Correlations between Molecular Subtyping and Serotyping of *Listeria monocytogenes*

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To define relationships between *Listeria monocytogenes* genetic lineages, ribotypes, and serotypes, 235 *L. monocytogenes* isolates were characterized by serotyping and automated *Eco*RI ribotyping. Genetic lineage predicted the following serovar clusters: lineage I, comprising serotypes 1/2b, 3b, 3c, and 4b; lineage II, comprising serotypes 1/2a, 1/2c, and 3a; and lineage III, comprising serotypes 4a and 4c. Some *Eco*RI ribotypes contained multiple serotypes; a subset of these isolates was further differentiated with *Pvu*II ribotyping. Of the 12 resultant *Eco*RI-*Pvu*II combination types, only 4 contained multiple serotypes, demonstrating the potential of ribotyping for serotype prediction.

Listeria monocytogenes is a food-borne pathogen associated with severe invasive diseases in humans and animals that is estimated to cause approximately 2,500 cases of human illness and 500 deaths annually in the United States (11). Accurate, reliable, and standardized subtyping methods provide epidemiological markers, which are critical to disease outbreak investigations.

A variety of subtyping methods have been used to differentiate *L. monocytogenes* beyond the species level (19). Although serotyping is not very discriminatory, it is a universal technique sometimes used as a prerequisite for other subtyping methods (18). Serology divides *L. monocytogenes* into 13 serotypes on the basis of somatic and flagellar antigens; this technique relies on high-quality, specific sera prepared with standardized strains and is currently performed in only a small number of reference laboratories. The vast majority of human listeriosis cases are caused by three serotypes (1/2a, 1/2b, and 4b), rendering this method minimally useful in epidemiologic investigations (19). The goal in using molecular methods for subtyping of *L. monocytogenes* is to attain greater discrimination of strains than is achieved by serotyping (19).

Ribotyping, a sensitive and reproducible subtyping method, is based on rRNA gene restriction fragment polymorphisms. Previous work has shown that *Eco*RI ribotyping can differentiate *L. monocytogenes* from other *Listeria* spp., a large database of *L. monocytogenes* ribotype patterns already exists, and a fully automated ribotyping system is commercially available (3, 10, 12). As traditional phenotypic methods are now being used in conjunction with or have been replaced by molecular subtyping for *L. monocytogenes* surveillance, we must define the relationships between subtypes determined by different methods. Genotypic analyses have consistently grouped *L. monocytogenes* into two major lineages. Multilocus enzyme electrophoresis divides the species into two primary subgroups, division I (serotypes 1/2b, 4a, and 4b) and division II (serotypes 1/2a and 1/2c) (14). Pulsed-field gel electrophoresis also yields

a binary division into group I (serotypes 1/2b, 3b, 4b, 4d, and 4e) and group II (serotypes 1/2a, 3a, 1/2c, and 3c) (2). Corresponding genetic subdivisions were also found with ribotyping; one group (RTα) contained serotypes 1/2a, 1/2c, and 3a, while a second group (RTβ) contained serotypes 1/2b, 3b, 4b, and 4ab (8). Ribotyping and virulence gene allelic analysis have been shown to subdivide *L. monocytogenes* into three lineages that may differ in pathogenic potential (10, 15, 21). Ribotyping is commonly used for subtyping of *L. monocytogenes* and has played an important role in outbreak detection (1, 3, 8). Hence, there is a need to define associations between *L. monocytogenes* serotypes and genetic lineages and ribotypes to further improve the diagnostic utility of this subtyping method. Understanding of correlations between ribotypes and genetic lineages with serotypes may also provide insight into the evolution of *L. monocytogenes* subtypes. We describe here the relationships among serotypes, ribotypes, and genetic lineages in a set of *L. monocytogenes* isolates predominantly from humans and animals.

Bacterial isolates. A total of 235 *L. monocytogenes* isolates from humans ($n = 161$), animals ($n = 72$), and foods ($n = 2$) were selected from the Cornell University *Listeria* Collection for inclusion in this study. Some of the isolates had previously been characterized by *Eco*RI ribotyping (10). All of the isolates were stored in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with 15% glycerol at −80°C.

Automated ribotyping. Bacterial isolates were streaked onto brain heart infusion agar plates and incubated at 37°C for 24 h, after which the plates were submitted for automated ribotyping. Ribotyping was performed with the RiboPrinter Microbial Characterization System (Qualicon, Wilmington, Del.) as previously described (3, 9). All isolates were ribotyped by using *Eco*RI chromosomal digests, and a subset was ribotyped by using *Pvu*II ($n = 32$). Isolates were assigned to genetic lineage I, II, or III based on *Eco*RI ribotypes as previously described (21).

Serotyping. All isolates were serotyped in accordance with the scheme for routine serodiagnosis of *L. monocytogenes* (20). Serotypes were designated based on agglutination reactions

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TABLE 1. Distribution of serotypes among *L. monocytogenes Eco*RI ribotypes

Lineage	*Eco*RI ribotype	No. of isolates of serotype:								
		1/2a	1/2b	1/2c	3a	3b	3c	4a	4b	4c
I	DUP-1038								41	
I	DUP-1042		26			1	1		19	1
I	DUP-1044	1							31	
I	DUP-1052		6			1			1	
I	DUP-1026		1							
I	DUP-1024		5						2	
I	DUP-1043		1							
I	DUP-1027		2							
II	DUP-1062	8								
II	DUP-1030	30		7	1					
II	DUP-1039	9		1	1					
II	DUP-1045	13								
II	DUP-1053	4								
II	DUP-1054	1								
II	DUP-1056	3								
II	DUP-1029	2								
II	DUP-1035	1								
II	DUP-1047	4								
III	DUP-1061							1		1
III	DUP-1059							4		1
III	DUP-10146	1								
III	DUP-10147									1
III	DUP-10145									1

with factor antisera. All serodiagnoses were performed blinded.

Correlations between serotypes, *Eco*RI ribotypes, and lineages. We identified eight serotypes among the 235 isolates tested; the majority of isolates were characterized as serotype 1/2a (33%), 1/2b (17%), or 4b (40%). *Eco*RI ribotyping differentiated these isolates into 24 distinct ribotypes; 8 fell within lineage I, 10 were in lineage II, and 6 were in lineage III (Table 1). Consistent with previous studies, genetic lineages predicted serovar clusters. Lineage I contained serotypes 1/2b, 3b, 3c, and 4b; lineage II contained serotypes 1/2a, 1/2c, and 3a; and lineage III included serotypes 4a and 4c. There also is a specific correlation between single antigens and lineages. Lineages II and III contain flagellar antigens a and c, while lineage I predominantly contains antigen b.

Two atypical lineage I isolates (one of ribotype DUP-1042 and one of ribotype DUP-1044) were of serotypes 4c and 1/2a, respectively. One atypical lineage III isolate was of serotype 1/2a. While it has previously been shown that serotyping may not always be highly reproducible (18), the occurrence of these atypical combinations of serotypes and molecular subtypes allows speculation that horizontal gene transfer may occur among *L. monocytogenes* or that point mutations could result in phenotypic shifts detectable by serodiagnosis.

Lineages I and II correspond to the primary divisions of *L. monocytogenes* uncovered by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis, whereas lineage III represents a distinct taxonomic unit that has been proposed to represent at least one new subspecies (15, 21). Our results are consistent with previous studies in grouping different *L. monocytogenes* serotypes into lineages I and II and further confirm that these two lineages represent distinct subgroups. Serotype 4a is unique to lineage III, and serotype 4c occurred only once outside lineage III. Our findings corroborate the conclusions of others that lineage III strains represent a distinct subset of *L. monocytogenes* (13, 17). Furthermore, previous studies have shown that lineage III strains are isolated significantly less frequently from humans (0.8%) than from animals (10.5%), indicating that lineage III may rarely cause human disease (10, 21). Other studies have also indicated that only 0 to 2% of human cases are caused by lineage III (12, 21) or serotype 4a and 4c strains (7). Similar findings led to the definition of *Listeria* serotype 5 strains (which predominantly causes disease in animals) as a new species, *L. ivanovii*, in 1985 (16). Further analyses, including total genomic DNA-DNA homology studies, are required, however, to clarify the taxonomic status of lineage III.

Lineage I isolates characterized as *Eco*RI ribotypes DUP-1042, DUP-1052, DUP-1024, and DUP-1044 contained more than one serotype. Also, two lineage II ribotypes (DUP-1030 and DUP-1039) contained multiple serotypes (1/2a, 1/2c, and 3a). Two of the lineage III ribotypes (DUP-1061 and DUP-1059) accommodated multiple serotypes (4a and 4c). Overall, we found four distinct genetic groups (two lineage II ribotypes and two lineage III ribotypes), each of which contains both a and c flagellar antigen groups. This observation indicates the importance of considering the distribution of single antigens among genetic subtypes or lineages, in addition to serotypes as a whole. The fact that both a and c flagellar antigens are present within closely related genetic groups possibly indicates that a single genetic event or, less likely, horizontal gene transfer could lead to conversion from a to c or vice versa. Currently, we have little knowledge of the molecular basis for the serotypes of *L. monocytogenes*. Understanding of the genetic determinants of flagellar and somatic antigenic groups would allow further probing of this issue.

Correlations between serotypes and *Pvu*II ribotypes. Eighteen *Eco*RI ribotypes appear to be predictive of a specific

a. *Pvu*II ribogroups for DUP-1042

RiboGroup		Serotype	RiboPrint(R) Pattern
NYSDOH/PVU11 106-1381-S-1		1/2b	
NYSDOH/PVU11 106-1381-S-1		1/2b	
NYSDOH/PVU11 106-1381-S-1	A	4b	
NYSDOH/PVU11 106-1381-S-1		4b	
NYSDOH/PVU11 106-1381-S-1		4b	
NYSDOH/PVU11 106-1381-S-1		4b	
NYSDOH/PVU11 121-735-S-6	B	4b	
NYSDOH/PVU11 116-400-S-2		1/2b	
NYSDOH/PVU11 116-400-S-2	C	1/2b	
NYSDOH/PVU11 121-735-S-3		1/2b	
NYSDOH/PVU11 106-1381-S-5		1/2b	
NYSDOH/PVU11 116-282-S-5	D	3c	

b. *Pvu*II ribogroups for DUP-1052

RiboGroup		Serotype	RiboPrint(R) Pattern
NYSDOH/PVU11 106-1381-S-1		1/2b	
NYSDOH/PVU11 106-1381-S-1	A	1/2b	
NYSDOH/PVU11 106-1381-S-1		1/2b	
NYSDOH/PVU11 106-1381-S-1		4b	
NYSDOH/PVU11 116-400-S-4	E	3b	

c. *Pvu*II ribogroups for DUP-1030

RiboGroup		Serotype	RiboPrint(R) Pattern
NYSDOH/PVU11 106-1381-S-2		1/2a	
NYSDOH/PVU11 106-1381-S-2		1/2a	
NYSDOH/PVU11 106-1381-S-2		1/2a	
NYSDOH/PVU11 106-1381-S-2	F	1/2a	
NYSDOH/PVU11 106-1381-S-2		1/2a	
NYSDOH/PVU11 106-1381-S-2		3a	
NYSDOH/PVU11 106-1381-S-7	G	1/2a	
NYSDOH/PVU11 106-1381-S-3		1/2c	
NYSDOH/PVU11 106-1381-S-3	H	1/2c	
NYSDOH/PVU11 106-1381-S-3		1/2c	

d. *Pvu*II ribogroups for DUP-1039

RiboGroup		Serotype	RiboPrint(R) Pattern
NYSDOH/PVU11 106-1381-S-2	F	1/2a	
NYSDOH/PVU11 106-1381-S-2		1/2a	
NYSDOH/PVU11 116-285-S-5	I	1/2a	
NYSDOH/PVU11 106-1381-S-3	H	1/2a	
NYSDOH/PVU11 106-1381-S-3		1/2c	

FIG. 1. *Pvu*II ribotype patterns found within *Eco*RI ribogroups, which contain multiple serotypes. (a) *Pvu*II ribogroups for DUP-1042 isolates. (b) *Pvu*II ribogroups for DUP-1052 isolates. (c) *Pvu*II ribogroups for DUP-1030 isolates. (d) *Pvu*II ribogroups for DUP-1039 isolates. The ribotype patterns shown represent normalized data, and gels were run from left to right. Ribotype patterns within each letter grouping (A to I) are considered the same.

serotype, while four *Eco*RI ribotypes in lineage I and two ribotypes each in lineages II and III contain multiple serotypes. *Pvu*II ribotyping has previously been shown to improve the discriminatory power of ribotype-based differentiation of *L. monocytogenes* strains over *Eco*RI ribotyping (6). Thus, a subset of isolates representative of *Eco*RI ribotypes that contained more than one serotype (32 isolates; DUP-1042, DUP-1052, DUP-1030, and DUP-1039) were further characterized by *Pvu*II ribotyping. These isolates gave rise to nine *Pvu*II patterns (A through I; Fig. 1), resulting in 12 combination types. The combination of *Eco*RI and *Pvu*II ribotypes improves serotype prediction, as only 4 of the 12 combination types contained multiple serotypes (DUP-1042/A, DUP-1052/A, DUP-1030/F, and DUP-1039/H). Specifically, within lineage I, DUP-1042 separated into four *Pvu*II ribogroups (A, B, C, and D) and DUP-1052 split into two groups (A and E; Fig. 1a and b). One *Pvu*II ribogroup (A) was common to both DUP-1042 and DUP-1052. Isolates of serotypes 3b and 3c each had unique

*Pvu*II ribogroup patterns, whereas *Pvu*II ribogroups generally did not differentiate serotypes 1/2b and 4b, with the exception of group B (serotype 4b) and group C (exclusively serotype 1/2b). Within lineage II, *Pvu*II ribotyping separated DUP-1030 and DUP-1039 into three groups each (F, G, and H and F, I, and H, respectively), two of which (F and H) were common to both (Fig. 1c and d). *Pvu*II ribotyping differentiated serotype 1/2a and 1/2c isolates, with the exception of one DUP-1039 serotype 1/2a isolate. Specifically, *Pvu*II types G and I contained only serotype 1/2a isolates, *Pvu*II type F contained five serotype 1/2a isolates and one 3a isolate, and *Pvu*II type H contained four serotype 1/2c isolates and one 1/2a isolate. Our results provide further evidence that ribotyping with two enzymes allows finer discrimination of strains and improved prediction of *L. monocytogenes* serotypes. Nevertheless, even when two restriction enzymes are used, a limited number of ribotypes still contain multiple serotypes.

Conclusion. Although both molecular subtyping methods and serotyping are valuable techniques for studying the epidemiology of bacterial pathogens, the connection between molecular subtypes and serotypes has yet to be defined for many organisms. Like listeriosis, outbreaks of cholera are caused primarily by specific serogroups of *Vibrio cholerae* and an analysis of 103 clinical *V. cholerae* strains yielded a low degree of correlation between ribotypes and serotypes (5). However, within group A *Streptococcus* isolates, serotypes correlate with ribotype patterns (with two restriction enzymes), although genetic heterogeneity has been demonstrated among certain *Streptococcus pyogenes* serotypes (4). Our results demonstrate the potential of ribotyping for the prediction of *L. monocytogenes* serotypes, although a small number of subtypes differentiated by *Eco*RI and *Pvu*II ribotyping contain more than one serotype. A complete understanding of the genetic determinants of flagellar and somatic antigenic groups is required to elucidate the complex relationships between subtyping methods and to provide further insight into the evolution of the serotypes of this food-borne pathogen.

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