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Phages for methicillin-resistant *Staphylococcus aureus*: an international trial

J. F. RICHARDSON[1]*, V. T. ROSDAHL[2], W. J. VAN LEEUWEN[3], A. M. VICKERY[4], A. VINDEL[5] AND W. WITTE[6]

[1] *Campylobacter Reference Unit, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK*
[2] *Statens Seruminstitut, Artillerivej 5, DK 2300 Copenhagen, Denmark*
[3] *Diagnostic Laboratory for Infectious Disease and Perinatal Screening, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, PO Box 1, 3720 BA Bilthoven, The Netherlands*
[4] *Department of Microbiology, Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Camperdown 28220, 2050 New South Wales, Australia*
[5] *Servicio de Bacteriologia, Centro Nacional de Microbiologia, Virologia e Immunologia Sanitarias, Majadahonda, Madrid, Spain*
[6] *Robert Koch-Institut, Bereich Wernigerode, Burgstrasse 37, Postfach 36643 Wernigerode, 03700 Wernigerode (Harz), Germany*

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SUMMARY

An internationally agreed and validated set of phages is used worldwide for the typing of strains of *Staphylococcus aureus* of human origin. However, because of the sometimes reduced susceptibility of methicillin-resistant strains (MRSA) to these phages, some of the national typing centres use locally isolated and characterized sets of experimental phages. In this trial, 42 such phages were distributed to 6 centres and tested against 744 isolates of MRSA with the intention of defining a phage set to augment the international set. The use of these experimental phages increased the percentage typability from 75% with the international set to 93% and the number of identifiable lytic patterns from 192 to 424. A subset of 10 experimental phages was selected. When this subset was compared with the experimental panel, the typability rate was 91% and 370 distinct patterns were obtained. This subset of phages has been distributed for international trial.

INTRODUCTION

Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) were first detected soon after the introduction of methicillin into clinical use in 1960 [1]. Throughout that decade they were the cause of outbreaks of hospital infection in many countries, but with the introduction of gentamicin and perhaps of improved infection control the incidence of MRSA decreased to about 3% of all hospital isolates of *S. aureus*. By the mid-1980s, however, several countries had reported outbreaks of hospital-acquired infection caused by local epidemic strains (affecting more than one hospital) and there was also documented international spread of some strains, the most notorious being the Eastern Australian strain which, in the UK, was designated EMRSA-01 [3, 4].

During the 1980s and 1990s, although the staphylococcal flora in some countries remained constant, there were changes in others. For example in the UK, where EMRSA-01 had, by 1991 largely disappeared, other prevalent strains in the mid-90s were EMRSA-03, EMRSA-15 and EMRSA-16 [5–7].

Phage typing of *S. aureus* is organized internationally through a subcommittee of the Inter-

* Author for correspondence.

national Union of Microbiological Societies and an agreed international set is used in 35 countries. In response to local epidemic strains of MRSA, supplementary experimental phage sets were developed by many typing centres, notably in Australia [8], Spain [9], the UK [10], The Netherlands and Germany, to increase typability rates and improve discrimination.

The primary aim of this study was to attempt to combine the different sets of experimental MRSA-phages in order to define a set which would type most current MRSA with both good reproducibility and high discrimination. Secondary aims were to determine the extent, if any, of international spread of local epidemic strains of MRSA and to see if phage sets developed to type strains prevalent in the 1980s were still effective in countries where these strains had changed.

MATERIALS AND METHODS

Six centres were involved in this trial: the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, Bilthoven, The Netherlands; Fairfax Institute of Pathology, Camperdown, Australia; Statens Serum Institut, Copenhagen, Denmark; Staphylococcus Reference Section, Central Public Health Laboratory, London, UK; Servicio de Bacteriologia, Centro Nacional de Microbiologia, Madrid, Spain, and Robert-Koch Institut, Wernigerode, Germany.

PHAGES

International

All centres typed their isolates using the international phages for *S. aureus* of human origin. This set is composed of 23 phages: 29, 52, 52A, 79, 80, 3A, 3C, 55, 71, 95, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 81, 94 and 96 [11].

Experimental

Forty-two experimental phages were distributed to the six participating centres. These were: MR6, MR8, MR12, and MR25 from The Netherlands; 47T, 56A, 56B, 56C, 1648, 67R, 87M, 13M, and 90A from Australia [8]; 83C, 88A, 89, 90, 932, 616, 617, 618, 620, 622, 623, 625, 626, 629 and 630 from England [10]; 30, 31, 32, 33, 34, 35, 37 and 38 from Spain [9]; and M1, M2, M3, M4, M5, and M6 from Germany. Instructions for the propagation of these phages were given to the participating laboratories by the originating centre.

REFERENCE METHOD FOR PHAGE TYPING

Isolates of *S. aureus* were subcultured in nutrient broth and incubated at 37 °C to give an inoculum of about 5×10^7 c.f.u./ml. Plates containing 0·7% nutrient agar supplemented with 400 μg/ml calcium ions were flooded with bacterial culture and allowed to dry open at room temperature. Phage suspensions at Routine Test Dilution (RTD) and $100 \times$ RTD were applied with a Lidwell applicator [12] and the plates allowed to dry. All participating centres used the international phages at both concentrations. After overnight incubation at 30 °C phage reactions were read on a scale of $\pm$ (up to 19 plaques) + (20–49 plaques), ++ (50 or more plaques) at both concentrations, and were coded as 2, 3 and 8 respectively. Additional codes at $100 \times$ RTD were 0 for inhibition and 9 for confluent lysis. It was assumed that isolates typable at RTD by the experimental phages would also be typable at $100 \times$ RTD by these phages and similarly isolates untypable at $100 \times$ RTD would have been untypable at RTD.

Strains

Participating laboratories were asked to phage type not more than 100 recent isolates of MRSA received since 1990 using the routine base medium and the phage concentrations they would in their reference service. The isolates were to fall into four categories with a maximum for each category of 25. These categories were:

(1) representative isolates of current major epidemic strains of MRSA and their phage variants;
(2) sporadic MRSA;
(3) non-typable MRSA;
(4) MRSA originating from abroad.

Centres were asked to use the experimental phages at the concentration that would be used for supplementary typing in their own laboratory. Copies of the typing results, as worksheets, were sent to the Staphylococcus Reference Section for analysis. Four centres also supplied copies of their results with the international phages.

Phages included in the study

One centre, The Netherlands, tested the experimental phages at RTD alone, whereas the Australian and Spanish centres tested them at $100 \times$ RTD alone; the

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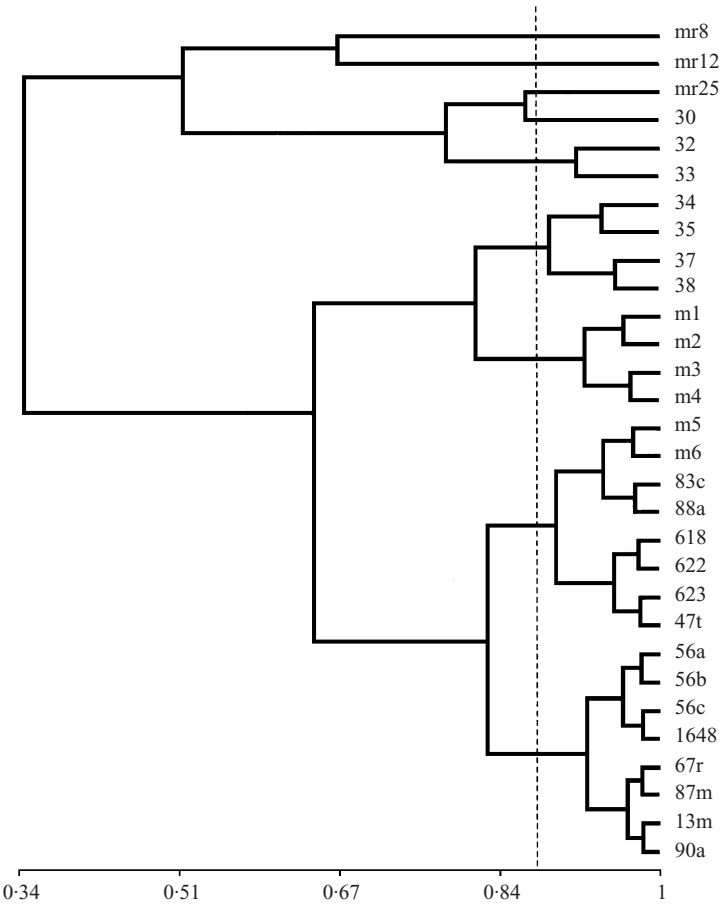

Fig. 1. Dendrogram showing similarities between 30 experimental *S. aureus* phages at 100 × RTD. The dendrogram was constructed using UPGMA cluster analysis and the *x*-axis computer generated. The dotted line indicates an arbitrary 90% similarity. Phages selected for inclusion in a trial set for international use were: MR8, MR12, MR25, 30, one from 32 and 33, one from 34–38, one from M1–M4, one from M5–47T and one from 56A–90A. Phage 622 was also included.

remaining three centres used them at both concentrations.

Thirty-five phages were used by all the centres that typed at RTD but only 30 phages by all the centres that typed at 100 × RTD. Phages not used at RTD were 618, which belongs to a series of phages that can be both difficult to propagate and unstable on storage [10], 33, 83C and 88A. Phages not used at 100 × RTD were 616, 617, 620, 625, 626, 629, 630, as they lost titre rapidly on storage, and phage 932. Analysis of the relationships between 30 phages was restricted to those used at 100 × RTD by all the participating centres which typed at this concentration.

Analysis of results

There were two aims in constructing the data files for analysis. The first was to determine whether these experimental phages increased the typability of, and aided discrimination between, strains of MRSA, and the second was to determine how much inter-country spread of strains of MRSA had occurred. In the first stage isolate details and typing results received from the participating laboratory were entered into a database.

For detailed analysis the decision was taken both to consider only strong reactions and to define identity of isolates as those showing no variation in lytic pattern. The same rules applied to both phage sets. The international type, when available, was coded independently for each isolate and sets of isolates were indexed, within country, on their international type. An international type corresponded to a strain. The exercise was repeated within a single international type, this time comparing experimental types and allowing a single strong reaction difference between them. One isolate of each experimental type per international type was then included in the main analysis. When no international type was given, or the strain was untypable by the international phages, one

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isolate of each experimental type was included with one strong reaction difference being allowed.

Reduction of the phage set

The patterns of lysis at 100 × RTD were used and only strong reactions (those graded 8 or 9) were considered. To determine whether two phages from different centres had the same spectrum of activity the number of occasions when both reacted together was counted. From this co-positivity, the frequency with which two phages reacted together, was calculated and a similarity matrix prepared using a statistical package (MVSP Plus 2.1, Kovach Computing Services, Pentreath Wales, UK). Approximately 90% similarity between phages was taken as an arbitrary point indicating relationship. This was estimated from the matrix. Representative phages were chosen (Fig. 1) from within clusters. Nine were selected. Preference was given to phages that were easy to propagate to high titre and stable at 4 °C. Phage 622 of the British set was also included so that there was a representative phage from all the experimental sets. Results were re-analysed using the reactions of these ten phages.

Comparison between countries

To compare strains from different countries the phage patterns were simplified, excluding all reactions of less than 50 plaques and concentrated into a single field. The file was then sorted on this field allowing direct comparison of core patterns. Analyses were carried out at RTD and 100 × RTD.

RESULTS

Comparison and selection of experimental phages

A total of 744 isolates of MRSA were examined by the six participating centres. A preliminary inspection of the data was performed to exclude repeat isolates of the same strain from the analysis set. This was to ensure that the analysis was limited to those phages with different spectra of activity. Four hundred and sixty-one distinct strains were identified by phage-typing patterns but 52 strains from The Netherlands were excluded as they had been examined at RTD only. There were therefore 409 strains for inclusion in this analysis. This data set was used to calculate co-positivity. A 90% similarity was estimated from the resulting matrix (Fig. 1). With the aid of this dendrogram a set of ten phages was selected: MR8,

Table 1. *Distinct strains of MRSA: Comparison of the discriminatory power of the international phages against international phages plus experimental phages sets. Results given by country*

Country	Experimental phages at	Isolates tested	Number of distinct strains: international phages	Number of distinct strains: experimental phages plus international phages	Number of distinct strains: 10 experimental phages plus international phages
Australia	100 × RTD	75	19 (0)	33 (0)	31 (0)
Denmark	RTD, 100 × RTD	114	48 (41)	95 (13)†	88 (18)†
England	RTD, 100 × RTD	109	42 (34)	55 (20)†	53 (20)†
Spain	100 × RTD	272	55 (62)	189 (5)†	156 (13)†
Germany	RTD, 100 × RTD	48	—	37* (2)	23* (7)
The Netherlands	RTD	126	28 (32)	52 (12)†	42 (12)†
	Total	744		461[a]/409[b]	379[a]/337[b]

( ): No. of isolates untypable by specified phage set(s); —, data not available; * experimental phages only; † untypable by the international phages and experimental phage sets combined. See text for the composition of the experimental sets. [a] total isolates, [b] isolates tested at 100 × RTD.

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Table 2. *Distinct strains of* S. aureus: *distribution by international phage group* (*the data excludes Germany*)

Phage group	Australia			Denmark			England			Spain			The Netherlands			Total		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
III	15	29	27	36	54	54	25	31	30	34	69	68	20	36	29	130	219	208
I+III	4	4	4	9	9	9	12	10	10	13	40	39	6	6	6	44	69	68
I	0	0	0	1	4	4	4	4	4	3	10	7	2	1	1	10	19	16
Other	0	0	0	2	2	2	1	1	1	5	19	13	0	0	0	8	22	16
Experimental phages only	—	0	0	—	13†	19†	—	9†	8†	—	15†	13†	—	9†	8†	—	95†	62†
Distinct strains	19	33	31	48	95	88	42	55	53	55	189	156	28	52	42	192	424	370
NT by all phages	0	0	0	41	13	18	34	20	20	62	5	13†	32	12	12	169	50	63

Distinct strains defined by: (a) the international phages; (b) the international phages and experimental phages; (c) the international phages and reduced set of experimental phages. See text for description of the experimental phage sets; † untypable by the international phages, but typable by experimental phages. See text for the composition of the experimental phage sets.

MR12, MR25, 30, 33, 38, M3, M5 and 56B; phage 622 was included to represent the British set. The choice of an individual phage from different clusters was biased towards those which propagated easily to high titre and were stable at 4 °C.

Typability and discriminatory power of the experimental phages

General

Of the 744 isolates examined, 696 had been tested against both the international and experimental phages (Tables 1, 2). The mean typability rate for the international phages was 76%, with a range between countries of 64–100% and 192 distinct patterns (strains) identifiable. When the international and experimental phage sets were combined, the mean typability rate increased to 93% (range 82–100%), allowing 646 isolates to be phage-typable and defining 424 distinct strains. Restriction of the experimental set to 10 phages reduced the mean typability to 91% (633) of isolates but the spread of percentage typability was unaffected. The number of distinct strains fell to 370.

Table 2 shows the variation between countries in phage typing and classifies the typable strains into phage groups. Sixty-eight percent of the 192 typable strains belonged to phage group III, and 23% were of group I+III. These figures excluded the German MRSA. The increase in recognizable strains was because 119 isolates untypable by the international phages became typable when the experimental phages were used. These were divisible into 95 distinct types. Some strains defined by the international phages could be further subdivided.

It was considered that, for reference use, centres would wish to apply these experimental phages at RTD and RTD × 100. To accommodate them into one block it was necessary to reduce the number of experimental phages to 10.

Reduction of the experimental set from 30 to 10 phages reduced the number of distinct (typable) strains from 424 to 370. This reduction was principally caused by the decrease in the number of distinct strains (33) which could be recognized among isolates untypable by the international phages but typable by the experimental phages. Only 13 isolates became non-typable when the phage number was reduced (Table 2).

National difference in typability and discriminatory power

There was no increase in the typability of the Australian isolates when the experimental phages were included because all were typable by the international phages. In countries except England where 18% of isolates remained non-typable, there was an increase in typability from about 70 to over 90% of isolates tested with the experimental phages. The number of distinct strains was nearly doubled when the experimental phage set was added to the international phage set. The increase was most marked among the Spanish isolates where distinct strains rose from 55 to 189 and least among the English isolates which increased from 42 to 55.

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Table 3. *Multicountry occurrence of strains of MRSA-defined by phage type (international phages and 30 experimental phages used at 100 × RTD)*

Strain	Country	Experimental type	International type
1 (2)	Germany	30/34/35/M1/M2/M3/M6	NT
	Denmark		85±
2 (3)	Denmark	30	85±
	Germany	30/67R	Not available
	Germany	30/618/67R	NT
3 (4)	Denmark	32	29/52+
	Denmark	32	29/52+
	Spain	32	29/52/52A/80
	Denmark	32/618	52
4 (11)	Denmark (5)	M3	NT
	Spain	M3	29
	Spain	M3	NT
	Germany	M3/M5	NT
	Germany	M3/M4/M5	NT
	Spain	M3/M4/M5	NT
	Germany	M3/56B	NT
5 (3)	Germany (2)	M6	Not available
	Denmark	M6	NT
6 (3)	England	623	NT
	Denmark (2)	623	85+/−93*

( ) number of isolates; * one isolate lysed by phage 93. One strain 6 from Denmark had been referred from Hong Kong. Strains 2 and 4 as defined here are groups of possibly related strains.

Comparison of patterns between countries

There were no inter-country matches at RTD but at 100 × RTD six strains were found in more than one country (Table 3). Strain 3 belonged to phage group I and strain 4 may also have been phage group I-related. Isolates of the other strains which had spread across national boundaries were either untypable by the international phages or lysed weakly by phage 85 of this set. Most of these strains appeared in continental Europe. Some isolates of strain 6 were defined in England, and others had been referred to the Danish typing centre from Hong Kong. In this study there was no evidence of spread of strains from Australia to Europe or *vice versa*.

DISCUSSION

We describe an international trial of phages developed to type national strains of MRSA. The phages had been developed in The Netherlands, Australia [8], England [10], Spain [9] and Germany in response to local problems in the 1980s and early 1990s so it was of interest not only to see if these phages would increase typability of MRSA outside their country of origin, but also if they were still active against current strains. The majority of the isolates included in this study belonged to phage groups III or I + III or were untypable by the international phages as had been expected from earlier studies [5–9]. Typability rates (Table 1) varied from 100% (Australia) to 60–70% of isolates tested (Denmark and England) and these figures refute a widely held view that the majority of MRSA are not typable by phage. The increase in discrimination within isolates from the same country when the inclusive experimental phage set was used in addition to the international phages was encouraging. With the exception of isolates from England, the number of recognizable strains almost doubled in three countries, and increased from 55 to 189 in Spain. This was expected because the phage set developed in Spain was primarily designed to distinguish between isolates of similar international type [9]. This phage set therefore increased discrimination within groups of typable isolates as well as increasing overall typability. There was a reduction in the number of distinct strains recognized when the experimental phage set was reduced to 10 and non-typability increased slightly. These decreases were considered acceptable.

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For many years bacteriophage (phage) typing has been the first technique used in epidemiological investigation of *S. aureus*. In recent years, there have been attempts to replace it with molecular methods [13] which work well and in practice may increase discrimination within a set of strains initially delineated by phenotyping [14, 15]. It is clear, however, that phage typing which is cheap, easy to perform, instrumentally simple, exportable and reproducible [16] still has a place in epidemiological investigation, particularly for initial screening of large numbers of isolates of *S. aureus*. The material selected by this screening could then be examined by the molecular methods of choice.

The set of 10 MRSA phages described in this paper, MR8, MR12, MR25, 30, 33, 38, M3, M5, 56B and 622, may increase the typability of, and discrimination between, many collections of MRSAs though local differences in typability rates are to be expected. An extended trial of these phages involving the national typing centres [16] is now under way.

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