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Mini-Review

Pulsed Field Gel Electrophoresis for Subtyping of *Listeria monocytogenes*

Sung Sik Jang*, Graham H. Fleet and Julian M. Cox

Food Science and Technology, School of Chemical Engineering and Industrial Chemistry, The University of New South Wales, Kensington, NSW, 2052, Australia

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***Listeria monocytogenes* is a high-risk foodborne pathogen responsible for foodborne listeriosis outbreaks, and is particularly dangerous to immuno-compromised people with mortality rate of about 30%. This review summarizes subtyping of *L. monocytogenes* using Pulsed Field Gel Electrophoresis, widely used to trace origin of foodborne outbreaks and to determine relationship between isolates.**

Key words: Listeria monocytogenes, *Listeriosis, Foodborne pathogenic bacteria, Subtyping, Pulsed Field Gel Electrophoresis*

Monitoring foodborne pathogenic bacteria through typing at the subspecies (strain) level is now an important part of the investigative processes, such as the epidemiological investigation of foodborne disease outbreaks or tracking of environmental sources of contamination.[1-3] Strain typing can be explained as "differentiation of bacterial and foodborne pathogens beyond the species level" or "determining the relationship between bacterial isolates." The terms "subtyping" and "fingerprinting" are used interchangeably with "strain typing."[2] The terms used in subtyping have been well defined by Tenover *et al.*,[4] and the concepts and methods of typing were recently reviewed.[1-3,5] In the present review, the methods used for typing the isolates of *Listeria monocytogenes* responsible for outbreaks of listeriosis are examined, with a particular focus on the use of pulsed field gel electrophoresis (PFGE).

Listeria monocytogenes

L. monocytogenes is a bacterial genus responsible for several outbreaks of foodborne listeriosis.[6-9] The mortality rate of listeriosis is about 30%, typically causing meningitis, still birth, abortion and septicemia.[6,7,10-13] In the United States, *L. monocytogenes* infections show the highest hospitalization rate (90%) among all foodborne pathogens, responsible for 30% of all reported deaths by foodborne illnesses[14]. The foods generally implicated in the outbreaks of listeriosis are milk, soft cheese, meat products, smoked sea foods, Pâté, and vegetable and fruit salads.[6,15-18]. Therefore, several countries have introduced legislative standards governing the presence of *L. monocytogenes* in foods.

Strain Typing Methods

Typing methods can be categorized into the more traditional or conventional phenotypic methods and the newer genotypic methods. The former are based on the expression of one or more characteristics, and include biotyping, serotyping, phage typing, and antibiotic resistance testing, while the latter involve direct analysis of chromosomal or extrachromosomal genetic materials.

As discussed by Farber[1] and Farber *et al.*,[5] conventional methods using phenotypic markers have several limitations, including unavailability to all bacterial species, irreproducibility due to unstable expression of markers under some environmental or culture conditions, difficulty in standardization, and lack of discriminatory power. On the other hand, molecular typing methods, based on DNA analysis, are more reproducible, have increased discriminatory power, are easier to interpret, and may provide speed of performance, technical simplicity, prospects for statistical data analysis, and ease of automation.[1,19]

Molecular Strain Typing Methods and *Listeria* species

Many molecular typing methods have been applied to the isolates of *Listeria* species, and the features of those most commonly used are summarized in Table 1. Comparative studies using a number of these typing methods have also been conducted.[20-23] The principles of each method except ribotyping and PFGE are not covered in this review. Instead, readers are referred to the recent reviews for an explanation of each method.[2,5] Plasmid typing, Restriction Endonuclease Analysis (REA), and Random Amplification of polymorphic DNA (RAPD) are quicker and easier to perform than other methods, while ribotyping and PFGE are more tedious and time-consuming. However, PFGE is considered to be more reproducible and have better discriminatory power.[1]

*Current address of Corresponding author: Centre for Agricultural Biomaterials, Seoul National University, San 56-1, Shillim-dong, Kwanak-gu, Seoul 151-921, Korea
Phone: +82-2-880-4890; Fax: +82-2-873-5260
E-mail: sungsjang@yahoo.co.kr

Abbreviations: PFGE, Pulsed Field Gel Electrophoresis; REA, Restriction Endonuclease Analysis; RAPD, Random Amplification of Polymorphic DNA; PMSF, Phenylmethylsulfonyl fluoride

Table 1. Advantages and disadvantages of various genotypic typing methods for *Listeria monocytogenes*

Method	Advantages	Disadvantages	Sample reference
Plasmid typing	Relatively quick and easy method Results can be standardized using known markers	Plasmids usually unstable Some organisms contain few or no plasmids Different plasmids can appear to be the same size	50-52)
Chromosomal DNA Restriction Endonuclease Analysis (REA)	Universally applicable Rapid, inexpensive, and relatively easy to perform	Genomic restriction fragments are usually too numerous and too closely spaced. Have to screen a number of restriction endonucleases (RE)	22,38)
Ribotyping	Single probe can be used to subtype all eubacteria Reproducible patterns obtained with reasonable number of fragments after probing Can be automated	A little more tedious and time-consuming (multiple steps) than other molecular typing methods Not as discriminating as some of the newer molecular methods (rrn operons cover only about 0.1 of chromosomal DNA) May not be useful for some bacteria that contain only 1 or 2 rrn loci	20,21,28-30,32,33,53)
Pulsed-Field Gel Electrophoresis (PFGE)	A tool for both taxonomic and epidemiological studies Highly reproducible and discriminatory Produces around 10-15 easily visible bands	More tedious and time-consuming than other molecular typing methods Restriction enzymes tend to be expensive Cost of equipment	20,34,39,43,45,50,54,55)
Random amplification of polymorphic DNA (RAPD)	Rapid, easy to perform Does not require isotopic labelling nor use of restriction endonucleases Does not require prior knowledge of DNA sequence Oligo-primers can be made in unlimited amounts anywhere, so can develop standard RAPD typing procedure without need for exchanging research materials	Reproducibility, if not well standardized Only looking at possible difference from a small % of total genome when using 1 primer Comparison of different intensity bands of the same size may be problematic	20,22,23,34,40,56,57)

Adapted from Farber.[1)]

PFGE has been widely adopted for differentiating the isolates of *L. monocytogenes*. Standard protocols for profiling *L. monocytogenes* isolates by PFGE have been developed and used in the USA as part of PulseNet, a network of public health laboratories that generate and share PFGE profiles, facilitating the investigation of disease outbreaks, particularly when multiple States are involved.[24, 60)]

Ribotyping

Ribotyping involves restriction enzyme digestion of chromosomal DNA, electrophoretic separation and transfer of fragments from gel onto a nitrocellulose or nylon membrane,[25)] and hybridization with an appropriately labelled 16+23S ribosomal RNA (rRNA) or rDNA probe.[26)] Generally, *Escherichia coli* rRNA[26)] or a cloned ribosomal operon (*rrn*B) of *E. coli*[27)] is used to probe *L. monocytogenes*. Ribotyping has been used widely to subtype *L. monocytogenes*,[20,21,28-35)] and *Eco*RI is generally used as the restriction endonuclease.

The Riboprinter (Qualicon, Wilmington, DE, USA), an automated ribotyping system, which can generate, analyze, and store riboprint patterns of bacteria, has also been used to type *Listeria*.[28,29,32)] More information on ribotyping can be found [1,21,30,33)].

Principles of Pulsed Field Gel Electrophoresis (PFGE)

This section describes the basic principles involved in the performance of PFGE, and its applicability to isolate *Listeria* species. The subsequent section examines the use of PFGE analysis of *Listeria* species in investigative processes.

The PFGE procedure for profiling of bacterial isolates is outlined schematically in Fig. 1.[60)] Bacterial cells, cultured under controlled conditions, are sedimented by centrifugation and suspended in low-melting agarose gel to avoid shearing the chromosomal DNA. They are then incubated with a lysing reagent to release DNA, and treated with proteinase K to digest the protein. Subsequently, phenylmethylsulfonyl fluoride (PMSF) is used to further inhibit the proteolytic

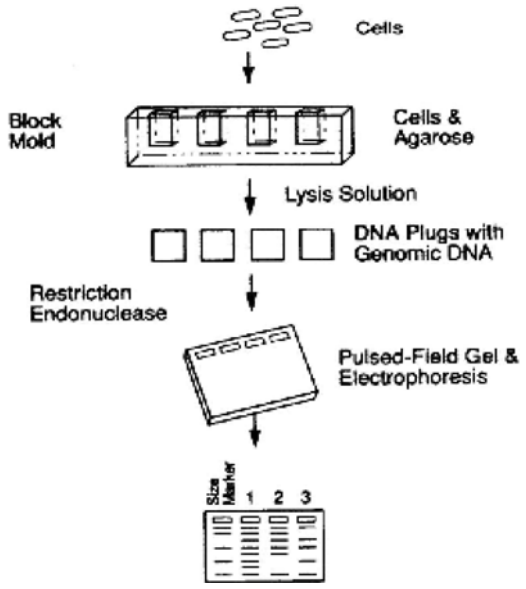

Fig. 1. Schematic illustration of Pulsed Field Gel Electrophoresis.

activity. The whole genomic DNA is then digested with one or more specific restriction endonucleases, and the DNA fragments produced are subjected to gel electrophoresis using an alternating 'zig-zag' or pulsed electric field, then visualised by staining, typically with ethidium bromide.

Bacterial typing by PFGE was developed to overcome the drawbacks of restriction enzyme analysis (microrestriction analysis) that produces hundreds of differently sized DNA fragments, which are difficult, if not impossible, to interpret after conventional, unidirectional gel electrophoresis. PFGE exploits restriction enzymes that recognize long sequences of bases and thus cut DNA infrequently, produce fewer (5-20) but larger (10-800kb) DNA fragments. While these large fragments cannot be resolved under conventional electrophoretic conditions, they can be clearly resolved and interpreted using pulsed electric fields. Many factors such as electric field strength, field angle and shape, agarose type and concentration, pulse time, ionic strength, and temperature are known to influence the resolution of these large-sized DNA fragments and thus need to be properly controlled.[1)]

For the differentiation of *Listeria* isolates, restriction enzymes such as *Apa*I,[18,22,23)] *Asc*I,[36)] and *Sma*I[37)] have been singularly used most often. However, a combination of two different enzymes such as *Apa*I + *Sma*I,[21,38-43)] *Apa*I + *Asc*I,[24,44,45)] *Asc*I + *Sma*I,[46)] and *Apa*I + *Not*I[41,47)] also yield acceptable results.

Consideration need to be given to the selection of enzyme and interpretation of results. Some specific enzymes do not generate readily discernable macrorestriction fragments in *Listeria*. Carriere *et al.*[47)] reported that *Apa*I and *Not*I did not digest DNA of many isolates of *Listeria* serovar 1/2c. Brosh *et al.*[44)] similarly noted that *Apa*I did not digest DNA of *Listeria* serovar 4c isolates. However, *Asc*I and *Apa*I are generally considered to be the most useful enzymes for DNA profiling of *Listeria* species.[36,44)]

Table 2. Criteria for interpreting PFGE profiles

Category	Number of genetic differences	Typical number of fragment differences
Indistinguishable	0	0
Closely related	1	2-3
Possibly related	2	4-6
Unrelated	≥3	≥7

Adapted from Tenover *et al.*[4)]

Some variations have also been observed in the genome size among different serotypes of *L. monocytogenes*. Carriere *et al.*[47)] demonstrated DNA polymorphism among 35 reference and field strains of *L. monocytogenes* which correlated with serotype. Calculation based on the sum of fragments showed the genome sizes of serovar 1/2a, 1/2b, 4b, and 1/2c strains to be about 2,660, 2,640, 2,710, and 2,340 kb, respectively. Serovar 1/2c was different from other serovars with respect to absence of restriction fragment length polymorphism and a chromosome that is 15% shorter.

As with many organisms, PFGE-based restriction analyses of *Listeria* species normally produce about 5 to 20 DNA fragments; however, even the same strain may produce slightly different DNA fragment profiles according to environmental or culture conditions. Tenover *et al.*[4)] indicated minor changes in profiles may readily occur through point mutation, resulting in loss of a restriction site, insertion of DNA into an existing restriction fragment, deletion of chromosomal DNA segments or loss or acquisition of extrachromosomal elements. Consequently, some minor variations in band profile should be taken into consideration when assessing strain relationships. A well-accepted basis for categorization of strains into "Indistinguishable," "Closely related," "Possibly related," and "Unrelated" has been suggested (Table 2).

Strain Diversity and Epidemiological Tracking using PFGE

PFGE has been used to examine strain diversity among samples of numerous food products as well as environmental samples.[23,34,48)]

The results of studies on *L. monocytogenes* and other *Listeria* species from different sources, using different restriction enzymes, are summarized in Table 3. The degree of diversity (%, number of found pulsotypes/number of tested isolates) ranges from 5[33)] to 86%[44)] depending on the source of *Listeria* isolates. In addition, although no conclusive trend could be observed, the degree of diversity within the isolate was also different according to the restriction enzymes used.

Autio *et al.*,[45)] upon investigation of the diversity among 295 *L. monocytogenes* isolates from various food items supplied by 41 producers from 10 countries using PFGE, found 66 different pulsotypes. Ten pulsotypes were common to two or more product types, and 17 were detected in foods from

Table 3. Diversity of *Listeria monocytogenes* and other *Listeria* species in foods, human, and environment analyzed by Pulsed Field Gel Electrophoresis

Species	%[a]	Number of isolates	Restriction enzyme - No. of pulsotypes	Source	Reference
L. monocytogenes	8.6 5	486	*Asc*I-42, *Sma*I-24	Food processing plant	33)
L. monocytogenes	30 29	90	*Apa*I-26, *Sma*I-27	Chicken, Salami, Sausage, Human, Würstel	58)
L. monocytogenes	20 16	295	*Apa*I-47, *Asc*I-61	Meat, Dairy, Fish, Poultry, Vegetable	45)
L. monocytogenes	20 18	153	*Apa*I-30, *Sma*I-28	Sheep, Cattle, Foodstuff, Human	49)
L. monocytogenes	5 11	155	*Apa*I-7, *Sma*I-17	Cold-smoked Salmon & Processing plants	39)
L. monocytogenes	60	5	*Apa*I-3	Live pigs, Slaughter house	59)
L. monocytogenes	9 10	131	*Apa*I-14, *Sma*I-12	Meat and Processing plant	54)
L. monocytogenes	2 3	303	*Asc*I-9, *Sma*I-7	Cold-smoked Rainbow Trout & Processing Plant	46)
L. monocytogenes	6	287	*Apa*I-17	Pork & Slaughtering house	22)
L. monocytogenes	15 20 17	41	*Apa*I-6, *Asc*I-8, *Sma*I-7	Ice cream plant	55)
L. monocytogenes	58 63 63 50	40	*Apa*I-23, *Asc*I-25, *Sma*I-25, *Sse8387*I-20	Beef, Human, Salmon, Cheese, Raw milk, Pork	48)
L. monocytogenes	30 30	10	*Apa*I-3, *Sma*I-3	Shrimp, Cheese, Fruitcake, Human	40)
L. monocytogenes	43 51	51	*Apa*I-22, *Sma*I-26	Human	21)
L. monocytogenes	71	45	*Asc*I-32	Raw milk, Foods	43)
L. monocytogenes	36 41	176	*Asc*I-63, *Apa*I-72	Human, Animal food, Environment	44)
L. monocytogenes	38 17 40	42	*Apa*I-16, *Not*I-7, *Sma*I-17	Human, Animal, Environment, Foods	41)
L. innocua	8 12	131	*Apa*I-15, *Sma*I-11	Meat and Processing plant	54)
L. welshimeri	5 5	131	*Apa*I-6 *Sma*I-6	Meat and Processing plant	54)
L. ivanovii	14 9	43	*Apa*I-6, *Sma*I-4	Food, Human, Animal	42)
Non- *L. monocytogenes*	82 86	22	*Apa*I-19, *Asc*I-18	Human, Animal food, Environment	44)

[a]: Number of pulsotypes/number of tested isolates

producers, who had no apparent association. Furthermore, the recurrence of some pulsotypes from the same product of the same producer was reported, suggesting the persistence of some strains in the production environment. This highlights the need to consider genotypic information in association with epidemiological evidence before attempting to establish anything but broad evolutionary relationships among strains. In Spain, Vela *et al.*[49] demonstrated substantial genetic diversity among the Spanish *L. monocytogenes* isolates, finding 55 pulsotypes among 153 isolates. Ramage *et al.*[42] surveyed the distribution of *L. ivanovii* in UK. They obtained only six different DNA profiles, and the majority of isolates (38 out of 45) belonged to one profile, suggesting that this species may be genetically homogeneous. Nakama *et al.*[48] used PFGE to examine 40 isolates of *L. monocytogenes* isolated from shredded cheese, the environment of the two cheese-producing factories, and other sources in Japan. Nine isolates from shredded cheese of different lots and four isolates from the cheese-processing environment were found to have the same genotype, which suggested that spreading of *Listeria* in cheese occurred through the cross-contamination from the cheese-processing environment.

Many examples of the use of PFGE to trace the origin of episodes of listeriosis have been reported.[37,40,49] Moore and Datta[37] analysed the DNA fragment profiles of isolates from two outbreaks and one sporadic case of listeriosis, and distinctive DNA fragment profiles were obtained from the strains of major foodborne disease serotypes 1/2a, 1/2b, and 4b. They also reported that, within a given episode, close similarity was found among isolates, and that distinct difference among strains from separate episodes could be demonstrated.

Another application of this technique is to trace the origin of continuous contamination in food production plants. Dauphin *et al.*[39] investigated three cold-smoked salmon processing plants. One strain of *L. monocytogenes*, which predominated and persisted in plant I, was found. In plant II, no *L. monocytogenes* was found in the smoked skinned salmon. On the other hand, in plant III, all smoked salmon samples were contaminated with a unique strain of *L. monocytogenes* that originated from the slicing process. Therefore, it was concluded that the contamination of final products originated from the processing environment and not from the bacterium found on raw salmons. Similarly, Autio *et al.*[46] investigated a cold-smoked rainbow trout processing plant and confirmed that *L. monocytogenes* on the final product was not from raw fish but from the processing areas (brining and slicing). Giovannacci *et al.*[22] collected 287 *L. monocytogenes* isolates from five French pork slaughtering and cutting plants and found the presence of closely related *L. monocytogenes* strains over a 1-year period in the environments of two plants, even after cleaning and disinfection procedures. In accordance with this result, they suggested some *L. monocytogenes* strains possibly persist in pork processing environments and questioned the efficiency of cleaning and disinfection procedures in pork slaughterhouses, particularly in the chilling and cutting rooms.

PFGE has also been utilized to compare the isolates of *L. monocytogenes* obtained by different detection methods. Loncarevic *et al.*[38] demonstrated that direct plating of food samples onto an agar medium could recover a greater diversity of strains/pulsotypes from foods than methods based on selective enrichment followed by plating. Some strains might not grow in the presence of certain selective agents, and this could lead to an underestimation of the presence of *L. monocytogenes* in some foods.

Standardization of PFGE Protocols

PFGE has been used to elucidate the relationship between *Listeria* isolates from various sources. Comparison of data from many different laboratories is difficult, because the analytical protocols have not been standardized. This is critical if typing is to play a role in the investigation of widespread foodborne disease outbreaks. The development of a highly standardized PFGE protocol by the US Centers for Disease Control and Prevention allowed the establishment of PulseNet, a nationwide alert system for *L. monocytogenes*, using pulsotype information obtained from many laboratories around the nation.[24]

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