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Original Article

Comparison of Genomic Structures in the Serovar 1/2a *Listeria monocytogenes* Isolated from Meats and Listeriosis Patients in Japan

Fukiko Ueda*, Kyoko Yugami[1], Mariko Mochizuki, Fumiya Yamada[2], Kunitoshi Ogasawara[3], Akikazu Fujima[4], Hiroshi Shoji[5] and Ryo Hondo

Department of Veterinary Public Health, Nippon Veterinary and Animal Science University, Tokyo 180-8602,
[1]Tokyo Metropolitan Institute of Public Health, Tama Branch Laboratory, Tokyo 190-0023,
[2]Saitama Institute of Public Health, Saitama 338-0824, [3]Niigata Quarantine Station, Niigata 950-0072,
[4]Kitasato Otsuka Biomedical Assay Laboratories Co., Ltd., Kanagawa 228-8555 and
[5]First Department (Neurology) of Internal Medicine, School of Medicine, Kurume University, Fukuoka 830-0011, Japan

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SUMMARY: Foodborne disease by *Listeria monocytogenes*, serovar 1/2a has recently been reported in many countries. Although contamination by this bacteria is also known to be gradually spreading among the marketed foods of Japan, there is little information on relation between listeriosis and food contamination. In the present study, the characteristics of the genomic structures of serovar 1/2a were compared among the isolates from marketed meats and listeriosis patients. Several isolates from meats purchased at the same shop on different days had the same genomic structure, and prolonged contamination was suggested by the conditions in the shop. Genomic structures of one strain isolated from meat were identical to those of two isolates from a patient. Another isolate was obtained from meats purchased at two different shops, and this isolate was also identical to that of the isolates from another patient. These findings suggest that the isolates from meat may have caused the listeriosis in the patients, and that the strains may have somehow traveled between the shops.

INTRODUCTION

Contamination by *Listeria monocytogenes* has been reported to induce a foodborne disease, listeriosis, in Europe and the U.S. since the 1980s (1-3). The incidence rate is different among districts (4,5), and the rates per million have been estimated to be 2 to 8 in the U.S. and Europe, 10.9 in Spain (6) and 1.6 to 14.7 in France (7), though the occurrence is usually lower than those of salmonellosis and vibriosis. In Japan, wide contamination by *L. monocytogenes* has also been found in marketed meats, fish and processed foods, and the incidence rate of listeriosis in Japanese has been estimated as 0.65 per million per year (8). In spite of these low incidence rates, listeriosis has a high mortality of more than 20% (9) due to the existence of high-risk groups (10) such as pregnant women, neonates and diabetics.

Thirteen serovar-types have been identified using the combination of somatic- and flagella-antigens, and the strain of serovar 1/2c is dominant (approximately 60%) in the environment (11). However, the isolation rates of serovars 1/2a, 1/2b and 1/2c in food are almost equal (approximately 20 - 30%). Further, the restricted serovars of 1/2a, 1/2b and 4b have frequently been isolated from patients (7,12-14). In human listeriosis, the isolation rates of serovars 4b and 1b (present serovar: 1/2b) in Japan were 60.6 and 31.0%, respectively, over the 26-year period of 1958 - 1984, but other serovars were rare during this period (15). These rates seem to have changed in recent years; the rates of serovars 4b, 1/2a and 1/2b in Japan were reported as 56, 26.5 and 18%, respectively (16). Similar phenomena have been observed in other countries. For example, the rates of serovars 4b, 1/2a and 1/2b were 29 - 89.7%, 3.4 - 41.9% and 3.5 - 42.3%, respectively, in Italy (17), and similar increased were observed in a study on 11 countries including Finland in the 1990s (18). The rates of serovar 4b were the highest in 11 of 12 countries and those of serovar 1/2a were the second in 10 countries. In Finland the rates of serovar 1/2a and 4b were 42.3 and 57.7% in 1989 (18). However, in another report (19), the number of human listeriosis cases caused by serovar 1/2a increased from 22% in 1990 to 67% in 2001, and those caused by serovar 4b decreased from 61 to 27%, respectively. Thus, elucidation of the relation between the serovar 1/2a strains from the field and those isolated from patients is needed to clarify the epidemiology of *L. monocytogenes* before the further spread of serovar 1/2a-induced listeriosis in Japan.

The *iap* gene encoding an extracellular protein p60 has genetic diversity in the EGD-*L. monocytogenes* strain (20,21). The authors have reported that the combination of restriction fragment length polymorphism (RFLP) analysis of the chromosomal DNA extracted from *L. monocytogenes* and analysis of the diversity in a part of the *iap* gene is useful for discrimination among the *L. monocytogenes* isolates from foods and humans (22-24). In the present study, the characteristics of the genomic structures of serovar 1/2a were compared among the isolates from marketed meats and listeriosis patients using the combination of RFLP analysis of the extracted chromosomal DNA and the multiple sequence alignments for the polymerase chain reaction (PCR) products of

*Corresponding author: Mailing address: Department of Veterinary Public Health, Nippon Veterinary and Animal Science University, Kyonan-cho 1-7-1, Musashino, Tokyo 180-8602, Japan. Tel: +81-422-31-4151 ext. 282, Fax: +81-422-30-7531, E-mail: fueda@nvau.ac.jp

the *iap* gene.

MATERIALS AND METHODS

Isolation, identification and serotyping of *L. monocytogenes*: **Meat-origin isolates**: A total of 20 *L. monocytogenes* isolates, all of serovar 1/2a, were used in the present study. Seventeen isolates were from unprocessed pork, chicken and beef purchased between June 1996 and June 2000 at 8 different shops in the Tokyo metropolitan area, Chiba and Saitama Prefectures. UVM broth (Becton, Dickinson and Company, Sparks, Md., USA) and Palcam (Merck, Darmstadt, Germany) agar were used for the pre-culture broth and the selection of *Listeria* spp., respectively. Identification was performed by the usual method described in the previous reports (22-24), and the serovar of the isolates was then determined by the Sahumy method (25).

Human-origin isolates: Two strains of serovar 1/2a were isolated in 1996 from the blood and feces of one listeriosis patient with septicemia using a method similar to that used here for meat. One isolate from the spinal fluid of a listeriosis patient with meningitis was provided in 2000 by the First Department (Neurology) of Internal Medicine, School of Medicine, Kurume University.

RFLP analysis of chromosomal DNA extracted from *L. monocytogenes* isolates: The chromosomal DNA was extracted and purified from the isolated strains as previously described (22-24). The DNA was digested with the restriction enzymes *Ban*III, *Pst*I, *Xba*I, *Sal*I, *BamH*I and *Bgl*II according to the manufacturer's instructions (Toyobo, Tokyo, Japan). The reactants were separated on 0.8% agarose gel followed by staining with ethidium bromide (EtBr). DNA fragments on the gel were visualized using an UV transilluminator, and a photograph was taken of each gel.

Determination of the nucleotide sequence: A part of the *iap* gene (810 bp) which contains the repeated variable nucleotide sequences was amplified by PCR using a pair of primers, SI3A (5′-ACTGGTTTCGTTAACGGTAAA-3′) and SI4B (5′-TTTAGTGTAACCAGAGCAATC-3′). The amplified products were then cycle-sequenced (554 bp) using a pair of dye* primers, SI4AD (5′-AATACGGTGTTTCTGT TCAAG*-3′) and SI4BD (5′-TTTAGTGTAACCAGAGCA ATC*-3′), with a DNA sequencer (Hitachi SQ-5500; Hitachi, Tokyo, Japan) as previously described (22-24). A part of the determined nucleotide sequences, 407 bp between positions 1116 and 1522, was used for a comparative analysis against the SV1/2a EGD (EGD) strain reported by Kohler et al. (20) (GenBank accession number X52268).

RESULTS

RFLP analysis of chromosomal DNA extracted from *L. monocytogenes* isolates: The chromosomal DNAs extracted from the 20 isolates were digested with the 6 restriction enzymes *Pst*I, *Xba*I, *Ban*III, *Sal*I, *BamHI* and *Bgl*II, and the RFLP analysis was then attempted by agarose gel electrophoresis. Figures 1a and 1b show the typical digested patterns by *Pst*I and by 3 restriction enzymes, respectively. The patterns from 20 isolates were classified into 8 different types, a to h, and the same classification was obtained after the digestion by all 6 restriction enzymes (data not shown). In the following description the numbers correspond to the strain numbers shown in the Figures.

The digested patterns in the 2 isolates (Nos. 1 and 2)

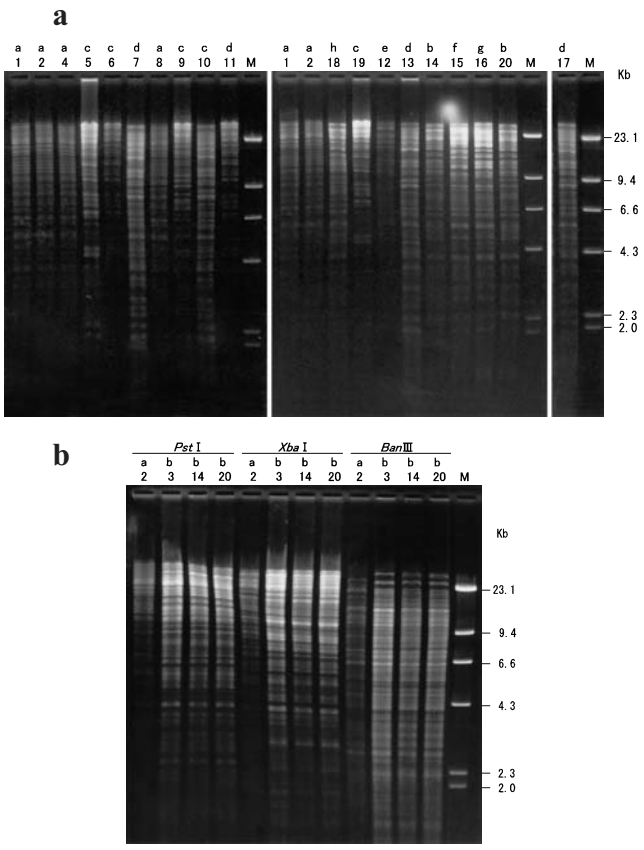

Fig. 1. RFLP analysis of the *Listeria monocytogenes* isolated from meats and patients. Typical digested patterns by *Pst*I (a) and *Pst*I *Xba*I and *Ban*III (b). Small letters a to d on the strain numbers show the same cleavage pattern. Number (in parentheses) and strain name: (1) L96-HM1, (2) L96-HM2, (3) L2K1-12H, (4) L96-11P1, (5) L98-76P2, (6) L98-78P5, (7) L98-104P5, (8) L98-80C1, (9) L98-89C5, (10) L99-183P1, (11) L99-213C1, (12) L99-173B3, (13) L99-221C1, (14) L99-186C1, (15) L2K-265C1, (16) L99-223C3, (17) L2K-268C1, (18) L2K-307C1, (19) L2K-312B1, (20) L99-188C3.

from one patient were identical (type a), but a different pattern (type b) were seen in isolate No. 3 from the other patient. The patterns of the former 2 human isolates were indentical to those of Nos. 4 and 8 from pork and chicken, respectively, purchased in the same store but in a different year. The latter pattern from No. 3 (the other human isolate) was indentical to those of 2 isolates, Nos. 14 and 20 from chicken purchased in the same year but from different stores. Although other patterns from the meat isolates were not matched with those of human isolates, a pattern (type c) was identified in 5 isolates, Nos. 5, 6 and 9 from one store and Nos. 10 and 19 from two other stores and a pattern (type d) was identified in 4 isolates, Nos. 7, 11, 13 and 17 from different stores. However, the RFLP patterns from the other 4 isolates, Nos. 12, 15, 16 and 18, were not matched with those of any other isolates, and thus were classified into types e to h, respectively.

Comparison of the nucleotide sequences: The sequences determined for all the strains are shown in Fig. 2 in comparison with that of the reference SV1/2a EGD strain. Although no nucleotide deletion was observed in the region of these isolates, the isolates showed variations in nucleotide substitutions and insertions, and in the number of repeated sequences.

A total of 12 nucleotide substitutions were observed in 15 of the isolates investigated compared with that of the EGD

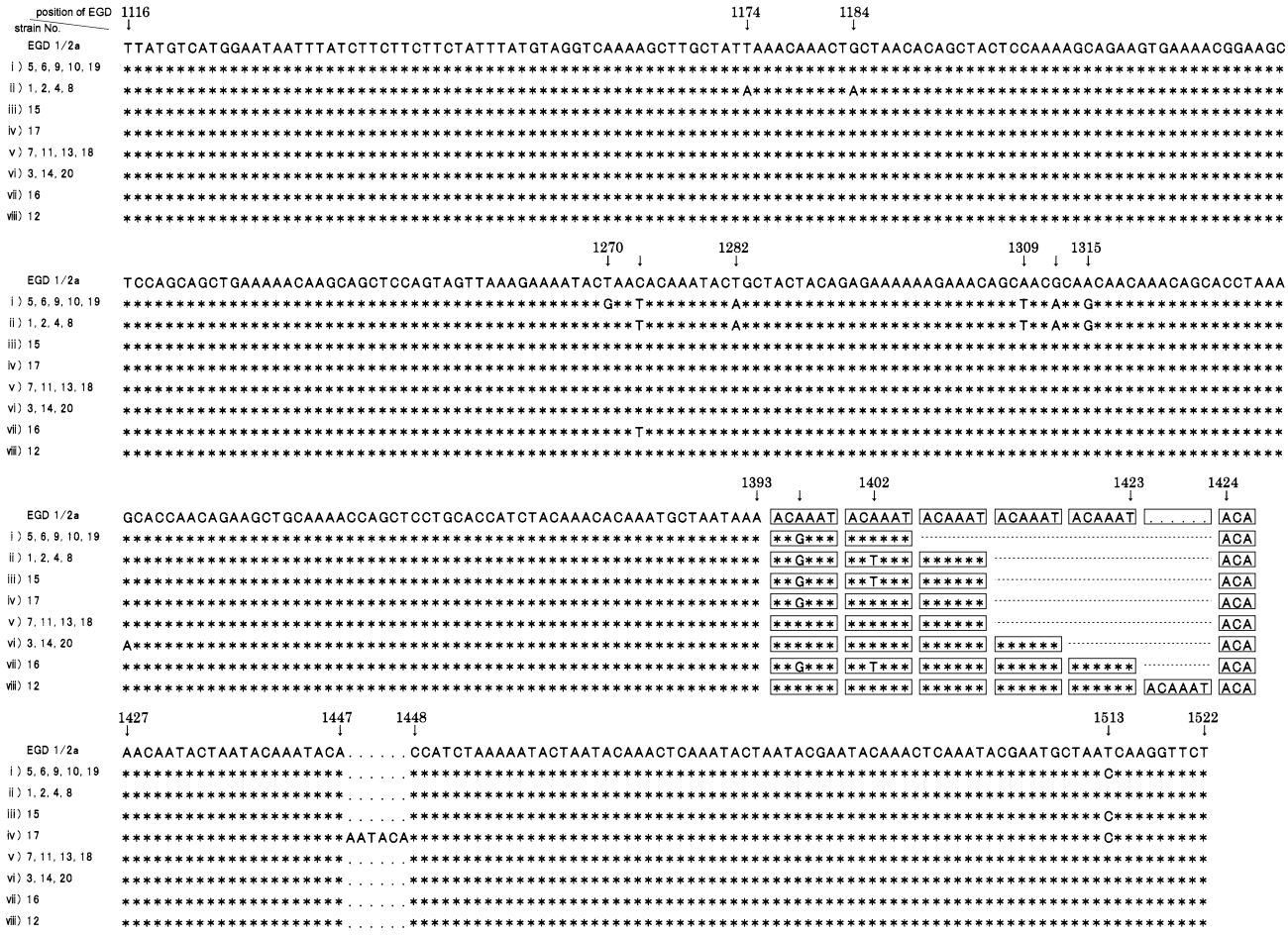

Fig. 2. Nucleotide sequences of *iap* region (407bp) in serovar 1/2a *Listeria monocytogenes* isolates. Number and strain name are in Fig. 1.

strain, while 5 isolates, Nos. 7, 11, 13, 18 (type v) and 12 (type viii) had no nucleotide substitution. The number of nucleotide substitutions in each isolate ranged from 1 to 9. The 20 isolates were consequently classified into 7 types from the nucleotide substitutions. Only one isolate, No. 17, had a 6-nucleotide insertion of AATACA between positions 1447 and 1448 downstream of the repeated sequence.

The repeated sequence consisted of 6 nucleotides (ACAAAT) for one unit and ended with ACA located at positions 1394 to 1426 in the EGD strain, and the EGD strain had 4 cycles. Nucleotide substitutions within the structure consisted of an A to G substitution at position 1396 in 12 isolates, and an A to T substitution at position 1402 in 6 of these 12 isolates. The number of repeated sequences ranged from 1 to 5, and the 20 isolates were classified into 5 types based on these repetitions.

Consequently, the 20 isolates were classified into 8 types based on the nucleotide substitutions, a single insertion and the repeated structures.

DISCUSSION

Table 1 shows the summary of the details for the isolates and the results. These isolates were classified into 9 types, I to IX, though 8 types were obtained from the RFLP analysis of chromosomal DNA. This is due to disagreements in the types of IV, V and VI. The cleavage pattern type d was divided into 2 types of IV containing 3 isolates and V of No. 17. This phenomenon is not strange because RFLP analysis cannot detect a 6-nucleotide insertion, and also perhaps 2-nucleotide substitution in No. 17 if the nucleotide does not relate with the cutting site. Then, although types IV and VI had the same nucleotide sequence, the type VI of No. 18 had a different cleavage pattern of h because the analysis of the nucleotide sequence cannot detect a difference in other regions due to the analysis of short PCR product. Although discriminations of bacteria using the RFLP and the PFGE analysis of a PCR product and/or a chromosomal DNA have frequently been performed in epidemiology after a foodborne disease such as salmonellosis (26) and listeriosis (27,28), the present results suggest that the analysis by RFLP only, and perhaps also that by PFGE only, are not sufficinet for an accurate discrimination among the strains. Thus, more detailed gene analysis for the discrimination of *L. monocytogenes* strains would be made possible by the combination of RFLP analysis of the chromosomal DNA and determination of the sequence for the PCR product.

In case A, 2 isolates, Nos. 4 and 8, and 3 isolates, Nos. 5, 6 and 9, were identified as the same type, types I and III, respectively, suggesting that contamination by these 2 strains was prolonged in this shop because these meats were purchased on different days. It is particularly important that the former strains of type I were identified as the same type with the isolates from a patient. A similar identification was also obtained among an isolate from the other patient, No. 14 and No. 20 from chicken (type II). The virulence of

Table 1. Genomic characteristics and details of *Listeria monocytogenes* used in the present study

Case	No.	Sample	Date	Strain	Cleavage pattern	Nucleotide substitution	Number of repeat structure	Insertion	Delation	Type
human	1	Blood	960318	L96-HM1	a	9	2	0	0	I
	2	Feces	960408	L96-HM2	a	9	2	0	0	I
	3	Spinal fluid	020904	L2K1-12H	b	1	3	0	0	II
A	4	Pork	960624	L96-11P1	a	9	2	0	0	I
	5	Pork	980210	L98-76P2	c	8	1	0	0	III
	6	Pork	980211	L98-78P5	c	8	1	0	0	III
	7	Pork	980417	L98-104P5	d	0	2	0	0	IV
	8	Chicken	980213	L98-80C1	a	9	2	0	0	I
	9	Chicken	980311	L98-89C5	c	8	1	0	0	III
B	10	Pork	990612	L99-183P1	c	8	1	0	0	III
	11	Chicken	990620	L99-213C1	d	0	2	0	0	IV
C	12	Beef	990613	L99-173B3	e	0	5	0	0	VII
	13	Chicken	990620	L99-221C1	d	0	2	0	0	IV
D	14	Chicken	990613	L99-186C1	b	1	3	0	0	II
	15	Chicken	000611	L2K-265C1	f	3	2	0	0	VIII
E	16	Chicken	990620	L99-223C3	g	3	4	0	0	IX
	17	Chicken	000611	L2K-268C1	d	2	2	1	0	V
F	18	Chicken	000619	L2K-307C1	h	0	2	0	0	VI
G	19	Beef	000611	L2K-312B1	c	8	1	0	0	III
H	20	Chicken	990614	L99-188C3	b	1	3	0	0	II

the isolates from meats was not examined in the present study. Although many Japanese do not eat raw meat except fish, our results show the risk of foodborne disease from meat. The chicken isolates may be related to sporadic listeriosis in Japanese because 3 of 4 isolates (Nos. 8, 14 and 20 in types I and II) were from chicken.

On the other hand, the same strains were isolated from meats purchased at different shops, suggesting that there were some routes for moving the strain between shops of D and H (type II), among shops A, B and G (type III), and among shops A, B and C (type IV). However, no information was obtained for these shops. Accumulation of information on the possible relation might be useful for surveillance and management of the risk, though the virulence is, of course, unknown for any of these isolates except type II. Further investigation will be needed for the management and/or HACCP of the meat safety in Japan.

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