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Molecular Typing of *Listeria monocytogenes* Isolated from Different Sources by Pulsed-Field Gel Electrophoresis

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A total of 30 *L. monocytogenes* strains from different sources including 13 strains isolated from the foreign imported meat were genotyped in order to establish their genetic relatedness and to compare them with the foreign isolates. PFGE analysis of genomic DNA showed the 11~16 fragments ranging in size from 38 to 504 kb. Eleven different PFGE types (1~11) were identified in the dendrogram at 75% similarity, and the two major PFGE types, type 1 and 2, contained 94% of domestic isolates (16/17). All isolates from domestic beef and pork carcass were grouped in each different type, however, isolates from chicken were clustered together with those from pork and beef. We also found all foreign strains were unrelated with each other, regardless of geographic criteria and that they could be differentiated from those from the domestic isolates by PFGE pattern. The PFGE pattern of one isolate from chicken wing, which the chicken meat was found to be imported from foreign country, was closely related to that of isolate from the Thailand.

Key Words: Genotyping, *Listeria monocytogenes*, Pulsed-field gel electrophoresis (PFGE)

INTRODUCTION

Foodborne disease has emerged as an important and growing public health problem in many countries during the past few decades. Among them, gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganism, including pathogens such as *Salmonella*, *Campylobacter jejuni*, enterohaemorrhagic *Escherichia coli*, *Listeria monocytogenes* and parasites (D'Aoust, 1994; Maslow et al., 1993). Approximately 1.8 million children in developing countries (excluding China) died from diarrheal disease in 1998, caused by microbiological agents, mostly originating from food and water. One person in three in industrialized countries may be affected by foodborne illness each year (Rocourt et al., 2003). In Korea, some 25 thousand persons of foodborne illness, are estimated to occur for the last 4 years (KFDA, 2002).

L. monocytogenes is a Gram-positive, nonspore-forming foodborne bacterium that can cause severe invasive disease manifestations, including abortion, septicemia, and meningitis (Salamina et al., 1996; Dalton et al., 1997). It occurs widely in both the agricultural and food processing environment. Although frequently present in raw foods of both food processing plant and animal origin, it also can be isolated in such foods due to post-processing contamination. There is evidence to suggest that it is a transitory resident of the intestinal tract in humans, with 2 to 10% of general population being carriers of the organism without any apparent health consequences. Various studies indicate that from 1 to 5% of common ready-to-eat foods may contain *L. monocytogenes*, and these foods may be widely distributed as a current marketing and distribution practices (Soriano et al., 2001). Traditional epidemiological surveillance alone may not detect many common source outbreaks, particularly if a limited number of cases occur over a wide geographic area because of the unique characteristics of foodborne listeriosis.

Subtyping methods for *L. monocytogenes* include phenotypic (e.g., serotyping and phage-typing) as well as different DNA-based molecular subtyping methods (Sauders et al., 2003). Such a typing scheme would help to trace epidemiological investigation and control the organism in

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the food production system. But serotyping could not discriminate well among strains because the three serotypes represent over 90% of human isolates. Also, phage-typing suffers from biological variability. More recently, molecular subtyping methods include multilocus enzyme electrophoresis (MLEE) (Norrung et al., 1993), polymerase chain reaction (PCR) (Salcedo et al., 2003), amplified fragment length polymorphism (AFLP) (Autio et al., 2003), automated ribotyping (Gendel et al., 2000) and pulsed field gel electrophoresis (PFGE) (Graves et al., 2001). This wide spread use of molecular typing has resulted in a plethora of techniques and protocols for subtyping even the same species of bacteria. Because each laboratory uses its own protocols for molecular typing and designations of patterns, the results cannot be compared with those of another laboratory, even if both laboratories have used essentially the same methods. This lack of comparability has greatly diminished the power of molecular subtyping methods.

Recent developments in PulseNet's PFGE protocols address some of these needs. PulseNet is a national network of public health and food regulatory laboratories in the US that perform standardized PFGE subtyping of bacteria that may be causative agents of foodborne disease (Graves et al., 2001; Swaminathan et al., 2001). PulseNet laboratories are able to rapidly compare PFGE patterns with national electric database of PFGE patterns maintained at the Centers for Disease Control and Prevention (CDC). Current PulseNet protocols for PFGE subtyping of *Escherichia coli* O157:H7, *Salmonella* spp. and *Shigella* spp. are based on a recently developed protocol (Gautom, 1997).

PFGE involves the use of rare-cutter restriction enzymes to generate a limited number of high molecular-weight restriction fragments. These fragments are then separated by agarose gel electrophoresis with programmed variations in both the direction and the duration of the electric pulsed field. The Korean Food & Drug Administration (KFDA) is also now implementing an active foodborne disease surveillance program. Its objectives were to estimate the burden of foodborne disease in the country, investigate the sources of infection in outbreaks, and build public health infrastructure for dealing with emerging foodborne disease issues.

Several epidemiological and molecular studies of human and animal listeriosis have been published during the last decade overseas (Miettinen et al., 1999; Vela et al., 2001; Autio et al., 2003; Wagner et al., 2003). However, few studies on genetic characterization of *L. monocytogenes* have been reported in Korea. The objective of this study was to describe the genetic relatedness of 30 *L. monocytogenes* isolates from different sources by PFGE and to provide the preliminary data to the PulseNet database to be built in this country.

MATERIALS AND METHODS

1. Bacterial strains

A total of 30 *L. monocytogenes* strains were used for this study. Among them 13 strains which isolated from the imported meat were included (Table 1). All strains were biochemically identified by using the commercial API system (bioMerieux, France).

2. Serotyping

Serotyping of *L. monocytogenes* isolates was performed according to the serotyping scheme of Seeliger and Hohne (1979). Commercial *Listeria* O antisera poly, O antisera 1 and 4 (Difco, USA) were used according to the manufacturer's instructions.

3. PFGE

Intact genomic DNA from *L. monocytogenes* isolates was prepared in agarose plugs using a CHEF bacterial genomic DNA plug kit (Bio-Rad, USA) with some modifications. Cells grown on Brain heart infusion agar plates were suspended in cell suspension buffer, and the cell density was adjusted to within a range of 7% T using colorimeter (bioMerieux, France). An equal volume of molten 1.2% w/v SeaKem Gold agarose (FMC bioproducts, USA) was mixed with 100 μl of bacterial cells, and the mixture was dispensed into 1.5-mm thick disposable molds. The agarose plugs were incubated in tubes containing lysozyme for 2 h at 37℃. The plugs were transferred to proteinase K after rinsing once with sterile water, and the tubes were incubated overnight at 50℃ without agitation. After proteolysis, the plugs were washed four times, 1 h each at room temperature with gentle agitation. Unless used immediately the plugs were stored at 4℃. Before digestion with restriction enzyme, the plugs were washed once again for 1 h. High-molecular-weight *L. monocytogenes* DNA in 1.5 mm plug slices were digested with *Apa*I (Roche Molecular Biochemicals, Swiss) in buffer solutions according to the

Table 1. Characteristics of 30 *L. monocytogenes* in this study

Isolate	Origin	Source	PFGE type	Serotype
B1/03	domestic	Beef	1c	4
B2/03	domestic	Beef	1c	4
B3/03	domestic	Beef	1c	4
B4/03	domestic	Beef	1c	4
B5/03	domestic	Beef	1c	4
B6/03	domestic	Beef	1b	4
B7/03	domestic	Beef	1c	4
B8/03	Austria	Beef	5b	4
B9/03	USA	Beef	8	4
B10/03	USA	Beef	8	4
P1/03	domestic	Pork	2c	4
P2/03	domestic	Pork	2b	4
P3/03	domestic	Pork	2b	4
P4/03	domestic	Pork	2c	4
P5/03	domestic	Pork	2d	4
P6/04	Belgium	Pork	5a	4
P7/01	Canada	Pork	6	4
P8/01	Denmark	Pork	10	4
P9/04	Finland	Pork	3b	4
P10/03	France	Pork	4	4
P11/03	France	Pork	4	4
P12/02	Hungary	Pork	11	4
P13/01	Netherlands	Pork	3a	4
C1/03	domestic	Chicken	2a	4
C2/03	domestic	Chicken	1a	4
C3/02	Thailand	Chicken	9	1
C4/02	Thailand	Chicken	9	1
M1/03	domestic	Chicken leg	2b	4
M2/03	domestic	Chicken breast	2e	4
M3/03	domestic	Chicken wing	7	4

manufacturer's instructions. Restriction was done at an enzyme concentration of 200 U per plug for 5 h at 30℃.

The samples were run on an 1% agaroge gel (Takara, Japan) in 0.5X TBE (0.5 M Tris, 0.1 M boric acid, 0.2 mM EDTA, pH 8.0) buffer with CHEF-mapper system (Bio-Rad, USA) under the following conditions: temperature 14℃; initial switch time, 0.1 s; final switch time, 40 s; run time, 23 h; angel 120℃; gradient, 6.0 V/cm; ramping factor, linear. Lambda ladder PFGE marker I was used as fragment size marker. After electrophoresis the gels were stained for 15 min in 250 ml of deionized water containing 25 μl of ethidium bromide (10 mg/ml) and destained by two washed of 20~30 min each using 500 ml of deionized water. A dendrogram was constructed with Analysis software (Biometra, Germany). The patterns were compared by means of the Dice coefficient of band-based similarity by unweighted pair group method using averages (UPGMA); a tolerance of 5% in the band position was applied. Any nonidentity in the presence, absence, or apparent mobility of a band was considered one difference from the pattern of the strain. Variation in band intensity was not counted as a difference.

RESULTS

Twenty eighty of 30 *L. monocytogenes* strains, except 2 isolates from Thailand which were serotyped as 4, were serotyped as 1. A total of 30 *L. monocytogenes* strains were analysed by PFGE after digestion with *Apa*I. PFGE analysis of genomic DNA showed the 11~16 fragments ranging in size from 38 to 504 kb. As depicted in Fig. 1, 11 different PFGE types (1~11) were identified in the dendrogram at 75% similarity, and two major PFGE type 1 and 2 predominated (16 strains, 53%). PFGE type 1 comprised 8 isolates (26.7%) and consisted of 3 different subtype, and PFGE type 2 comprised 8 isolates (26.7%) and consisted of 5 different subtype. Two and 7 PFGE types were represented by two and single isolates, respectively. All 13 isolates from 10 overseas countries were clustered into 8 PFGE types. Also, 16 of 17 domestic isolates were grouped together in the two major PFGE type 1 and 2. One isolate was clustered separately in type 7.

We defined the minimum size of a cluster as three (A-C) closely related PFGE types in order to determine whether the 17 domestic strains were clustered according to their sources from the beef, pork and chicken (Fig. 2). Cluster A contained 1 isolate from chicken wing. Its PFGE pattern was closely related to that from imported meat. Cluster B contained 8 isolates from 5 porks, 1 chicken, 1 chicken leg and 1 chicken breast. These two isolates from chicken processed meats showed an identical or very close PFGE patterns with strains from pork. Cluster C contained all 7 isolates from beef and 1 isolate from chicken.

Thirteen isolates from 10 foreign countries were genotyped in order to establish their genetic relatedness and to compare them with the domestic isolates. As shown in Table 1 and Fig. 3, 8 different PFGE types were clustered. Six PFGE types included 1 isolate each from Canada, Denmark, France, Hungary, Thailand and USA. Other 2 PFGE

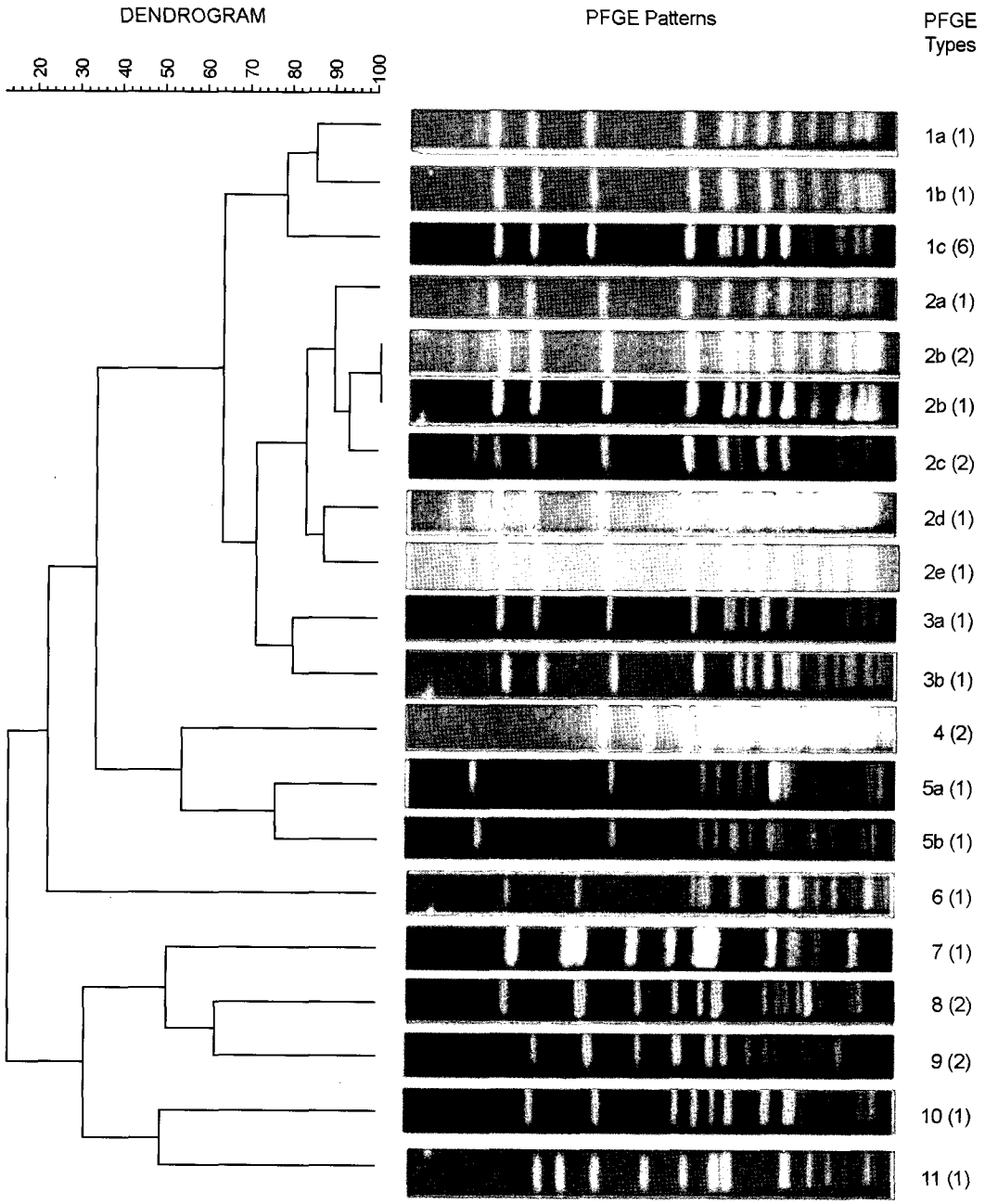

Fig. 1. Dendrogram and 11 representative PFGE types of 30 *L. monocytogenes* strains. Numbers in parenthesis indicate the numbers of strains belonging to a particular PFGE types.

types included 2 isolates each, from Netherlands and Finland, Belgium and Austria, respectively. One isolate from Canada (lane 2) was most unrelated with the others. Two isolates each from France, Thailand and USA showed an identical PFGE pattern, respectively.

In terms of PFGE types with different sources, 10 beef, 13 pork, and 4 chicken strains were characterized by 3, 8, and 2 different PFGE types, respectively (Fig. 4). All seven isolates from domestic beef carcass contained two fragments of 136 and 143 kb, which were not detected in any of other domestic strains, but detected in some of foreign strains. And, a size of 82 kb fragment was detected in all 3 foreign beef isolates, which were not detected in domestic beef isolates.

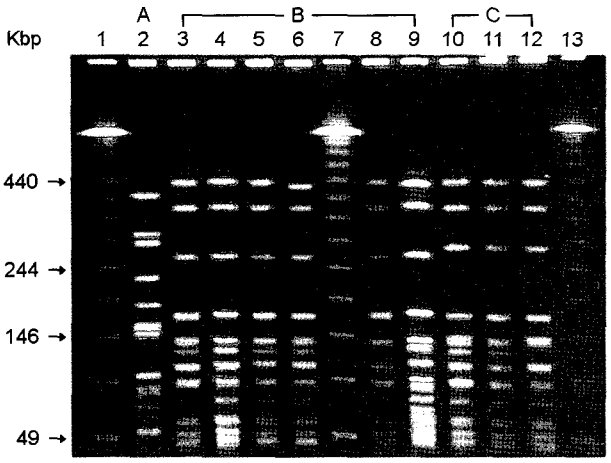

Fig. 2. PFGE profiles of 17 *L. monocytogenes* domestic isolates. Lane 1, 7 and 13, lambda ladder marker; Lane 2, M3/03; Lane 3, P1/03 (P4/03); Lane 4, M1/03; Lane 5, M2/03; Lane 6, P2/03 (P3/03); Lane 8, C1/03; Lane 9, P5/03; Lane 10, B1/03 (B2/03, B3/03, B4/03, B5/03, B7/03); Lane 11, C2/03; Lane 12, B6/03. Isolates in parenthesis indicate the same PFGE patterns. A, B and C indicate each cluster.

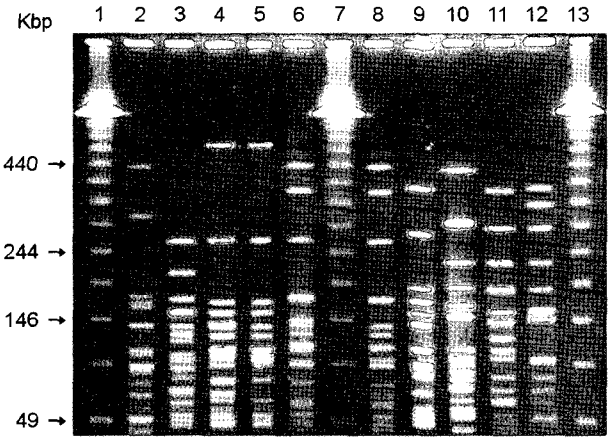

Fig. 3. PFGE profiles of 13 *L. monocytogenes* foreign isolates. Lane 1, 7 and 13, lambda ladder marker; Lane 2, P7/01 (Canada); Lane 3, P10/03 and P11/03 (France); Lane 4, P6/04 (Belgium); Lane 5, B8/03 (Austria); Lane 6, P13/01 (Netherlands); Lane 8, P9/04 (Finland); Lane 9, P8/01 (Denmark); Lane 10, B9/03 and B10/03 (USA); Lane 11, C3/02 and C4/02 (Thailand); Lane 12, P12/02 (Hungary). Name of country in parenthesis indicates the origin of isolate (s).

DISCUSSION

Although several methods have been used for typing *L. monocytogenes*, PFGE was used in this study because of its great discriminatory power (Aarnisalo et al., 2003). Restriction endonuclease *ApaI* was selected because other

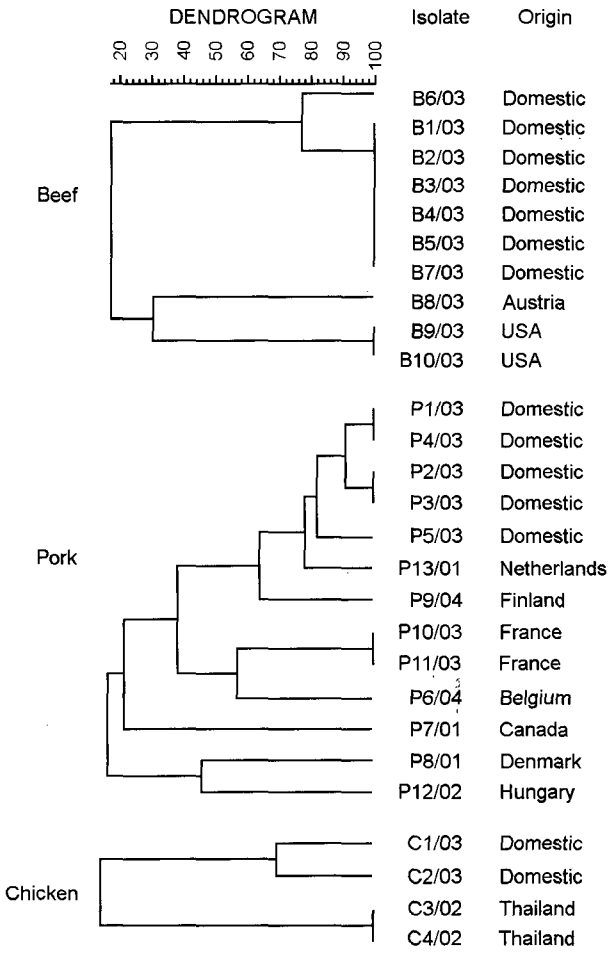

Fig. 4. Dendrogram of 27 *L. monocytogenes* isolates from different sources.

investigators have shown excellent discrimination for *L. monocytogenes* (Buchrieser et al., 1993; Broch et al., 1994) Macrorestriction patterns with 11~16 fragments ranging in size from 38 to 504 kb in this study were comparable to those with 12~17 fragments ranging in size from 20 to 557 kb by Vela et al. (2000), and with 11~16 fragments ranging in size from 50 to 500 kb by Nakama et al. (1998). This study also detected fragments size of more than 510 kb, which were unclear and unstable, so we did neglected them.

Serotyping was of lower discriminatory power simply owing to the limited 13 number of serovars on the basis of somatic and flagella antigens. However, it was sometimes used as a prerequisite for other subtyping methods. The majority of human listeriosis was usually caused by three serotypes, 1/2a, 1/2b and 4b. Brosch et al. (1994) reported a correlation of serotyping with molecular typing; binary division into group I (serotype 1/2b, 3b, 4b, 4d and 4e) and

group II (serotypes 1/2a, 3a, 1/2c and 3c) by PFGE. This study could not analyze a correlation between them in this study because 28 of 30 *L. monocytogenes* strains, except 2 isolates from Thailand chicken serotyped as 4, were serotyped as 1.

The two most common PFGE types, type 1 and 2, contained 94% of domestic isolates (16/17). Seven isolates from domestic beef and 1 of 2 isolates from chicken were grouped in type 1. Other 8 isolates including all 5 from domestic pork, 1 from chicken and 2 from chicken processed meat, were clustered in type 2. The fact that all isolates of beef and pork carcass were grouped in each different type implies that a genetically high homogeneous *L. monocytogenes* between animal source might exist at least in a province because the carcass samples were collected from the several different slaughter house. Interestingly, 2 isolate, 1 each from Netherlands and Finland pork, were genetically moderate related with the domestic isolates. The two countries belong to top ten countries where this country imports the pork meat annually. All these facts confirmed the close genetic relation of *L. monocytogenes* between the different source of the food chain and its spread through several countries. However, the result that 2 isolates from chicken were clustered together with those from pork and beef in this study agrees with previous report. Revazishvili et al. (2004) reported it was unlikely that a perfect division of clusters could be identified according to different sources since *L. monocytogenes* was acquired by the consumption of contaminated foods originated from diverse animals. Additional studies with more samples from different source, such as cattle, pig and chicken, would elucidate the genetic relation among different source.

Among the 3 isolates from chicken processed meats, 1 isolate from chicken leg showed an identical PFGE pattern with those from pork. As an epidemiological investigation of the chicken processing factory where the chicken foods were processed, we found that the factory also manufactured pork processed meat. This result raised the possibilities that the chicken processing factory was cross-contaminated with *L. monocytogenes* from between 2 sources or that a clone of *L. monocytogenes* might exist in the factory. The PFGE pattern of an isolate from chicken wing, which the chicken meat was found to be imported from foreign country, was closely related to that of isolate from the Thailand. The Thailand is one of the two countries where this country imports the chicken meat annually.

The 13 isolates from the imported meats of 10 foreign countries were clustered into 8 PFGE types, and the two isolates each from Thailand chicken, USA beef and France pork showed an identical pattern, respectively. A Canadian isolate showed the most heterogeneous genetic background among them, which was characterized by the three unique fragments of 164, 174 and 309 kb. That diverse PFGE types of *L. monocytogenes* are distributed across the border was confirmed in this study. We found all foreign strains were unrelated with each other, regardless of geographic criteria and that they could be differentiated from those from the domestic isolates by PFGE pattern. To facilitate tracing of this organism for international distribution across the world a nation and worldwide surveillance program is encouraged such as PulseNet.

Our finding that 3, 8 and 2 different PFGE patterns were characterized from 10 beef, 13 pork and even 4 chicken strains indicate a great diversity among the strains of *L. monocytogenes* studied. This is consistent with previous report for these species (Swaminathan et al., 2001). These differences, however, were moderately slight between strains from different source of domestic origin. Maslow et al. (1993) have reported that strains with a 1~2 band shift in PFGE could be considered as clonally related.

So, our study demonstrated that the isolates from domestic origin were genetically homogeneous and that isolates from foreign origins yielded low degree of genetic relations with those from domestic isolates. The presence of a diverse genetic background in several countries from strains associated with the human outbreak has been reported in previous studies (Dalton et al., 1997; Miettinen et al., 1999; Wagner et al., 2003). Further studies on the collection of human listeriosis associated strains and monitoring of their genetic lineage of *L. monocytogenes* combined with those from foodstuffs will be needed for veterinary and human public health in the future.

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