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Improved Procedure for Bacteriophage Typing of *Listeria* Strains and Evaluation of New Phages

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A modified technique in listerial phage typing, termed reversed phage typing procedure, was developed. Ready-to-use typing plates were prepared by preapplication of phage suspensions on tryptose agar plates. The new procedure offers a number of substantial advantages as compared with the conventional method, being much more reliable, efficient, and convenient to use. More than 1,000 strains of *Listeria* have thus far been typed with the reversed phage typing procedure, employing an extended set of 21 genus-specific bacteriophages. The overall typability of strains was 89.5%.

Breaking down listerial species into phagovars has provided the epidemiologist with a very precise and delicate means of tracing the foodborne origin and course of outbreaks of listeriosis (3, 6, 11, 12). Furthermore, persistent listerial contaminations within food plants and/or processing equipment could clearly be separated from sporadically occurring impacts of *Listeria* species (9).

Prior to this investigation, a set of 16 specific bacteriophages and the conventional spot-on-the-lawn technique were employed in phage typing of *Listeria* species (8). However, the limited number of phages as well as the rather laborious typing procedure were somewhat unsatisfactory. Therefore, this study was conducted to simplify phage typing by introducing a modified technique which greatly reduces the time and material required.

Phage typing procedure. In the conventional procedure, plates of tryptose agar (E. Merck AG, Darmstadt, Federal Republic of Germany), fortified with 1.25 mM $CaCl_2$, were flooded with 4-h broth cultures of the *Listeria* strains to be typed. Subsequently, the phage suspensions were applied onto the growing bacterial lawn (8, 15).

In the reversed phage typing procedure, the sterile, fairly dried agar plates (no moisture on the surface) are preinoculated with the phage suspensions. A self-constructed, multisyringe applicator is used for this purpose. Up to 25 phage preparations can be dispensed simultaneously on one plate. In addition, a marker dye spot (e.g., India ink) was placed alongside the plate to identify the order of the phage macroplaques. The suspended drops contained as little as 4 to 6 μl. Therefore, up to 200 ready-to-use phage typing plates can be rapidly prepared from 1 ml of each of the phage suspensions. This represents a twofold increase in efficiency as compared with the conventional method.

Because of the glycerol added to the phage preparations (10%, vol/vol) (8), the applied drops were rapidly absorbed into the agar. The prepared plates can be used directly or stored at 4°C until use. No loss in phage activity was observed within storage up to 2 weeks.

In order to standardize the cellular densities of the listerial cultures, they were prepared by simply diluting 2 ml of overnight broth cultures (tryptose broth; Merck) with 6 ml of sterile broth, which was followed by another 2 h of incubation at 30°C. This procedure yields fairly consistent culture densities (approximately 2×10^8 cells per ml), which is crucial for comparable and reproducible typing results. Prior to phage typing, the prepared plates were allowed to reach room temperature. The agar surface was then flooded with 2 ml of a broth culture of the *Listeria* strains in question.

Following overnight incubation at 30°C, the plates were evaluated with the aid of an oblique lighting apparatus (LIS 01; Dinkelberg, Neu-Ulm, Federal Republic of Germany). The phage particles were not found to diffuse along the agar surface; they actually remained in the circular area coincident with the original phage drop.

Isolation, preparation, and evaluation of new phages. It was necessary to search for new phages to increase the typability of strains and to further subdivide some predominately observed phagovars (8). The methods used to obtain *Listeria* phages from environmental sources as well as from lysogenic strains have already been explained in detail (2, 4, 8, 17, 18, 20). In this study, one phage (C722) was isolated from sewage. In addition, the UV-induction technique, as described earlier (8, 10), was used to isolate 16 phages (A006, B051, B052, B053, B054, B055, B056, B101, B110, B111, A118, A153, D441, A538, B545, and B653) from lysogenic *Listeria* strains.

Six of the tested strains (*Listeria innocua* WSLC 2051, WSLC 2052, WSLC 2053, WSLC 2054, WSLC 2055, and WSLC 2056) were already shown to carry prophages (18) (the original designations of the phages derived thereof are indicated in Table 1). Five tested strains of *Listeria monocytogenes* (SLCC 1806, SLCC 1807, SLCC 1090, SLCC 1652, and SLCC 2671), one strain of *L. innocua* (SLCC 5375), and one strain of *Listeria ivanovii* (SLCC 4719) were previously reported as lysogens (1, 2, 18). However, in this trial they were not found to release any temperate phage, although a set of 72 selected *Listeria* indicator strains was used.

Phage A006 could be released from the parent strain only by increasing the UV exposure time from 60 s to at least 120 s. Very few phage particles were then detectable.

All 17 phages were assigned to a selected host strain and subsequently purified, propagated, and titrated as reported earlier (8, 19). For application in typing, the phage preparations were adjusted to 100 times the routine test dilution. This was defined as the highest dilution that produced just semiconfluent lysis on the respective host strain, when assayed in the typing procedure as described above.

Phage typing and analysis of results. The reversed phage typing procedure and this set of 17 phages were evaluated by

TABLE 1. Properties of phages, parent strains, and propagating strains of the extended phage typing set

Bacteriophage		Parent strain		Propagating strain	
Code[a]	Source[b]	Species	Serovar	Species	Serovar
A511	Sewage			*L. monocytogenes*	4b
A118	1118	*L. monocytogenes*	1/2a	*L. monocytogenes*	1/2a
A502	Sewage			*L. monocytogenes*	1/2a
A006	1006	*L. monocytogenes*	1/2a	*L. monocytogenes*	4b
B653	2653	*L. innocua*	6b	*L. ivanovii*	5
B054 (4286)	2054	*L. innocua*	6a	*L. ivanovii*	5
B051 (4211)	2051	*L. innocua*	6b	*L. ivanovii*	5
B055 (4295)	2055	*L. innocua*	6b	*L. ivanovii*	5
B025	2025	*L. innocua*	6b	*L. ivanovii*	5
D441	4441	*L. seeligeri*	4c	*L. ivanovii*	5
B545	2545	*L. innocua*	6b	*L. ivanovii*	5
B053 (4277)	2053	*L. innocua*	6b	*L. ivanovii*	5
B056 (5337)	2056	*L. innocua*	6b	*L. ivanovii*	5
B101	2101	*L. innocua*	6b	*L. ivanovii*	5
B110	2110	*L. innocua*	6b	*L. monocytogenes*	4c
C707	Sewage			*L. ivanovii*	5
B024	2024	*L. innocua*	6b	*L. ivanovii*	5
B012	2012	*L. innocua*	6b	*L. ivanovii*	5
B035	2035	*L. innocua*	6b	*L. ivanovii*	5
A020	1020	*L. monocytogenes*	4a	*L. ivanovii*	5
A500	ATCC[b]			*L. monocytogenes*	4b

[a] Numbers in parentheses are designations of phages given by Rocourt et al. (18).
[b] Numbers are from the Weihenstephan Listeria Collection. ATCC indicates the American Type Culture Collection (ATCC 23074-B1).

typing 150 *Listeria* reference strains from international strain collections. After computer-aided cluster analysis of the obtained results (host ranges; data not shown), the phage set was combined with the earlier set of 16 phages (8). It was then reduced from 33 to 21 phages, since 12 phages were determined to be almost identical to others in means of host ranges. Hence, they were of no particular use in phage typing. The final phage set derived is detailed in Table 1.

This set was then used to type 937 *Listeria* isolates which had been collected during routine investigation of various foods, dairy products, and silage as well as during sampling of a variety of dairy plants.

The results of phage typing of 1,087 strains of *Listeria* spp. are shown in Fig. 1. The overall typability increased from 84.5% to almost 90%, which was exclusively due to the new phages included in the set. In particular, phages A118, B110, and B653 were able to lyse strains which were untypable with the first phage set (8). Other recently published data

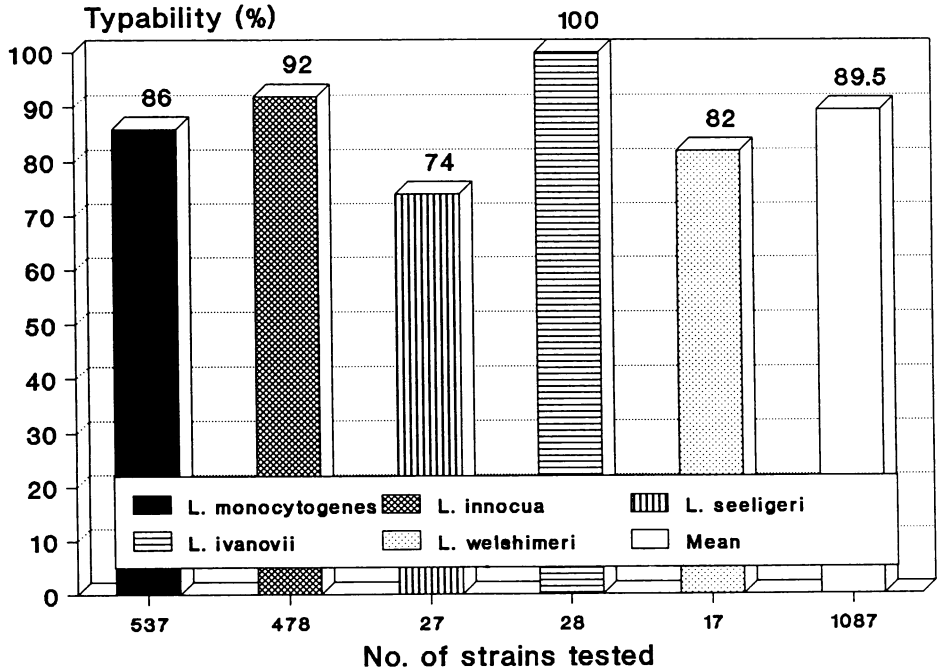

FIG. 1. Typability values of *Listeria* spp. strains examined with the extended set of 21 bacteriophages.

report 58% typability for *L. monocytogenes* (5) and 45% (13), 60% (16), and 68% (17) typable strains among all five relevant species.

The discriminatory quality of the typing system was also substantially improved by the addition of the new phages. Many previously unseen phagovars were observed (data not shown).

With respect to the various origins of the cultures typed in this investigation, the highest typability was noted among strains isolated from silage, with 97% being lysed by at least one phage.

The lytic spectrum of each phage was never solely restricted to strains of the same serovar as the parent or propagating strain. This holds especially true for phage A511, which lysed over 80% of all cultures tested. Further evidence for the conclusion that this phage species is the first virulent *Listeria* bacteriophage described was obtained by investigating phage-resistant mutants derived. A511 is apparently not able to lysogenize, whereas all other phages in the set gave rise to lysogenized mutants resistant to homologous phage (unpublished data).

Some phages (e.g., B054 and B653) needed 40 to 48 h of incubation to produce clearly visible macroplaques on some strains because of the small size of their single plaques. Therefore, an incubation period of at least 40 h is recommended, until final results are recorded. However, the macroplaques produced by A511 might be already visible after 5 to 6 h of incubation. This could aid in generic identification of *Listeria* spp.

The reversed phage typing procedure offers a number of substantial advantages compared with the conventional procedure applied in listerial phage typing. Because of the ease in which this procedure can be performed, it should be of interest to the food microbiologist dealing with phage typing. Furthermore, the distribution of prepared, ready-to-use typing plates could avoid the necessity of cloning and titration of phages within each laboratory, which present a major difficulty.

The evaluation of reversed phage typing procedure results (i.e., reading of the plates) was simplified by the fact that so-called inhibitory reactions were substantially reduced. These are rather common in listerial phage typing and might be due to the presence of bacteriocins (7, 14, 20, 21) or other inhibitory substances and/or proteins liberated by the lysed host cells.

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