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Molecular Epidemiological Study on *Listeria monocytogenes* in
Dairy and Beef Cattle in Japan

(我が国の乳牛および肉牛におけるリステリア菌の分子疫学的研究)

天使大学大学院看護栄養学研究科

栄養管理学専攻

長谷川めぐみ

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General Introduction

Listeria is a regular gram-positive rod with a rounded end; it is an aerobic, microaerophilic, facultatively anaerobic organism that is catalase positive and oxidase negative. *Listeria* does not produce spores and capsules are not formed. *Listeria* usually grows well on most commonly used bacteriological media and can multiply over a wide range of temperatures (1–45°C). The genus *Listeria* contains ten species: *Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. fleischmannii*, *L. marthii*, *L. rocourtiae*, and *L. weihenstephanensis*. *L. monocytogenes* and *L. ivanovii* are naturally and experimentally pathogenic. *L. monocytogenes* is the causative agent of listeriosis, a serious invasive illness that affects both humans and animals; *L. ivanovii* is mainly responsible for abortion in animals (68). *L. monocytogenes* has traditionally been regarded as pathogenic at the species level, with a generally accepted belief that all *L. monocytogenes* isolates are potentially virulent and capable of causing diseases. However, from experimental data collected in recent years, it has become clear that *L. monocytogenes* demonstrates enormous serotype/strain variation in virulence and pathogenicity. *L. monocytogenes* comprises 13 serovars, of which serotypes 1/2a, 1/2b, 1/2c, and 4b account for the vast majority of cases of human disease (67).

Listeriosis commonly affects pregnant women, neonates, elderly individuals, and immunosuppressed individuals. Unlike other foodborne illnesses, which rarely result in fatalities, the mortality rate of listeriosis is approximately 30% (45). The consumption of contaminated food is believed to be the principal cause of infection.

In the United States, human listeriosis affects approximately 1,600 individuals annually, causing 255 deaths each year (72). The European Center for Disease Prevention and Control reported that listeriosis had the highest impact in the elderly (age \geq 64 years), with the highest confirmed case rates and mortality being reported in 2009 (18). In Japan, *Listeria* detection has not been routinely performed on samples from people with diarrhea, and the outbreak trend of listeriosis has still not been determined. In the single foodborne listeriosis case reported in Japan, *Listeria*-contaminated food was first detected during routine monitoring, and epidemiological studies were later carried out on people who consumed the food (49).

Raw milk has been suggested to be a source of *L. monocytogenes* in the dairy processing environment (68). The organism is excreted for months in the milk of healthy cows over several lactation periods, and direct contamination of bulk milk may occur as a result of udder infections (84). Indirect contamination may occur if the organism is present on the udder surfaces because of contaminated feed, feces, bedding, and other environmental sources.

The bovine colostrum is an important source of nutrients and immune factors for neonatal calves; however, the colostrum may also be the earliest source of exposure of dairy calves to infectious agents. This exposure is a matter of concern because pathogenic bacteria present in the colostrum may cause diseases such as diarrhea or septicemia and because the bacteria may interfere with the absorption of immunoglobulin (26). Meanwhile, the value of bovine colostrum as a biological

product with medicinal benefits has been well documented in clinical trials and supported by relatively large databases. Clinical trials have shown that concentrated bovine colostrum may enhance the immune systems of patients with weakened immunity (77). Therefore, the popularity of colostrum products has been increasing because of an increased demand for functional foods and dietary supplements. The medicinal uses of bovine colostrum have been studied; however, the microbial diversity present in dried commercial bovine colostrum products has not yet been completely investigated (34). Knowledge of the prevalence of pathogenic bacteria in the colostrum is necessary because of the importance of colostrum immunoglobulins to the health of neonatal calves. Many studies have detected the presence of *L. monocytogenes* isolates in raw milk (69, 88, 89); however, there have been no documented cases of *L. monocytogenes* isolates in the bovine colostrum.

Meat products have been suggested as the cause of listeriosis outbreaks in many countries (9, 27, 73). It is generally thought that cattle feces may be an important source of foodborne pathogens for beef meat contamination in processing plants (1); therefore, reduction of *L. monocytogenes* at the farm level is important for decreasing human exposure to the bacterium. Investigations of the molecular ecology and genetic diversity of isolates collected from dairy farms have been previously conducted (4, 35, 44, 83). However, only a few studies have investigated the prevalence and molecular characteristics of *L. monocytogenes* isolated from beef cattle farms (7, 46, 52). Specifically in Japan, no studies have been performed on the

molecular epidemiology of *L. monocytogenes* found in beef cattle farms. Furthermore, molecular characterization of *L. monocytogenes* isolates from beef meat has not been performed, although the prevalence of the isolates in beef meat has been investigated in Japan (37, 60).

Objectives of this study

The objectives of this thesis were as follows. Chapter 1 described work that determined the prevalence and molecular characteristics of *L. monocytogenes* isolates in the bovine colostrum. In addition, colostrum products were examined to determine their biological safety. In Chapter 2, the prevalence and molecular characteristics of the bacterium in black beef cattle feces collected from farms across Japan were assessed in order to provide basic data for the control of *L. monocytogenes* at the farm level. The work described in Chapter 3 focused on understanding the distribution subtypes of *L. monocytogenes* from beef meat and examined the relatedness among the isolates from black beef cattle, beef meat, and human clinical cases. The overall objective of this thesis was to study molecular characteristics of *L. monocytogenes* isolates, and to provide data that contribute to developing methods to control *L. monocytogenes*.

Chapter 1

Prevalence and Characteristics of *Listeria monocytogenes* in Bovine Colostrum in Japan

Introduction

Listeria monocytogenes is the causative agent of listeriosis, a serious invasive illness that affects both humans and animals. In humans, listeriosis commonly affects pregnant women, neonates, elderly individuals, and immunosuppressed individuals. Unlike other foodborne illnesses, which rarely result in fatalities, the mortality rate of listeriosis is approximately 30% (45). The consumption of contaminated food is believed to be the principal cause of the infection. Raw milk has been suggested to be a source of *L. monocytogenes* in the dairy processing environment (69), and milk and milk-related products have been implicated in many listeriosis outbreaks (10, 47). The organism is excreted in the milk of healthy cows for months over several lactation periods, and direct contamination of bulk milk may occur as a result of udder infections (84). Indirect contamination may occur if the organism is present on the udder surfaces because of contaminated feed, feces, bedding, and other environmental sources.

In the United States, human listeriosis affects approximately 1,600 individuals, causing 255 deaths each year (72). The European Center for Disease Prevention and Control reported that listeriosis had the highest impact in the elderly (those over 64 years) with the highest confirmed case rates and high mortality in 2009 (18). In Japan, no outbreaks of listeriosis have been reported, except for one case of foodborne listeriosis caused by consumption of natural cheese in 2001 (49). Okutani et al. (62) collected data on Japanese foods contaminated with *L. monocytogenes* and found that the prevalence was almost the same as that in the United States,

France, and Canada, although the annual incidence of listeriosis is lower in Japan than in those countries (63). Thus, it has been suggested that food-borne listeriosis might occur at the same level in Japan as in these countries.

Bovine colostrum is an important source of nutrients and immune factors for neonatal calves; however, colostrum may also be the point of the earliest exposure of dairy calves to infectious agents. This exposure is a matter of concern because the pathogenic bacteria present in colostrum may cause diseases such as diarrhea or septicemia. It is also a matter of concern because the bacteria in colostrum may interfere with the absorption of immunoglobulin (26). Streeter et al. (76) reported that *Mycobacterium paratuberculosis* was isolated from colostrum obtained from clinically normal cows and there was a higher prevalence of isolation from colostrum than from milk. Houser et al. (36) reported that the mean standard plate count; preliminary incubation count; laboratory pasteurization count; and *Staphylococcus aureus*, coagulase negative staphylococci, streptococci, coliforms, and non-coliforms counts in colostrum were considerably higher than those in raw bulk tank milk counts. Thus, colostrum may have an increased risk of bacterial contamination.

The value of bovine colostrum as a biological product with medicinal benefits has been well documented in clinical trials and supported by relatively large databases. Colostrum has antimicrobial properties and is known to modulate immune responses (77). Clinical trials have shown that concentrated bovine colostrum may enhance the immune systems of patients with weakened immunity(77). Therefore,

colostrum products have been growing in popularity because of an increased demand for functional foods and dietary supplements. The medicinal uses of bovine colostrum have been studied; however, the microbial diversity present in dried commercial bovine colostrum products has not yet been completely investigated (34).

Knowledge of the prevalence of organisms in colostrum is necessary because of the importance of colostral immunoglobulins to the health of neonatal calves. Many studies have detected the presence of *L. monocytogenes* isolates in raw milk (69, 88, 89); however there have been no documented cases of *L. monocytogenes* isolates in bovine colostrum. Therefore, the aim of this study was to determine the prevalence and molecular characteristics of *L. monocytogenes* isolates in bovine colostrum. In addition, colostrum products were examined to determine their biological safety.

Materials and Methods

Sample collection

Colostrum samples were collected from 210 dams in 21 dairy farms in Hokkaido prefecture, Japan within 24 h of parturition. Approximately 50 ml of colostrum was collected using a new sterile glove after forestripping and predipping with a 0.5% iodine-based teat dip, drying the teat ends with a clean paper towel, and scrubbing all the teat ends with an alcohol-soaked gauze pad. The sample was then aseptically stripped directly into a sterile 50 ml plastic sampling vial with approximately equal amounts of milk collected from the four quarters. Fecal samples were collected from

42 neonatal calves (age, 2–8 days) and the 42 cows from which were collected colostrum samples. All the samples were chilled and transported to the laboratory for microbiological analysis.

Colostrum supplements

Ninety-three samples, i.e., 3 lots of 31 bovine colostrum supplement products (prepared in the United States, New Zealand, Australia, and Japan), were purchased online to investigate the prevalence of *L. monocytogenes*. These colostrum supplements were powders or tablets.

Bacterial isolates

In the preliminary experiment, the use of the cold-enrichment process before antibiotic enrichment yielded more *L. monocytogenes* isolates from colostrum than the use of antibiotic enrichment alone. Thus, colostrum samples (25 ml) were enriched at 4°C for 9 weeks. Colostrum supplements (25 g) were added to 225 ml of tryptose broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 0.1% pyruvic acid and enriched at 4°C for 2 weeks. Next, 5 ml of the enriched samples were added to 45 ml of University Vermont Modified *Listeria* Enrichment Broth (UVM; BD, Franklin Lakes, NJ). Five grams of fecal samples were added to 25 ml of Nutrient Broth (Nissui Seiyaku Co., Ltd.) and enriched at 4°C for 2 weeks, and 5 ml of the enriched culture was added to 45 ml of UVM *Listeria* Enrichment Broth. After incubation at 30°C for 24 h, 0.1 ml of the culture was transferred to 10 ml of Fraser Broth (BD) with Fraser Selective Supplement (Oxoid, Basingstoke, UK) and incubated at 35°C for either 24 or 48 h. Next, 0.1 ml of each culture was streaked

onto Oxford Medium Base (BD) with *Listeria* Selective Supplement (Oxoid), and both were incubated at 35°C for 24–48 h. Typical *Listeria*-like colonies with black halos were selected from these plates. Putative colonies were spotted onto CHROMagar *Listeria* plates (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 24–48 h. Five blue colonies with halos were characterized using the Christie, Atkins, Munch-Peterson test, β -hemolysis reaction, catalase reaction, Gram staining, and motility test in semisolid media. *L. monocytogenes* isolates were stored in Brain Heart Infusion (BHI; Difco, Detroit, MI) medium with 10% glycerol at –80°C.

Bacterial strains

A total of 19 *L. monocytogenes* isolates from human listeriosis cases were used. *L. monocytogenes* isolates were kindly provided by the following researchers: Dr. Makino, S. I., Obihiro University of Agriculture and Veterinary Medicine, 3 isolates (49, 63); Dr. Ueda, F., Nippon Veterinary and Life Sciences University, 1 isolate (59); Dr. Yoshida, T., Nagano Environmental Conservation Research Institute, 10 isolates; Dr. Nakama, A., Tokyo Metropolitan Institute of Public Health, 1 isolate; Dr. Ito, M., Sapporo Clinical Laboratory Inc., 2 isolates; Dr. Kobayashi, K., Daiichi Clinical Laboratories Inc., 2 isolates. The isolates comprised 8 strains of serotype 1/2b and 11 of serotype 4b. Two isolates were isolated from the feces of raccoons, which were caught in another dairy farm (N farm) in Hokkaido, using the same laboratory protocols as described above.

Serotyping of the isolates

Serotyping was performed using commercial *Listeria* antisera (Denka Seiken Co., Ltd., Tokyo, Japan), according to the manufacturer's recommendations.

Pulsed field gel electrophoresis (PFGE) analysis

The author followed the Center for Disease Control and Prevention PulseNet protocol for PFGE (28). The chromosomal DNA of *L. monocytogenes* was restriction digested using *AscI* (New England BioLabs, Beverly, MA) and *ApaI* (Takara, Shiga, Japan). PFGE was performed on a CHEF-DRII electrophoresis system (Bio-Rad Laboratories, Hercules, CA) with recirculated 0.5× tris–borate–ethylenediaminetetraacetic acid (TBE) extended-range buffer (Bio-Rad Laboratories) at 14°C. The macrorestriction fragments were resolved on a 1% SeaKem Gold Agarose (Cambrex, Rockland, ME) gel in 0.5× TBE buffer. *XbaI*-digested *Salmonella* Braenderup H9812 DNA was used as a molecular weight marker. The pulse time was increased from 4.0 to 40.0 s during an 18 h run at 6.0 V/cm. The PFGE patterns were compared using the BioNumerics program (version 5.0; Applied Maths, Kortrijk, Belgium). Similarities among the restriction fragments of isolates were determined using the unweighted pair group method with arithmetic mean.

PCR of L. monocytogenes virulence-associated genes and epidemic clone markers

DNA was extracted from overnight BHI cultures using a commercially prepared extraction preparation (InstaGene Matrix; Bio-Rad Laboratories), following the manufacturer's instructions. The primers for 11 *L. monocytogenes*

virulence-associated genes (*actA*, *hly*, *iap*, *inlA*, *inlC*, *mpl*, *plcA*, *plcB*, *opuCA*, *prfA*, and *clpC*) have been described previously (50). The PCR protocol consisted of an initial denaturation step (94°C for 2 min) followed by 40 cycles of denaturation (94°C for 30 s), annealing (53.5°C for 30 s with *hly*; 55.5°C for 30 s with *actA*, *inlA*, *inlC*, *prfA*, *opuCA*, *plcA*, and *clpC*; and 59.5°C for 30 s with *iap*, *mpl*, and *plcB*), extension (72°C for 30 s), and a final extension (72°C for 2 min) step. The primers used for the identification of isolates with the *L. monocytogenes* epidemic clone (EC) I and II markers have been described previously (11). The PCR protocol consisted of an initial denaturation (94°C for 2 min) step followed by 30 cycles of denaturation (94°C for 30 s), annealing (51°C for 30 s with ECI; 55°C for 30 s with ECII), extension (72°C for 50 s), and a final extension (72°C for 2 min) step. The PCR products were electrophoresed using a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed for 48 of the 80 *L. monocytogenes* isolates. Fourteen different antimicrobials were selected for susceptibility testing using the microbroth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) standards (13) for the following antimicrobial agents (dilution ranges): penicillin (0.12 to 256 µg/ml), ampicillin (0.12 to 256 µg/ml), oxacillin (0.12 to 128 µg/ml), amoxicillin (0.12 to 64 µg/ml), gentamicin (0.12 to 128 µg/ml), kanamycin (0.12 to 128 µg/ml), streptomycin (0.12 to 128 µg/ml), erythromycin (0.12 to 128 µg/ml), vancomycin (0.12 to 128 µg/ml),

tetracycline (0.12 to 64 µg/ml), chloramphenicol (0.12 to 64 µg/ml), fosfomicin (0.12 to 128 µg/ml), ciprofloxacin (0.12 to 64 µg/ml), and trimethoprim/sulfamethoxazole (0.08 to 80 µg/ml). The microbroth dilution panels were purchased in a frozen 96-well format (Eiken Chemical Co., Ltd., Tokyo, Japan). The panels were stored at –80°C and thawed immediately before use. The panels were incubated for 20 to 24 h at 35°C. The minimum inhibitory concentrations (MICs) were read manually. The MIC for 50% of the strains (MIC₅₀), and the MIC for 90% of the strains (MIC₉₀) were determined for each antibiotic. The breakpoints for the susceptibility of *L. monocytogenes* to penicillin and ampicillin were obtained from the CLSI guidelines (13). Other antibiotics had bimodal MIC distributions with no breakpoints specified in these guidelines, so the microbiological breakpoints were also applied based on the research results of the Japanese Veterinary Antimicrobial Resistance Monitoring System (55). The microbiological breakpoint is defined as the intermediate MIC between the two peak distributions. The breakpoint was not determined if the MIC distribution was monomodal.

Results

Prevalence of L. monocytogenes in bovine colostrum

In this study, the author surveyed 210 colostrum samples from 21 farms in Hokkaido prefecture. The prevalence of *L. monocytogenes* in bovine colostrum is shown in Table 1. *L. monocytogenes* was found in six (28.6%) farms. Of the 210 samples, 16 (7.6%) were positive for *L. monocytogenes*. At KB, KO, and KU farms,

the isolation rate of the bacterium was higher (66.7, 50.0, and 50.0%, respectively) than on H, T, and YW farms (6.3, 11.8, and 3.7%, respectively). The bacterium was not isolated in 129 samples collected from 15 farms.

Prevalence of L. monocytogenes in feces from cows and their neonatal calves

The author analyzed 42 fecal samples from 42 of the 210 cows from which were collected the colostrum samples. Furthermore, the author investigated 42 fecal samples from 42 neonatal calves that were born and bred from the cows. *L. monocytogenes* was isolated from two (4.8%) fecal samples from cows and three (7.1%) fecal samples from their neonatal calves. Ten cows were found to shed *L. monocytogenes* in their colostrum. However, no cows shed the bacterium in both their feces and colostrum. One of 10 calves whose dam shed *L. monocytogenes* was positive for the bacterium (data not shown).

Serotyping of the isolates

A total of 80 *L. monocytogenes* isolates were recovered from colostrum samples, and the *L. monocytogenes* serotypes are summarized in Table 1. The serotype distribution of the *L. monocytogenes* isolates in the colostrum was as follows: 44 (55%) were serotype 1/2b and 36 (45%) were serotype 4b (Table 1). Serotypes 1/2b and 4b were identified in the KB, KO, and KU farms. These two serotypes were also identified in colostrum samples from cows KB9 and KO1 (data not shown).

PFGE typing of the isolates

PFGE characterization of the 80 isolates from bovine colostrum detected six different PFGE types (Fig. 1). Of the six subtypes detected, three (50%) were

serotype 1/2b and three (50%) were serotype 4b. Four of the PFGE types were isolated from KB farm. Two of the PFGE types were isolated from KO and KU farms. One PFGE type was isolated from H, T, and YW farms. Multiple PFGE types were isolated from the individual colostrum samples from three cows and a maximum of three different PFGE types were isolated from one colostrum sample. PFGE types I and II were isolated from KB farm. PFGE type III was isolated from KB, KO, and KU farms. PFGE type IV was isolated from KB, KO, KU, and T farms. PFGE type V was isolated from H farm. PFGE type VI was isolated from YW farm. One colostrum isolate from farm KB was identical to a fecal isolate from a neonatal calf. One fecal isolate from a raccoon captured on another dairy farm was identical to a colostrum isolate from KB farm. PFGE types I and III corresponded to the isolates from two human clinical cases. PFGE type IV shared 86% similarity with outbreak strains in Hokkaido.

Virulence-associated genes and EC markers in the isolates

Twenty of 80 *L. monocytogenes* isolates from bovine colostrum were investigated. Among these isolates, 17 (85.0%) possessed all 11 virulence genes (Table 1). The remaining isolates lacked *iap*, *mpl*, or *opuCA*. The ECI marker was not detected in the *L. monocytogenes* isolates in colostrum. PFGE type I isolates contained the ECII marker. The raccoon-derived isolate shared high similarity with the PFGE type I isolate with the ECII marker (Fig. 1).

Antimicrobial resistance phenotypes

The MIC distributions of penicillin (<0.12 to 0.5 µg/ml), oxacillin (4 to 8 µg/ml),

ampicillin (0.25 to 1 µg/ml), amoxicillin (<0.12 to 0.5 µg/ml), gentamicin (<0.12 to 0.5 µg/ml), kanamycin (0.5 to 4 µg/ml), streptomycin (2 to 8 µg/ml), erythromycin (0.25 µg/ml), vancomycin (0.5 to 1 µg/ml), tetracycline (0.5 to 1 µg/ml) chloramphenicol (4 to 8 µg/ml), ciprofloxacin (1 to 4 µg/ml), and trimethoprim/sulfamethoxazole (0.31 to 1.25 µg/ml) were monomodal, suggesting that all isolates were susceptible to these antibiotics (Table 2). The MIC distributions of fosfomycin (>128 µg/ml) were higher than the range the author surveyed. The MIC distribution of oxacillin was that for resistance of staphyrococci. (14). It was described that *L. monocytogenes* is naturally resistant to oxacillin and fosfomycin (80).

Prevalence of L. monocytogenes in bovine colostrum supplements

Many different types of colostrum supplements are sold online. The author purchased 93 colostrum supplements, which had been made in the United States (n = 45), New Zealand (n = 42), Australia (n = 3), and Japan (n = 3), and investigated the prevalence of *L. monocytogenes*. The bacterium was not isolated from any of the colostrum supplements.

Discussion

The author conducted a survey to determine the prevalence and characteristics of *L. monocytogenes* in bovine colostrum collected from dairy farms in Hokkaido, Japan. Hokkaido is a major dairy region and approximately 60% of the Japanese dairy cattle are bred in this area. In the present study, 210 samples were collected from 21 dairy farms. Sixteen (7.6%) of the 210 samples and six (28.6%) of the 21

farms were positive for *L. monocytogenes*. Many previous studies have detected *L. monocytogenes* in raw milk and milk products but the prevalence of *L. monocytogenes* in bovine colostrum has not yet been investigated. This survey provides the first data of the prevalence and characteristics of *L. monocytogenes* in bovine colostrum.

In the United States, 861 bulk tank milk samples were collected from farms in 21 states as part of the National Animal Health Monitoring System for dairy during 2002. The survey indicated that *L. monocytogenes* was present in 6.5% of the bulk tank milk samples (81). Other surveys have reported the isolation of *L. monocytogenes* from 4.0% of the 150 raw milk samples from 1989 to 1990 in Japan (70), 0.3% of the 943 bulk tank milk samples from 1990 to 1991 in Japan (89), 5.1% of the 2009 bulk tank milk samples from 1992 to 1993 in England and Wales (58), 1% of the 294 farm bulk tank milk samples from 1997 to 1998 in Sweden (83), 2.8% of 248 bulk tank milk samples from 2001 to 2002 in the United States (40), and 6.1% of the 98 bulk tank milk samples during 2005 in Spain (82). Thus, the isolation rates of the bacterium from bulk tank milk samples were 0.3–6.5%. Several studies have surveyed *L. monocytogenes* contamination in bulk tank milk samples, however, the prevalence of *L. monocytogenes* in colostrum and milk from individual cows has not yet been fully investigated worldwide. Compared with previous studies, this survey provides new data.

L. monocytogenes can cause mastitis in cows, and it can be shed into the milk of asymptomatic cows (84). Bourry et al. (5) induced chronic subclinical mastitis via

intramammary inoculation with a single dose of *L. monocytogenes* and reported that the infections were usually subclinical, and the udder appeared normal. Improperly fermented silage is considered a common cause of ruminants shedding *L. monocytogenes* in their feces. Normal healthy cattle may occasionally shed the bacterium in their feces, with a prevalence rate of up to 52% (84). Mohammed et al. (51) reported that the likelihood of *Listeria* detection was three times higher in bedding samples than in silage, and the pathogen appeared to be most prevalent in feed bunks, water troughs, and bedding. In this study, the author assumed that bacteria penetrating the teat canal could cause *L. monocytogenes* teat colonization from the bedding or local environment. As calving approached and colostrum formation occurred, the gland became susceptible to infection. Despite the increased milk leukocyte concentration in colostrum, the mammary gland is still susceptible to new infections during the prepartum period (54). The author did not determine the somatic cell count in the colostrum. Whether inflammation occurred was unclear. More work is needed to determine whether contamination of the colostrum develops into mastitis. Streeter et al. (76) reported that *M. paratuberculosis* was isolated from colostrum of clinically normal cows and there was higher prevalence of isolation from colostrum than from milk. *M. paratuberculosis* can survive in macrophages for prolonged periods *in vitro*. Macrophages are the predominant cell type in noninfected bovine mammary glands during the peripartum period. It has been hypothesized that the long duration of the nonlactating period and accumulation of large numbers of macrophages during this period may account for

the higher prevalence of isolates from colostrum than from milk. *L. monocytogenes* can also survive and proliferate within macrophages (43). The present study may support a similar hypothesis. Fedio and Jackson (20) reported that contamination from within the udder is likely to be rare. Thus, the other bacteria found in the samples must be identified to exclude the possibility of colostrum being contaminated from outside the teat. Fox et al. (22) reported a correlation between the level of hygiene standards in farms and the occurrence of *L. monocytogenes*. A sanitary dairy farm environment is essential for preventing colostrum contamination.

L. monocytogenes isolates of serotypes 1/2b and 4b were detected from colostrum samples. In previous studies in other countries have shown that serotypes 1/2a, 1/2b, and 4 were recovered frequently from dairy farms (4, 22, 81). The results of this study are in agreement with these findings. In Japan, serotypes 1a, 1/2a, 4ab, and 4b were detected in raw milk samples and dairy farm environment (70, 79, 89). Serotype 1/2c was isolated mainly from the carcass surfaces of cattle and their intestinal contents (62). However, the author did not detect serotype 1/2a nor 1/2c. This study was conducted in a limited area of Japan; therefore, it is necessary to investigate the epidemiology of this bacterium at a larger scale.

The isolation rate of *L. monocytogenes* varied according to the farm. There were farms from which *L. monocytogenes* was never isolated. However, *L. monocytogenes* was isolated from approximately half of the cows investigated in some farms. The

prevalence of *L. monocytogenes* in KB, KO, and KU farms was higher than that in the other farms. Four different PFGE types and both serotypes were detected on KB farm. Multiple PFGE types were detected in the isolates from three farms; therefore, these locations probably were continuously contaminated with *L. monocytogenes*. In other countries, there have been reports of genetic diversity of *L. monocytogenes* being detected in a single dairy farm. Borucki et al. (4) reported that 57 different PFGE types were detected in dairy farm environments, and the maximum number of PFGE types and serotypes isolated from the fecal sample of one cow were six and four, respectively. Ho et al. (35) reported that a single cow can harbor more than one *L. monocytogenes* ribotype, and 20 distinct subtypes were isolated in a single herd. In Japan, several studies have surveyed *L. monocytogenes* contamination in dairy farms but the prevalence of *L. monocytogenes* has not yet been fully investigated in dairy farms. This is the first report on the detection of multiple PFGE types and serotypes from a single farm.

To determine whether the *L. monocytogenes* isolates in colostrum were related to isolates from human listeriosis cases, the author characterized the colostrum isolates by PFGE profiles. Some *L. monocytogenes* isolates in colostrum had identical to those of human clinical isolates, suggesting that bovine colostrum could be a significant reservoir of *L. monocytogenes* that cause human infections. In other countries, the PFGE profiles of food animal isolates from ruminant farms, foods, and different environments were compared with the profiles of pathogenic human isolates to identify any possible correlations (24, 64). In Japan, Nakama et al. (53)

reported that some PFGE types from foods on retail sale were recognized isolates of clinical origin. In this study, the genetic diversity of *L. monocytogenes* strains was less than that reported by Borucki et al. (4) and Ho et al. (35). However, these results cannot be compared directly because the sampling method was different from their studies, i.e., the study of Borucki et al. (4) was undertaken on farms where bovine listeriosis cases occurred, whereas Ho et al. (35) investigated fecal samples for approximately one month. The genetic diversity could be related to the low incidence of listeriosis in Japan. Further investigation is required to reveal the genetic diversity of the bacterium from different sources.

Of note, the *L. monocytogenes* isolates in colostrum possessed an ECII marker. To date, most documented human outbreaks of foodborne listeriosis have involved a small number of closely related strains, primarily of serotype 4b: ECI and ECII (12). ECI includes isolates from large listeriosis outbreaks that occurred in European countries, Canada, and the United States, while ECII includes isolates from two listeriosis outbreaks in the United States that were linked to the consumption of contaminated hot dogs and turkey. The ECI and ECII markers may be associated with the increased pathogenicity of the outbreak-associated *L. monocytogenes* 4b strains, making them particularly dangerous to humans. Serotypes 1/2a, 1/2b, and 4b are responsible for most human listeriosis cases (45). The isolates from bovine colostrum were classified as serotypes 1/2b and 4b. The presence of *L. monocytogenes* virulence-associated genes, and the PFGE types and serotypes of the bovine *L. monocytogenes* isolates in colostrum, suggested that these strains

may be able to invade host cells and cause listeriosis. These data reinforce previous reports implicating farms as possible reservoirs for human epidemic *L. monocytogenes* strains (56). A fecal isolate from a raccoon captured at another dairy farm was identical to a colostrum isolate, and it possessed the ECII marker. The raccoon is native to North America, but many have been imported to Japan as pets. In Hokkaido, the intentional release and escape of pet raccoons has led to a naturalized population. These raccoons could contribute to the amplification and dispersal of *L. monocytogenes* into the farm environment and thus to increase in the number of human listeriosis cases. The PFGE profiles of fecal isolates from neonatal calves were identical to those of the colostrum isolates from their dams; therefore, bovine colostrum might cause neonatal cattle listeriosis. Thus, cattle must be kept healthy, to prevent the contamination of bovine colostrum.

L. monocytogenes was not isolated from the colostrum supplement products that the author investigated. Hayes et al. (34) reported that *Bacillus*, *Pseudomonas*, *Kocuria*, and *Enterococcus* species were identified in dried colostrum nutraceutical products, although the presence of these species did not indicate the origin of these organisms or the history of the processing of the products. Sanitary manufacturing practices are necessary to prevent the contamination of bovine colostrum products with *L. monocytogenes* and other pathogenic organisms.

The *L. monocytogenes* isolates from bovine colostrum were susceptible to antimicrobial agents, except oxacillin and fosfomycin. Until recently, *L. monocytogenes* was considered to be susceptible to antibiotics that are effective

against gram-positive bacteria. However, many antibiotic-resistant strains of *L. monocytogenes* have been reported since 1988 (65). Srinivasan et al. (75) reported that 15% of the *L. monocytogenes* isolated from dairy farm environments were multidrug resistant. Harakeh et al. (30) reported a high percentage of resistance to penicillin, ampicillin, and chloramphenicol in *L. monocytogenes* isolated from dairy-based food products. There have been very few studies on antibiotic resistance of *L. monocytogenes* in Japan (61). Antimicrobial agents are used for growth promotion to improve animal husbandry in Japan. Veterinary antimicrobial use is a selective force that promotes the appearance and prevalence of antimicrobial-resistant bacteria in food-producing animals (39, 55). *L. monocytogenes* becomes resistant to antibiotics through the acquisition of mobile genetic elements (65). *L. monocytogenes* isolates in Japan could acquire antibiotic resistance genes, thereby functioning as an antimicrobial resistance gene pool for other commensal and pathogenic bacteria on dairy farms. Thus, public health can be protected by continuously monitoring antibiotic resistance in *L. monocytogenes* isolates from dairy farms.

Further investigations are needed to reduce the contamination of bovine colostrum with *L. monocytogenes*. This is the first study on the prevalence and serological and molecular characteristics of *L. monocytogenes* in bovine colostrum from dairy farms. Although this study was limited to a region of Japan, these findings have important implications for improving public health, and they provide a significant advancement in the understanding of the molecular epidemiology of *L.*

monocytogenes in bovine colostrum.

Summary

This study determined the prevalence and characteristics of *L. monocytogenes* in bovine colostrum in Japan. The author collected bovine colostrum samples from 210 dams from 21 dairy farms in Hokkaido prefecture in Japan between March and June 2009. *L. monocytogenes* was detected in six (28.6%) of the 21 farms. Of the 210 samples, 16 (7.6%) were positive for *L. monocytogenes*. The author recovered 80 *L. monocytogenes* isolates. Forty-four (55%) isolates were classified as serotype 1/2b and 36 (45%) as serotype 4b. PFGE characterization of 80 isolates identified six different PFGE types. Two PFGE types corresponded to human listeriosis cases. Most *L. monocytogenes* isolates possessed virulence-associated genes (*actA*, *hly*, *iap*, *inlA*, *inlC*, *mpl*, *plcA*, *plcB*, *opuCA*, *prfA*, and *clpO*). One PFGE type isolate possessed an epidemic clone II marker. These findings suggest that isolates from bovine colostrum may have the potential to cause human and animal listeriosis. The isolates were susceptible to penicillin, ampicillin, amoxicillin, gentamicin, kanamycin, streptomycin, erythromycin, vancomycin, tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim/sulfamethoxazole. This is the first study on the prevalence and characteristics of *L. monocytogenes* isolated from bovine colostrum obtained from dairy farms. The results of this study have important implications for improving public health and elucidating the molecular epidemiology of *L. monocytogenes* in bovine colostrum.

Table 1. Prevalence and characteristics of *L. monocytogenes* in bovine colostrum from Hokkaido, Japan

Farm	No. of samples examined	No. (%) of positive samples	No. of isolates	Serotypes(s) (No. of isolates)	PFGE type(s) (No. of isolates) ^a	Virulence gene(s) ^b
Positive farms (by name)						
H	16	1 (6.3)	5	1/2b (5)	V (5)	<i>mpl</i> ^c
KB	9	6 (66.7)	30	1/2b (6), 4b (24)	I (10), II (4), III (10), IV (6)	+, <i>opuCA</i> ^d , <i>iap</i> ^e
KO	2	1 (50.0)	5	1/2b (3), 4b (2)	III (2), IV (3)	+
KU	10	5 (50.0)	25	1/2b (15), 4b (10)	III (10), IV (15)	+
T	17	2 (11.8)	10	1/2b (10)	IV (10)	+
YW	27	1 (3.7)	5	1/2b (5)	VI (5)	+
Negative farms (combined)						
15	129	0				
Total farms						
21	210	16 (7.6)	80	1/2b (44), 4b (36)		

^aCombined profile for two restriction enzymes: *AscI* and *ApaI*

^bGenes *actA*, *hly*, *iap*, *inlA*, *inlC*, *mpl*, *plcA*, *plcB*, *opuCA*, *prfA*, and *clpC*. (+): strains harbor all 11 genes.

^cIsolate with *mpl* deficiency

^dIsolate with *opuCA* deficiency

^eIsolate with *iap* deficiency

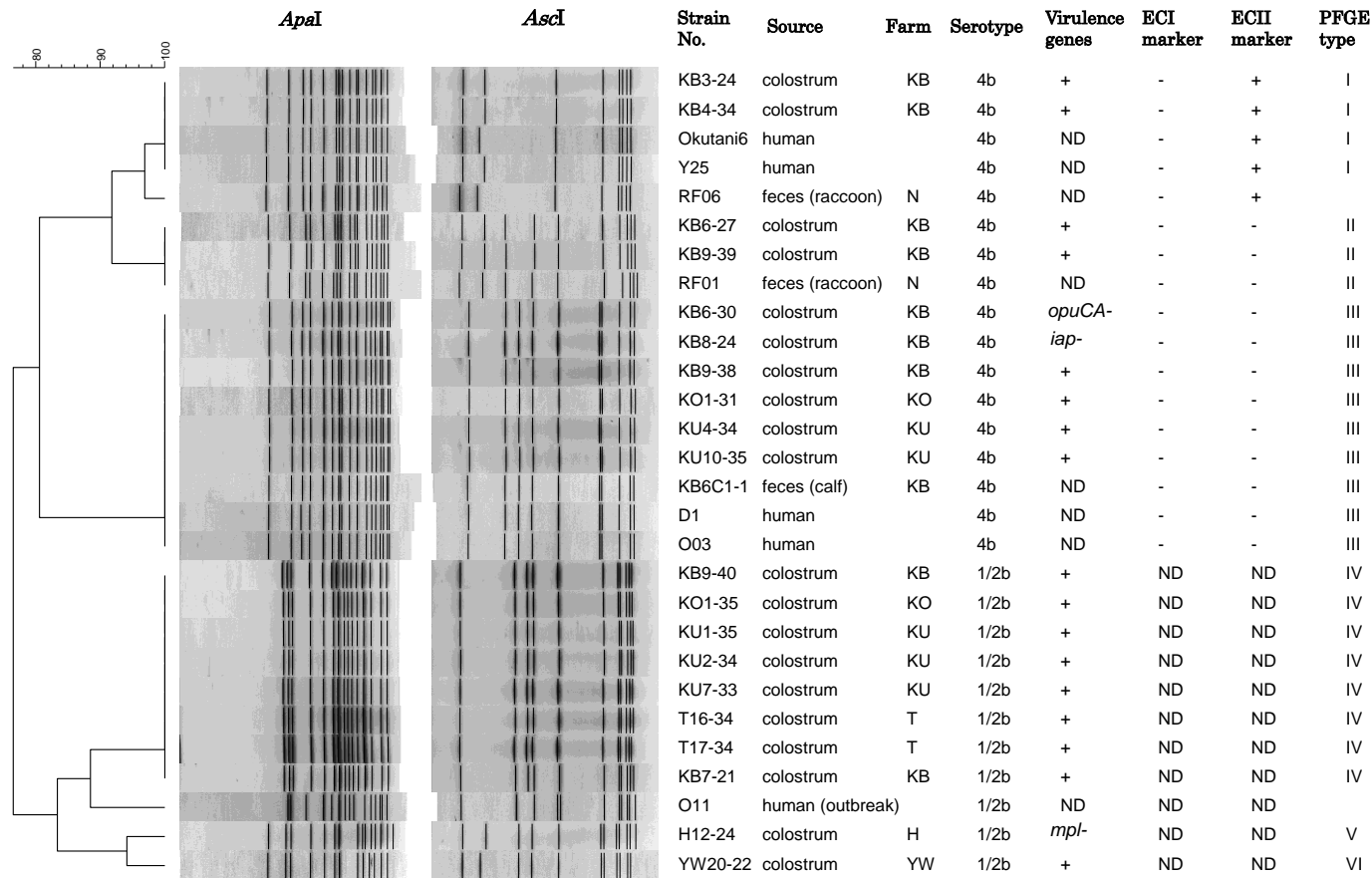


Figure 1. Dendrogram of the *L. monocytogenes* PFGE types for isolates from bovine colostrum, human clinical cases, and the feces of calves and raccoons. ND, not done. Virulence genes are *actA*, *hly*, *iap*, *inlA*, *inlC*, *mpl*, *plcA*, *plcB*, *opuCA*, *prfA*, and *clpC*. Isolates with an *mpl* deficiency (*mpl*-), an *opuCA* deficiency (*opuCA*-), and an *iap* deficiency (*iap*-) are indicated.

Table 2. Antimicrobial susceptibility of 48 *Listeria monocytogenes* isolates from bovine colostrum in Hokkaido, Japan ^a

Antimicrobial agent	No. of isolates susceptible at MIC ($\mu\text{g/ml}$) of :																	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
	0.16	0.31	0.62	1.25	2.5	<0.12	0.25	0.5	1	2	4	8	16	32	64	128	256		
Penicillin	ND	ND	ND	ND	ND	2	28	18	0	0	0	0	0	0	0	0	0	0.25	0.5
Oxacillin	ND	ND	ND	ND	ND	0	0	0	0	0	47	1	0	0	0	0	ND	4	4
Ampicillin	ND	ND	ND	ND	ND	0	18	29	1	0	0	0	0	0	0	0	0.5	0.5	
Amoxicillin	ND	ND	ND	ND	ND	1	43	4	0	0	0	0	0	0	0	ND	ND	0.25	0.25
Gentamicin	ND	ND	ND	ND	ND	17	23	8	0	0	0	0	0	0	0	0	ND	0.25	0.5
Kanamycin	ND	ND	ND	ND	ND	0	0	1	21	23	3	0	0	0	0	0	ND	2	2
Streptomycin	ND	ND	ND	ND	ND	0	0	0	0	3	36	9	0	0	0	0	ND	4	8
Erythromycin	ND	ND	ND	ND	ND	0	48	0	0	0	0	0	0	0	0	0	ND	0.25	0.25
Vancomycin	ND	ND	ND	ND	ND	0	0	2	46	0	0	0	0	0	0	0	ND	1	1
Tetracycline	ND	ND	ND	ND	ND	0	0	11	37	0	0	0	0	0	0	ND	ND	1	1
Chloramphenicol	ND	ND	ND	ND	ND	0	0	0	0	0	1	47	0	0	0	ND	ND	8	8
Fosfomycin	ND	ND	ND	ND	ND	0	0	0	0	0	0	0	0	0	0	0	48 (>128)	>128	>128
Ciprofloxacin	ND	ND	ND	ND	ND	0	0	0	45	2	1	0	0	0	0	ND	ND	1	1
Trimethoprim-Sulfamethoxazole	0	1	46	1	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.62	0.62

^a Antimicrobial susceptibility tests were performed for 48 of the 80 *L. monocytogenes* isolates. MIC₅₀ and MIC₉₀ are the MICs for 50 and 90% of the isolates, respectively. ND, analysis not done.

Chapter 2

Prevalence and Characteristics of *Listeria monocytogenes* in Feces of Japanese Black Beef Cattle Reared in Three Geographically Distant Areas in Japan

Introduction

Listeria monocytogenes is the causative agent of listeriosis, a serious invasive illness that affects both humans and animals. Unlike other foodborne illnesses, which rarely result in fatalities, the mortality rate of listeriosis is approximately 30% (45). In the United States, human listeriosis is known to affect approximately 1,600 individuals and cause 255 deaths every year (72). In Japan, an average of 83 cases of listeriosis per year has been reported (63). However, accurate numbers of the incidence of listeriosis are not available owing to the lack of a mandatory notification system.

Meat products have been implicated as sources of listeriosis outbreaks in many countries (9, 27, 73). It is generally thought that cattle feces may be an important source of foodborne pathogens for beef meat contamination in processing plants (1); therefore, the reduction of *L. monocytogenes* at the farm level is important for decreasing human exposure to the bacterium.

Investigations of molecular ecology and genetic diversity of isolates collected from dairy farms have previously been conducted to prevent contamination of *L. monocytogenes* in milk products and listeriosis outbreaks (4, 35, 44, 83, 88-90). Although deli meats were implicated in 25% of the 24 listeriosis outbreaks during 1998–2008 in the United States (8), few studies have investigated the prevalence and characteristics of *L. monocytogenes* isolated from beef cattle farms (7, 46, 52). Specifically in Japan no studies on molecular epidemiology of *L. monocytogenes* in

beef cattle farms have ever been carried out. To provide basic data for control of *L. monocytogenes* at the farm level, the objective of this study was to determine the prevalence and molecular characteristics of the bacterium in black beef cattle feces collected from farms across Japan.

Materials and Methods

Sample collection

Japanese black beef cattle feces were collected from farms located in 3 areas of Japan: northern (Hokkaido prefecture), central (Gifu and Mie prefectures), and southern (Oita, Miyazaki, and Kagoshima prefectures; Fig. 2) between April and June (spring) 2011. Furthermore, fecal samples were collected from northern area between July and September (summer) 2011. The numbers of tested farms and cattle are shown in Table 3. Fecal samples were obtained from apparently healthy cattle directly through the rectum of each cattle by using a clean plastic sleeve for each sample. Samples were chilled and transported to the laboratory, and the samples were analyzed within 12 h.

Bacterial isolates

Cold enrichment followed by selective enrichment was used to isolate *L. monocytogenes* from fecal samples (16). Briefly, fecal samples (1 g) were added to 5 ml of Nutrient Broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan) and enriched at 4°C for 2 weeks. Next, 5 ml of enriched samples were added to 45 ml of University of

Vermont Modified *Listeria* Enrichment Broth (BD, Franklin Lakes, NJ). After incubation at 30°C for 24 h, cultures (0.1 ml) were incubated with 10 ml of Fraser Broth (BD) containing Fraser Selective Supplement (Oxoid, Basingstoke, UK) at 35°C for either 24 or 48 h. Each culture (0.1 ml) was then streaked onto CHROMagar *Listeria* plates and incubated at 37°C for 24–48 h. Four to 10 typical *Listeria*-like colonies with halos were selected from plates and characterized by the Christie, Atkins, Munch-Peterson test, β -hemolysis reaction, catalase reaction, Gram staining, and motility test in semisolid media. *L. monocytogenes* isolates were stored in BHI with 10% glycerol at –80°C.

Serotyping of the isolates

Serotyping was performed using commercial *Listeria* antisera (Denka Seiken Co., Ltd., Tokyo, Japan), according to the manufacturer's recommendations.

Pulsed-field gel electrophoresis (PFGE) analysis

PFGE was carried out by following the Center for Disease Control and Prevention PulseNet protocol (28). Chromosomal DNA of *L. monocytogenes* was digested with restriction enzymes, *AscI* (New England BioLabs, Beverly, MA) and *ApaI* (Takara, Shiga, Japan). PFGE patterns were compared using the BioNumerics program (version 5.0; Applied Maths, Kortrijk, Belgium). Similarities among restriction fragments of isolates were determined using unweighted pair group method with arithmetic mean.

PCR of L. monocytogenes epidemic clone (EC) II and III markers

DNA was extracted from overnight BHI cultures using a commercially prepared extraction preparation (InstaGene Matrix; Bio-Rad Laboratories), according to manufacturer's instructions. The primers used for identification of isolates of the *L. monocytogenes* EC II and III markers have been described by Chen and Knabel (11). The cycling program and electrophoresis conditions have been described previously (31). Since none of the black beef cattle isolates had identical PFGE profiles to those of ECI clone, ECI marker was not investigated.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed for 315 out of 996 *L. monocytogenes* isolates. Fourteen different antimicrobials, including penicillin, ampicillin, oxacillin, amoxicillin, gentamicin, kanamycin, streptomycin, erythromycin, vancomycin, tetracycline, chloramphenicol, fosfomycin, ciprofloxacin, and trimethoprim/sulfamethoxazole, were selected for susceptibility testing using the microbroth dilution method, according to the Clinical and Laboratory Standards Institute standards (13). The breakpoints were determined as described previously (31).

Bacterial strains

In total, 35 *L. monocytogenes* isolates from human listeriosis cases, livestock that had a diagnosis of listeriosis, and wild animals were used to study the relatedness with the isolates from black beef cattle (Table 4).

Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More precisely, it is a modified version of R commander (version 1.6-3) designed to add statistical functions that are frequently used in biostatistics. A chi-squared test was used to compare the prevalence of *L. monocytogenes*. Differences were considered significant at a significance level of $p < 0.05$.

Results

Prevalence of L. monocytogenes among feces of black beef cattle

The author surveyed farms from three areas of Japan, including northern, central, and southern areas. Prevalence of *L. monocytogenes* among feces of black beef cattle for each area were as follows: northern, 11.4% of 651; central, 2.8% of 572; and southern, 2.9% of 515 (Table 3). Based on these data, the isolation rate in the northern area was significantly higher than that in the central or southern area ($p < 0.01$). In the northern area, the isolation rate in spring was significantly higher than in summer ($p < 0.01$).

Serotyping of the isolates

Serotyping of 996 isolates identified 1/2b as the most prevalent serotype (40.5%), followed by 1/2a (36.9%), 4b (21.6%), and 4ab (1.0%) (Table 5). In the northern area,

12.1% of the isolates were classified as serotype 4b, whereas in the central and southern area, 46.3% and 44.8%, respectively, were classified as serotype 4b, indicating a significantly lower rate of serotype 4b in the northern area ($p < 0.01$). Notably, in the northern area, multiple serotypes were isolated from 60% of *L. monocytogenes*-positive farms. In addition, multiple serotypes were isolated from individual fecal samples from 18 cattle (data not shown), with a maximum of three different serotypes isolated from one sample (Fig. 3). However, in the central and southern area, only one kind of serotype was isolated from individual farms.

PFGE typing of the isolates

Of 996 *L. monocytogenes* isolates identified from black beef cattle, 239 were analyzed by PFGE. As shown in Table 5, 48 different PFGE types were detected. Isolates from the northern, central, and southern areas were characterized to consist of 43, five, and six different PFGE types, respectively. The author found that the isolates from the northern area were genetically diversified, with multiple PFGE types identified from two-thirds of positive farms in the northern area. Furthermore, nine PFGE types were isolated from the N11 farm, five of which were isolated from the same animal (Fig. 3). Dendrogram of *L. monocytogenes* PFGE types for isolates from black beef cattle, human clinical cases, animal clinical cases, and wild animals is shown Figure 4. The author observed that 5 of the isolates (strain nos. H12, D1, O03, MMS-03174, and Y25) derived from human clinical cases showed identical PFGE patterns to those from black beef cattle. It is worth noting

that two isolates from dairy cattle clinical cases (strain nos. BC07 and BC08) and one isolate from a sheep clinical case (strain no. BC11) were also identical to those from black beef cattle. Importantly, one isolate from a black beef cattle clinical case (strain no. BC02) shared 97.9% similarity with the isolates from black beef cattle. The isolates from deer (strain no. Y21) and crow (strain no. Y22) were identical to those from black beef cattle, as well as to those from human clinical cases.

EC markers in the isolates

The isolates with PFGE type 47 were found to possess ECII marker (data not shown). These isolates were collected from five farms, three of which were in the northern area and two were in the central area. Interestingly, the isolate from a black beef cattle clinical case (strain no. BC02) also possessed ECII marker (data not shown). Moreover, the isolate with PFGE type 12 collected from the northern area was found to possess ECIII marker.

Antimicrobial resistance phenotypes

The MIC distributions of penicillin (<0.12 to 0.5 µg/ml), oxacillin (2 to 8 µg/ml), ampicillin (<0.12 to 1 µg/ml), amoxicillin (<0.12 to 0.5 µg/ml), gentamicin (<0.12 to 1 µg/ml), kanamycin (0.5 to 8 µg/ml), streptomycin (1 to 16 µg/ml), erythromycin (<0.12 to 0.5 µg/ml), vancomycin (0.5 to 1 µg/ml), tetracycline (0.25 to 1 µg/ml), chloramphenicol (4 to 8 µg/ml), fosfomicin (32 to >128 µg/ml), ciprofloxacin (0.5 to 4 µg/ml), and trimethoprim/sulfamethoxazole (0.16 to 0.62 µg/ml) were found to be monomodal, suggesting that all isolates tested were susceptible to these antibiotics

(Table 6). The MIC distributions of fosfomycin were higher than the range the author surveyed. *L. monocytogenes* has been described to be naturally resistant to oxacillin and fosfomycin (80).

Discussion

In this study, to determine the prevalence and molecular characteristics of *L. monocytogenes*, fecal samples were collected from black beef cattle in farms from three geographically distant areas of Japan: northern, central, and southern areas. In the northern area, the isolation rate was found to be significantly higher than in the central or southern area, and black beef cattle were shedding genetically diversified clones in feces. Five isolates from human clinical cases and three isolates from animal clinical cases were identical to the isolates from black beef cattle.

Previous studies conducted in the United States and elsewhere have shown that the prevalence of the bacterium in feces of beef cattle ranges from 0% to 8.3% (2, 7, 17, 46, 48, 52). In Japan, although *L. monocytogenes* was previously isolated in 0% to 3.4% of cattle fecal samples (37, 38, 71, 78), the prevalence of *L. monocytogenes* in beef cattle among farms throughout Japan has never been examined. Since the investigation of livestock animals on farms is very important to elucidate the contaminant source of pathogenic bacteria and to reduce the carrier animals, this survey provides crucial data for control of *L. monocytogenes* at the farm level.

In this study, the isolation rate in the northern area was found to be higher than

that in the central and southern areas. In a previous survey of beef-processing plants in the United States, *Listeria* species were prevalent among the hides of cattle presented for slaughter at plants in cooler climates and during the winter and spring seasons (29). These observations may be attributed to the ability of *Listeria* species to outcompete and exclude other bacterial species at low temperatures. The higher prevalence in the northern area is associated with cooler weather, consistent with the ability of *Listeria* species to grow at lower temperatures.

It has been reported that silage is a source of *L. monocytogenes* in dairy cattle (35, 84). In this study, the bacterium was also isolated from the silage-fed cattle. Meanwhile, the bacterium was also isolated from feedlot cattle that were not fed with the silage (data not shown). In feedlot operations, feeder cattle that were introduced to feedlot farms might be the source of pathogenic bacteria. The author next observed that the isolates from wild animals were identical to those from black beef cattle. It is speculated that black beef cattle farms might be the source of *L. monocytogenes* in wild animals, and that the wild animals might spread the bacterium to other farms. As other studies have shown (84), it is thought that there might be various sources of the bacterium. Therefore, further investigation would be needed to reduce carrier animals of the pathogenic bacteria.

In the northern area, the prevalence of serotype 4b was lower than that in the central and southern areas. In the southern area, the prevalence of serotype 1/2a was lower than that in the northern and central areas. It is hypothesized that the

difference in the distribution of serotypes may arise from the characteristics of each serotype. Buncic et al. (6) reported that serotype 1/2a isolates tended to be more resistant to the bacteriocins at 4°C than serotype 4b isolates. It was also reported that there is varied distribution of serotypes among *L. monocytogenes* isolates from bulk tank milk in different regions of the United States (81). Therefore, difference in the characteristics of various serotypes might vary their abilities to get established in the environment. Another hypothesis is that the difference in the serotype distribution may be explained by the sample shortage in this investigation. Further investigation on a larger scale would be needed.

Isolates from northern farms were genetically diverse, compared to those from central and southern farms. It is not yet clear as to why the isolates from northern farms, but not central and southern farms, were genetically diverse. It was postulated that low temperatures might affect the genetic diversity of *L. monocytogenes* in northern farms as follows. First, low temperatures might inhibit the growth of competing bacteria. Second, *L. monocytogenes* might grow more easily in silage, manure, and field soil, and therefore, persist in the environment. In addition, other strains might be introduced by feed, wild animals, or other contributing factors in these areas. Third, more than half of the dairy cattle in Japan are reared in the northern area, and the ratio of the ranch area in the northern area is the highest among the 3 areas. It has been reported that dairy cattle contribute to the amplification and the dispersal of *L. monocytogenes* into the

farm environment, and the bovine-farm ecosystem maintains a high prevalence of *L. monocytogenes* (56).

In this study, five PFGE types were isolated from a fecal sample from one black beef cattle. A similar result was reported by a previous survey, in which six PFGE types were identified from a fecal sample of one dairy cow (4). Hence, investigating more than 10 isolates from a sample is a more reliable method to gauge the genetic diversity of *L. monocytogenes*. Because the author did not characterize all of the isolates from the black beef cattle with PFGE, further characterization of the isolates might be needed to elucidate more precisely the genetic diversity of *L. monocytogenes* in the black beef cattle farms.

The isolates possessing ECII marker were isolated from five farms, three of which were northern farms and two were central farms, whereas the isolate possessing ECIII marker was isolated from a northern farm. A previous study showed that epidemic clonal markers might be correlated with the pathogenic potential and environmental persistence of the strains (23). In addition, ECII strains have been shown to be resistant to broad-host-range phages, when grown at temperatures lower than 37°C; such an advantage of ECII bacteria may enhance their fitness in a cooler environment (42). Based on these findings, the isolates possessing ECII marker might have the ability to survive in various environments.

The results show that the *L. monocytogenes* isolates from black beef cattle were susceptible to all antimicrobial agents tested, except oxacillin and fosfomycin.

Antimicrobial agents are used for growth promotion by improving animal husbandry. Unfortunately, veterinary antimicrobial use is a selective force that promotes the appearance and prevalence of antimicrobial-resistant bacteria in food-producing animals (39, 55, 75). In the Japanese black beef cattle, 44.4% of 1,397 *Escherichia coli* isolates were resistant to at least one type of antibiotic (87). It was also shown that *L. monocytogenes* became resistant to antibiotics through the acquisition of mobile genetic elements (65). To date, there have been very few studies on antibiotic resistance of *L. monocytogenes* in Japan (61); therefore, it is important to protect public health by continuously monitoring antibiotic resistance of *L. monocytogenes* on black beef cattle farms.

In conclusion, this study suggests that the black beef cattle in Japan may be a reservoir of genetically diversified *L. monocytogenes*. Moreover, it was observed that the prevalence of *L. monocytogenes*, the distribution of serotypes, as well as the diversity of PFGE types varied according to the area. In the northern area, it is necessary to monitor pathogenic bacteria that can grow at low temperature such as *L. monocytogenes*.

Summary

This study was conducted to determine the prevalence and molecular characteristics of *L. monocytogenes* in the feces of black beef cattle reared in geographically distant areas in Japan. The author surveyed 129 farms in the

following three areas: northern (Hokkaido prefecture), central (Gifu and Mie prefectures), and southern (Oita, Miyazaki, and Kagoshima prefectures) areas and collected 1,738 fecal samples. The data showed the following isolation rate for each area: northern, 11.4% of 651; central, 2.8% of 572; and southern, 2.9% of 515, indicating that the isolation rate in the northern area was significantly higher than that in the central or southern areas ($p < 0.01$). Moreover, serotyping of 996 isolates identified 1/2b as the most prevalent serotype (40.5%), followed by 1/2a (36.9%), 4b (21.6%), and 4ab (1.0%). In the northern area, multiple serotypes were isolated from 60% of *L. monocytogenes*-positive farms. In addition, multiple serotypes were isolated from individual fecal samples from 18 cattle. PFGE characterization of 239 isolates detected 48 different PFGE types. The author found that isolates from northern farms were genetically diverse compared to those from central and southern farms. Five isolates from human clinical cases and three isolates from animal clinical cases were identical to isolates from black beef cattle. Furthermore, the isolates from northern and central farms were characterized to possess EC II or III markers. The author next showed that the isolates were susceptible to penicillin, ampicillin, amoxicillin, gentamicin, kanamycin, streptomycin, erythromycin, vancomycin, tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim/sulfamethoxazole. Taken together, this survey provides crucial data regarding the prevalence and characteristics of *L. monocytogenes* in black beef cattle farms throughout Japan.

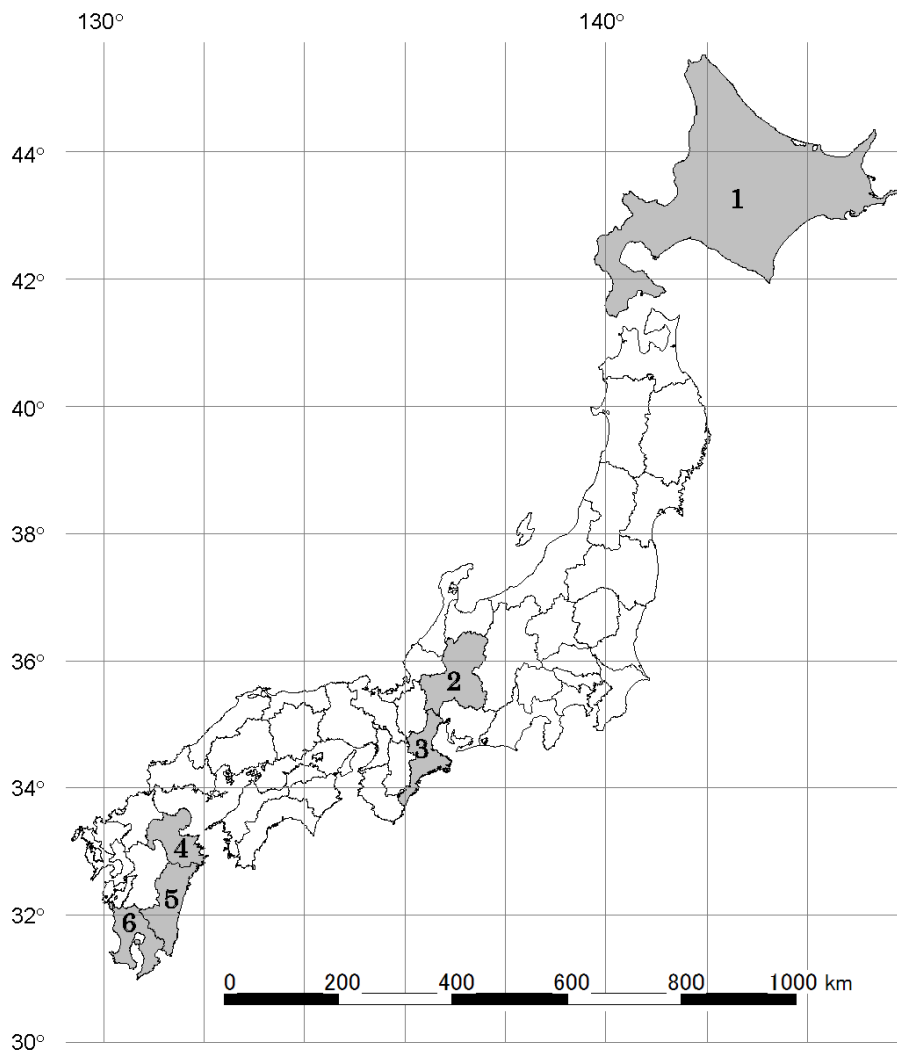


Figure 2. Map of Japan. The prefectures the author selected are shown in gray and are coded as follows: 1, Hokkaido prefecture (northern area); 2, Gifu prefecture (central area); 3, Mie prefecture (central area); 4, Oita prefecture (southern area); 5, Miyazaki prefecture (southern area); 6, Kagoshima prefecture (southern area)

Table 3. Prevalence of *Listeria monocytogenes* in Japanese black beef cattle

Area ^a	Season ^b	No. of farms examined	No. of positive farms (%)	No. of cattle examined	No. of positive cattle (%)
Northern	Spring	9	4 (44.4)	175	30 (17.1)
Northern	Summer	21	11 (52.4)	476	44 (9.2)
Northern (total)		30	15 (50.0)	651	74 (11.4)
Central	Spring	52	5 (9.6)	572	16 (2.8)
Southern	Spring	47	4 (8.5)	515	15 (2.9)
Total		129	24 (18.5)	1,738	105 (6.0)

^aNorthern, Hokkaido prefecture; central, Gifu and Mie prefectures; southern, Oita, Miyazaki, and Kagoshima prefectures

^bSpring, April through June; summer, July through September

Table 4. Sources and serotypes of *Listeria monocytogenes* isolates tested

Source	Serotypes (no. of isolates)	References
Human listeriosis cases	1/2a (2), 1/2b (9), 4b (11)	Makino et al. (49) Ochiai et al.(59)
Animal listeriosis cases (dairy cattle, black beef cattle,and sheep)	1/2a (7), 1/2b (1), 4b(1)	Provided by Agricultural Administration Division, Department of Agriculture, Hokkaido, Japan
Wild animals (crows, deer, and raccoon dog)	1/2b (1), 4b (4)	Yoshida et al. (91)

Table 5. Prevalence and characteristics of *Listeria monocytogenes* from black beef cattle feces

Farm	No. of cattle examined	No. (%) of positive cattle	No. of isolates	Serotype (no. of isolates)	PFGE type
Northern^a					
Positive farms (by name)					
N01	26	1 (3.8)	10	1/2b (10)	27
N03	51	7 (13.7)	70	1/2a (31), 1/2b (39)	2, 6, 14, 25, 28, 31
N05	25	3 (12.0)	30	1/2a (10), 1/2b (20)	1, 33
N06	44	3 (6.8)	30	1/2b (20), 4ab (10)	38, 48
N07	60	20 (33.3)	200	1/2a (148), 1/2b (52)	8, 9, 10, 24, 26
N11	4	4 (100)	39	1/2a (25), 1/2b (12), 4b (2)	3, 4, 11, 12, 21, 22, 23, 30, 46
N13	29	1 (3.4)	10	1/2a (10)	7
N16	33	1 (3.0)	10	1/2a (10)	11
N17	23	8 (34.8)	65	1/2a (3), 1/2b (52), 4b (10)	15, 24, 32, 33, 35, 45
N18	20	1 (5.0)	10	4b (10)	40
N19	20	5 (25.0)	45	1/2a (10), 1/2b (5), 4b (30)	5, 36, 41, 43, 47
N21	21	4 (19.0)	39	1/2b (39)	19, 20, 33
N27	27	6 (22.2)	60	1/2b (49), 4b (11)	33, 37, 47
N28	30	8 (26.7)	80	1/2a (31), 1/2b (26), 4b (23)	9, 13, 33, 34, 39, 42, 47
N30	10	2 (20.0)	15	1/2b (15)	33
Negative farms (combined)					
15	228	0			
Total farms					
30	651	74 (11.4)	713	1/2a (278), 1/2b (339), 4b (86), 4ab (10)	
Central^b					
Positive farms (by name)					
C01	20	7 (35.0)	70	1/2a (70)	17
C26	10	1 (10.0)	10	1/2a (10)	18
C28	6	1 (16.7)	10	4b (10)	47
C29	10	1 (10.0)	10	4b (10)	41, 44
C49	10	6 (60.0)	49	4b (49)	47
Negative farms (combined)					
47	516	0			
Total farms					
52	572	16 (2.8)	149	1/2a (80), 4b (69)	
Southern^c					
Positive farms (by name)					
S07	14	7 (50.0)	60	4b (60)	43
S27	24	1 (4.2)	10	1/2b (10)	21
S35	10	6 (60.0)	54	1/2b (54)	25, 29, 34
S39	20	1 (5.0)	10	1/2a (10)	16
Negative farms (combined)					
43	447	0			
Total farms					
47	515	15 (2.9)	134	1/2a (10), 1/2b (64), 4b (60)	
Total of 3 areas					
129	1,738	105 (6.0)	996	1/2a (368), 1/2b (403), 4b (215), 4ab (10)	

^aNorthern, Hokkaido prefecture; ^bCentral, Gifu and Mie prefectures; ^cSouthern, Oita, Miyazaki, and Kagoshima prefectures

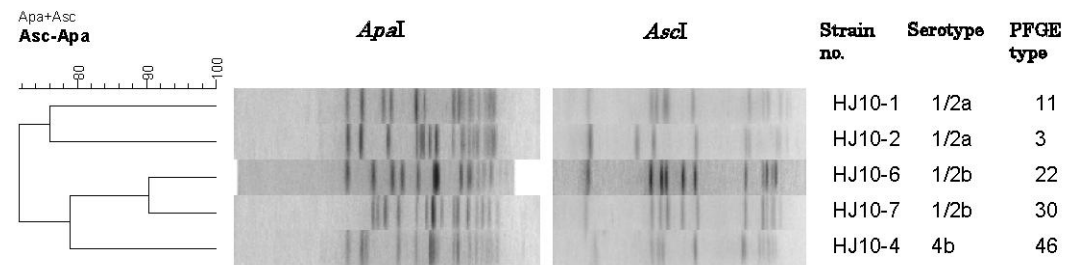


Figure 3. PFGE types and serotypes of *Listeria monocytogenes* isolates from cattle on the N11 farm

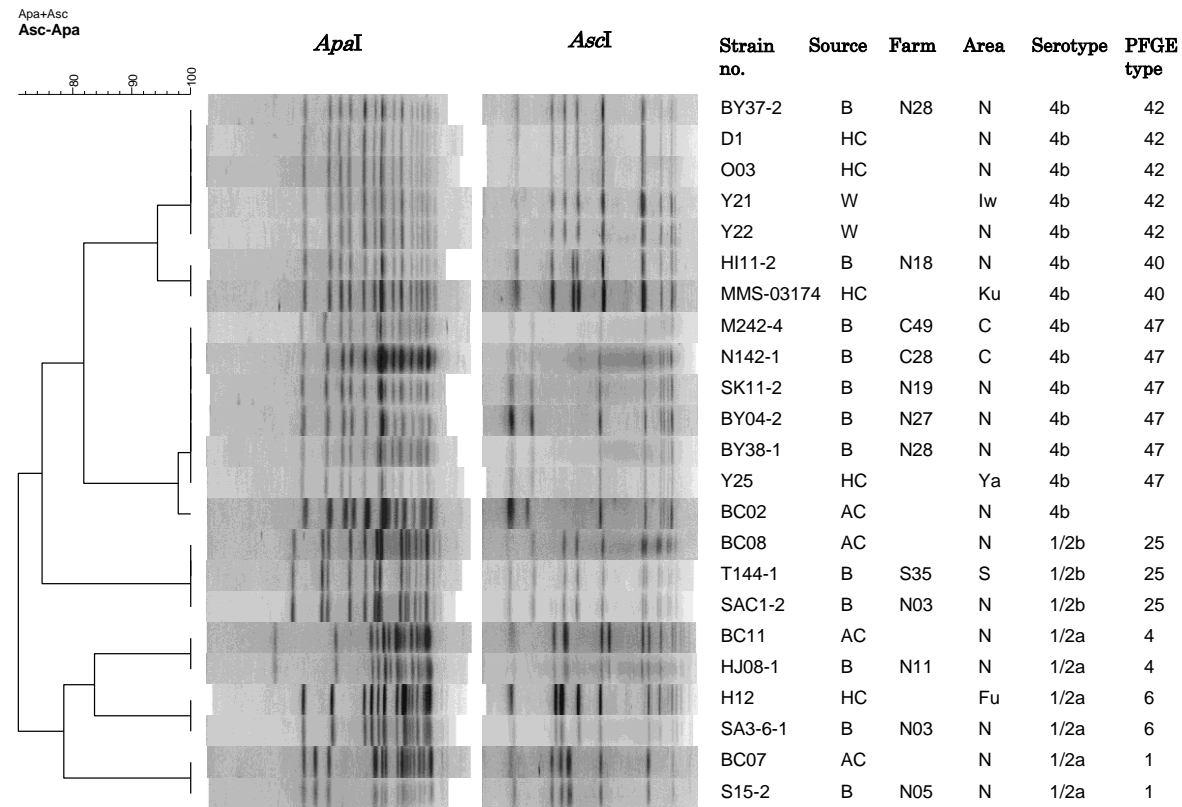


Figure 4. Dendrogram of *Listeria monocytogenes* PFGE types for isolates from black beef cattle (B), human clinical case (HC), animal clinical cases (AC), and wild animals (W). Areas are coded as follows: N, northern; C, central; S, southern; Fu, Fukuoka prefecture; Ku, Kumamoto prefecture; Iw, Iwate prefecture; Ya, Yamagata prefecture

Table 6. Antimicrobial susceptibility of 315 *L. monocytogenes* isolates from black beef cattle in Japan ^a

Antimicrobial agent	No. of isolates susceptible at MIC ($\mu\text{g/ml}$) of:																MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
	0.16	0.31	0.62	1.25	<0.12	0.25	0.5	1	2	4	8	16	32	64	128	256		
Penicillin	ND	ND	ND	ND	17	223	75	0	0	0	0	0	0	0	0	0	0.25	0.5
Oxacillin	ND	ND	ND	ND	0	0	0	0	6	281	28	0	0	0	0	ND	4	4
Ampicillin	ND	ND	ND	ND	7	145	155	8	0	0	0	0	0	0	0	0	0.5	0.5
Amoxicillin	ND	ND	ND	ND	9	272	34	0	0	0	0	0	0	0	ND	ND	0.25	0.5
Gentamicin	ND	ND	ND	ND	75	189	47	4	0	0	0	0	0	0	0	ND	0.25	0.5
Kanamycin	ND	ND	ND	ND	0	0	7	130	139	36	3	0	0	0	0	ND	2	4
Streptomycin	ND	ND	ND	ND	0	0	0	1	49	194	70	1	0	0	0	ND	4	8
Erythromycin	ND	ND	ND	ND	2	295	18	0	0	0	0	0	0	0	0	ND	0.25	0.25
Vancomycin	ND	ND	ND	ND	0	0	6	309	0	0	0	0	0	0	0	ND	1	1
Tetracycline	ND	ND	ND	ND	0	14	192	109	0	0	0	0	0	0	ND	ND	0.5	1
Chloramphenicol	ND	ND	ND	ND	0	0	0	0	0	58	257	0	0	0	ND	ND	8	8
Fosfomycin	ND	ND	ND	ND	0	0	0	0	0	0	0	0	9	14	5	287(>128)	>128	>128
Ciprofloxacin	ND	ND	ND	ND	0	0	15	278	20	2	0	0	0	0	ND	ND	1	1
Trimethoprim- Sulfamethoxazole	1	176	138	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.31	0.62

^a Antimicrobial susceptibility tests were performed for 315 of the 996 *L. monocytogenes* isolates. MIC₅₀ and MIC₉₀ are the MICs for 50 and 90% of the isolates, respectively. ND, analysis not done.

Chapter 3

Prevalence and Characteristics of *Listeria monocytogenes* Isolates From Raw Meat of Japanese Black Beef Cattle in Japan

Introduction

Listeria monocytogenes is the causative agent of listeriosis, a serious invasive illness that affects both humans and animals. *L. monocytogenes* is composed of 13 serovars, of which serotypes 1/2a, 1/2b, 1/2c and 4b account for the vast majority of cases of human disease. In humans, listeriosis commonly affects pregnant women and neonates, as well as elderly and immunosuppressed individuals. Unlike other foodborne illnesses, which rarely result in fatalities, the mortality rate of listeriosis is approximately 30% (45).

In the United States, human listeriosis affects approximately 1,600 individuals and causes 255 deaths each year (72). In 2011, according to the World Organization for Animal Health, *L. monocytogenes* caused 282 human listeriosis cases and 50 deaths in France and 169 human listeriosis cases and 32 deaths in United Kingdom (86). In Japan, an average of 83 cases of listeriosis per year has been reported (63). However, accurate data for the incidence of listeriosis are not available, owing to the lack of a mandatory notification system.

Meat products have been implicated as sources of listeriosis outbreaks in many countries. For instance, a multistate outbreak of listeriosis occurring in the United States in 2002 was linked to turkey deli meat (9), and a nationwide outbreak of listeriosis taking place in Canada in 2008 was associated with ready-to-eat meat products (27). In addition, a listeriosis outbreak in Denmark in 2009 was caused by infected beef meat from a meals-on-wheels delivery service (73). In the United

States, deli meats have the highest predicted relative risk of causing listeriosis (19). Zoonotic pathogens in meat have to be controlled through a complete and continuous farm-to-fork system. It is of the utmost importance to control direct and indirect fecal contamination of carcasses through efficient hygiene management systems (57).

It is well-known that environmental persistence is a key factor for food contamination by *L. monocytogenes* and biofilms contribute to persistence (15). Biofilms consist of bacterial cells encapsulated in an exopolysaccharide matrix which allows them to adhere to each other and to surfaces, and also protects them from adverse conditions (74).

In a previous study, the isolation rate of *L. monocytogenes* from Japanese black beef cattle in the northern region of the country was found to be higher than that in the central and southern areas of Japan. Beef cattle may be a reservoir of genetically diversified *L. monocytogenes* in Japan (32). The prevalence of *L. monocytogenes* in beef meat has been investigated in Japan (37, 60). However, molecular characterization of those isolates had not yet been performed. Thus, the purpose of this study was to determine the prevalence of *L. monocytogenes*, to understand the distribution subtypes of *L. monocytogenes* from beef meat, and to examine relatedness among the isolates from black beef cattle, beef meat, and human clinical cases (33). Furthermore, biofilm-forming ability of the *L. monocytogenes* isolates from beef meat and beef cattle was tested to evaluate a risk

of food contamination.

Materials and Methods

Sample collection

A total of 315 samples of retailed Japanese beef meat were obtained from 107 retail stores in three areas of Japan: northern (Hokkaido prefecture), central (Gifu, Aichi, and Mie prefectures), and southern (Fukuoka, Oita, Miyazaki, Kumamoto, and Kagoshima prefectures). The sampling was performed between September 2011 and March 2012. Samples were chilled and transported to the laboratory for further microbiological analysis.

Bacterial isolates

Isolation and identification of *L. monocytogenes* were carried out as previously described (31). Meat samples (25 g) weighed into filtered stomacher bags were mixed for 1 min with 225 ml of University of Vermont Modified *Listeria* Enrichment Broth (BD, Franklin Lakes, NJ) in a Masticator Classic (IUL S.A., Barcelona, Spain). After the samples were incubated at 30°C for 24 h, cultures (0.1 ml) were incubated with 10 ml of Fraser Broth (BD) containing Fraser Selective Supplement (Oxoid, Basingstoke, UK) at 35°C for either 24 or 48 h. Each culture (0.1 ml) was then streaked onto CHROMagar *Listeria* plates (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 24–48 h. Ten typical *Listeria*-like colonies with halos were selected from plates and characterized by the Christie, Atkins,

Munch-Peterson test, β -hemolysis reaction, catalase reaction, Gram staining, and motility test in semisolid media. *L. monocytogenes* isolates were stored in Brain Heart Infusion medium (BHI; Difco, Detroit, MI) with 10% glycerol at -80°C.

Serotyping of the isolates

Serotyping was performed using commercial *Listeria* antisera (Denka Seiken Co. Ltd., Tokyo, Japan), according to the manufacturer's recommendations.

Pulsed-field gel electrophoresis (PFGE) analysis

PFGE was carried out by following the Center for Disease Control's PulseNet protocol and a previously described protocol (31). Chromosomal DNA of *L. monocytogenes* was digested with the restriction enzymes *AscI* (New England BioLabs, Beverly, MA) and *ApaI* (Takara, Shiga, Japan). *XbaI*-digested *Salmonella* Braenderup H9812 DNA was used as a molecular weight marker. PFGE patterns were compared using the BioNumerics program (version 5.0; Applied Maths, Kortrijk, Belgium). Similarities among restriction fragments of isolates were determined using the unweighted pair group method with arithmetic mean.

PCR of L. monocytogenes epidemic clone (EC) I marker

DNA was extracted from overnight BHI cultures using a commercially prepared extraction preparation (InstaGene Matrix; Bio-Rad Laboratories), according to manufacturer's instructions. The primers used for identification of isolates of the *L. monocytogenes* ECI marker have been described by Chen and Knabel (11). The cycling program and electrophoresis conditions have been described previously (31).

Bacterial strains

A total of 22 *L. monocytogenes* isolates from human listeriosis cases were used. *L. monocytogenes* isolates were kindly provided by the following researchers: Dr. Makino, S. I., Obihiro University of Agriculture and Veterinary Medicine, four isolates (49, 63); Dr. Ueda, F., Nippon Veterinary and Life Sciences University, two isolates (59); Dr. Yoshida, T., Nagano Environmental Conservation Research Institute, 10 isolates; Dr. Nakama, A., Tokyo Metropolitan Institute of Public Health, two isolates; Dr. Ito, M., Sapporo Clinical Laboratory Inc., two isolates; and Dr. Kobayashi, K., Daiichi Clinical Laboratories Inc., two isolates. The isolates from human listeriosis cases consisted of two strains of serotype 1/2a, nine strains of serotype 1/2b and 11 strains of serotype 4b. In a previous study, the author detected 48 different PFGE types of isolates from black beef cattle, 18 of which were serotype 1/2a, 20 were serotype 1/2b, nine were serotype 4b, and one was serotype 4ab (32). A total of 47 PFGE-typed *L. monocytogenes* isolates (except serotype 4ab) from black beef cattle were used to study relatedness to isolates from beef meat and to study biofilm-forming ability.

Evaluation of biofilm-forming ability by crystal violet (CV)

The ability of *L. monocytogenes* strains to form biofilms was assayed using a previously described method, with slight modifications (3). This method involves forming biofilms on microtiter plates, staining them with CV, and then solubilizing the bound dye to measure its absorbance. Briefly, cells were grown overnight in BHI

broth at 37°C and diluted to an optical density (OD) of 0.5 at 620 nm. Bacterial cultures (50 µl) were transferred to 5 ml of BHI and vortexed. An aliquot (100 µl) of each isolate culture was inoculated into each of the six wells of a microtiter plate and incubated for 24 h at 37°C. BHI broth was used as the negative control. After incubation, the culture was removed from each well, and the plate was washed five times with 150 µl of sterile distilled water and air-dried. A 0.1% crystal violet (CV) solution (150 µl) was added to the wells and incubated at room temperature for 45 min. The wells were then rinsed thoroughly five times with distilled water. Biofilms were quantified by dissolving the remaining CV with 95% ethanol, and the absorbance was measured at 590 nm (OD₅₉₀).

Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More precisely, it is a modified version of R commander (version 1.6.3), designed to add statistical functions that are frequently used in biostatistics. A chi-squared test was used to compare the prevalence of *L. monocytogenes*. In addition, a *t*-test was used to compare biofilm-forming abilities. Differences were considered significant at a significance level of $p < 0.05$.

Results

Prevalence of L. monocytogenes in beef meat

The author surveyed 315 beef meat samples from 107 retail stores in three areas of Japan: northern, central, and southern areas. The following isolation rates were observed: northern, seven (7.1%) of 98; central, 11 (9.2%) of 120; and southern, five (5.2%) of 97 (Table 7).

Serotyping of the isolates

A total of 230 *L. monocytogenes* isolates were recovered from beef meat; the serotype distribution is summarized in Table 7. The predominant serotype was 1/2c (62.6%), followed by 4b (26.1%) and 1/2a (11.3%). Serotype distribution of the isolates differed among the three areas: serotype 4b was not isolated from the northern area, and 1/2a was not isolated from the southern area. Multiple serotypes, that is, 1/2a and 1/2c, were isolated from one beef meat sample obtained from store N18. Serotype 1/2c was isolated from beef meat obtained 2 and 3 weeks later from store N18; however, 1/2a was not isolated from the sample (Fig. 5).

PFGE typing of the isolates

Of 230 *L. monocytogenes* isolates identified from beef meat, 96 were analyzed by PFGE. As shown in Figure 6, the author detected 12 different PFGE types, three of which were serotype 1/2a, six were serotype 1/2c, and three were serotype 4b. The isolates serotyped 1/2c had >95% similarity each other. PFGE types 4, 5, and 10 were isolated from multiple samples sold in different stores. Strains Me045-10 and

Me115-1 showed 97.8% similarity; those strains were isolated from samples of beef meat sold in store N18 on different dates (Fig. 5). The same genotypic clones of *L. monocytogenes* were isolated from beef meat samples, which had different production sites, sold in the same store (Fig. 7).

ECI marker in the isolates

One beef meat isolate (strain no. Me305-3) had 98.3% similarity with a strain isolated from a human clinical case (strain no. H11), for which ECI had been reported (Fig. 6) (59). Furthermore, the beef meat isolate had >94% similarity to a strain isolated from black beef cattle (strain no. SK19-1) (Fig. 6). These two isolates from beef meat and black beef cattle were found to possess an ECI marker.

Biofilm-forming ability of the isolates from beef meat

Biofilm-forming ability was tested by CV. This method is based on the adsorption of bacteria followed by staining by CV, and measuring the absorbance. A total of 60 isolates were tested: 12 PFGE types from beef meat, 47 PFGE types from black beef cattle, and one ECI strain. The mean OD₅₉₀ of 1/2c was higher than that of 1/2a or 4b isolates from beef meat (Table 8); however, the difference was not significant. The mean OD₅₉₀ of 1/2c isolates from beef meat was higher than that of 1/2b isolates from black beef cattle; however, the difference was not significant. The OD₅₉₀ of the ECI strain was lower than the mean OD₅₉₀ of the isolates from beef meat.

Discussion

This study was conducted to determine the prevalence and molecular characteristics of *L. monocytogenes* in retailed beef. A total of 315 samples were purchased from retail stores in northern, central, and southern areas of Japan. *L. monocytogenes* was isolated from 7.3% of 315 beef samples. Some difference was observed in serotype distribution among the three areas. Furthermore, ECI strains were isolated from beef meat and black beef cattle.

Previous studies conducted in the United States and elsewhere have shown that the prevalence of the bacterium in raw beef meat ranges from 1.6% to 24% (25, 66, 85). In Japan, a study conducted during 1988 and 1994 showed that the prevalence of the bacterium in retailed beef was 34.2% (37), and another survey conducted during 1998 and 2003 showed that the prevalence of the bacterium in retailed beef meat was 15.5% (60). The prevalence determined in the present study was lower than that in other surveys conducted in Japan, although the results cannot be compared directly because of the difference between the methods. A similar tendency has been reported for *Escherichia coli* O157: the prevalence of *E. coli* O157 in beef carcasses decreased after 2003 (21). Microbial contamination of beef meat from feces might have decreased in Japan because of hygiene management strategies in beef processing environment adopted to prevent outbreaks of foodborne pathogens.

The predominant serotype of beef meat isolates was 1/2c, followed by 4b and 1/2a.

Serotype 1/2c was not isolated from black beef cattle, and serotype 1/2b was the predominant serotype in black beef cattle isolates (Chapter 2, (32)). This finding suggests that there might be a contamination source of *L. monocytogenes*, other than intestinal contents of Japanese black beef cattle. Similarly, a study reported that *L. monocytogenes* serotypes 1/2a, 1/2b and 4b were isolated from the hides of cows and bulls at processing plants and that serotypes 1/2a, 1/2c, and 4b were found on postintervention carcasses (29). The PFGE types of 1/2c isolates were highly similar, and the biofilm-forming ability of 1/2c isolates was relatively high. Furthermore, 1/2c clones were isolated from samples obtained from the same store on different dates, and the PFGE types of these isolates showed high similarities. These results suggest that the 1/2c clones might persist through beef processing via selection for persistence in that environment. It has been reported that in a simulated gastrointestinal system, the relative survival of *L. monocytogenes* serotypes 4b and 1/2a strains was higher than that of serotype 1/2c (41). This finding suggests that the difference in environment between the gastrointestinal tract and food processing plants affects the distribution of serotypes. Therefore, serotype 1/2c might be predominant in beef meat isolates but not in black beef cattle isolates.

The same PFGE types of *L. monocytogenes* clones were isolated from beef meat samples, which varied in their production sites and were sold in the same store. This finding suggests the spread of the clones via the process of slicing or packing.

The serotype distribution of beef meat isolates differed among the three studied areas: serotype 4b was not isolated from the northern area and 1/2a was not isolated from the southern area. In a previous study on black beef cattle feces, a similar tendency was observed: the isolation rate of serotype 4b was significantly lower from the northern farms than from central and southern farms, and the isolation rate of serotype 1/2a was significantly lower from southern farms than from northern and central farms. Furthermore, ECI strains were isolated from beef meat and black beef cattle. These results suggest that beef cattle might be the contamination source of pathogenic bacteria for beef meat. ECI was implicated in numerous outbreaks in Europe and North America (12). In Japan, ECI was isolated from a case of human listeriosis and from chicken (59); however, an outbreak caused by ECI has never been reported. Surveillance of EC strains has hardly been performed in Japan (59). It is generally thought that ECI strains may have relatively high fitness in foods compared to other serotype 4b strains and that contamination may be established and may persist in processing plants (12). The biofilm-forming ability of ECI strains was not as high as that of other strains, although biofilm-forming ability may be associated with *L. monocytogenes* persistence (3). ECI strains might have other ability to survive in the food-processing environment. Because this study found ECI strains in beef meat and black beef cattle, more extensive surveillance and characterization of such organisms in food should be performed in Japan. In the food-processing

environment, it is important to prevent the contamination of *L. monocytogenes* from raw beef meat to cooked products.

In conclusion, this study showed that ECI strains were isolated from beef meat and black beef cattle and that the serotype distribution of 1/2a and 4b in beef meat and black beef cattle varied by region. These results suggest that black beef cattle might be the contamination source of pathogenic bacteria for beef meat. Meanwhile, there might be a contamination source of *L. monocytogenes*, other than the intestinal content of black beef cattle. More extensive surveillance is needed to control the pathogenic bacteria through a complete and continuous farm-to-fork system.

Summary

L. monocytogenes, a foodborne pathogen, is known to cause invasive disease in humans and animals. In a previous study of Chapter 2, the prevalence of *L. monocytogenes* in black beef cattle reared in northern farms was higher than that of cattle from central and southern farms (32). Further, the *L. monocytogenes* isolates from northern farms showed more genetic diversity than isolates from central and southern farms. To determine the risk of contamination of beef meat by fecal *L. monocytogenes*, the prevalence and molecular characteristics of *L. monocytogenes* in retail beef meat were examined. The author obtained retail beef meat from three areas of Japan: northern, central, and southern areas. *L. monocytogenes* was

isolated from 7.3% of 315 beef meat samples. The isolates possessing the ECI marker came from beef meat and beef cattle. Some difference was observed in serotype distribution among the three areas. These findings suggest that fecal *L. monocytogenes* might contaminate beef meat. Gut bacteria from black beef cattle might be the contamination sources causing human listeriosis. The predominant serotype was 1/2c (62.6%), followed by 4b (26.1%) and 1/2a (11.3%). The 1/2c serotype was not isolated on analysis of black beef cattle. Therefore, there might be a contamination source of *L. monocytogenes*, other than the intestinal content of black beef cattle. Because genotypically similar *L. monocytogenes* clones were consistently isolated from the same retail store, beef meat might have been contaminated during the process of slicing or packaging. Further efforts for reducing the contamination of meat and meat products by pathogenic bacteria are needed.

Table 7. Prevalence and serotype of *Listeria monocytogenes* in beef meat

Area	No. of samples examined	No. (%) of positive samples	No. of isolates	No. of isolates (%)		
				1/2a	1/2c	4b
Northern	98	7 (7.1)	70	16 (22.9)	54 (77.1)	0
Central	120	11 (9.2)	110	10 (11.1)	60 (54.5)	40 (36.4)
Southern	97	5 (5.2)	50	0	30 (60.0)	20 (40.0)
Total	315	23 (7.3)	230	26 (11.3)	144 (62.6)	60 (26.1)

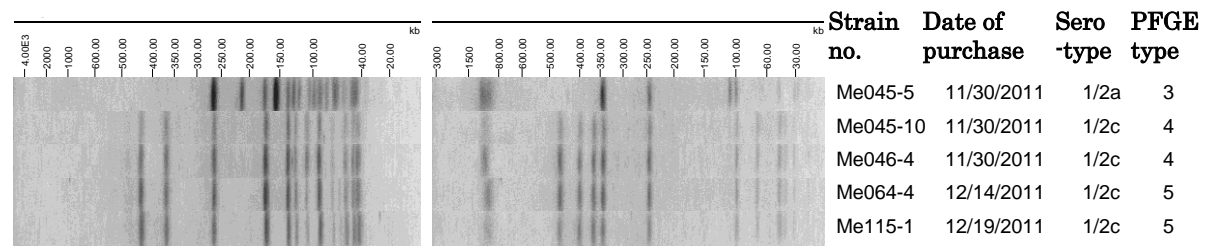


Figure 5. PFGE types of *Listeria monocytogenes* isolates from beef meat purchased from store N18

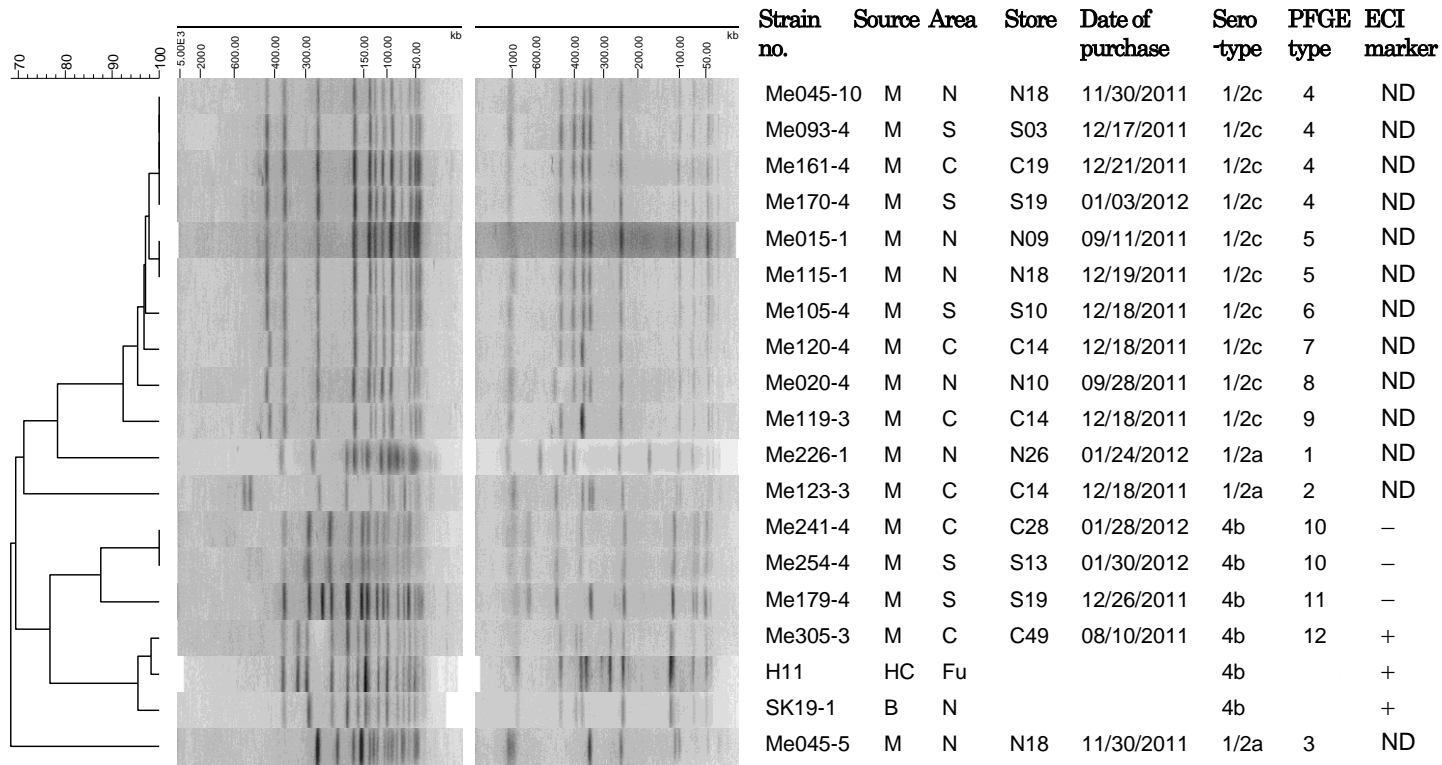


Figure 6. Dendrogram of *Listeria monocytogenes* PFGE types for isolates from beef meat (M), human clinical cases (HC), and black beef cattle(B). Areas are coded as follows: N, northern; C, central; S, southern; Fu, Fukuoka prefecture. ND, not done.

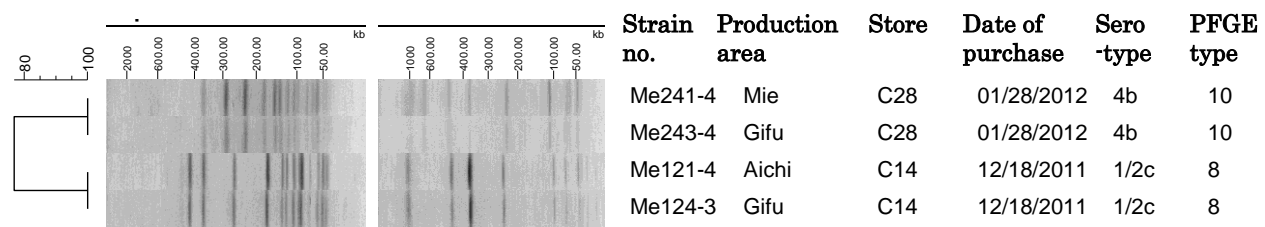


Figure 7. PFGE types of *Listeria monocytogenes* isolates from beef meat purchased from stores C14 and C28

Table 8. Average OD₅₉₀ for serotypes of *Listeria monocytogenes* isolates from beef meat and black beef cattle, based on a crystal violet destining biofilm assay

Source	Assay results			
	Mean OD ₅₉₀	SD	Range	Sample size
Beef meat				
1/2a	0.331	0.176	0.211–0.533	3
1/2c	0.416	0.117	0.294–0.591	6
4b	0.236	0.159	0.104–0.412	3
Black beef cattle				
1/2a	0.376	0.147	0.182–0.614	18
1/2b	0.344	0.126	0.048–0.562	20
4b	0.380	0.088	0.205–0.506	9
Human listeriosis case				
ECI	0.197			1

General Discussion

L. monocytogenes is the causative agent of listeriosis, a serious invasive illness that affects both humans and animals. Unlike other foodborne illnesses, which rarely result in fatalities, the mortality rate of listeriosis is approximately 30%. In Japan, accurate data for the incidence of listeriosis are not available, owing to the lack of a mandatory notification system. The limited amount of epidemiological data available in Japan represents a problematic issue. Therefore, the aim of this study is to determine the prevalence and molecular characteristics of *L. monocytogenes* isolates in dairy and beef cattle to provide basic data for the control of *L. monocytogenes*.

Chapter 1 provides information on analysis of the bovine colostrum for determining the prevalence and molecular characteristics of *L. monocytogenes*. Bovine colostrum samples were collected from dairy farms in Hokkaido, Japan. Sixteen (7.6%) of the 210 samples and six (28.6%) of the 21 farms were positive for *L. monocytogenes*. In many previous studies, *L. monocytogenes* has been detected in raw milk, but the prevalence of *L. monocytogenes* in the bovine colostrum has not been investigated. This survey is the first to provide data on the prevalence and characteristics of *L. monocytogenes* in the bovine colostrum. *L. monocytogenes* isolates of serotypes 1/2b and 4b were detected from colostrum samples. Multiple PFGE types were detected in the isolates from three farms; therefore, it is likely that these locations were continuously contaminated by *L. monocytogenes*. In Japan, several studies have surveyed *L. monocytogenes* contamination on dairy farms, but

the prevalence of *L. monocytogenes* has not yet been fully investigated on dairy farms. This is the first report on the detection of multiple PFGE types and serotypes from a single farm. Some *L. monocytogenes* isolates in the colostrum had PFGE profiles identical to those from human clinical isolates. This observation suggested that bovine colostrum could be a significant reservoir of *L. monocytogenes* that causes human infections. The *L. monocytogenes* isolates in colostrum possessed an ECII marker. The isolates from bovine colostrum were classified as serotypes 1/2b and 4b. The presence of *L. monocytogenes* virulence-associated genes and the PFGE types and serotypes of bovine *L. monocytogenes* isolates in the colostrum suggested that these strains may be able to invade host cells to cause listeriosis.

Chapter 2 describes the analysis involving Japanese black beef cattle for determining the prevalence and molecular characteristics of *L. monocytogenes*. Fecal samples were collected from black beef cattle in farms from three geographically distant areas of Japan: northern, central, and southern areas. In the northern area, the isolation rate was found to be significantly higher than that in the central or southern area, and black beef cattle shed genetically diversified clones in their feces. In the northern area, the prevalence of serotype 4b was lower than that in the central and southern areas. In the southern area, the prevalence of serotype 1/2a was lower than that in the northern and central areas. The isolates possessing the ECII marker were isolated from five farms, three of which were northern farms and two were central farms, whereas the isolate possessing the ECIII marker was isolated from a northern farm. Five isolates from human clinical

cases and three isolates from animal clinical cases were identical to the isolates from black beef cattle. The prevalence of *L. monocytogenes* in beef cattle among farms throughout Japan has never been examined. Since investigation of livestock animals on farms is very important for elucidating the contamination source of pathogenic bacteria and to reduce the number of carrier animals, this survey provides crucial data for the control of *L. monocytogenes* at the farm level. This study suggests that the black beef cattle in Japan may be a reservoir of genetically diversified *L. monocytogenes*. In the northern area, it is necessary to monitor pathogenic bacteria that can grow at low temperatures, such as *L. monocytogenes*.

Chapter 3 provides information regarding the analysis of the prevalence and molecular characteristics of *L. monocytogenes* in retail beef meat, beef cattle, and the human listeriosis case, performed to determine the risk of contamination of beef meat by fecal *L. monocytogenes*. The author obtained retail beef meat from three areas of Japan: northern, central, and southern. *L. monocytogenes* was isolated from 7.3% of 315 beef meat samples. The isolates possessing the ECI marker were isolated from beef meat and black beef cattle. A difference was observed in serotype distribution among the three areas. These findings suggest that gut bacteria from black beef cattle might be a contamination source and a cause of human listeriosis. Further examination will be required to clarify the relatedness between the isolates of beef cattle and beef meat. There was some difference in serotype distribution between beef meat and black beef cattle isolates. Therefore, there might be a contamination source of *L. monocytogenes*, other than the intestinal contents of

black beef cattle. Because genotypically similar *L. monocytogenes* clones were consistently isolated from the same retail store, beef meat might have been contaminated during the process of slicing or packaging. Further efforts for reducing the contamination of meat and meat products by pathogenic bacteria are required.

The present study elucidates the molecular epidemiology of *L. monocytogenes* in farm animals in Japan. The results of this investigation will be useful in controlling *L. monocytogenes* through a complete and continuous farm-to-fork system. There is a lack of molecular epidemiological studies on *L. monocytogenes* in the food-processing environment of Japan. Further examination of *L. monocytogenes* and sanitary criteria, based on risk assessment, will be required to prevent outbreaks of listeriosis.

Conclusion

The objective of this thesis is to determine the prevalence and molecular characteristics of *L. monocytogenes* isolates in dairy and beef cattle, in order to provide basic data for control of *L. monocytogenes*. The results obtained are summarized as follows:

1. Sixteen (7.6%) of the 210 bovine colostrum samples and six (28.6%) of the 21 farms were positive for *L. monocytogenes*. Characterization of *L. monocytogenes* isolates from bovine colostrum indicated that the isolates might have potential for causing human and animal listeriosis.
2. Prevalence of *L. monocytogenes* in black beef cattle reared in northern farms was higher than that in cattle from central and southern farms. Further, the *L. monocytogenes* isolates from northern farms were more genetically diverse than those from central and southern farms.
3. *L. monocytogenes* isolates from black beef cattle were found to consist of 48 different PFGE types. Furthermore, EC strains were isolated from black beef cattle. The black beef cattle in Japan may be a reservoir of genetically diversified *L. monocytogenes*.
4. *L. monocytogenes* isolates from beef cattle might contaminate food through beef meat and cause human listeriosis.

The global prevalence of *L. monocytogenes* in the colostrum and milk from individual cows has not yet been fully investigated. This study is the first to reveal the prevalence of *L. monocytogenes* in bovine colostrum and provided crucial data to

help produce safe bovine colostrum products. This study is also the first to reveal the prevalence of *L. monocytogenes* in beef cattle among farms throughout Japan. The examination of *L. monocytogenes* isolates from black beef cattle across Japan revealed the difference in prevalence among the areas and provided extremely important data for performing hygienic management. Furthermore, genotyping data, which were obtained in this study, can be compared with the data for other countries and will be critical in determining the source of future foodborne listeriosis cases.

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Japanese Abstract

Listeria monocytogenes (LM)は、ヒトを含む多くの動物に病原性を示し、患者由来株の血清型は、1/2a、1/2b、1/2c および 4b が主である。LM による食中毒は髄膜炎、敗血症、流産などを引き起こし、死亡率は約 30%である。アメリカでは、年間約 1,600 人がリステリア症に罹患し、約 255 人が死亡している。一方、我が国では、リステリア症の発生頻度についての統計はなく不明である。また、集団食中毒は、北海道におけるナチュラルチーズの1事例のみである。海外では乳および食肉製品が LM による食中毒の主な原因食品と推定されているが、分子疫学的研究は殆ど行われていない。このため、乳牛および肉牛におけるLMの汚染状況と分離株の分子疫学的性状を解析し、LM を制御するための基礎資料を提供する。

北海道の 21 農場よりホルスタイン初乳 210 検体を採取した。北海道、中部および九州の 129 農場より黒毛和牛の新鮮糞便 1,738 検体を採取し、また、各地域の販売店より和牛肉 315 検体を購入した。各検体より LM を分離し、血清型別およびパルスフィールドゲル電気泳動法による遺伝子型別を行い、さらに、流行株 (EC) マーカー遺伝子の保有状況を解析した。初乳および黒毛和牛由来株は薬剤感受性試験を、黒毛和牛および和牛肉由来株はバイオフィルム産生能試験を行った。

初乳の 7.6%から LM が分離された。血清型は 1/2b (55%) および 4b (45%) であった。黒毛和牛糞便の 6.0%が陽性であり、北海道、中部および九州の黒毛和牛の保菌率は、それぞれ 11.4、2.8 および 2.9%で、北海道の保菌率は他と比較して有意に高かった。また、血清型は 1/2b (40.5%) が最も多く、次いで、1/2a (36.9%)、4b (21.6%)、4ab (1.0%) の順であった。遺伝子型別の結果、北海道の分離株は遺伝子型が極めて多様で、1農場から9種の遺伝子型の LM が分離された例もあった。市販和牛肉の 7.3% が陽性であった。血清型は、1/2c (62.6%) が最も多く、次いで 4b (26.1%)、1/2a (11.3%) の順であった。EC マーカー遺伝子

を保有する株が、初乳、黒毛和牛および牛肉より分離された。初乳および黒毛和牛由来株には、ヒト症例由来株と遺伝子型が一致する株もあった。初乳および黒毛和牛由来株は、12種の薬剤に対して感受性であった。牛肉由来の1/2cは、遺伝子型の多様性が低く、バイオフィルム形成能が高い傾向を示した。また、同じ店舗で2および3週間隔で購入した牛肉から、相同性の非常に高い遺伝子型の株が、継続して分離された例もあった。

血清型別および遺伝子型別の結果から、初乳、黒毛和牛および牛肉由来株は、ヒトにリステリア症を発症させる可能性が高いことが示された。北海道の黒毛和牛の保菌率が極めて高く、多様な遺伝子型が肉牛飼養環境に定着していると考えられる。このため、低温環境におけるLMに対する対策が重要である。ECマーカー遺伝子を保有する株が黒毛和牛および和牛肉から分離されたことは、腸管由来菌が牛肉を汚染し、リステリア症を発症させる可能性が高いことを示している。一方、黒毛和牛と和牛肉由来株の血清型分布が異なることから、腸管内容物以外の汚染源も考えられた。また、同一店舗より継続して相同性の高いLM株が分離されたことは、和牛肉のスライス・包装工程で、継続汚染した可能性を強く示唆している。

本研究では、初乳中のLMによる汚染状況を初めて明らかにし、安全な初乳製品を製造するための重要なデータを提供した。また、肉牛のLMに関する全国的な調査から、地域による汚染状況および分子疫学的性状の相違を明らかにし、衛生管理方法を策定するための極めて重要な資料を提供した。さらに、今回得られた遺伝子型のデータは、我が国の標準株として、他国由来株との比較を可能にし、食中毒発生の際には、汚染源を推定する極めて重要な資料となり得る。