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The evaluation of a phage-typing system for *Listeria monocytogenes* for use in epidemiological studies

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Summary. A typing system for strains of *Listeria monocytogenes* based on the lytic properties of 28 phages has been evaluated with a set of strains isolated in the UK and tested in a blind trial. The system was highly reproducible and discriminatory, and 64% of all the strains tested could be typed.

Introduction

Since the initial description of *Listeria monocytogenes* as a pathogen in a wide range of animals including man (Gray and Killinger, 1966) the epidemiology of listeriosis has remained poorly understood. A serological typing scheme was described by Donker-Voet (1959) and Seeliger (1961) but almost all the strains from cases of human listeriosis fall into one of two serogroups; therefore another means of subtyping was necessary.

Bacteriophages active against *Listeria* were first described by Schultz (1945), and several attempts have since been made to use phages in typing this genus (Sword and Pickett, 1961; Tubylewicz, 1963; Jasinska, 1964; Maupas *et al.*,1976; Audurier *et al.*, 1977*a*; Durst, 1980; Ortel, 1981; Rocourt *et al.*, 1982). An international multicentre study to evaluate different phage sets and methods has been undertaken (Rocourt *et al.*, 1985).

One of us described sets of 15 and 20 phages tested with strains isolated in France (Audurier *et al.*, 1977*b* and 1979). This report evaluates a set of 28 phages tested blindly against strains of *L. monocytogenes* isolated in the UK during the period 1967–1983. We describe criteria for the application of the results in epidemiological investigations.

Materials and methods

Bacterial strains

Isolates of *L. monocytogenes* from human infections in the UK were received during 1967–1983 and confirmed as *L. monocytogenes* by the criteria listed in table I (Seeliger and Hohne, 1979; Rocourt *et al.*, 1983) and serotyped as described by Seeliger and Hohne (1979) into serogroup 1/2 (O Factor I), serogroup 3 (O Factor IV) or serogroup 4 (O Factor V/VI).

Table I. Criteria for the identification of *L. monocytogenes*

Test	Result
Gram's stain, morphology	Positive, coccus
Haemolysis on horse blood agar	Positive
Tumbling motility at room temperature	Positive
VP test	Positive
Urease production	Negative
Catalase production	Positive
Oxidase production	Negative
Reduction of nitrates	Negative
CAMP test *Staphylococcus aureus*	Positive
CAMP rest *Rhodococcus equi*	Negative
Hydrolysis of aesculin	Positive
Acid but no gas from D-glucose	Positive
Acid from D-salicin	Positive
Acid from L-rhamnose	Positive
Acid from α methyl D-mannoside	Positive
Acid from D-xylose	Negative
Acid from D-mannitol	Negative

Isolates from a total of 475 cases were sent to Tours for phage typing; 153 were from infections involving perinates (a mother-and-baby pair constituted one case) and 322 were from cases not involving perinates. Six of the non-perinatal cases represented only three individual patients, each of whom had two separate episodes of listeriosis; the times between the pairs of episodes were between 3 months and 2 years (McLauchlin *et al.*, 1986). The distribution of serogroups and the numbers of strains isolated from the cases and sent for phage typing are shown in table II. A total of 807 subcultures in 22 coded batches, each containing about 50 strains, was despatched to Tours for phage typing over a period of approximately 3 years. All strains were stored on nutrient-agar slopes at room temperature.

Phage typing

Phage typing was performed in Tours by the method

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Table II. Origin and serogroups of 807 strains of *L. monocytogenes*

Serogroup	Number of perinatal cases	Number of non-perinatal cases	Total number of cases	Subcultures phage-typed
1/2	59	118	177	308
3	1	8	9	13
4	93	196	289	486
Total	153	322	475	807

of Audurier *et al.* (1977*a*, 1979). The phage preparations were used at 100 times their routine test dilutions (the highest dilutions that produced confluent or semi-confluent lysis when 0·02 ml was placed on a lawn of the propagating strain). After 18 and 42 h at 30 C the lytic reactions were read as described for phage typing *Staphylococcus aureus* by Williams and Rippon (1952), and were recorded as follows: + + = > 50 plaques—strong lysis; + = 20–49 plaques—moderate lysis; ± = < 20 plaques—weak lysis; 0 = no plaques—no lysis.

Initially 27 phages were used, but two were later omitted and a newly isolated phage included in the routine typing set. In table III the phages are classified into three groups (as previously described by Audurier *et al.*, 1979) based on the serogroup of the parent and propagating strain, and, with a few exceptions in groups 1 and 2, the lytic spectrum of each phage is restricted to strains of the parent serogroup. The phage-typing results were returned to Colindale for decoding and analysis with a Sirius 1 microcomputer (Sirius Systems Technology, Scotts Valley, CA, USA) and dBase II data files (Victor, Scotts Valley, CA, USA).

Table III. Properties of the phage-typing set

Phage analysis number	Phage Designation	Phage Serovar of parent strain	Host propagating strain Designation	Host propagating strain Serovar
Phage-group 1				
1	1967	1/2a	PS 8615	1/2a
2	2685	1/2b	PS 1803	1/2c
3	4477	1/2b	PS 1803	1/2c
4	575	1/2b	PS 3926	1/2b
5	1652	1/2a	PS 4044	1/2a
6	12029	1/2	PS IP31	1/2
7	1806	3c	PS 1803	1/2c
Phage-group 2				
8	2671	4b	PS 10401	4b
9	1444	4b	PS 4136	4b
10	2425	4b	PS 4262	4b
11	3551	4b	PS 7413	4b
12	3552	4b	PS 6917	4b
13	1317	4b	PS 4525	4b
14	2389	4b	PS 1089	4e
15	3274	4b	PS 1782	4b
28	2425A	4b	PS 83339	4b
Phage-group 3				
16	1090	6b	PS 1815	5
17	1807	4ab	PS 1810	4c
18	184	6a	PS 10401	4b
19	16	5	PS 46	5
20	4277	6b	PS 4202	6b
21	5337	6b	PS 4292	6a
22	4211	6b	PS 5290	6b
23	4295	6b	PS 5337	6b
24	4207	6a	PS 4275	6a
25	4276	6b	PS 4202	6b
26	4292	6b	PS 4213	6b
27	4286	6a	PS 5326	6a

Phages 19 (16) and 25 (4276) were used in tests with batches 1–15 and 20.
Phage 28 (2425A) was used only in tests with batches 13–19, 21 and 22.

Evaluation of the phage-typing system

The evaluation of phages for typing *L. monocytogenes* was considered in three ways:

(i) *Proportion of strains typed.* A single strain was selected for each patient as described below and the proportion that were strongly lysed by at least one phage preparation were recorded as phage-typable.

(ii) *Variability between phage patterns.* The number of differences in phage reactions obtained when typing two cultures from the same, similar or related sources was recorded. From this assessment of variability it is possible to estimate the minimum number of differences in phage senstivities needed before two different cultures can be regarded as unrelated.

The variability of phage patterns was analysed by a method similar to that of Williams and Rippon (1952) and was assessed initially between sets of cultures from the same individual, a mother being a separate individual from her baby. The following variability categories were investigated: (a) subcultures from the same isolate when different colonial types were selected; (b) subcultures from the same isolate phage typed in the same batch; (c) subcultures from the same isolate phage typed in different batches; (d) subcultures from specimens taken from different sites in the same individual.

A difference between the phage patterns of two cultures was recorded when a major phage difference of between a 0 and a + + lytic reaction occurred. Other differences (e.g., 0 and ±, + and ±, 0 and +) were not considered to be major differences in phage reaction.

An analysis for all the sets of subcultures within any one category was obtained as follows. An index phage type was first selected. This was either the modal phage type (that most commonly occurring) on the basis of + + and + phage lysis only, or if this was not possible (as when two or more different patterns were obtained at the same frequency), the phage pattern obtained from the first phage typing. The variation between subcultures within each category was assessed by recording the

number of major phage differences between the phage type of the index strain with each of the other strains in that category. Equal weight was given to each comparison. Thus, in a set of two strains, one point was entered in the appropriate line for the "number of major phage differences" in the "variation in typing results" tables. Similarly, in a set of six strains of which, for example, three were classed as having no major phage differences and two classed as having two major phage differences from the index strain, three points would be entered in the "none" and two in the "two major phage differences" lines respectively in the "variation in typing results" table. The comparison was performed in a hierarchical manner in order of reproducibility, i.e., starting with the comparison of different colonial types from the same isolate and ending with subcultures from different sites within the same patient. For example, a between-batch analysis was performed by counting the number of major phage differences between the "between-batch index strain" and the "within-batch index strains". Where a comparison was made between batches and a phage was not used in one of the batches, the resulting reactions with this phage were not considered in this part of the analysis. The values expressed in this manner were taken as averages of the numbers of major differences by which two cultures from the same source may be expected to vary.

The variability was finally assessed between the index strains from mother-and-baby pairs within the same case, and between strains isolated from both individuals in a pair of twins. A single index strain was then selected for each case as described above (with a mother-and-baby pair as a single case), and this was used in subsequent analysis.

Because of the restricted lytic spectrum of the phages (i.e., serogroup-1/2 strains react only with phages 1–7 and serogroup-4 strains do not), only the reactions between serogroup-1/2 strains and the first seven phages, and serogroup-4 strains and the remaining 20 (or 19) phages were considered even though each strain was tested against all 27 (or 26) phages.

Cultures from 62 sets of serogroup-1/2 strains (phage typed on 169 occasions) and cultures from 90 sets of serogroup-4 strains (phage typed on 270 occasions) were used in a reproducibility analysis. The variability between isolates from mothers and babies within the same case was also assessed for 14 cases due to serogroup-1/2 strains and 14 cases due to serogroup-4 strains. Strains of *L. monocytogenes* serogroup-1/2 from two pairs of twins were also included.

The variability for each individual phage was assessed from the number of major phage-lysis differences between the index strain and other strains from within a case. The frequency with which strong phage lysis occurred against all the strains, and the ratio of the number of strongly lytic phage reactions to the total number of phage-lytic reactions (strong + moderate + weak degrees of lysis) for the set of index strains was also calculated for each individual phage.

All + + and + degrees of lysis were given equal weight, and the percentage of any pair of phage-lytic reactions which occurred together and of any lytic phage reaction which occurred in the absence of any other lytic reaction was calculated and expressed in the form of a histogram as described by Williams and Rippon (1952). This allows an indication of the usefulness of each phage. For example, a phage may have properties distinct from other phages and be the sole phage in the set to cause lysis of a test strain, and thus be a useful component of the routine typing set. Other phages may have similar specificities and frequently give similar reactions, and are therefore less useful in the routine typing set.

(iii) *The discrimination of the system.* The number and frequency of occurrence of phage-lysis patterns was determined. All degrees of lysis (+ +, + and ±) for the single index strain from each case that were typable were given equal weight, and their phage-lysis patterns considered.

Results

Proportion of strains typed

The overall proportion of the strains typed in each of the serogroups is shown in table IV. Of the 164 serogroup-4 strains phage typed when phages 19 and 25 but not phage 28 were used, 134 (82%) were typable. Of the remaining 125 strains typed when phage 28 but not phages 19 and 25 were used, 104 (83%) were typable. Of the latter group of typable strains, 11 (9%) out of 125 were lysed by phage 28 only, i.e., only 74% would have been typable had phage 28 not been used.

Variation in phage patterns

The assessment of variability within each set of strains (tables V and VI) shows that phage typing was highly reproducible. For those strains that were typed within the same batch, patterns showing no major phage differences were obtained on 21 out of 25 occasions (84%) for serogroup-1/2

Table IV. Proportion of cases with phage-typable strains*

Serogroup	Number tested	Number (%) typable by phages
1/2	177	65 (37)
3	9	0 (0)
4	289	238 (82)
Total	475	303 (64)

* A single index strain was selected from each case as described in *Materials and methods*.

Table V. Variation in typing results with serogroup-1/2 strains

Number of major phage differences*	Number of strains with the given number of differences in: subcultures derived from the same strain: phage typed in the same batch	subcultures derived from the same strain: phage typed in different batches	subcultures derived from the same strain: different colony type	different isolates derived from the same patient	isolates from both mother and baby within the same case	isolates from each of a pair of twins
Non-typable on all occasions	8	44	1	10	8	1
0	13	16	1	3	5	1
1	3	7	1	0	1	0
⩾2	1	1	0	1	0	0

* Includes tests with typable results on at least one occasion, and non-typable results on another.

Table VI. Variation in typing results with serogroup-4 strains

Number of major phage differences*	Number of strains with the given number of differences in: subcultures derived from the same strain: phage typed in the same batch	subcultures derived from the same strain: phage typed in different batches	subcultures derived from the same strain: different colony type	different isolates derived from the same patient	isolates from both mother and baby within the same case
Non-typable on all occasions	4	6	0	1	2
0	33	39	4	13	5
1	9	23	1	8	3
2	2	12	0	5	3
3	0	2	1	0	0
>4	0	3	2	2	1

* Includes tests with typable results on at least one occasion and non-typable results on another.

strains, and on 37 out of 48 occasions (77%) for those of serogroup-4 (this figure includes strains non-phage-typable on all occasions). Similar results were obtained for the other variability categories although the results obtained with serogroup-4 strains typed in different batches were the least reproducible. However, a residual number of strains from within each of the variability categories did not give exactly the same phage pattern on repeat testing. Therefore a limit to the number of major phage differences obtained between strains was defined, only beyond which subcultures were considered to be indistinguishable. With the criteria that >1 major phage-reaction differences for serogroup-1/2 strains, and >2 major phage-reaction differences for serogroup-4 strains constituted significant differences, there was a small probability of strains from the same source being considered distinguishable, especially if they were phage typed in the same batch. Even the results obtained when the strains were phage typed in different batches were well reproducible within these limits (tables V and VI). Of the 28 sets of cultures from mother-and-baby pairs within a case, 27 were indistinguishable by the above criteria and this is further discussed (McLauchlin *et al.*, 1986).

Tables VII and VIII show the variability of lysis with each phage, the frequencies with which each phage lytic reaction occurred, the total number of times each phage lysed a strain, and the proportion of strong (++) measures of lysis.

The percentage of strains lysed by any one phage and by any other phage at the same time, and the percentage of strains lysed by any phage alone out of the 65 phage-typable index serogroup-1/2 strains and the 238 phage-typable index serogroup-4 strains is shown in figs. 1 and 2 respectively.

The discrimination of the system

In the 65 phage-typable serogroup-1/2 strains, 25 patterns of phage sensitivity were observed. Three patterns were most common, each exhibited by 22 strains (34% of those phage-typable; table IX), and of these patterns two would be considered indistinguishable from each other (lysis by phage 4, and

Table VII. Variability, occurrence and frequency of reaction of phages 1–7 against serogroup-1/2 strains

Phage no.	Frequency of variability* (Variability data—173 subcultures)	Number (%†) of strong reactions (A single index strain selected from each of the 176 cases)	Total number of lytic reactions‡	Proportion of strong reactions§
1	1/19	21 (12)	27	0·8
2	1/21	8 (5)	15	0·5
3	4/24	23 (13)	27	0·9
4	3/37	28 (16)	34	0·8
5	1/8	10 (6)	11	0·9
6	5/28	21 (12)	24	0·9
7	1/2	1 (0·6)	4	0·3

* Number of major phage-reaction differences between index strain and other strains from within a case/number of strong (+ +) lytic reactions.
† Number of strong lytic reactions/total number of strains tested (%).
‡ Number of strong (+ +) and moderate (+) and weak (±) lytic reactions.
§ Total number of strong reactions/total number of reactions (+ +, + and ±).

Table VIII. Variability, occurrence and frequency of reaction of phages 8–28 against serogroup-4 strains (for explanations of headings see table VII)

Phage no.	Frequency of variability (%) (Variability data—270 subcultures*)	Number (%) of strong reactions (A single index strain selected from each of the 297 cases†)	Total number of lytic reactions	Proportion of strong reactions
8	4/98 (4)	95 (32)	121	0·8
9	3/38 (8‡)	61 (21)	79	0·8
10	5/81 (6)	71 (24)	95	0·7
11	10/79 (13)	96 (32)	117	0·8
12	26/90 (29)	74 (25)	118	0·6
13	27/62 (44)	67 (23)	110	0·6
14	9/79 (11)	67 (23)	87	0·8
15	9/35 (26‡)	40 (13)	54	0·7
18	18/17 (121‡)	16 (5)	45	0·4
20	1/10 (10‡)	13 (4)	24	0·5
24	6/16 (38‡)	16 (5)	40	0·4
27	23/45 (51‡)	42 (14)	67	0·6
28	3/63 (5)	57 (44)	64	0·9

* Phage 28 tested on 115 subcultures, and phages 19 and 25 tested on 155 subcultures.
† Phage 28 tested on 130 subcultures, and phages 19 and 25 tested on 167 subcultures.
‡ Percentage calculated on <50 observations.
Phages 16, 17, 19, 21, 22, 23, 25 and 26 gave lytic reactions <2 occasions and are not included.

by phages 2 and 4) because they differed by only a single phage reaction. A further six strains (9% of those phage-typable) exhibited a single difference from at least one of the three commonest phage patterns. The remaining 37 strains (57% of the typable strains) that did show more than a single difference from the three commonest phage patterns, gave a further 18 patterns.

Similarly in the 227 phage-typable serogroup-4 strains, 119 patterns of phage sensitivity were recorded. Five patterns predominated, and were exhibited by a total of 48 strains (21% of those typable; table X). Of these common patterns, three were considered to be indistinguishable from each other (those with phage 11, phage 14 and phages 10 and 14) because they differed by $\leqslant 2$ reactions. A further 88 strains (39% of those phage-typable) gave $\leqslant 2$ phage reactions different from those five common patterns and would thus be considered indistinguishable from at least one of these patterns. The remaining 91 strains (40% of those phage-typable) that did show >2 phage reactions different from at least one of the five common patterns, formed a further 67 patterns. In this analysis the lytic activity of phage 28 has not been considered because only some strains were tested

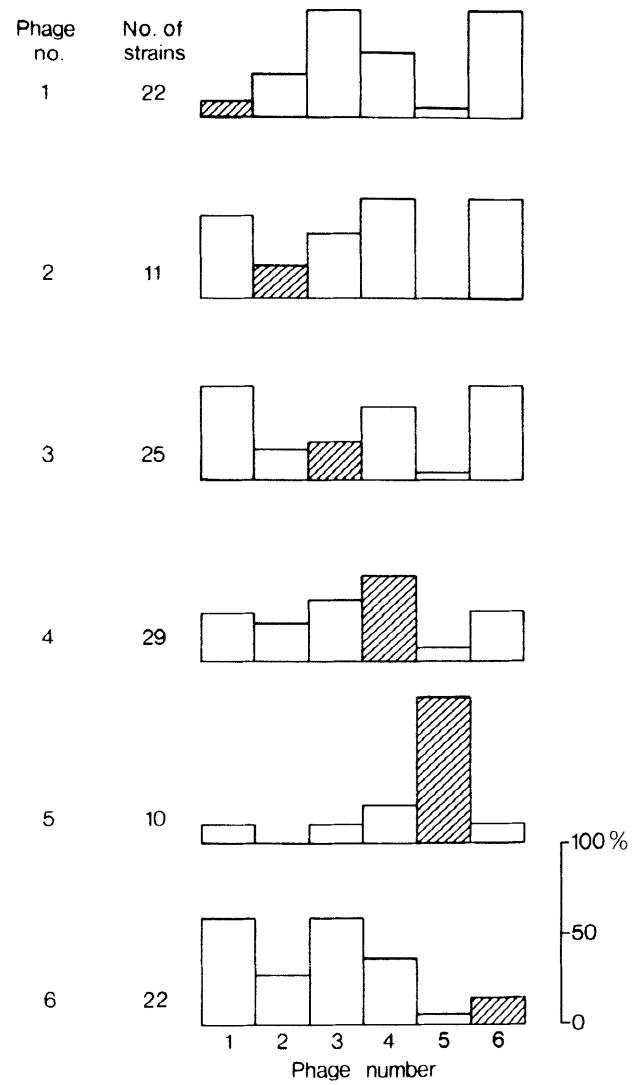

Fig. 1. Frequency of occurrence of pairs of lytic phage reactions for 65 phage-typable serogroup-1/2 index strains (as described in *Materials and methods*). Shaded blocks indicate occurrence of a phage reaction without any other phage reaction. Phage 7 reacted on only one occasion and is not included.

against it. Eleven of the 238 phage-typable strains were lysed only by phage 28, and only the remaining 227 were used in this analysis of the discrimination.

Discussion

The ability of a typing system to distinguish between strains of different origin depends not only upon the typability of the species, but also on the reproducibility and discrimination of the test. We have attempted to assess these characters with strains of *L. monocytogenes* isolated in the UK from 477 cases of human infection and with a set of 28 phages.

An overall typability of 64% for strains isolated in the UK was obtained—37% for serogroup-1/2 strains and 82% for serogroup-4 strains. These percentages are lower than those previously reported. Audurier *et al.* (1977*b*) used a set of 15 phages with strains of *L. monocytogenes* isolated in France and Germany (the majority from France) and were able to type 68% of strains (35% of serogroup 1/2 strains, and 89% of serogroup-4 strains). With an extended set of 20 phages (Audurier *et al.*, 1979) and strains isolated in France, an overall typability of 78% was obtained (57% of serogroup-1/2

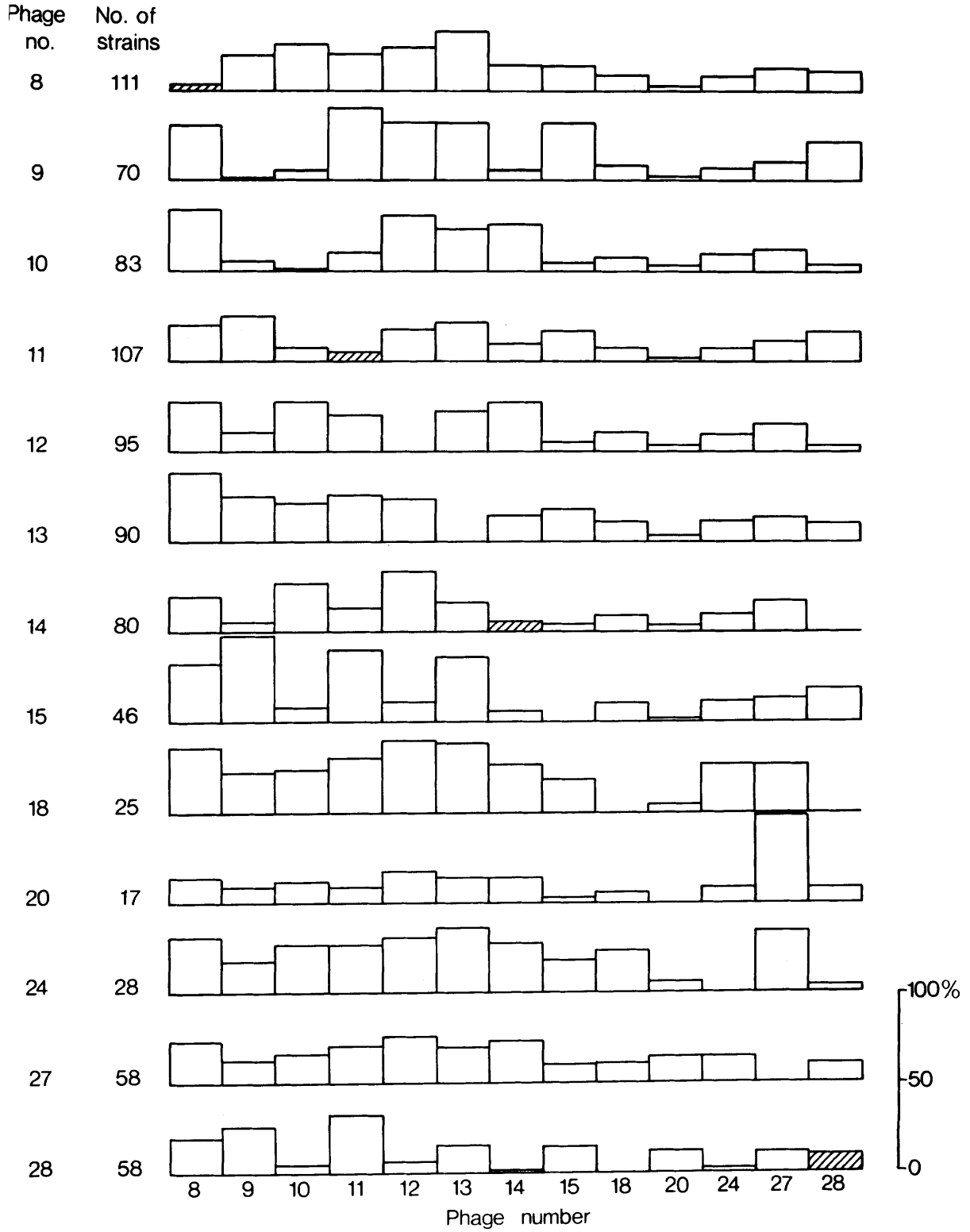

Fig. 2. Frequency of occurrence of pairs of phage reactions for 238 phage-typable serogroup-4 index strains (as described in *Materials and methods*). Shaded blocks indicate occurrence of a phage reaction without any other phage reaction. Phages 16, 17, 19, 21, 23, 25 and 26 did not react with these strains and are not included. (Phage 28 was tested on only 167 occasions.)

Table IX. Occurrence of three most common phage lytic patterns in the 65 typable serogroup-1/2 strains

Phages in common lytic pattern	Numbers of strains showing common phage-lysis pattern	Numbers of strains showing one lytic reaction different (i.e., indistinguishable) from common phage pattern
4	12	8
5	5	4
2,4	5	14

Table X. Occurrence of five most frequent phage lytic patterns in the 227 typable serogroup-4 strains

Phages reacting in common phage-lysis pattern	Numbers of strains showing common phage-lysis pattern	Numbers of strains showing $\leqslant 2$ lytic reactions different (i.e., indistinguishable) from common phage pattern
11	17	30
10, 14	9	55
8, 9, 11, 12, 13, 15	8	26
14	7	46
8, 10, 12, 13	7	28

strains and 88% of serogroup-4 strains). The lower percentage typability of strains isolated in Britain than of strains from France and the different frequencies of patterns of phage lysis (Audurier *et al.*, 1979) suggest that at least in part the British strains were significantly different from those isolated in France. Different geographical patterns of phage specificity have been observed for other organisms, e.g., *S. aureus* (Parker, 1972), group-A streptococci (Skjold and Wannamaker, 1976) and group-B streptococci (Stringer, 1980).

The reason for the decreased number of typable serogroup-4 strains when phage 28 was used could be due either to the bias introduced by the selection of index strains (i.e., because of the selection of typable strains from earlier batches), or to the unequal distribution of phage-lytic patterns observed over relatively short periods of time (McLauchlin *et al.*, 1986) as the strains typed with phage 28 included a large proportion of recent isolates which were phage typed in the later stages of the study.

When strains are phage typed in the same batch and the criteria of >1 major difference in phage reaction for distinguishing between serogroup-1/2 strains, and >2 major phage-reaction differences in the case of serogroup-4 strains are used, there is a low probability that strains isolated from related sources will appear unrelated. Some bias may have been introduced in the analysis of variability because index strains were selected from within the batches, hence the commonest phage pattern for that strain was compared with the patterns of strains phage typed in different batches. However, this bias is probably small, and useful information concerning the epidemiology of this organism has been gained in a further analysis (McLauchlin *et al.*, 1986).

There are however limitations in using a prescribed number of major phage-lysis differences to define indistinguishable strains. Most importantly this assumes, first, that each phage-lytic reaction is reproduced at a similar frequency and second, that preparations of different phages show significantly different properties. Some phages, e.g., phage 6 in group 1 and phages 12, 13 and 27 in groups 2 and 3, showed a higher frequency of variability than most of the other phages. It is possible that these phages are inherently less stable on storage, although phage typing of the propagation strains performed regularly during a period of 6 years has not shown variations greater than those indicated above and preparations are stable to the extent of a loss in potency of 1 log after a period of storage of 1 year at 4°C. Alternatively, those phages that gave less 'reproducible' results may be more prone to observer error (i.e., their results may have been more difficult to read) and, indeed, these phages all gave a lower proportion of strong lytic reactions, i.e., weak and moderate degrees of lysis were recorded more frequently. These phages give plaques of an unusually small size (Audurier, unpublished observation) and this may contribute to their poor reproducibility. Furthermore, no two phage preparations (at least in groups 1 and 2) appeared to be sufficiently similar to exclude them from a routine typing set (figs. 1 and 2). However, because the number of times each phage was tested for variability was small, and because an assessment of the frequency of reproducibility for each phage would lead to a complicated system of reporting, the "traditional" single value for the number of phage differences defining indistinguishable strains provides a basis for investigating the epidemiology of this organism.

Some phages lysed *L. monocytogenes* strains from human sources very rarely (e.g., phages 7 and 10) or never (e.g., phages 16, 17, 19, 21, 22, 23, 25 and 26) and these may be excluded from the routine typing set (Rocourt *et al.*, 1982), as phages 19 and 25 were in the later stages of this study.

Some of these "redundant" phages react more frequently with strains isolated from animals, and with other serogroups and species of *Listeria*, and hence may still be useful in typing isolates from other sources and for taxonomic investigations.

The most recent addition to the typing set is phage 28. This was isolated together with phage 10 and propagated in a strain of *L. monocytogenes* isolated in Boston, USA (Fleming *et al.*, 1985) but it appears significantly different from phage 10 (fig. 2) and is a useful addition to the routine set.

The discrimination of the system was difficult to quantify because of the inherent variability of phage patterns, and also because strains tended to be susceptible to certain combinations of phages giving relatively few discrete lytic patterns. The equal weighting of all the degrees of lysis had the effect of reducing the number of discrete patterns of phage lysis (results not given) and giving more groups of strains with no major phage differences between them.

Bias may have been introduced in this complete analysis towards some patterns because strains were included from related cases, i.e., where cross-infection or recurrent episodes or common source outbreaks of listeriosis may have taken place. An unequal distribution of phage patterns over certain periods of time was observed which is further discussed (McLauchlin *et al.*, 1986).

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