phage set combined at RTD.)

Category of the difference in phage pattern	(1) No. of pairs of cultures	(2) Percentage of 782	(3) Recalculated percentage (of 713)*
1. None	71	9·1	9·9
2. Strength of reaction only	43	5·5	6·0
3. Loss or gain of + or ± reactions	285	36·5	40·0
4. Loss or gain of one + +, with or without change in + and ± reactions	155	19·9	21·7
5. Loss or gain of two + +, with or without change in + and ± reactions	74	9·5	10·4
6. Loss or gain of three + +, with or without change in + and ± reactions	85	10·4	11·9
7. No common reactions	28	3·6	.
8. One culture untypable	29	3·8	.
9. Both cultures untypable	12	1·7	.

* Excluding categories 7, 8 and 9.

Table 15. *Number of distinct patterns of phage lysis among cultures* of* Staph. aureus *typed by three methods*

(*a*) with the basic set at RTD; '2-differences' rule applied. (*b*) with the combined set at RTD; '3-differences' rule applied. (*c*) with the basic set at RTD and RTD × 1000; '2-differences' rule applied.)

	No. of patterns	No. of cultures typable
Cultures typed by the basic set at RTD and the '2-differences' rule applied	136	183
Cultures typed by the combined set at RTD and the '3-differences rule' applied	150	192
Cultures typed by the basic set at RTD and RTD × 1000 and the '2-differences' rule applied	154	204

* Two hundred and eleven cultures were isolated in batches in one hospital on 10 occasions at an interval of one week; patterns for separate weeks were not compared.

There is a potential danger that the use of a 3-differences rule might reduce unacceptably the number of subdivisions which can be made between different cultures of staphylococci. The results of typing all staphylococci isolated from one ward of the St Bartholomew's Hospital survey in 10 consecutive weeks were then examined; these cultures formed part of the series of 2000 staphylococci previously typed. Numbers of distinct patterns of lysis observed in each separate week were added together (Table 15); patterns from separate weeks were not compared. On typing with the basic set alone at RTD, 136 patterns of phage

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lysis were differentiable by the 2-differences rule; with the basic set at RTD and RTD × 1000, 154 patterns emerged. With the combined phage set at RTD, and the proposed 3-differences rule, 150 patterns were differentiable. In this example, 204 of 211 cultures were typable by the present system of phage-typing and 192 cultures were typable by the combined set of phages at RTD only. Thus, a roughly equal number of cultures were typable and differentiable with the two systems of typing and the different criteria for culture differentiation.

DISCUSSION

A system of phage-typing which relies in part on the use of phages at a thousand times their routine test dilution has four major disadvantages. First, inhibition reactions frequently occur and lead to confusion. Williams & Rippon (1952) found that some undiluted phage lysates caused inhibition of growth. When these lysates were progressively diluted, the effect was lost without passing through a dilution that produced plaques. Whether inhibition is a specific adsorption phenomenon or a non-specific lysis from without (Ralston, Baer, Lieberman & Krueger 1957) is still open to doubt. Burnet & Lush (1935) showed that coagulase-positive staphylococci adsorb all staphylococcal bacteriophages whether or not they were lysed by them. Rountree (1947) showed that coagulase-positive staphylococci adsorb all typing phages irrespective of their lytic activity and that this adsorption may be lethal. Ralston *et al.* (1957) described 'lysis from without' of *Staph. aureus* K_1 with phage-cell ratios greater than 17; this was independent of phage infectivity. It is probable that in staphylococcus phage-typing the ratio of phage to coccus in the area of the drop at RTD × 1000 is between 12:1 and 25:1.

The production of inhibition reactions depends on the relative concentration of phage and coccus (E. H. Asheshov, personal communication). Increasing the bacterial concentration may eliminate inhibition reactions and lowering it may increase the number and strength of these reactions. Beard & Rountree (1965) found that inhibition was due to phage/cell interaction when the phage/cell ratio was approximately 1:1. Most of the infected cocci were killed and a minority produced a few phage particles with a longer latent period. These phages are released and can only adhere to already dead cocci. There is therefore a generalized thinning of the bacterial growth. The evidence thus suggests (E. H. Asheshov, personal communication; Beard & Rountree, 1965) that inhibition reactions are very poorly reproducible and that very little epidemiological information is to be gained from them.

Secondly, at least 75% of the propagating strains are themselves lysogenic (Lowbury & Hood, 1953; Rosenblum & Dowell, 1960; Rountree, 1949) and concentrated lysates may be expected to contain not only the propagated phage but one or more temperate phages originally carried by the propagating strains.

Thirdly, phage preparations may also contain host-range mutants which may produce plaques on cultures insusceptible to the majority population of the phage. Dilution of the preparation to RTD will render the effect of these mutants negligible.

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Fourthly, plating concentrated phage lysates on lysogenic bacteria may cause prophage induction with resultant plaque formation. In some cases this may be the cause of plaques appearing in an area of inhibited growth; these plaques must therefore be ignored. Blair & Williams (1961) suggested ignoring lesser degrees of lysis than 50 plaques and also all inhibition reactions, when typing at RTD × 1000.

In our experience, it is possible to type at least as many cultures of staphylococci untypable by the basic set at RTD by the use of 20 extra phages at RTD as were typed by the basic set at RTD × 1000, but these findings may not be applicable to cultures of *Staph. aureus* found in other parts of the world. Before any change in the internationally accepted procedure for phage-typing is undertaken a trial on a much bigger scale would have to be carried out.

With the growing doubts that the retyping of cultures of staphylococci at RTD × 1000 yields much additional information, it has been suggested that the use of a somewhat lower concentration of phage might improve reproducibility without reducing the percentage of typable cultures (Report, 1967). Jevons & Skalova at the Staphyloccocus Reference Laboratory (see Parker, 1966) studied the reproducibility of phage-typing at various concentrations of phage. They found that the reproducibility of phage-typing was much better at RTD × 100 than at RTD × 1000. An international study group has been formed to investigate typing results from all national laboratories at these two strengths of phage.

Degré (1966, 1967) has suggested that the use of the phages only at RTD × 100 is preferable to the present system. He found only slight degrees of inhibition at RTD × 100 and a greater reproducibility of the weak reactions. Zierdt & Marsh (1962), using this strength of phage, found that it 'tended to stabilize and complete phage patterns'. They also noted a marked absence of inhibition reactions. Whilst this stabilization of phage patterns might in some cases be advantageous, too much completion of phage patterns would tend to lump potentially unrelated cultures together and so reduce the epidemiological value of phage-typing.

The results of this investigation suggest that it might be preferable to substitute a number of extra phages at RTD for the secondary typing at RTD × 1000 of cultures untypable by the basic set at RTD. Cultures could either be typed by all the phages on the first day and differentiated by the suggested 3-strong differences rule, or the basic set and extra phages could be used on successive days and the 2-strong differences rule applied separately. The increased amount of spare room in the two typing blocks produces an incidental advantage in that there is more room for extra phages which may only have a local use for a restricted time. Future work on any expanded phage-typing system must be in the form of a comparative trial of a large number of phages in several countries. Adoption of any change in the method of phage-typing is a lengthy business and the proposed change, by using two blocks containing at least 40 phages, would necessarily require a world-wide trial.

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SUMMARY

As many cultures of *Staph. aureus* untypable by the international basic-set phages at RTD were typed by a block of 20 extra phages at RTD as by the basic set at RTD × 1000. Reproducibility of the lytic patterns was as good with the extra phages at RTD as with the basic-set phages at RTD, and better than with the basic-set phages at RTD × 1000.

A method of assessing redundancy in any set of phages is presented, and a '3-strong differences' rule is suggested for discriminating between cultures of staphylococci from patterns of phage lysis by the combined set of phages at RTD.

Experience with the combined set of phages suggests that it may be possible to improve the phage-typing of staphylococci by substituting a larger series of phages used only at RTD, for the present set of 22 phages used at RTD and RTD × 1000.

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