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Association between *Mycoplasma* pneumonia outbreaks in calves and *Mycoplasma* mastitis in milking cows on dairy farms

Megumi Itoh[1)], Takahiro Aoki[1)], Miyuki Furuoka[2)], Yukihiro Baba[2)], Tetsu Saitoh[2)], Eiji Hata[3)], Yuuki Hirano[4)], Kazuhiro Senna[4)], Norio Yamagishi[1)] and Kazutaka Yamada[5,*)]

[1)] Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan
[2)] Milk Testing Laboratory, Tokachi Federation of Agricultural Cooperative Associations, Obihiro, Hokkaido 080–0024, Japan
[3)] Hokkaido Research Station, National Institute of Animal Health, National Agriculture and Food Research Organization, Sapporo, Hokkaido 062–0045, Japan
[4)] Animal Research Center, Hokkaido Research Organization, Shintoku, Hokkaido 081–0038, Japan
[5)] Department of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 252–5201, Japan

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Abstract

To clarify the possibility of *Mycoplasma* mastitis in milking cows from *Mycoplasma* pneumonia outbreaks in calves, *Mycoplasma bovis* was isolated over time from nasal swabs of calves and bulk tank milk at two dairy farms where no cases of *Mycoplasma* mastitis occurred. Isolates were genotypically analyzed using pulsed-field gel electrophoresis. At both farms, *Mycoplasma bovis* was first detected in bulk tank milk after pneumonia outbreaks in calves, and the genotypes of the isolates from nasal swabs and milk were consistent. These findings indicate that outbreaks of *Mycoplasma* pneumonia in calves is one of the possible etiologies of *Mycoplasma* mastitis in dairy herds.

Key Words: mastitis, *Mycoplasma bovis*, pneumonia

Mycoplasma mastitis is a contagious disease disseminated within dairy herds during milking[4)]. In Japan, the prevalence of *Mycoplasma* mastitis in large-scale dairy farms has increased[11)]. *Mycoplasmas* are known to be causative agents of pneumonia and arthritis, which are often isolated from the respiratory and urogenital systems of healthy cattle[2,12)]. Biddle *et al.*[1)] reported that the pulsed-field gel electrophoresis (PFGE) patterns of the *Mycoplasma* strains isolated from the milk of mastitic cows were identical to those obtained from the nasal cavity of the same animal, suggesting internal transmission of *Mycoplasma*. Also, calves that drink the *Mycoplasma*-infected milk from infected mother cows were often known to develop pneumonia[5,17)]. On the other hand, it is possible that the transmission of *Mycoplasma* from the discharge of calves to the udder of milking cows and the subsequent developing mastitis. However, it has hardly been verified

*Corresponding author: Kazutaka Yamada, Department of Veterinary Medicine, Azabu University, 1–17–71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252–5201, Japan
E-mail: kyamada@azabu-u.ac.jp
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about it. In this study, we investigated the relationship between *Mycoplasma* pneumonia calves and the occurrence of mastitis cows.

The study was conducted at two farms in the Tokachi District of Hokkaido, Japan. Prior to the investigation, bulk tank milk at both the farms tested negative for *Mycoplasma*. Farm A had approximately 70 milking cows that were milked using either a milking parlor (approximately 30 cows) or a robotic system (approximately 40 cows), and milk obtained from each system was stored in different bulk tanks. After calving, all cows were confirmed to be negative for *Mycoplasma* in the milk. Calves and heifers were kept separately in suckling calves, growing calves, non-pregnant heifers and pregnant heifers. Farm B had approximately 700 milking cows that were milked using a milking parlor. Calves and heifers were kept separately in suckling calves, growing calves and pregnant heifers. At both the farms, there was no direct contact between the calves and milking cows. At both farms, colostrum and milk for suckling calves were pasteurized at 60°C for 30 min before feeding. Data regarding the incidence of respiratory diseases (characterized by fever, harsh lung sounds, coughing and nasal discharge) in calves and heifers were collected from the farm medical records or veterinarians. At both the farms, samples were collected over three periods (designated I–III) at 1–2-month intervals. Nasal samples were collected using sterile swabs from suckling calves, growing calves, non-pregnant heifers and pregnant heifers in farm A, and from suckling calves, growing calves and pregnant heifers in farm B. At the same time, samples from the bulk tank milk were collected. At farm A, individual milk was collected when the bulk tank milk tested positive for *Mycoplasma*, and the nasal swabs were collected from each *Mycoplasma*-positive cow. At farm B, individual milk was not collected because owner did not permit sampling due to large population of milking cows. Instead, environmental samples were collected from feeder troughs, waterers and floors of barns that housed the suckling and growing calves.

For detection and identification of *Mycoplasma*, nasal swabs, environmental swabs and milk samples (100 µl) were inoculated into 3 ml of modified DNA-supplemented Hayflick broth (*Mycoplasma* NK Medium; Miyarisan Pharmaceutical, Tokyo, Japan) and cultured at 37°C and 5% CO_2 for 3 days. DNA was extracted using a commercial kit (Cica Geneus DNA Extraction Reagent; Kanto Chemical, Tokyo, Japan), and *Mycoplasma* was detected using a polymerase chain reaction (PCR) kit (Cica Geneus Bovine *Mycoplasma* HI Screening kit; Kanto Chemical, Japan). Samples of *Mycoplasma*-positive cultures (10 µl) were plated onto modified DNA-supplemented Hayflick agar plates (*Mycoplasma* NK Agar Medium, Miyarisan Pharmaceutical, Japan) and were incubated at 37°C and 5% CO_2 for 5–7 days. Colonies were sub-cultured into 3 ml of modified DNA-supplemented Hayflick broth and were incubated at 37°C and 5% CO_2 for 3 days, and the *Mycoplasma* species were identified by species-specific PCR[10,16], SDS-PAGE[13], and 16S rRNA sequencing[14]. Liquid cultures identified as *Mycoplasma bovis* (*M. bovis*) were stored at −80°C. For PFGE analysis, *Mycoplasma* DNA was prepared in agarose plugs, as previously reported by Hata *et al.*[7] DNA plugs were digested with 45 U of *Bam*HI (Takara Bio, Shiga, Japan) at 37°C for 3 h. Electrophoresis was conducted in a 1% agarose gel using a CHEF Mapper Pulsed-Field Electrophoresis System (Bio-Rad, Hercules, CA, USA), as previously reported[7]. Images of PFGE gels stained with ethidium bromide were captured using an ultraviolet transilluminator. FPQuest Software, ver. 5.1 (Bio-Rad), was used for genotypic analysis of the PFGE patterns, and the Jaccard coefficient was selected for similarity; the unweighted pair group method using arithmetic averages was employed for dendrogram calculations. All experiments were approved by the ethics committee of Hokkaido Research Organization Animal Research Center.

The results at farm A are shown in Table 1.

Table 1. *Mycoplasma bovis*-positive samples during each period at farm A

Groups of cows	Sources*	*Mycoplasma bovis*-positive samples/all samples (positive rate) collected at each period		
		I	II	III
Suckling calves	NAS	2/9 (22.2%)	5/8 (62.5%)	10/12 (83.3%)
Growing calves	NAS	2/10 (20.0%)	2/9 (22.2%)	1/7 (14.3%)
Milking cows	BTM-mp	−	+	+
	BTM-rs	−	−	−

*Sources: NAS, nasal swab.
BTM-mp, bulk-tank milk of milking parlor.
BTM-rs, bulk-tank milk of robotic system.
+: *M. bovis* positive. −: *M. bovis* negative.
Underlining: groups in which pneumonia was prevalent.

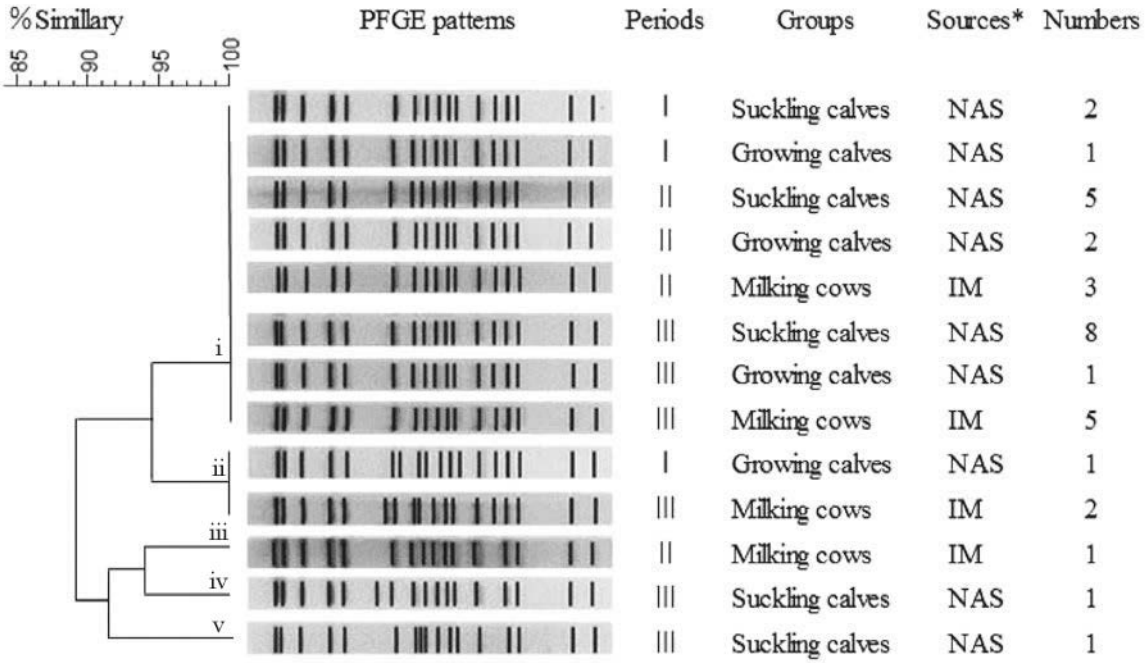

Fig. 1. Dendrogram based on *Bam* H1 pulsed-field gel electrophoresis (PFGE) patterns of *Mycoplasma bovis* at farm A. The dendrogram was constructed by using Jaccard similarity coefficients (2.0% optimization and 2.0% tolerance)
*Sources: NAS, nasal swab; IM, individual milk

During period I, *M. bovis* was isolated from nasal swabs from suckling (22.2%) and growing (20.0%) calves; however, bulk tank milk samples tested negative for *M. bovis*. During periods II and III, pneumonia was prevalent among suckling calves, and the occurrence of *M. bovis* from nasal swabs of suckling calves increased to 62.5% and 83.3%, respectively. *M. bovis* was not isolated from nasal swab samples from *Mycoplasma*-positive milk cows, pregnant heifers and non-pregnant heifers at farm A. In addition, *M. bovis* was detected in bulk tank milk collected from the milking parlor but not from the robotic system. Among individual milk from the milking parlor, *M. bovis* was detected in four samples in period II and seven samples in period III (Fig. 1). The results at farm B are shown in Table 2. During period I, *M. bovis* was isolated from nasal swabs from suckling (40.0%) and growing (20.0%) calves. During period II, pneumonia was prevalent in

Table 2. *Mycoplasma bovis*-positive samples during each period at farm B

Groups of cows	Sources*	*Mycoplasma bovis*-positive samples/all samples (positive rate) collected at each period		
		I	II	III
Suckling calves	NAS	2/5 (40.0%)	NS	3/3 (100%)
Growing calves	NAS	3/15 (20.0%)	NS	3/7 (42.9%)
Milking cows	BTM-mp	−	−	+

*Sources: NAS, nasal swab.
BTM-mp, bulk-tank milk of milking parlor.
+: *M. bovis* positive. −: *M. bovis* negative.
NS: no sample (samples could not be collected).
Underlining: groups in which pneumonia was prevalent.

Table 3. *Mycoplasma bovis*-positive samples in the environment during each period at farm B

Groups of cows	Sources	*Mycoplasma bovis*-positive samples/all samples (positive rate) collected at each period		
		I	II	III
Suckling calves	ENV	0/2 (0%)	NS	0/3 (0%)
Growing calves	ENV	0/12 (0%)	NS	2/8 (25.0%)

ENV: environments (feeding trough or waterer).
NS: no sample (samples could not be collected).
Underlining: groups in which pneumonia was prevalent.

suckling and growing calves. During period III, the rate of *M. bovis* detection from nasal swabs increased in suckling (100%) and growing (42.9%) calves. *M. bovis* was not isolated from nasal swab samples from pregnant heifers at farm B. In bulk tank milk samples, *M. bovis* was detected during period III, but not during periods I and II. In environmental samples, *M. bovis* was not detected during period I, but it was detected in samples from feeder troughs and waterers of growing calves (25.0%) during period III (Table 3).

The PFGE pattern of *M. bovis* isolated from farm A revealed five genotypes (designated i–v). Genotype i was the major strain detected in the nasal swabs from suckling calves (period I: n = 2, II: n = 5, III: n = 8), growing calves (period I: n = 1, II: n = 2, III: n = 1), and individual milk samples (period II: n = 3, III: n = 5). Genotype ii was isolated from nasal swabs of growing calves (period I: n = 1) and individual milk samples (period III: n = 2). Three other genotypes (iii, iv, v) were isolated from an individual milk sample (period II: n = 1) and nasal swabs (period III: n = 2) in the suckling calves group. The similarity between these strains was in the range of 90%–100% (Fig. 1). At farm B, *M. bovis* was detected from nasal swabs of suckling (period I: n = 2, III: n = 3) and growing (period I: n = 3, III: n = 3) calves, the environment of the growing calves (period III: n = 2) and bulk tank milk (period III: n = 1). These isolates detected from farm B showed the same PFGE patterns (Fig. 2).

Mycoplasma species, especially *M. bovis*, are highly pathogenic microorganisms that cause diseases in cattle[6]. Because many dairy cows respond poorly to antibiotic treatment for *Mycoplasma* mastitis[8], efforts to reduce the introduction of *Mycoplasma* into dairy herds are important for the prevention of mastitis[12]. The route of introduction of mastitis-causing *Mycoplasma* in the milking herds is generally thought inner transmission from other body parts (e.g. respiratory organ, urogenital system, arthritis) of the cow to the mammary gland[1]. However, calves that developed *M. bovis* pneumonia are known to shed large amounts of

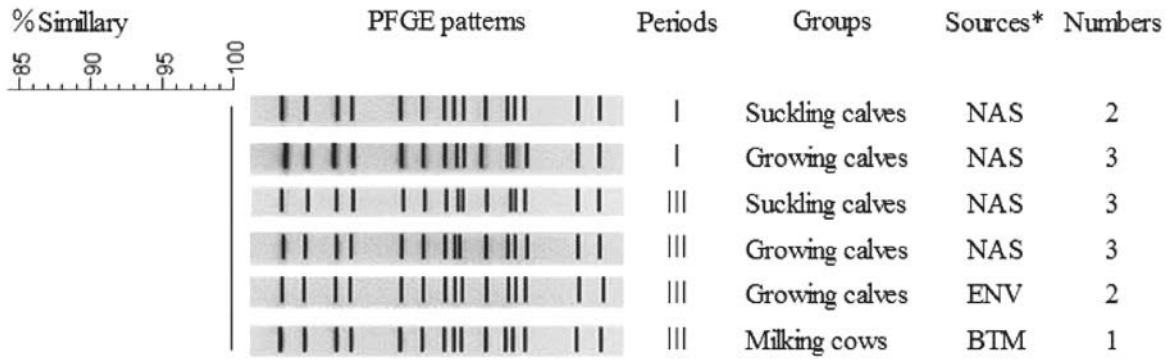

Fig. 2. Dendrogram based on *Bam* H1 pulsed-field gel electrophoresis (PFGE) patterns of *Mycoplasma bovis* at farm B. The dendrogram was constructed by using Jaccard similarity coefficients (2.0% optimization and 2.0% tolerance)
*Sources: NAS, nasal swab; BTM, bulk-tank milk; ENV, environments

Mycoplasma in their nasal discharge and saliva[12,15], which cannot be ruled out as the potential source of infection. In this study, *M. bovis* was detected in bulk tank milk samples after the outbreak of pneumonia in suckling and growing calves, and the genotypes of these strains in calf nasal discharge and bulk tank milk samples were identical. Furthermore, at farm B, *M. bovis* of the same genotype as the isolates from the calf nasal discharge and milk samples were detected from environment after pneumonia outbreak. As *Mycoplasma* can survive for long periods in farm environments[9,18], workers in contact with infected milk or animals, contaminated equipment, or any type of improperly cleaned material are potent fomites for *Mycoplasma* transmission[2]. Direct contact between calves and milking cows was unlikely at both farms. However, workers or materials were possibly involved in the transmission of *Mycoplasma* from the calves to milking cows. Especially, at farm A, *M. bovis* was detected only in milk samples from the milking parlor and not from the robotic system. This result suggested that the risk of *Mycoplasma* infection in milk from robotic systems is low because farm workers do not participate in the milking process. *M. bovis* was shed to the environment due to the occurrence of pneumonia, which appears to be the cause for the positive detection of *Mycoplasma* in the cow's milk. Thorough cleansing and disinfection of boots, clothing, gloves and other equipment is considered effective for preventing *Mycoplasma* mastitis at dairy farms, especially during pneumonia outbreaks.

The detection of *Mycoplasma* and results of PFGE analyses suggested that *Mycoplasma* pneumonia outbreaks in calves might be an etiology for *Mycoplasma* mastitis in dairy cows on the same farm. To the best of our knowledge, this is the first report to show a possibility of within-farm transmission of *M. bovis* from calves to cows.

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