Epidemiol. Infect. (1993), **111**, 71–79

Comparison of multilocus enzyme electrophoresis (MEE), ribotyping, restriction enzyme analysis (REA) and phage typing for typing of *Listeria monocytogenes*

B. NØRRUNG[1] AND P. GERNER-SMIDT[2]

[1] *Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Bülowsvej 13, 1870 Frederiksberg C, Denmark*

[2] *Department of Clinical Microbiology, Statens Seruminstitut, Artillerivej 5, DK-2300, Copenhagen S, Denmark.*

(*Accepted 18 February 1993*)

SUMMARY

The discriminatory power of four methods for typing of *Listeria monocytogenes* was compared. The four methods were multilocus enzyme electrophoresis (MEE), ribotyping, restriction enzyme analysis (REA), and a newly developed Danish phage typing system. Ninety-nine human clinical, food and slaughterhouse isolates of *Listeria monocytogenes* were typed by each method. The most discriminatory single typing method was phage typing with an overall discriminatory index (DI) of 0·88 followed by REA, MEE and ribotyping with DI-values at 0·87, 0·83 and 0·79 respectively. Considering strains from each of the two predominant O-serotypes alone, serotype 1 was best discriminated by the molecular typing methods, in particular REA, which showed a DI of 0·92. The serotype 4 strains were best discriminated by phage typing (DI = 0·78). If two or more typing methods were combined, the combination of REA and MEE were found to be the most discriminatory combination. The DI values were 0·96, 0·74 and 0·90 for serotype 1, 4, and both combined, respectively. Phage typing is a rapid and inexpensive typing method but not as reproducible as the molecular typing methods. It is the most suitable method for mass screening. In situations where results are required to be highly reliable, i.e. when studying the relationships between only a few strains, a single or a combination of molecular typing methods should be used, preferable MEE and REA.

INTRODUCTION

The ubiquitous nature of the foodborne pathogen *Listeria monocytogenes* makes discriminatory typing systems essential for the investigation of its epidemiology. Several typing systems are used in the epidemiological investigation of *L. monocytogenes*. The most widely used methods have relied upon phenotypic characteristics such as O and H antigens (serotyping) and bacteriophage lysis patterns (phage typing). Recently several new molecular typing methods, based on the characterization of the genotype as well as on phenotype, have been developed [1, 2].

The quality of a typing system is normally evaluated by its typability,

reproducibility and discriminatory power. The typability is the proportion of strains investigated that can be assigned to a specific type by the method, and reproducibility is the proportion of strains that are assigned to the same type on repeated testing. A discriminatory index (DI), stating the probability that two epidemiologically unrelated strains will be distinguished by a typing method, has been described by Hunter and Gaston [3]. The DI is determined by the number of types defined by the method in question and the relative frequency of these types.

Among the methods used to type *L. monocytogenes*, serotyping is the least discriminatory. Almost all strains isolated from humans, foods and environments fall into one of the serovars 1/2a, 1/2b or 4b. Phage typing and molecular typing methods provide a much more detailed subdivision of *L. monocytogenes* [4]. We have, however, no exact knowledge of the discriminatory power of each of these typing methods relatively to each other. In this study we have therefore compared the discriminatory power of the four typing methods phage typing, multilocus enzyme electrophoresis (MEE), ribotyping, and restriction enzyme analysis (REA) by using the DI to estimate the discriminatory power.

MATERIALS AND METHODS

A total of 99 *L. monocytogenes* strains were used in this study. Sixty-nine of the strains were derived from clinical cases of human listeriosis and represented all but four human clinical strains recovered in Denmark during 1989 (32 cases) and 1990 (37 cases). Apart from eight strains (2930–3060, 14613–14614, 15811–15847, 14675–14676) which were double isolates, i.e. derived from four cases of human listeriosis, all strains were presumed to be epidemiologically unrelated. Seventeen strains were derived from different kinds of sausages and raw meat, produced by five different Danish manufacturers and 13 strains were derived from meat, meat offal and environmental samples from a single slaughterhouse.

For serological identification, bacto *Listeria*-O-antisera against serotype 1 (Difco, 2300–50) and serotype 4 (Difco, 2301–50) were used. The terms serotype 1 and serotype 4 refer to the somatic antigens, commonly also referred to as serogroup 1/2 and serogroup 4.

Multilocus enzyme electrophoresis was performed by studying the mobility of the following 12 enzymes: adenylate kinase, nucleoside phosphorylase, 6-phosphogluconate dehydrogenase, alanine dehydrogenase, mannose phosphate isomerase, glucose 6-phosphate dehydrogenase, NADP-dependent glutamate dehydrogenase, phosphoglucose isomerase, alpha-naphtyl acetate esterase, alpha-naphtyl acid phosphate, L-phenylalanyl-L-leucine peptidase and catalase. Although four of the enzymes (adenylate kinase, nucleoside phosphorylase, glutamate dehydrogenase and phosphoglucose isomerase) in a previous study were enzymes of single relative electrophoretic mobility [5], these enzymes were examined because they might be polymorphic in new strains examined. Thus in this study only 2 of the 12 enzymes (adenylate kinase and phosphoglucose isomerase) were found to be monomorphic. Preparation of enzyme extracts were performed as described by Nørrung [5]. The enzyme extracts were electrophoresed in 11·4% starch gels at a voltage of 130V/cm. Tris-citrate (pH8) was used as buffer

in the gels. The gels were stained as described by Selander and colleagues [6]. Specific staining procedures for catalase were performed by using the method of Harris and Hopkinson [7].

Strains were phage typed using a newly developed Danish *L. monocytogenes* phage typing system [8]. A total of 26 different phages were used. Twelve phages were used to type strains of serotype 1 and 14 phages were used to type strains of serotype 4. Phage reactions were tested at 100 × Routine Test Dilution (RTD) for all strains and also at RTD for serotype 4 strains. The additional use of RTD for serotype 4 strains, increased the discrimination of these strains [8]. RTD is the phage dilution that produces near confluent lysis on the propagating strain of the phage. Strains were considered different if they showed more than one major difference in the phage reactions at each dilution. A major difference is considered to be a difference between a strong (more than 50 plaque forming units) and an absent phage reaction (less than 5 plaque forming units). At this level of discrimination the reproducibility of phage typing is at least 90%.

REA- and ribotyping were carried out as described elsewhere [9]. Briefly, DNA was extracted by an EDTA/SDS lysis, phenol/chloroform extraction procedure. After cutting the DNA with the appropriate restriction enzyme, the restriction fragments were separated by electrophoresis in an agarose gel, stained in ethidium bromide and photographed over an UV-transilluminator. For REA-typing the banding patterns were analysed visually at this stage. For ribotyping, the fragments were vacuum blotted onto a nylon membrane and hybridized with a digoxigenin-11-dUTP labelled probe made by random priming of *Escherichia coli* 16S and 23S ribosomal RNA using reverse transcriptase. The detection procedure used an alkaline phosphatase mediated colour reaction of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (x-phosphate) after coupling of alkaline phosphatase labelled anti-digoxigenin antibodies to the hybrids. The banding patterns were compared visually. The restriction enzyme *Hha*I was used for REA-typing as this enzyme was shown to be very discriminatory [10]. In a pilot study using 10 strains with different phage types, 8 restriction enzymes were tested for ribotyping (*Bam*HI, *Eco*RI, *Hha*I, *Hae*III, *Hin*DIII, *Pst*I, *Sal*I, and *Sma*I). Of these, *Eco*RI was by far the most discriminatory and was therefore chosen for the rest of the study.

The reproducibility of MEE was carried out by typing of 11 reference strains [5] kindly provided by Dr J. C. Piffaretti, Instituto Cantonale Batteriologico, Lugano, Switzerland. Ten strains showing at least one difference between each other in the position of a single restriction band, were selected for reproducibility testing of REA- and ribotyping. Differences in the intensity of the restriction bands were not considered. The reproducibilities of all three typing methods were evaluated by typing the selected strains at least twice read independently by two different persons.

The discriminatory power of each typing method was determined by calculating the discriminatory index, according to a modification of the numerical index method [11]. The discriminatory index is given by the formula:

$$\mathrm{DI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j,$$

https://doi.org/10.1017/S0950268800056697 Published online by Cambridge University Press

Table 1. *Strain designation, source, serotype, ribo-type, REA-type and MEE-type of 99 strains of L. monocytogenes*

Strain	Source	Sero-type	Ribo-type	REA-type	MEE-type
1542	Hum[a]	4	1	1	1
1571	Hum	4	1	1	1
1619	Hum	4	1	1	1
1645	Hum	4	1	1	1
1785	Hum	4	1	1	1
2264	Hum	4	1	1	1
2374	Hum	4	1	1	1
2433	Hum	4	1	1	1
2502	Hum	4	1	1	1
2930	Hum	4	1	1	1
3060	Hum	4	1	1	1
3861	Hum	4	1	1	1
5558	Hum	4	1	1	1
5626	Hum	4	1	1	1
7176	Hum	4	1	1	1
7595	Hum	4	1	1	1
7814	Hum	4	1	1	1
8333	Hum	4	1	1	1
8845	Hum	4	1	1	1
9084	Hum	4	1	1	1
10315	Hum	4	1	1	1
10542	Hum	4	1	1	1
11984	Hum	4	1	1	1
12410	Hum	4	1	1	1
13408	Hum	4	1	1	1
15374	Hum	4	1	1	1
15631	Hum	4	1	1	1
15632	Hum	4	1	1	1
6668	Hum	4	1	1	24
12400	Hum	4	1	1	24
10136	Hum	4	1	1	26
5223	Hum	4	2	2	4
429	Hum	4	1	3	1
7751	Hum	4	2	4	4
14613	Hum	4	2	4	4
14614	Hum	4	2	4	4
15811	Hum	4	2	4	4
15847	Hum	4	2	4	4
15950	Hum	4	2	4	4
1670	Hum	4	2	5	4
8160	Hum	4	2	5	4
9050	Hum	4	2	5	4
9618	Hum	4	2	5	4
12621	Sau[b]	4	2	5	4
12624	Sau	4	2	5	4
12627	Min[c]	4	2	5	4
12630	Sau	4	2	5	4
12352	Hum	4	4	6	32
10165	Hum	4	3	7	27
751	Sla[d]	1	7	8	9
770	Sla	1	7	8	9
5500	Hum	1	13	8	9
750	Sla	1	7	8	15

https://doi.org/10.1017/S0950268800056697 Published online by Cambridge University Press

Table 1 (*cont.*)

Strain	Source	Sero-type	Ribo-type	REA-type	MEE-type
760	Sla	1	7	8	15
930	Sla	1	7	8	15
910	Sla	1	7	8	16
12992	Hum	1	13	8	16
6227	Hum	1	7	9	9
7785	Hum	1	14	10	25
12622	Sau	4	2	11	4
12623	Sau	4	2	11	4
12628	Sau	4	2	11	4
12629	Sau	4	2	11	4
12982	Sau	4	2	11	4
12983	Sau	4	2	11	4
12984	Sau	4	2	11	4
12987	Sau	4	2	11	4
12997	Sau	4	2	11	4
12998	Sau	4	2	11	4
335	Hum	1	12	12	4
9709	Hum	1	5	13	4
10511	Hum	1	5	13	4
11539	Hum	1	5	13	4
12625	Sau	1	5	13	4
14675	Hum	1	5	13	4
14676	Hum	1	5	13	4
9495	Hum	1	7	14	7
11463	Hum	1	7	15	13
11464	Hum	1	7	15	13
3648	Hum	1	9	16	9
14387	Hum	1	9	16	11
730	Sla	1	7	17	2
740	Sla	1	7	17	9
3853	Hum	1	7	17	9
12353	Hum	1	8	18	5
10472	Hum	1	14	19	28
10585	Hum	1	7	20	8
11049	Hum	1	10	21	29
12626	Min	1	7	22	9
12993	Min	1	7	22	11
12994	Min	1	7	22	11
3820	Hum	1	6	23	5
5700	Hum	1	13	24	9
4718	Hum	4	1	25	1
710	Sla	1	7	26	9
741	Sla	1	6	26	9
922	Sla	1	7	26	9
920	Sla	1	7	26	11
11819	Hum	1	11	34	31

[a] Hum, human; [b] Sau, sausage; [c] Min, minced meat; [d] Sla, slaughterhouse.

where DI is the discriminatory index, N is the number of strains investigated and a_j is the number of strains with a type indistinguishable from the type of the jth strain. The DI was calculated on an IBM-compatible PC using a program developed by Gerner-Smidt, Statens Seruminstitut, Copenhagen.

https://doi.org/10.1017/S0950268800056697 Published online by Cambridge University Press

RESULTS

REA, MEE and ribotyping

In the present study, the molecular typing methods REA, MEE and ribotyping showed a typability and a reproducibility of 100%. All of the strains investigated, their serotype, and the type designation within the three other typing systems can be seen from Table 1. By the use of REA-typing, 27 different types were identified among 99 strains of *L. monocytogenes*, while MEE and ribotyping identified 20 and 14 different types, respectively. The type designations used within the different typing systems are serially numbered in order of discovery. The reason that some designation numbers are higher than the number of types occurring in this study is that each typing method has been used in investigations of strains not included in this study.

REA and ribotyping were found to be serotype specific, i.e. all REA- or ribotypes were found to comprise strains from only one serogroup. In contrast one type, the electrophoretic type 4 (ET 4), identified by MEE, was found to comprise strains belonging to both serogroup 1 and serogroup 4. The most striking differences among the results from the three typing methods were the occurrence of several different REA- and ribotypes within the ETs 4 and 9 as well as the occurrence of several different REA types and ETs within ribotype 7 (Table 1). The number of types and discriminatory indices of the three typing methods in relation to the serotypes of the 99 *L. monocytogenes* strains can be seen from Table 2.

Within the three typing methods, the overall best discriminatory power was achieved by the use of REA which showed a DI of 0·87. As MEE was found not to be serotype specific, more types were identified by combining MEE with serotyping and therefore, when both methods were considered together, the discriminatory power of MEE was found to be 0·83. The lowest index of discrimination was given by the use of ribotyping which showed a DI of 0·79. Considering both serogroups separately, all three molecular typing methods showed significantly lower DI-values within strains of serogroup 4 than within strains of serogroup 1.

The DI achieved by combining the three molecular typing methods can be seen from Table 3. Overall, the best discriminatory power indicated by a DI of 0·90 was achieved by combining MEE and REA. This DI was not improved further by the incision of ribotyping as an additional method (Table 3).

Phage typing

The typability of the phage typing system was found to be 92% and 98% within strains of serogroups 1 and 4, respectively. The overall typability was found to be 95%. The number of different phage-patterns, strong and weak reactions considered, as well as the DI-values in relation to serotypes, can be seen from Table 2. The overall DI of the phage typing was found to be 0·88. As the typing system is not 100% reproducible this DI cannot directly be compared to the DI values obtained by the molecular typing methods, which were found to be 100% reproducible. A lower DI-value at 0·71 within strains of serogroup 1, compared with a DI at 0·78 within strains of serogroup 4, was observed for phage typing.

Table 2. *Number of types and discrimination indices (DI) of four typing methods used to type 99 strains of* L. monocytogenes

Typing method*	Serotype 1 (39 strains) No. of types	DI	Serotype 4 (60 strains) No. of types	DI	Total (99 strains) No. of types	DI
Ribo	10	0·72	4	0·53	14	0·79
REA	18	0·92	9	0·69	27	0·87
MEE	14	0·87	6	0·58	19	0·83
Phage†	NA‡	0·71	NA	0·78	NA	0·88

* Ribo, Ribotyping; REA, Restriction enzyme analysis; MEE, Multilocus enzyme electrophoresis; Phage, Phage typing.

† The number of different phage-patterns, strong and weak reaction considered were 28, 56 and 84 for serotype 1 strains serotype 4 strains and all strains respectively.

‡ NA, Not applicable.

Table 3. *Number of types and indices of discrimination, for combinations of four* L. monocytogenes *typing methods*

Combinations of methods	Serotype 1 (39 strains) No. of types	DI	Serotype 4 (60 strains) No. of types	DI	Total (99 strains) No. of types	DI
Ribo + REA	20	0·94	9	0·69	29	0·88
Ribo + MEE	21	0·93	6	0·58	27	0·84
MEE + REA	24	0·96	11	0·74	35	0·90
Ribo + REA + MEE	26	0·97	11	0·74	37	0·90
Phage + Ribo	ND*	0·85	ND	0·78	ND	0·90
Phage + REA	ND	0·94	ND	0·81	ND	0·92
Phage + MEE	ND	0·91	ND	0·81	ND	0·92
Phage + Ribo + REA	ND	0·95	ND	0·81	ND	0·92
Phage + REA + MEE	ND	0·97	ND	0·84	ND	0·94
Phage + Ribo + REA + MEE	ND	0·98	ND	0·84	ND	0·94

* ND, not determined.

The DI-values achieved by combining phage typing with the different molecular typing methods are shown in Table 3. The overall highest DI-value (0·94) was achieved by combining phage typing with REA and MEE. This DI was not further improved by the inclusion of ribotyping.

DISCUSSION

Although the strains examined were not a random selection or representative of the wide diversity encountered within *L. monocytogenes*, the discriminatory index allows comparative assessment of the discriminatory power of the different typing systems within the population of test strains. In this case, however, the population of test strains includes those strains most frequently isolated from human cases of listeriosis in Denmark. For all typing methods, a high DI-value is desirable. A high DI-value, however, does not indicate that the typing methods is useful in every

epidemiological situation. The number of types, the prevalence of the dominating type, the reproducibility and the typability of each method are parameters which play a more or less important role, depending on the purpose of bacterial typing.

In this study, within strains of serotype 1, the most discriminatory single typing method was REA typing with a DI of 0·92. Within strains of serotype 4, phage typing was found to be the most discriminatory method (DI = 0·78). Considering both serotype 1 and serotype 4, also the phage typing system was the most discriminatory typing method with a DI at 0·88. As, however, the reproducibility of phage typing is not 100%, this method should not be the method of choice in situations where results are required to be highly reliable, i.e. when studying the relationships between only a few strains. In such situations, other more reproducible methods are preferable unless the discriminatory power of these methods is significantly lower than that of phage typing. In the present study, however, REA showed an almost identical DI (0·87) compared with phage typing. This DI value could be further increased to 0·90 by combining REA with MEE. This combination of typing methods was thus found to be optimal for comparing, for example, the relationships of a *L. monocytogenes* strain from a human patient with a strain from a suspected food source.

The implications of the finding that the type of an isolate from a patient and the type of an isolate from a suspected source are identical, varies depending on whether the type in question is rare or prevalent in the population. This makes it of the utmost importance, that, for any typing method to be used, the variability of the background bacterial population is known with regard to the typing method used. By combining phage typing with REA and MEE an overall DI-value of 0·94 was achieved. The reproducibility of a combination of methods are at most the same as the lowest reproducibility represented by a single method included in the combination. The combination of phage typing, REA and MEE has a reproducibility equivalent to that of phage typing alone.

In studies of bacterial epidemiology, typing methods are generally used to distinguish between genetically unrelated strains. The possibility that a typing method may distinguish also between genetically related strains, resulting in false high DI-values, however, has to be considered. The calculation of DI-values in relation to combination of methods, has been performed on the assumption that two strains are genetically unrelated if they differ in type, using a single typing method only. Further studies dealing with typing of epidemiologically related strains by each method used in a given study, are required, to ensure that the typing methods are expressing 'the true state of nature' and thus whether this assumption is correct.

The discriminatory power of each molecular typing method was strikingly different between strains belonging to serotype 1 and strains belonging to serotype 4. Among strains belonging to serotype 4, there were significantly fewer types and lower DI-values than among strains of serotype 1. A reason for this observation may be that the typing methods used were not sufficient to distinguish between genetically unrelated strains within serotype 4 strains. It is also possible, and maybe more likely, that it reflects a higher degree of genetic homogeneity among strains of serotype 4 than among strains belonging to serotype 1.

A higher DI value among strains of serotype 4 compared with strains of

serotype 1, was observed for phage typing. This is probably partly caused by the occurrence of a higher number of non-typable strains within serotype 1, compared with serotype 4, as all non-typable strains were considered a single type in the calculation of the DI.

A major advantage of the molecular typing methods is that the same set of reagents and equipment can be used for many different bacteria. In contrast phage typing requires a battery of phages and indicator strains and is standardized for only one bacterial species. On the other hand, once established in the laboratory, phage typing is inexpensive and a large number of bacteria (around several hundred) can be typed quickly. When a large number of strains is being investigated, the reproducibility of phage typing becomes less important thereby making this method suitable for mass screening of *L. monocytogenes*. Molecular typing methods are costly and time-consuming as only a lower number of strains can be typed per week. However, the better reproducibility of the molecular typing methods, compared with phage typing, and the 100% typability of these methods make them more suitable when only few strains need to be examined or when the reliability of results is of importance.

ACKNOWLEDGEMENTS

This work was supported by grant from the Danish Ministry of agriculture.

REFERENCES

1. Nocera D, Bannerman E, Rocourt J, Jaton-Ogay K, Bille J. Characterization by DNA restriction endonuclease analysis of *Listeria monocytogenes* strains related to the Swiss epidemic of listeriosis. J Clin Microbiol 1990; **28**: 2259–63.
2. Bibb WF, Gellin BG, Weaver R, et al. Analysis of clinical and foodborne isolates of *Listeria monocytogenes* in United States by multilocus enzyme electrophoresis and application of the method to epidemiological investigations. Appl Environ Microbiol 1990; **56**: 2133–41.
3. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of a typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988; **26**: 2465–6.
4. Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev 1991; **55**: 476–511.
5. Nørrung B. Characterisation of Danish isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis. Int J Food Microbiol 1992; **15**: 51–9.
6. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol 1986; **51**: 873–84.
7. Harris H, Hopkinson DA. Handbook of enzyme electrophoresis in human genetics. New York: Elsevier Science Publishers, 1976.
8. Gerner-Smidt P, Rosdahl VT. Frederiksen W. A new Danish *Listeria monocytogenes* phage typing system. APMIS. In press.
9. Gerner-Smidt P. Ribotyping of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex. J Clin Microbiol. In press.
10. Wesley IV, Ashton F. Restriction enzyme analysis of *Listeria monocytogenes* strains associated with food-borne epidemics. Appl Environ Microbiol 1991; **57**: 969–75.
11. Gaston MA, Hunter PR. Efficient selection of tests for bacteriological typing schemes. J Clin Pathol 1989; **42**: 763–6.