

Rapid Discrimination of *Listeria monocytogenes* Strains by Microtemperature Gradient Gel Electrophoresis

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Microtemperature gradient gel electrophoresis (μ -TGGE) was examined for use for the rapid subtyping of *Listeria monocytogenes* strains. Comparison of genomes between *L. monocytogenes* strains F2365 and H7858 identified a sequence encoding a portion of the PRT/PTS system IIA 2 protein domain as appropriate for μ -TGGE analysis. Thirty-one strains belonging to 10 different serovar types were tested by PCR, and sequence analysis of the amplified products revealed that the strains comprise 11 groups. All 55 possible pairs within the 11 groups were examined by μ -TGGE analysis. Of these, 47 pairs could be successfully discriminated, with a total electrophoresis time of only 7 min. Moreover, Cy3/Cy5 labeling allowed rapid identification of the sequence type in unknown strains of *L. monocytogenes* isolated from meat. These findings collectively indicate that μ -TGGE can be used for the rapid analysis of *L. monocytogenes* strains, facilitating determination of routes of contamination when these bacteria are found in food products.

Listeria monocytogenes is considered one of the most dangerous food-borne pathogens because it causes approximately 2,500 cases of listeriosis per year in the United States, with a mortality rate of ~20% (22). Listeriosis outbreaks have been associated with *L. monocytogenes* contamination of foods such as coleslaw, cheese, and milk (8). Therefore, it is critical for food producers and administrators to be able to quickly trace the route of contamination when *L. monocytogenes* is detected in the final food product (42). Such field tests, however, can be complicated by the existence of *L. monocytogenes* in the natural environment.

L. monocytogenes strains comprise 13 serovars grouped into three lineages (lineages I, II, and III) (14, 31, 43). Among these, serovars 1/2a, 1/2b, and, especially, 4b are implicated in many cases of human listeriosis (38). Various molecular typing methods beyond serovar typing have been investigated, including ribotyping (26), pulsed-field gel electrophoresis (PFGE) (34, 44), randomly amplified polymorphic DNA (RAPD) analysis (21), PCR-electrophoresis-based methods (15, 36), microarrays (4), esterase typing (9), amplified fragment length polymorphism analysis (1, 17), and multilocus enzyme electrophoresis (11). Among these, ribotyping and PFGE have better discriminatory powers and have been widely used (16). Recently, sequence typing (ST) and its extension, multilocus sequence typing (MLST), have been applied for analysis of *L. monocytogenes* (5, 23, 29, 32, 33, 45). These methods have the benefit of delivering relatively standardized data, allowing easy comparisons between laboratories or against databases. In addition, MLST has been shown to be superior to ribotyping and PFGE in terms of discrimination power (32, 45). Unfortunately, ST and MLST are relatively laborious and time-consuming for analyses of large numbers of samples, limiting their usefulness in larger contexts.

Temperature gradient gel electrophoresis (TGGE) and de-

naturing gradient gel electrophoresis (DGGE) are methods for the effective separation of DNA fragments having the same length but different sequences. These methods are based on variances in the electrophoretic mobilities of the partially melted DNA molecules (24, 25, 27, 30). When a DNA fragment reaches its melting temperature (T_m) in TGGE or its melting point in a gel containing a linear gradient of DNA denaturants in DGGE, the fragment rapidly melts and its migration in the gel is slowed. Many studies have used these methods to analyze microbiological populations based on differences in amplified regions of the 16S rRNA gene (24, 25). They have also been used to discriminate *L. monocytogenes* from other *Listeria* species, such as *Listeria innocua* (6, 20). Although TGGE/DGGE has adequate discrimination power and is reproducible, it is time-consuming, generally requiring ~4 h per run. Recently, Biyani and Nishigaki developed a miniaturized version of the TGGE apparatus, called micro-TGGE (μ -TGGE) (2, 3), that dramatically shortens the analysis time without a loss in data quality (2).

Here, μ -TGGE analysis was applied to the discrimination and general identification of *L. monocytogenes* strains. The PCR-amplified region was selected by comparison of the genomes of *L. monocytogenes* serovar 4b strains F2365 and H7858; it was able to separate 31 strains into 11 groups. Of 55 possible pairs, as many as 47 could be distinguished by μ -TGGE analysis in less than 10 min. Moreover, four groups that showed adequate separation were selected and PCR amplified with a Cy5-labeled primer. Rapid identification of the sequence type could be done by mixing them with Cy3-labeled PCR products from strains isolated from food, examining them by μ -TGGE analysis, and investigating the overlap patterns of the profile allowed. These findings suggest that this procedure could be effective for elucidation of contamination routes during outbreaks of food poisoning.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and isolation of *L. monocytogenes*. The 31 strains of *L. monocytogenes* used in this study are listed in Table 1. In addition to

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TABLE 1. *L. monocytogenes* strains used in this study

Strain ^a	Origin	Serotype	Group ^d
RIMD 1205022	Human	1/2a	D
ATCC BAA - 679	Rabbit	1/2a	D
ATCC 51772	Cheese	1/2a	D
ATCC 51775	Cheese	1/2a	F
ATCC 35152	Guinea pig	1/2a	H
ATCC 51773	Cheese	1/2a	H
ATCC 19111	Poultry	1/2a ^c	H
ATCC 15313	Rabbit	1/2a	H
ATCC 51774	Human	1/2a	I
ATCC 51780	Cheese	1/2b ^c	B
ATCC BAA - 751	NA ^e	1/2b ^c	C
RIMD 1205001	NA	1/2c	D
RIMD 1205020	Human	1/2c	D
ATCC 51779	Cheese	1/2c	D
ATCC 19112	Human	1/2c	D
ATCC 51782	Cheese	3a	G
ATCC 19113	Human	3a	H
ATCC 19114	Sheep	4a	J
RIMD 1205023	Human	4b	A
RIMD 1205024	Human	4b	A
ATCC 13932	Human	4b	A
ATCC 51776	Cheese	4b	A
ATCC 19115	Human	4b	A
ATCC 51777	Cheese	4b	A
ATCC 51778	Cheese	4b	C
RIMD 1205005	NA	4b	E
RIMD 1205021	Human	4b	E
ATCC 19116	Chicken	4c	K
ATCC 19117	Sheep	4d	K
ATCC 19118	Chicken	4e	A
NCTC 10890	Faeces	7	C
STC 1	Chicken liver	1/2b ^c	A
STC 4	Chicken liver	1/2b ^c	A
STC 7	Chicken liver	1/2b ^c	A
STC 9	Chicken leg	1/2a ^c	D
STC 10	Chicken leg	1/2a ^c	D
STC 11	Chicken leg	1/2b ^c	A
STC 12	Chicken leg	1/2a ^c	D
STC 14	Chicken leg	1/2a ^c	D
STC 16	Chicken leg	1/2a ^c	D
STC h-1	Pork minced	1/2b ^c	A
STC h-2	Pork minced	1/2b ^c	A
STC h-3	Pork minced	1/2b ^c	A
EGD-e ^b	Rabbit	1/2a	D
F6854 ^b	Frankfurter	1/2a	F
F2365 ^b	Cheese	4b	A
H7858 ^b	Frankfurter	4b	K

^a RIMD, Research Institute for Microbial Diseases, Osaka University; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; STC, isolated in this study.

^b Not experimental.

^c Serovar determination was performed in this study.

^d Group was defined according to the sequence type of the PCR-amplified products.

^e NA, information not available.

L. monocytogenes strains, *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. grayi* ATCC 25401, *L. seeligeri* ATCC 35967, and *L. welshimeri* ATCC 35897 were also used. All bacteria were grown in brain heart infusion (Oxoid, Hampshire, United Kingdom) agar or broth at 37°C. Isolation of *L. monocytogenes* from food was performed essentially as described previously (7). Briefly, meat samples were incubated in 225 ml of half-strength Fraser broth (Oxoid) for 24 h at 30°C. Next, 0.1 ml of the culture broth was mixed with 10 ml of full-strength Fraser broth, and the mixture was incubated for 24 h at 37°C. Finally, a 0.1-ml sample of this secondary culture was streaked onto CHROMagar Listeria (CHROMagar Microbiology, Paris, France) and incubated for 24 to 48 h at 37°C. Blue colonies with white halos were considered putative colonies of *L. monocytogenes*. *L. monocytogenes* was identified by morphological, cultural, and biochemical anal-

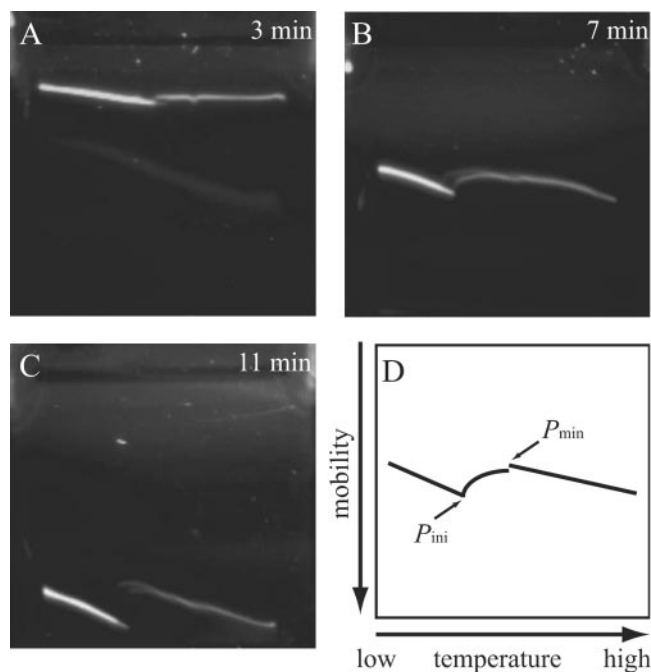


FIG. 1. μ -TGGE analysis of mixed PCR products of *L. monocytogenes* strains RIMD 1205005 (group E) and RIMD 1205023 (group A) (A to C). The electrophoresis was performed for 3 min (A), 7 min (B), and 11 min (C). (D) Schematic picture of the profile analyzed by μ -TGGE. P_{ini} , the position for DNA melting initiation; P_{min} , the position for strand dissociation (27, 30). Each experiment was repeated two times, and representative results are shown.

yses. API-Listeria strips (bioMérieux, Marcy l'Etoile, France) were also used for biochemical tests. Serotyping was performed by using *Listeria* O and H antisera (Denka Seiken, Tokyo, Japan), in accordance with the manufacturer's instructions.

DNA isolation and PCR conditions. Genomic DNA was isolated by using an ISOPLANT kit (Nippongene, Tokyo, Japan), according to the manufacturer's instructions. The files containing the genomic DNA sequence of *L. monocytogenes* strains F2365 and H7858 were obtained from The Institute for Genomic Research (<http://www.tigr.org/>). The PCR-amplified region was selected by comparing the files. The 135-bp portion encoding the PRT/PTS system IIA 2 protein domain (in strain F2365) or the Lmo 0297 (in strain EGD-e)-encoding region of *L. monocytogenes* was amplified by using primers SNP19F (5'-ATA AAG CCG GGC GAT ATA GC-3') and SNP19R (5'-GCA TAT CGC CGT TTT AAT TG-3'). The 5' end was labeled with Cy3 or Cy5 when necessary. PCR was performed in a final volume of 50 μ l containing 1 \times amplification buffer, 0.2 mM of mixed deoxynucleoside triphosphates, 2.5 U of ExTaq DNA polymerase (TAKARA BIO, Shiga, Japan), 25 pmol of each primer, and \sim 500 ng of the template DNA. The amplification was carried out in an i-Cycler instrument (Bio-Rad, Tokyo, Japan) under the following conditions: 3 min at 95°C, followed by 35 cycles of 0.5 min at 95°C, 0.5 min at 50°C, and 0.5 min at 72°C, with a final incubation for 10 min at 72°C. DNA sequencing was performed at Hitachi Science Systems (Tokyo, Japan).

μ -TGGE. μ -TGGE was performed essentially as described previously (2, 3, 39). Briefly, \sim 50 ng of each PCR-amplified fragment was mixed with loading buffer and loaded onto a 6% polyacrylamide gel (25 by 25 mm) containing 6.5 M urea. The gel was set on the μ -TGGE apparatus (μ -TG; Taitec, Saitama, Japan), and TGGE was performed at 100 V for 3 to 11 min, with a temperature gradient from 20°C to 60°C running perpendicular to the direction of electrophoresis. The gel was directly visualized by using a Typhoon 9400 fluorescence imager (Amersham Biosciences, Tokyo, Japan) or stained with ethidium bromide (0.5 μ g ml⁻¹) and examined under UV transillumination.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB219411 to AB219421 and AB248880 to AB248911.

TABLE 2. Number of nucleotide differences of the PCR products among *L. monocytogenes* strains

Group	Serovar	No. of nucleotide differences between two groups ^a										G+C content ^b	
		A	B	C	D	E	F	G	H	I	J		
A	4b, 4e												47 (35)
B	1/2b	2 ×											48 (36)
C	1/2b, 4b, 7	13 °	15 °										52 (39)
D	1/2a, 1/2c	20 °	19 °	14 ×									54 (40)
E	4b	21 °	19 °	8 (°)	14 ×								56 (41)
F	1/2a	20 °	18 °	13 °	6 (°)	15 ×							56 (41)
G	3a	23 °	21 °	14 °	8 (°)	10 ×	12 ×						56 (41)
H	1/2a, 3a	21 °	19 °	14 °	7 °	16 (°)	1 ×	13 (°)					57 (42)
I	1/2a	23 °	21 °	14 °	12 °	6 (°)	13 (°)	6 (°)	14 (°)				58 (43)
J	4a	21 °	20 °	13 °	11 °	5 (°)	12 °	5 °	13 (°)	1 (°)			59 (44)
K	4b, 4c, 4d	23 °	21 °	14 °	12 °	6 °	13 °	6 °	14 °	2 (°)	1 ×		60 (44)

^a °, clear distinction under ethidium bromide staining; (°), distinguishable under fluorescence labeled conditions; ×, indistinguishable.

^b Values are in base pairs. The numbers in parentheses indicate percent G+C content.

RESULTS

Screening of optimum genome region analyzed by μ -TGGE.

For successful strain differentiation by μ -TGGE analysis, the amplified region of the genome must be selected so that different strains have distinct G+C contents, resulting in differences in the T_m and the initial DNA melting point (P_{ini}) (Fig. 1D) (27). The genomic sequences of *L. monocytogenes* strains EGD-e (serovar 1/2a), F6854 (serovar 1/2a), F2365 (serovar 4b), and H7858 (serovar 4b) have been publicly released (10, 28). Because human listeriosis is mainly caused by serovar 4b strains (8), the first goal was to discriminate serovar 4b strains and perhaps distinguish groups within this serovar. To this end, the genomes of strains F2365 and H7858 were compared, and the region encoding the PRT/PTS system IIA 2 protein domain was identified as having adequate interstrain differences in G+C content. The G+C contents of the 135-bp fragment were 35% in F2365 and 44% in H7858. This region was amplified from nine serovar 4b strains (Table 2). Comparison of the sequences revealed that the nine strains tested could be distinguished into three distinct groups, one of which was coincident with sequences of F2365 and the second and third of which differed from those of both F2365 and H7858. Moreover, it turned out that all 31 tested strains converged into 11 groups (groups A to K) at this sequence, with G+C contents ranging from 35% to 44% (Table 2). The following represen-

tative strains were selected for further experiments: RIMD 1205023 (group A), ATCC 51780 (group B), ATCC 51778 (group C), RIMD 1205001 (group D), RIMD 1205005 (group E), ATCC 51775 (group F), ATCC 51782 (group G), ATCC 35152 (group H), ATCC 51774 (group I), ATCC 19114 (group J), and ATCC 19117 (group K). Although PCR-amplified bands were detected by using SNP primers for all *L. monocytogenes* strains tested, no amplified bands were detected by PCR for the five other species of *Listeria* (data not shown).

Discrimination of strains by μ -TGGE analysis. To examine whether the amplified DNA fragments could be separated by μ -TGGE, the relevant PCR products of groups A and E were mixed and analyzed. The separation was inadequate after 3 min of electrophoresis but was sufficient after 7 or 11 min (Fig. 1A to C); therefore, a 7-min electrophoresis was used in subsequent analyses. The electrophoresis profiles were notably different between the two strains with respect to P_{ini} (which reflects the T_m), the most retarded point (P_{min}), and the migration profiles between P_{ini} and P_{min} (which reflect the three-dimensional structures of partially melted double-stranded DNA; Fig. 1D). Based on this promising result, all remaining combinations were tested (Table 2). Of the 55 possible pairs, 34 could successfully be discriminated (an example is shown in Fig. 2A), even if the difference between two PCR products was only 6 bp (Fig. 2B). The electrophoresis profile, however, was

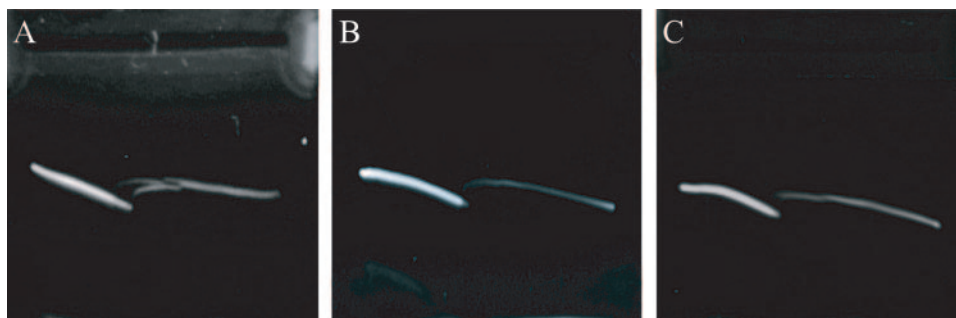


FIG. 2. μ -TGGE analysis of mixed PCR products of *L. monocytogenes* strains RIMD 1205023 (group A) and ATCC 19114 (group J) (A), RIMD 1205005 (group E) and ATCC 19117 (group K) (B), and RIMD 1205005 (group E) and ATCC 19114 (group J) (C). Each experiment was repeated two times, and representative results are shown.

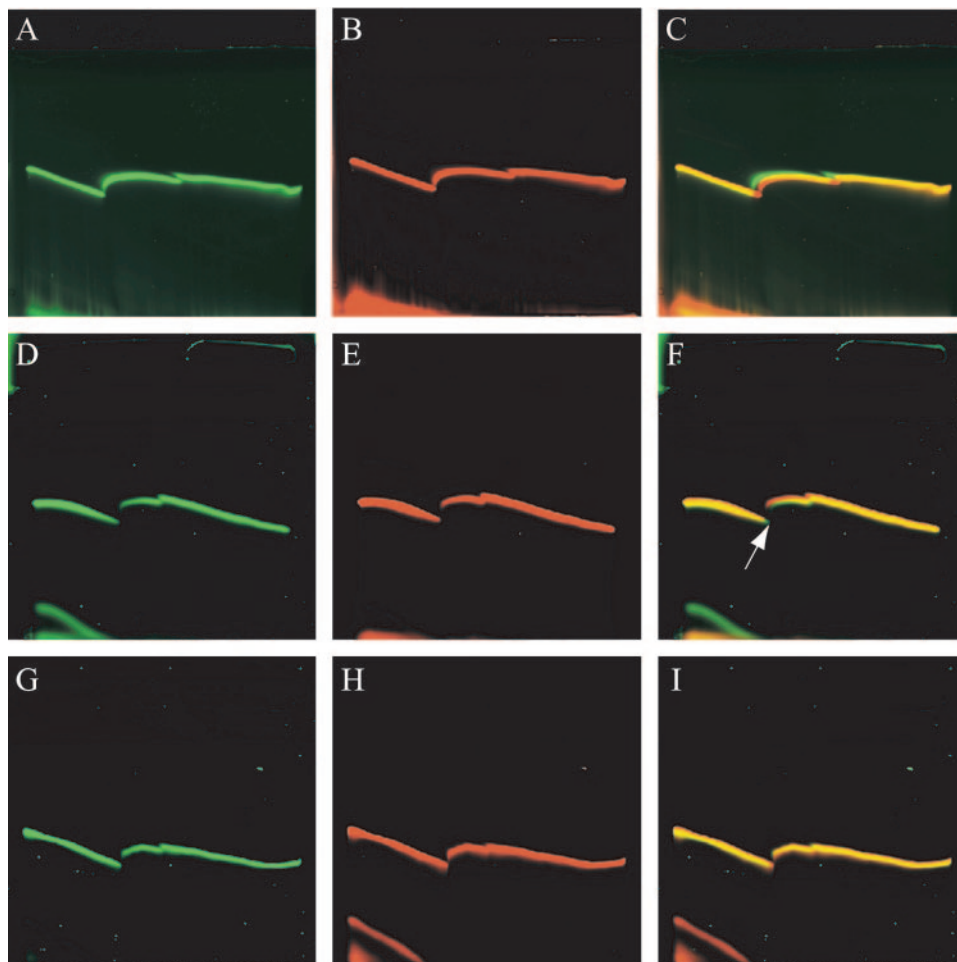


FIG. 3. Fluorescent images of the μ -TGGE separation profile. (A to C) The PCR product of *L. monocytogenes* strain RIMD 1205005 (group E) was labeled with Cy3 (A), and that of ATCC 19114 (group J) was labeled with Cy5 (B). (C) Overlapped image of the images in panels A and B. (D to F) The PCR product of *L. monocytogenes* strain ATCC 19117 (group K) was labeled with Cy3 (D), and that of ATCC 51774 (group I) was labeled with Cy5 (E). (F) Overlapped image of the images in panels D and E. (G to I) The PCR product of *L. monocytogenes* strain ATCC 51778 (group C) was labeled with Cy3 (G), and that of RIMD 1205001 (group D) was labeled with Cy5 (H). (I) Overlapped image of the images in panels G and H. Alternatively, the replacement of Cy3 labeling with Cy5 labeling and vice versa resulted in the same profiles.

ambiguous or indistinguishable for the remaining 21 pairs by ethidium bromide staining (Fig. 2C). The results are summarized in Table 2.

Discrimination of strains by μ -TGGE analysis of fluorescent PCR products. Previous studies revealed that the use of fluorescent dye labels can improve the precision of μ -TGGE analysis (39). To investigate whether the 21 indistinguishable pairs could be separated in this way, the respective

amplicons were labeled with Cy3 or Cy5 and mixed together, and the mixture was subjected to μ -TGGE. As shown in Fig. 3A to C, this method apparently distinguished some pairs that were ambiguous by ethidium bromide staining (Fig. 2C). Figure 3D to F shows that as little as a 2-bp difference could be detected, depending on the difference in P_{ini} (arrow in Fig. 3F). On the other hand, some pairs could not be separated, even if the difference between the ampli-

FIG. 4. Fluorescent images of the μ -TGGE profiles of strains isolated from food. (B, E, H, K, N) μ -TGGE profiles of Cy5-labeled PCR products of *L. monocytogenes* strains RIMD 1205023 (group A), ATCC 51778 (group C), ATCC 51775 (group F), and ATCC 19117 (group K). (A) μ -TGGE profiles of Cy3-labeled PCR products of *L. monocytogenes* strains STC 1, STC 4, and STC 7. (C) Overlapped image of the images in panels A and B. (D) μ -TGGE profile of Cy3-labeled PCR products of *L. monocytogenes* strains STC 9, STC 10, STC 11, STC 12, STC 14, and STC 16. (F) Overlapped image of the images in panels D and E. (G) μ -TGGE profiles of Cy3-labeled PCR products of *L. monocytogenes* strains STC h-1, STC h-2, and STC h-3. (I) Overlapped image of the images in panels G and H. (J) μ -TGGE profiles of Cy3-labeled PCR products of *L. monocytogenes* strain STC 11. (L) Overlapped image of the images in panels J and K. (M) μ -TGGE profiles of Cy3-labeled PCR products of *L. monocytogenes* strain STC 12. (O) Overlapped image of the images in panels M and N. The same results obtained with STC 12 were obtained with strains STC 9, STC 10, STC 14, and STC 16. Alternatively, the replacement of Cy3 labeling with Cy5 labeling and vice versa resulted in the same profiles.

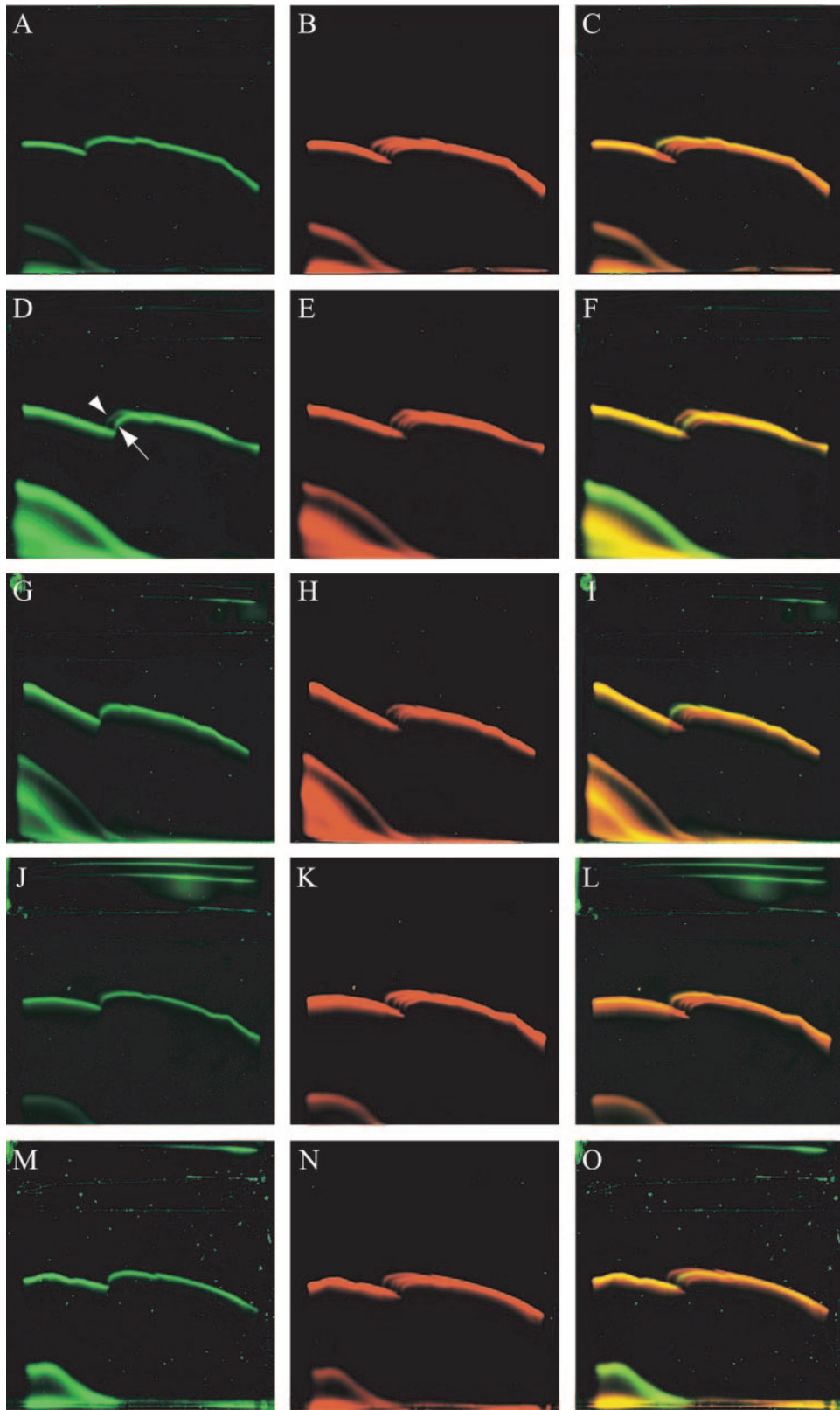


FIG. 4

cons was 14 bp (Fig. 3G to I). This fluorescent method distinguished 13 of the 21 pairs tested, so that, along with the nonfluorescent method, a total of 47 of 55 pairs could be distinguished by μ -TGGE analysis (Table 2).

Rapid identification of the sequence types of *L. monocytogenes* strains isolated from food. To examine whether μ -TGGE analysis could infer unidentified sequence types, it was performed to analyze *L. monocytogenes* strains isolated from several foods. Various types of beef, pork, and chicken were provided as sources of bacterial strains. Of these strains, three from chicken livers (STC 1, STC 4, and STC 7), six from chicken legs (STC 9, STC 10, STC 11, STC 12, STC 14, and STC 16), and three from minced pork (STC h-1, STC h-2, and STC h-3) were identified as *L. monocytogenes*. PCR with Cy3 (or Cy5)-labeled primers was performed with genomic DNA extracted from each strain. Also, PCR with Cy5 (or Cy3)-labeled primers was performed with genomic DNA from groups A, C, F, and K as reference markers that were adequately separable (Table 2). Next, the PCR products of the strains isolated from the same source were mixed together with the PCR products of a reference marker and analyzed by μ -TGGE. The profiles of the PCR products from the strains isolated from chicken liver overlapped with the reference marker corresponding to group A (Fig. 4A to C). Thus, all sequence types for strains isolated from chicken liver appeared to belong to group A or B. The same result was obtained for strains isolated from minced pork (Fig. 4G to I). On the other hand, the profiles of the PCR products for strains isolated from chicken legs mainly seemed to lie between reference markers corresponding to groups C and F or overlapped with both markers (arrow in Fig. 4D). Moreover, a thin band that overlapped with the reference marker for group A was detected (arrowhead in Fig. 4D). By investigating the profiles of one strain at a time, it became clear that only strain STC 11 (Fig. 4J to L) showed profiles different from those of the other five strains (Fig. 4M to O). When the DNA sequences of the PCR products for the isolated strains were examined, it was found that STC 11 and all of the strains isolated from chicken liver and minced pork belonged to group A, whereas all strains isolated from chickens leg except STC 11 belonged to group D, showing that the prediction according to μ -TGGE analysis was correct.

DISCUSSION

In this paper, it was shown that a less than 10-min μ -TGGE analysis could discriminate 11 of 31 groups of *L. monocytogenes* strains. In previous studies, TGGE/DGGE analysis of 16S rRNA or *inl* gene fragments was used to detect the presence of *L. monocytogenes* in certain foods (6, 20). These studies sought to distinguish *L. monocytogenes* from other *Listeria* spp., such as *L. innocua*. In contrast, the present investigation sought to use TGGE to type *L. monocytogenes* at the strain level. Therefore, it was necessary to choose a PCR-amplifiable region that differed among strains rather than species. The genomes of *L. monocytogenes* strains F2365 and H7858 were compared, and the region encoding the PRT/PTS system IIA 2 protein domain was chosen because there were great differences between strains in their G+C contents. Sequence analysis of this region revealed that the 31 strains could be combined into 11 groups.

According to previous studies, serovar 1/2b, 3b, 4b, 4d, and 4e strains are classified as lineage I; serovar 1/2a, 1/2c, 3a, and 3c strains as lineage II; and serovar 4a, 4c, and partial serovar 4b strains as lineage III (18, 26, 29, 42). Lineages I and II were deeply separated, with little horizontal gene transfer observed (29). No putative lineage I and II strains were grouped together in this study, consistent with the findings described in previous reports. In addition, like the serovar 1/2b strain, *L. monocytogenes* NCTC 10890 (serovar 7) belonged to group C, in agreement with the results of amplified fragment length polymorphism analysis (17). There was only one unexpected classification: a serovar 4d strain belonged to group K, which was made up of putative lineage III strains. Although it was necessary to investigate the lineage, the sequence classification performed in this study agreed with that presented in previous reports with respect to strain lineage. For other *Listeria* species, no PCR amplification was observed; however, a BLAST search suggested that *L. innocua* lin0325 was homologous and that it differs by only 2 bp from group K. There was a 1-bp mismatch in the forward primer so that it might lead to a failure in the PCR amplification.

Standard TGGE/DGGE analysis requires, on average, 4 h for electrophoresis (6, 20), whereas in the current study, the miniaturized μ -TGGE apparatus required only 7 min to resolve the amplicons. Even taking into account the time required to extract the genomic DNA and perform the PCR amplification, it was possible to complete the entire process within 2 h, which is a substantial improvement over the times required for other typing methods. For example, ribotyping requires 8 h (26), PFGE requires 20 to 24 h (34), and microarray typing requires an overnight hybridization (4). RAPD analysis and some of the PCR-electrophoresis-based methods can be performed in a time frame similar to that required for μ -TGGE (15, 21, 36), making μ -TGGE one of the fastest typing methods. In addition, μ -TGGE showed minimal problems with respect to reproducibility, which can be an issue in RAPD analysis (41). Thus, the method described here appears to have improved efficiency and reproducibility compared to those of previous methods.

Additional work, however, may be necessary to refine the discriminatory power of μ -TGGE. Of 55 pairs tested, it could not detect any profile difference in 8 pairs. In particular, the profiles of groups C and D, D and E, and E and F could not be distinguished even with the fluorescent dye, even though there were 14- to 15-bp differences between the two groups. This was probably due to the 0- to 2-bp differences in G+C contents and little deviation in the T_m s for the amplicons, resulting in overlapping P_{ini} s. In addition, the three-dimensional structures of the fragments might be too similar to result in differences in mobility, as reflected by P_{ini} and P_{min} . It may be possible to overcome this limitation in the future by using a GC clamp attached to the primer, which would increase the sensitivity of μ -TGGE by inhibiting complete strand separation until higher temperatures are reached, thus affecting the mobility between P_{ini} and P_{min} (35).

Although this system has problems that remain to be solved, it was possible to identify sequence types by overlapping the μ -TGGE profiles with those of four reference markers. At that time, strains that were isolated from the same food were analyzed together. By using this procedure, the μ -TGGE profiles

could clearly determine whether the isolated colonies that spoiled the particular food were from homogeneous or heterogeneous groups. In practice, a single μ -TGGE trial was able to show that strains isolated from chicken livers and minced pork were homogeneous, whereas those isolated from chicken legs were heterogeneous. Although the contaminating *L. monocytogenes* strains from the three meat-producing districts were different, the downstream distribution route was the same. This suggested that contamination with serovar 1/2b strains commonly isolated from the three foods occurred downstream of the distribution process, whereas contamination with serovar 1/2a strains, which were isolated only from chicken legs, occurred upstream of the distribution process.

Many studies have sought to trace the sources and routes of *L. monocytogenes* contamination in meat, poultry, seafood, and smoked fish production plants (12, 13, 37, 40). For practical use, ST and MLST currently have the greatest discriminatory powers and best data standardization (5, 19, 23, 29, 32, 33, 45), but these methods become time-consuming and laborious when large numbers of samples must be tested. Thus, it may be advantageous to perform an initial μ -TGGE analysis to prioritize the samples, followed by a more detailed analysis by ST or MLST. In this way, the novel combination of μ -TGGE analysis and subsequent MLST may provide the most rapid and accurate elucidation of the contamination route, leading to the prevention of the spread of food poisoning in the future.

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