Pulsed-field gel electrophoresis patterns of *Mycoplasma* isolates from various body sites in dairy cattle with *Mycoplasma* mastitis

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Objective—To determine whether *Mycoplasma* strains typically associated with mastitis in dairy cattle can be isolated from body sites other than the mammary gland.

Design—Prospective clinical trial.

Animals—7 Holstein cows in various stages of lactation with intramammary *Mycoplasma* infection.

Procedure—Milk samples, antemortem swab specimens from various body sites, and postmortem swab and tissue specimens were submitted for *Mycoplasma* culture. Pulsed-field gel electrophoresis (PFGE) was performed on chromosomal digests of all *Mycoplasma* isolates. Isolates with the same number and size of chromosomal digest bands were considered to be of the same type.

Results—For each cow, all isolates obtained from milk, mammary gland parenchyma, and supramammary lymph nodes had the same PFGE pattern. All cows had at least 1 isolate from nonmammary system tissues that had the same PFGE pattern as isolates from the mammary system. Overall, 44 of the 70 (63%) *Mycoplasma* isolates obtained from body sites other than mammary system sites had the same PFGE pattern as did mammary system isolates.

Conclusions and Clinical Relevance—Results confirmed our hypothesis that *Mycoplasma* strains isolated from the milk of dairy cattle with *Mycoplasma* mastitis frequently have PFGE patterns identical to those for strains isolated from other body sites, suggesting that there is at least a potential for internal transmission of *Mycoplasma* organisms. (*J Am Vet Med Assoc* 2005;227:455–459)

Mycoplasma mastitis is an important emerging infectious disease that is a threat to dairy cattle worldwide.[1-3] The prevailing thought has been that transmission of contagious mastitis pathogens such as *Mycoplasma* spp likely occurs as a result of direct contact between the teat orifice and contaminated milking machines or the contaminated hands of milking personnel[4] and that these contagious pathogens can be controlled through the use of proper milking hygiene.[5] Despite the implementation of control methods, however, the prevalence of *Mycoplasma* infection in dairy herds continues to increase, suggesting that the organism may spread through some means other than fomite transmission during milking time.

Mycoplasma organisms have been shown to colonize tissues other than the mammary gland and have been isolated from the urogenital, respiratory, and musculoskeletal systems.[6] In a previous study,[4] for instance, culture of nasal swab specimens collected from calves fed contaminated milk yielded *Mycoplasma bovis*, and nasal colonization persisted for several months after calves were no longer fed contaminated milk.

In addition, *Mycoplasma* mastitis can potentially result from infection or colonization of a body site other than the mammary gland and spread of the organism via the circulatory or lymphatic system.[6] For example, in a study[7] of previously uninfected cattle in which *M bovis* was inoculated into only 1 mammary quarter, the organism could be isolated from all 4 quarters within 17 to 19 days, despite the use of strict milking hygiene and a specially built milking machine designed to prevent quarter-to-quarter communication of milk and air.[7] It was thought that the organisms had spread hematogenously because *M bovis* could be isolated from blood samples. Similarly, in a study[8] of cows in various stages of lactation in which *Mycoplasma* organisms were inoculated aseptically via the teat canal, organisms could subsequently be isolated from all noninoculated mammary quarters and could intermittently be isolated from the nasal cavity, eye, rectum, vagina, urine, and blood.

In these previous studies, however, *Mycoplasma* isolates from various body sites were compared only on the basis of various phenotypic characteristics. In contrast, **pulsed-field gel electrophoresis (PFGE)** of chromosomal digests can be used to used to specifically compare isolates to determine whether they are the same strain.[9] The purposes of the study reported here, therefore, were to determine whether *Mycoplasma* strains typically associated with mastitis in dairy cattle can be isolated from body sites other than the mammary gland and whether isolates obtained from the milk of cows infected with *Mycoplasma* mastitis have the same PFGE pattern as isolates from other body sites.

Materials and Methods

Cows—Seven Holstein cows in various stages of lactation from 4 commercial dairies in Washington State were used in the study. In all cows, intramammary *Mycoplasma* infection had been diagnosed 1 to 3 weeks prior to enrollment in the study by a commercial milk quality laboratory[a] that used standard procedures advocated by the National Mastitis Council.[10] Cows were transported from their herd of origin to Washington State University prior to the study. During the study, cows were milked twice daily with a portable milking unit. They were slaughtered at the Washington State

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University Meat Laboratory at the end of the 28-day study. The study protocol was approved by the Washington State University Animal Care and Use Committee.

Collection and culture of milk samples—Milk samples from individual mammary quarters and composite milk samples were collected aseptically from all cows on the same day of the week once a week for 4 weeks. Personnel wore sterile gloves during collection of milk samples. Samples were held on ice for 1 hour until transported to the laboratory for culture. Milk samples were vortexed vigorously, and 100 µL was inoculated into 10 mL of *Mycoplasma* enrichment medium.[b] Tubes were incubated at 37°C in 10% CO_2 for 4 days, and on the fourth day, 100 µL of the enrichment medium was streaked on a *Mycoplasma* agar plate prepared according to National Mastitis Council guidelines.[10] Plates were incubated at 37°C in 10% CO_2 for 10 days before examination. On the 10th day, agar plates were examined with a 15× dissecting microscope for colonies with the distinctive "fried-egg" appearance.[10] Results were considered positive if any *Mycoplasma* colonies were seen and negative if there was no evidence of *Mycoplasma* growth. All *Mycoplasma* isolates obtained from composite and quarter milk samples were sent to the California Animal Health and Food Safety Laboratory in Davis, Calif, for speciation by means of an indirect immunoperoxidase method.[11]

Collection and culture of specimens from other body sites—Once a week on the same day each week for 4 weeks, swab specimens were collected from the mucosal surfaces of the right and left eyes, the right and left nasal cavities, the right and left ears, the suburethral diverticulum, the vestibular fossa, the vaginal wall, and feces. Personnel wore sterile disposable gloves during collection of swab specimens. For collection of specimens, swabs moistened with *Mycoplasma* enrichment medium were used to swab the surface of the selected site. Swabs were then placed in enrichment medium and transported to the laboratory. Tubes of enrichment medium containing swab specimens were incubated at 37°C in 10% CO_2 for 4 days. On the fourth day, 100 µL of enrichment medium was streaked on a *Mycoplasma* agar plate. Plates were incubated at 37°C in 10% CO_2 for 10 days, then examined with a 15× dissecting microscope for colonies with the distinctive "fried-egg" appearance.[10] Results were considered positive if any *Mycoplasma* colonies were seen and negative if there was no evidence of *Mycoplasma* growth.

Immediately after slaughter, swab and tissue samples were collected from all cows. Swab specimens were collected from the mucosal surfaces of the right and left eyes, the right and left nasal cavities, the right and left pharyngeal tonsils, the right and left auditory tubes, the right and left nasal turbinates, the right and left external ears, the right and left primary bronchi, the right and left tertiary bronchi, the sinuses, the pleura, the pericardial sac, the mucosal surface of the urinary bladder, the suburethral diverticulum, the vestibular fossa, the vaginal wall, the right and left tarsal joints, the right and left stifle joints, and feces. Swabs were immediately placed in 10 mL of enrichment medium. Tissue samples (approx 1 cm^3) were collected with sterile forceps and scissors from the right and left retropharyngeal tonsils, right and left conjunctivas, right and left lung parenchyma, right and left bronchial lymph nodes, mesenteric lymph node, spleen, right and left supramammary lymph nodes, and parenchyma of all 4 mammary quarters. Tissue samples were placed in sterile bags[c] and transported to the laboratory. At the laboratory, tissue samples were briefly exposed to a flame to reduce the likelihood of surface contamination and then macerated. Macerated tissue samples were placed in tubes containing 10 mL of enrichment medium, and tubes were incubated at 37°C in 10% CO_2 for 4 days. On the fourth day, 100 µL of enrichment medium was streaked on a *Mycoplasma* agar plate. Plates were incubated at 37°C in 10% CO_2 for 10 days, then examined with a 15× dissecting microscope for colonies with the distinctive "fried-egg" appearance.[10] Results were considered positive if any *Mycoplasma* colonies were seen and negative if there was no evidence of *Mycoplasma* growth. *Mycoplasma* isolates were sent to the California Animal Health and Food Safety Laboratory for speciation.

PFGE—For specimens with positive *Mycoplasma* culture results, the leading edge of the colony that grew from the isolation streak was cut out of the agar plate and placed in a tube with enrichment medium. Tubes were incubated at 37°C in 10% CO_2 for 4 days. On the fourth day, 1.6 mL of enrichment medium was mixed by means of manual rotation with 0.4 mL of glycerol in a 2-mL cryopreservation tube. Tubes were stored at –85°C in duplicate.

For PFGE, cryopreservation tubes were allowed to thaw on ice and 500 µL of each sample was put into a tube containing 5 mL of *Mycoplasma* enrichment medium. Tubes were incubated at 37°C in 10% CO_2 for 6 days. On the sixth day, samples were centrifuged at 1,200 × *g* at 4°C for 10 minutes to pellet the bacteria. The supernatant was poured off, and the bacteria were suspended in 200 µL of buffer solution. Ten microliters of proteinase K (20 mg/mL) was added, and the DNA was embedded in 200 µL of agarose. Plugs were cast and lysed as described.[12] Plugs were digested with *Sal*I according to the manufacturer's recommendations.[d] Pulsed-field gel electrophoresis was performed with a commercial unit[e] in 0.5× TBE electrophoresis buffer. Electrophoresis was performed at 14°C for 20.2 hours at a setting of 6 V/cm of gel and a linear pulse ramp of 1 to 12.9 seconds. After electrophoresis, gels were stained with ethidium bromide (0.001%) for 30 minutes, washed twice in distilled water for 30 minutes, and then photographed with UV light. Sizes of DNA fragments were determined by comparing bands with standard markers (0.1 to 200 kb and 225 to 2,200 kb)[f] by use of standard software.[g] Isolates from a cow that had the same number and size of chromosomal digest bands were considered to be of the same type.[13]

For comparison with *Mycoplasma* isolates obtained from cows in the study and to ensure that the *Sal*I restriction enzyme would be able to distinguish among *Mycoplasma* strains, 10 *M bovis* isolates were obtained from the Washington Animal Disease and Diagnostic Laboratory and PFGE was performed as described.

Statistical analyses—For purposes of data analysis, specimens were grouped on the basis of origin (mammary system, respiratory system, urogenital system, and other) and subgrouped according to whether specimens could be obtained antemortem or only postmortem. Mammary system specimens that could be obtained antemortem included composite and quarter milk samples; mammary system specimens that could only be obtained postmortem included supramammary lymph node and mammary parenchyma specimens. Respiratory system specimens that could be obtained antemortem included nasal cavity specimens; respiratory system specimens that could only be obtained postmortem included pharyngeal tonsil, retropharyngeal tonsil, primary bronchus, tertiary bronchus, lung parenchyma, sinus, nasal turbinate, pleura, and bronchial lymph node specimens. Urogenital system specimens that could be obtained antemortem included vaginal, vestibular fossa, and suburethral diverticulum specimens; there were no urogenital system specimens that could only be obtained postmortem. Other specimens that could be obtained antemortem included the mucosal surface of the eye, ear, and fecal specimens; other specimens that could only be obtained

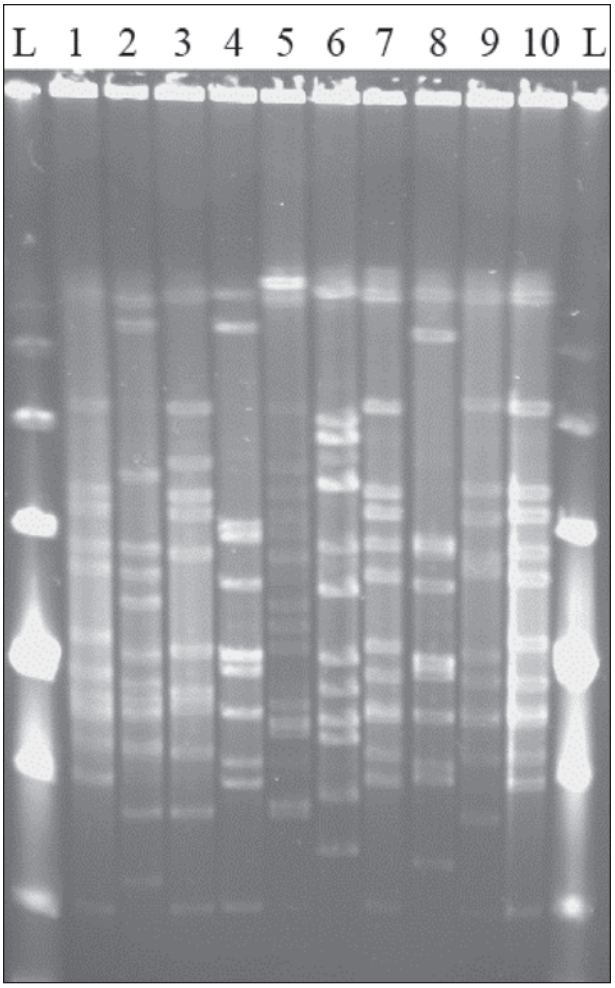

Figure 1—Pulsed-field gel electrophoretograms of 10 strains of *Mycoplasma bovis* following digestion with the restriction enzyme *Sal* I. Lanes L = DNA ladders; lane 1 = tracheal swab specimen; lanes 2, 3, 6, 9, and 10 = lung specimens; lane 4 = thoracic fluid specimen; lanes 5 and 8 = nasal swab specimens; and lane 7 = joint fluid specimen.

postmortem included conjunctiva, urinary bladder, stifle joint, tarsal joint, pericardial sac, mesenteric lymph node, spleen, and auditory tube specimens.

For each system group, the proportion of isolates that had the same PFGE pattern as did isolates obtained from milk samples was computed. Because variable numbers of observations were obtained for each system group, weighted least squares ANOVA followed by the Fisher least significant difference test[14] was used to compare proportions among system groups. Values of $P < 0.05$ were considered significant.

Results

The PFGE patterns for the 10 historic *M bovis* isolates were all unique, indicating that the *Sal*I restriction enzyme would allow differentiation of *Mycoplasma* isolates (**Figure 1**). All *Mycoplasma* isolates obtained from milk, mammary gland parenchyma, and the supramammary lymph nodes were identified as *M bovis* or *Mycoplasma californicum*.

For each cow, all isolates obtained from mammary system specimens (ie, milk, mammary gland parenchyma, and supramammary lymph nodes) had the same PFGE pattern. Ninety percent (28/31) of isolates obtained from the urogenital system and other sites had PFGE patterns identical to the patterns of mammary system isolates. In 6 cows, at least 1 isolate each from respiratory system specimens, urogenital system specimens, and other specimens had the same PFGE pattern as did an isolate from the mammary system specimens (**Table 1**; **Figure 2**). In the remaining cow (cow 1), none of the isolates obtained from respiratory system specimens had PFGE patterns that matched the pattern for mammary system isolates; however, isolates from the urogenital and other systems had patterns that matched the pattern for mammary system isolates. Overall, 44 of the 70 (63%) *Mycoplasma* isolates obtained from body sites other than mammary system sites had the same PFGE pattern as did mammary system isolates. Isolates from most of the accessible body sites had the same pattern. Moreover, the accessible body sites were likely to have the same pattern as the milk isolate.

Table 1—Results of pulsed-field gel electrophoresis of *Mycoplasma* isolates from various body sites in 7 cows with *Mycoplasma* mastitis.*

		Respiratory system			Other systems	
Cow No.	Mammary parenchyma	Antemortem	Postmortem	Urogenital system	Antemortem	Postmortem
1	3/3	0/1	0/9	1/1	1/2	0/1
2	2/2	1/1	2/2	0/0	0/0	1/1
3	5/5	3/3	1/7	3/3	1/2	4/4
4	0/0	1/1	0/0	3/3	2/2	0/0
5	2/2	0/0	4/4	1/1	2/2	2/2
6	0/0	3/3	0/7	0/0	3/3	3/3
7	1/1	1/1	0/0	1/1	0/0	0/0
Total	**13/13**	**9/10**	**7/29**	**9/9**	**9/11**	**10/11**

*Within each cell, the denominator represents the number of samples from which mycoplasma were isolated and the numerator represents the number of mycoplasma isolates that had a pulsed-field gel electrophoresis pattern the same as the pattern for the milk isolate from that cow. Mammary parenchyma included specimens from the mammary parenchyma and supramammary lymph node obtained postmortem. Antemortem respiratory system specimens included nasal cavity swab specimens; postmortem respiratory system specimens included pharyngeal tonsil, retropharyngeal tonsil, primary bronchus, tertiary bronchus, lung parenchyma, sinus, nasal turbinate, pleura, and bronchial lymph node specimens. Urogenital system specimens included vaginal, vestibular fossa, and suburethral diverticulum specimens. Antemortem specimens from other systems included mucosal surface of the eye, ear, and fecal specimens; postmortem specimens from other systems included conjunctiva, urinary bladder, stifle joint, tarsal joint, pericardial sac, mesenteric lymph node, spleen, and auditory tube specimens.

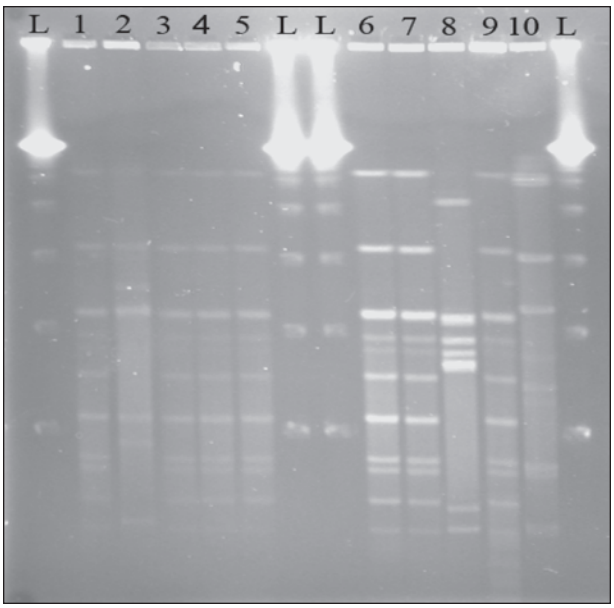

Figure 2—Pulsed-field gel electrophoretograms of *M bovis* isolates from 2 cows with *Mycoplasma* mastitis. Lanes 1 through 5 represent samples from 1 cow, and lanes 6 through 10 represent samples from a second cow. Lanes L represent DNA ladders. For the first cow, notice the pattern for the isolate from a composite milk sample (lane 1) matches patterns for isolates from swab specimens of the eye (lane 3), suburethral diverticulum (lane 4), and vagina (lane 5) but does not match the pattern for an isolate from a nasal swab specimen (lane 2). For the second cow, notice that the pattern for the isolate from a left front mammary gland milk sample (lane 6) matches patterns for isolates from swab specimens of the ear (lane 7) and feces (lane 9) but does not match the patterns for isolates from the pharyngeal tonsil (lane 8) or conjunctiva (lane 10).

For specimens obtained from sites that could be sampled antemortem, proportions of respiratory system, urogenital system, and other system isolates that had the same PFGE pattern as did the mammary system isolate were not significantly different from each other. For specimens obtained from sites that could only be sampled postmortem, the proportion of respiratory system isolates that had the same PFGE pattern as did the mammary system isolate was significantly lower than the proportion of other system isolates that did.

Discussion

It was our hypothesis that *Mycoplasma* spp causing mastitis frequently colonize body sites other than the mammary gland and that for individual infected cows, strains isolated from the milk would have PFGE pattens identical to those for strains isolated from other body sites. Results of the present study confirm this hypothesis. In particular, 28 of the 31 (90%) isolates obtained from the urogenital system and other sites had PFGE patterns identical to patterns of mammary system isolates. There was less agreement between PFGE patterns for mammary system isolates and patterns for respiratory system isolates. Still, 16 of the 39 (41%) respiratory system isolates had PFGE patterns identical to the pattern for mammary system isolates.

Our finding that PFGE patterns for *Mycoplasma* isolates from the milk and mammary glands were often identical to patterns for isolates from other body sites could suggest that in individual infected cows, a specific *Mycoplasma* strain colonized the mammary gland, causing intramammary infection, and was subsequently transmitted to other body sites through hematogenous or lymphatic routes, as proposed by others.[7] Alternatively, it is possible that a predominant *Mycoplasma* strain existed on the farms from which these cows were obtained and independently infected the mammary gland and these other body sites. However, results from a previous study[8] support our premise that transmission between internal body sites can occur. In that study, *Mycoplasma* organisms were experimentally inoculated into the mammary glands of 4 previously uninfected cows, and within a matter of days or weeks, an antigenically similar strain could be isolated from the nose, eye, and vagina of all 4 cows. On the other hand, although results of the present study may suggest that systemic transmission could occur, they do not indicate the route of any such transmission.

Other authors[15,16] have reported that systemic transfer of *M bovis* from the lungs to the mammary gland is possible. In 1 study,[16] *M bovis* was introduced into dairy herds through the purchase of animals with respiratory tract infection. When isolates were DNA typed, similar subtypes were recovered from animals of various ages in each herd with a variety of diseases, such as respiratory tract disease, arthritis, and mastitis.

An additional finding from the present study was that colonization of body sites other than the mammary gland was common, and isolates from these sites were almost always of the same type as isolates obtained from the mammary gland. If transmission were to occur from these other sites to the mammary gland, then it might be possible to predict development of *Mycoplasma* mastitis through collection of swab specimens from 1 or more of these other body sites.

a. Dr. Allan Britten, Udder Health Systems, Bellingham, Wash.
b. Hardy Diagnostics, Santa Maria, Calif.
c. Whirl Pak bags, Nasco, Modesto, Calif.
d. Invitrogen, Carlsbad, Calif.
e. CHEF DRII electrophoresis unit, BioRad, Hercules, Calif.
f. Sigma-Aldrich, St Louis, Mo.
g. BioNumerics gel analysis software, BioSystematics, Tavistock, UK.

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