Jpn. J. Med. Sci. Biol., 50, 63 - 71, 1997.

DISCRIMINATION OF *LISTERIA MONOCYTOGENES* STRAINS OF SEROTYPE 4b BY RESTRICTION ENZYME ANALYSIS OF CHROMOSOMAL DNA

Akinobu SAITO, Takuo SAWADA¹, Yoshikazu TOKUMARU
and Ryo HONDO²

Division of Food Hygiene, Saitama Institute of Public Health, 639-1, Kamiokubo, Urawa-shi, Saitama 338, ¹Department of Veterinary Microbiology and ²Department of Veterinary Public Health, Nippon Veterinary and Animal Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180

(Received December 26, 1997. Accepted March 21, 1997)

SUMMARY: Epidemiologically related cheese and environmental strains and epidemiologically unrelated strains of *Listeria monocytogenes* serotype 4b were examined by restriction enzyme analysis of chromosomal DNA with a total of 10 restriction enzymes. The DNA fingerprint patterns generated from each restriction enzyme digest of total DNA of all strains were classified. The restriction enzyme patterns of seven strains recovered from cheese and environmental samples in the same plant were identical to each other, but differed from those of seven epidemiologically unrelated strains. Two, originating from sporadic human patients, of eight epidemiologically unrelated strains exhibited the identical restriction enzyme patterns. Excepting these two strains, restriction enzyme analysis of the chromosomal DNA of *L. monocytogenes* serotype 4b can discriminate serologically indistinguishable strains.

斎藤章暢・徳丸雅一 (埼玉県衛生研究所食品衛生部食品微生物科 埼玉県浦和市上大久保639-1)

澤田拓士 (日本獣医畜産大学獣医微生物学教室 武蔵野市境南町1-7-1)

本藤 良 (日本獣医畜産大学獣医公衆衛生学教室 武蔵野市境南町1-7-1)

INTRODUCTION

Listeria monocytogenes is a gram-positive, short rod-shaped microorganism that has been acknowledged as the cause of listeriosis in human and various animal species. Listeriosis has also been recognized as a food-borne disease, since several major food-borne epidemics have occurred in North America in the 1980s (1-3). Although no epidemic has been verified as being caused by the food-borne listeriosis in Japan (4), *L. monocytogenes* is widespread in many foods and the environment in Japan (5-7). This indicates that the risk of food-borne listeriosis in Japan is the same as that in North America, therefore, it has become increasingly important to discriminate among *L. monocytogenes* strains for tracing the source of infection and for epidemiological study of listeriosis. Serotyping is commonly used as a discriminating method. Thirteen serotypes have been recognized, but 95% of the listeriosis cases have been caused by a small number of serovars (1/2a, 1/2b, or 4b) (8). Molecular epidemiological analyses, such as multilocus enzyme electrophoresis (MEE) (9,10), ribotyping (11,12) and restriction enzyme analysis (13,14), have hardly been used to serologically characterize identical strains from epidemics. We previously reported that six-base restriction enzymes were useful for restriction enzyme analysis of chromosomal DNA of *L. monocytogenes*, and that *L. monocytogenes* strains of serotype 1/2a could be discriminated among the serologically indistinguishable strains (15). The present study was designed to improve the applicability of restriction enzyme analysis to differentiation of chromosomal DNA of *L. monocytogenes* derived from epidemiologically unrelated strains and that from those related strains of serotype 4b which has been the most frequently detected serotype from patients.

MATERIALS AND METHODS

Bacterial strains: The *L. monocytogenes* strains used in the present study and their sources are listed in Table I. A total of 14 strains of *L. monocytogenes* serotype 4b were examined, including (i) a reference strain (Laboratory for Culture Collection, the Institute of Medical Science, the University of Tokyo, Tokyo), (ii) six wild strains, and (iii) four isolates from different lots of cheese manufactured in the same plant and three from environmental samples obtained in the cheese plant when the source of the cheese contamination was investigated. All strains were identified according to the conventional procedures and serotyped by

Table I. *Listeria monocytogenes* serotype 4b strains used in the study

Strain*	Reference	Source
IID571	Reference strain	Sheep
H2	Sporadic clinical isolate	Human
H6	Sporadic clinical isolate	Human
SC32	Epidemiologically unrelated	Poultry
Hu80	Epidemiologically unrelated	Raw hamburger
Hu120	Epidemiologically unrelated	Raw hamburger
CH6	Epidemiologically unrelated	Soft cheese
CH115	Different lot from the same plant	Shredded cheese
CH188	Different lot from the same plant	Shredded cheese
CH190	Different lot from the same plant	Shredded cheese
CH191	Different lot from the same plant	Shredded cheese
CHF201	Cheese plant	Environment swab
CHF208	Cheese plant	Environment swab
CHF231	Cheese plant	Drainage

*IID, Laboratory for Culture Collection, the Institute of Medical Science, the University of Tokyo.

Dr. M. Terao, Niigata Institute of Public Health and Pollution and Dr. T. Iida, Tokyo Metropolitan Research laboratory of Public Health. Cultures were maintained on nutrient agar slants.

DNA extraction: The DNA isolation procedure of Makino and his coworkers (16) was adopted for *L. monocytogenes* strains. These strains were inoculated into 1.5 ml of brain heart infusion broth, and incubated with shaking at 35 C for 24 hr. The pelleted cells ($20,000 \times g$, 1 min, 4 C) were suspended in 200 μ l of TES buffer [1 mM EDTA, 10 mM Tris, 100 mM NaCl (pH 8.0)]. The cells were lysed by treatment with 10 μ l of lysozyme (Seikagaku Co., Tokyo) at 10 mg/ml and 50 μ l of N-acetylmuramidase SG (Seikagaku) at 1 mg/ml. After incubation (37 C, 30 min), 100 μ l of TES buffer, 50 μ l of proteinase K (Promega Co., Madison, WI) at 1 mg/ml and 50 μ l of a sodium dodecyl sulfate solution [10% (wt/vol) in H₂O] were added and gently mixed. The lysate was incubated overnight at 55 C. The cell lysate was extracted with one volume of TE-saturated phenol [1 mM EDTA, 10 mM Tris, (pH 8.0)] and chloroform:isoamylalcohol (24:1). The aqueous phase of the lysate was separated, and DNA was precipitated with 1 ml of 99.5% ethanol. DNA was collected by centrifugation at $20,000 \times g$ for 3 min and washed three times in 0.5 ml of 70% ethanol. The DNA was suspended in 200 μ l of distilled wa-

ter and stored at 4 C. The concentration and purity of the DNA were determined by UV spectroscopy (Shimadzu UV160 Spectrophotometer, Kyoto).

Restriction enzyme analysis: Ten of six-base restriction enzymes, *Aat*I, *Bam*HI, *Bam*III, *Bq*III, *Eco*RI, *Kpn*I, *Pst*I, *Sac*I, *Sal*I and *Xba*I, were selected (15). These enzymes were purchased from Toyobo Co, Tokyo. For each restriction digestion, approximately 2 µg of DNA was used in a 25 µl reaction mixture containing 2.5 µl of × 10 restriction buffer, 1 µl each of BSA and RNase and 5 to 20 units of enzyme. The reaction mixtures were then incubated at 37 C for one hour. *Hind*III-digested bacteriophage λ DNA was used as the molecular weight standard. DNA fragments were electrophoresed in 1.0% horizontal agarose gel (200 × 150 × 5 mm) at 30 mA for 17 hr in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA (pH 7.8). After electrophoresis, the DNA bands were located under a short-wave UV light and photographed.

RESULTS

Differences in Restriction Fragment Length Polymorphism

The classification results for the DNA fingerprint patterns generated from the 10 restriction enzymes were identified. *Ban*III, *Pst*I and *Xba*I were particularly well recognizable. Six of the eight *L. monocytogenes* serotype 4b strains, which were epidemiologically unrelated, exhibited different restriction enzyme pattern, but two strains H2 and H6 derived from human patients exhibited the same restriction enzyme pattern, and two strains, Hu120 derived from raw hamburger and CH115 derived from cheese, exhibited similar restriction enzyme patterns, after digestion of their DNA with the 10 restriction enzymes. Strain H2 was isolated from the cerebrospinal fluid of a meningitis patient in 1989. Strain H6 was isolated from the cerebrospinal fluid of a newborn in 1995. All of the seven *L. monocytogenes* serotype 4b strains, derived from cheese and environmental samples and epidemiologically related, exhibited the same restriction enzyme pattern after digestion of their DNA with the 10 restriction enzymes. Typical results with restriction enzyme *Xba*I are shown in Fig. 1. Each restriction enzyme pattern was denoted with a different letter in Table II.

Fig. 1. DNA fingerprint patterns generated from *Xba*I digests of total DNA. [A] Epidemiologically unrelated strains of *L. monocytogenes* serotype 4b. [B] Epidemiologically related raw milk strains of *L. monocytogenes* serotype 4b. [A] Lanes: 1, SC32; 2, Hu80; 3, Hu120; 4, CH6; 5, CH115; 6, H2; 7, H6; 8, IID571; M, molecular weight marker, λ DNA digested with *Hind*III. [B] Lanes: 1, CH115; 2, CH188; 3, CH190; 4, CH191; 5, CHF201; 6, CHF208; 7, CHF231; M, molecular weight marker, λ DNA digested with *Hind*III.

DISCUSSION

To investigate the epidemiological relationship among *L. monocytogenes* strains, it is important to make experiments with epidemiologically related strains. In this study, we examined *L. monocytogenes* strains derived from epidemiologically related cheese and environmental samples; seven strains of *L. monocytogenes* serotype 4b were isolated from four different lots of cheese, two processing environments, and one drainage at the same cheese plant when investigating the cause of cheese contamination. The source of contaminant was not ascertained, but the present study supported the finding that *L. monocytogenes*

Table II. Classification* of DNA fingerprint patterns generated from 10 six-base restriction enzyme digests of total DNA from *Listeria monocytogenes* serotype 4b

Strain	Aat I	Bam HI	Ban III	Bgl II	Eco RI	Kpn I	Pst I	Sac I	Sal I	Xba I
IID571	A	A	A	A	A	A	A	A	A	A
H2	B	B	B	B	B	B	B	B	B	B
H6	C	C	C	C	C	C	C	C	C	C
SC32	D	D	D	D	D	D	D	D	D	D
Hu80	E	E	E	E	E	E	E	E	E	E
Hu120	F	F	F	F	F	F	F	F	F	F
CH6	G	G	G	G	G	G	G	G	G	G
CH115	H	H	H	H	H	H	H	H	H	H
CH188	H	H	H	H	H	H	H	H	H	H
CH190	H	H	H	H	H	H	H	H	H	H
CH191	H	H	H	H	H	H	H	H	H	H
CHF201	H	H	H	H	H	H	H	H	H	H
CHF208	H	H	H	H	H	H	H	H	H	H
CHF231	H	H	H	H	H	H	H	H	H	H

*Districtive electrophoretic patterns were designated with consecutive letters.

serotype 4b strains originating from the contaminated cheese were widespread and prevalent for a long time.

Strains H2 and H6 exhibited the same restriction enzyme patterns of DNA. Although we have no detailed information about these two strains, there was no particular relationship between the lives of the two patients' families and there was a 6-year difference between the times when the two strains were isolated.

We previously reported that the *L. monocytogenes* serotype 1/2a strains were discriminated by restriction enzyme analysis of chromosomal DNA with six-base restriction enzymes (*Ban*III, *Pst*I and *Xba*I were particularly well-recognizable) (15). In the present study, epidemiologically unrelated *L. monocytogenes* strains of serotype 4b, which is the most common serotype derived from the patients, also exhibited different restriction enzyme patterns except two strains with 10 of the six-base restriction enzymes and the above three restriction enzymes, *Ban*III, *Pst*I and *Xba*I, were particularly well recognizable. Thus, we confirmed that restriction enzyme analysis of *L. monocytogenes* chromosomal DNA using the six-base restriction enzymes, especially *Ban*III, *Pst*I and *Xba*I, could also be useful for differentiation of serologically indistinguishable strains of *L. monocytogenes* serotype 4b.

ACKNOWLEDGEMENTS

We thank Dr. Michinori Terao, the Niigata Institute of Public Health and Pollution and Dr. Takashi Iida, the Tokyo Metropolitan Research Laboratory of Public Health, for the serotyping of *L. monocytogenes* strains used in this study, and Mr. Susumu Funahashi, Higashi-Matsuyama Health Center, for collecting the materials.

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