NOTE ***Bacteriology***

Detection of Mycoplasma in Mastitic Milk by PCR Analysis and Culture Method

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ABSTRACT. *Mycoplasma alkalescens, M. bovigenitalium, M. bovirhinis* and *M. bovis* were directly detected from milk specimens by a polymerase chain reaction (PCR) when milk specimens were centrifuged and treated with mycoplasmal lysis buffer. The sensitivity of this PCR method was 110 to 1,400 colony forming units (CFU). This method was useful for the detection of mycoplasmas in milk specimens from cows at an early stage of mycoplasmal mastitis since a small amount of mycoplasma could be detect in milk without culture. The results were available within 12 hr, which is faster than conventional culture techniques. *M. bovirhinis* was detected in more than 70% of mastitic milk specimens when mycoplasmas were detected in milk specimens from 30 cows with mastitis by this PCR method.
KEY WORDS: mastitis, mycoplasma, PCR.

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Mycoplasma mycoides subsp. *mycoides, M. bovis, M. alkalescens, M. bovigenitalium, M. dispar* and others are the causative agents of several diseases in cows and calves, such as mastitis, pneumonia, arthritis, genital disorders and abortions [1, 3, 6, 12, 15]. *M. bovirhinis* is a troublesome agent as a secondary invader in respiratory diseases, and it is the mycoplasma that is the most common isolate from the nasal cavity of cattle with respiratory disease [8]. Since the diseases are largely resistant to chemotherapy [6, 24], it is necessary to have rapid and reliable diagnostic methods to detect the agents at an early stage, so that effective control measures can be introduced in time. However, current methods for the detection of mycoplasmas are restricted to culture and serology, and both of these diagnostic methods are time consuming, laborious and difficult. Isolation and identification of mycoplasmas may take weeks, and few laboratories have the capabilities required to routinely culture mycoplasmas. Serodiagnosis requires the demonstration of increasing antibody titers that are reached only ten to fourteen days after the onset of clinical symptoms [19]. Consequently, the pathogen cannot be detected during the incubation period. Moreover, the serological cross reactions among the mycoplasma species are a critical problem [19]. Therefore, the absence of practical and rapid detection methods and resistance to therapy hamper the effective control of mycoplasma infections.

Gonzalez [7] and Hatanaka *et al.* [9] reported cases of mycoplasmal mastitis caused by *M. bovis* derived from the nasal secretion of cows with pneumonia, emphasizing the relationship between mycoplasmal pneumonia and mastitis. During 1996–1997, we performed nationwide research on the isolation of mycoplasma from the nasal secretions of calves with respiratory diseases, and the result was that a strain of mycoplasma was isolated from about 30% of calves with respiratory diseases. The majority of the isolates consisted of the following 4 species, *M. alkalescens, M. bovigenitalium, M. bovirhinis* and *M. bovis* (Data not shown). Therefore, we restrict discussion to these four species in this study.

The purpose of this study was the development of an assay for the detection of mycoplasmas in milk based on *in vitro* amplification of the 16S rRNA gene for the detection of *M. alkalescens, M. bovigenitalium, M. bovirhinis* and *M. bovis*.

The mycoplasma strains used were 4 strains of *M. alkalescens*, 3 strains of *M. bovigenitalium*, 5 strains of *M. bovirhinis* and 5 strains of *M. bovis*. These strains included the type strain of each species (*M. alkalescens* PG51, *M. bovigenitalium* PG11, *M. bovirhinis* PG43 and *M. bovis* PG45) and field isolates. The above mycoplasmas were cultured in M-broth [5, 13]. The number of organisms per milliliter in the broth was determined by the M-agar [5, 13]. Organisms were stored in M-broth at –80°C until use [13].

One milliliter of each well-grown suspension culture of mycoplasma was adjusted to a concentration of 10^8 CFU/m*l* with phosphate buffered saline (PBS) for the positive control, or with milk for the simulated specimen. Serial 10-fold dilutions of the organisms with PBS or milk were used to compare the PCR and culture methods as described below. Furthermore, type strains were used to test the sensitivity of the PCR assay. Twenty and ten milk specimens from 30 cows affected with mastitis were collected from farms B and C, respectively. The specimens were transported within 24 hr and kept at 4°C until use.

PCR samples were prepared as below. Milk sample: The simulated specimen was directly used as a template DNA on PCR. Lysate sample: One milliliter of the simulated specimen was centrifuged at 12,000 rpm for 10 min. The pellet was resuspended with 1.0 m*l* of PBS and centrifuged at 9,000 rpm for 10 min. The pellet was resuspended with 1.0 m*l* of PBS and centrifuged at 6,000 rpm for 10 min. The pellet was resuspended with a 50 m*l* aliquot of mycoplasmal lysis buffer [14] containing 200 μg/m*l* of proteinase K, incubated at 60°C for 1 hr, heated at 100°C for 5 min to inactivate the proteinase K, rapidly chilled on ice, and stored at –20°C until use. This sample was also used as a template

DNA on PCR.

The oligonucleotide primers used are described below. As primers of *M. alkalescens (Mak)*, we used *Mak*F (5'-GCTGTTATAGGGAAAGAAAACT-3') and *Mak*R (5'-AGAGTCCTCGACATGACTCG-3') primers [13]. As primers of *M. bovigenitalium (Mbg)*, we used *Mbg*F (5'-CGTAGATGCCGCATGGCATTTACGG-3') and *Mbg*R (5'-CATTCAATATAGTGGCATTTCCTAC-3') primers [13]. As primers of *M. bovirhinis (Mbr)*, we used *Mbr*F (5'-GCTGATAGAGAGGTCTATCG-3') and *Mbr*R (5'-ATTACTCGGGCAGTCTCC-3') [13]. As primers of *M. bovis (Mbo)*, we used *Mbo*F (5'-GGCTCTCATTAA-GAATGTC-3') and *Mbo*R (5'-TTTTAGCTCTTTTTGAA-CAAAT-3') primers [10].

In vitro amplification by PCR for the detection of mycoplasma was carried out with 5 μl of PCR sample in 20 μl of reaction mixture containing RTG PCR beads (Amersham Pharmacia Biotech) and 20 pmol of each primer. The PCR cycles consisted of pre-heating at 94°C for 9 min, denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 1 min. The amplifications were performed for 35 cycles in a model 2,400 thermocycler (Perkin Elmer) with a final extension step at 72°C for 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by staining with SYBR Gold (Molecular Probes) [22].

The culture of clinical specimens was carried out as follows. For each specimen, a loop of the milk specimen (approx. 5 μl) was inoculated onto an M-agar plate. In addition, a pellet of 1.0 m*l* of the milk specimen centrifuged at 12,000 rpm for 10 min was inoculated into M-broth (2.7 m*l*). After culturing for 3–7 days, the M-broth was used to inoculate additional M-agar plates. Colonies that appeared on the M-agar plates were identified by growth inhibition (GI) and/or metabolism inhibition (MI) tests [20]. Colonies that were insensitive to digitonin were regarded as *Acholeplasma* spp. [17, 21].

In the positive control specimens diluted with PBS, PCR products were detected for all the mycoplasmal species and strains (data not shown). On the other hand, in the simulated specimens diluted with milk, PCR products were not detected for any of the mycoplasmal species and strains (Table 1). However, PCR products were detected for all mycoplasmal species or strains by use of the lysate sample (Table 1). In the negative control specimens containing only milk, PCR products were not detected using any of the primers (data not shown).

When a lysate sample was used as a PCR sample, the detection limits of type strain of *M. alkalescens, M. bovigenitalium, M. bovirhinis* and *M. bovis* were 1.4×10^3, 1.7×10^2, 1.1×10^2 and 4.0×10^2 CFU, respectively, whereas those of *M. alkalescens, M. bovigenitalium, M. bovirhinis* and *M. bovis* in the culture method were 1.4×10^4, 1.7×10^2, 1.1×10^3 and 4.0×10^3 CFU, respectively. These findings indicate that the sensitivity is almost equal between PCR and the culture method or tended to be higher in PCR.

Mycoplasmas in clinical milk specimens with major or minor mastitis pathogens were detected in almost cases (ex. Staphylococci, Streptococci) by PCR as shown in Table 2. *M. bovirhinis* was most frequently detected in the milk specimens. *M. bovirhinis* was detected by PCR in 22 of 30 (73.3%) cows with mastitis. *M. bovis* was detected by PCR in 2 of 30 (6.7%) cows. In two cows, two species, *M. bovirhinis* and *M. bovis*, were simultaneously detected by PCR. However, by the culture method, *M. bovirhinis* and *M. bovis* were isolated from eight (26.7%) and one (3.3%) cows, respectively.

Bovine mycoplasmal mastitis is very infectious and intractable, and thus causes great economical loss [9]. Although a large scale incidence was reported in Japan [9], it is difficult to establish a routine testing system because of complex testing procedures. Therefore, we investigated a method of detecting mycoplasma in milk by PCR to establish an early diagnosis method for bovine mycoplasmal

Table 1. Detection of mycoplasmas in milk by PCR

Bacteria	No. of strain tested	No. of PCR positive strain	
		Simulated milk samples	Lysate from the simulated samples
Mycoplasma alkalescens	4	0	4
Mycoplasma bovigenitalium	3	0	3
Mycoplasma bovirhinis	5	0	5
Mycoplasma bovis	5	0	5

Table 2. Detection of mycoplasmas by PCR and culture techniques

Bacteria	No. of strain detected by:					
	PCR			Culture		
	Farm B (n=20)	Farm C (n=10)	Total (n=30)	Farm B (n=20)	Farm C (n=10)	Total (n=30)
M. alkalescens	0	0	0 (0%)	0	0	0 (0%)
M. bovigenitalium	0	0	0 (0%)	0	0	0 (0%)
M. bovirhinis	18	4	22 (73.3%)	6	2	8 (26.7%)
M. bovis	2	0	2 (6.7%)	1	0	1 (0%)

mastitis and routine testing.

In this study, in attempt to process a number of specimens as rapidly and readily as possible, we investigated a method using the milk specimen as template DNA. When mycoplasmal culture fluid was used for the template DNA, all mycoplasmal species were detected, while none of the mycoplasma species were detected in the simulated specimens diluted with milk. However all mycoplasma species could be detected when mycoplasmas were collected from the simulated specimens by centrifugation and lysed with a mycoplasmal lysis buffer. Because a large number of milk components remain in template DNA in milk samples, these components may have interfered with the PCR reaction [2, 16, 18]. Therefore, we tried to eliminate milk components from the simulated specimens by centrifugation.

The detection limits of the mycoplasmas in the culture method was obtained and compared to those in PCR performed after processing by centrifugation and lysis with a mycoplasmal lysis buffer (lysate sample). The detection limits were almost equal in the two methods or tended to be lower in PCR for all mycoplasmas. This detection limit of mycoplasma was clearly lower than the amount of mycoplasma (10^4 CFU) in milk from diseased cows reported by Hotzel *et al.* [10], indicating that the PCR detection system for mycoplasmas in milk in this study is practical for clinical testing.

The detection rate for mycoplasmas in the culture method using clinical mastitis milk is significantly lower compared with that of PCR analysis, and these results were not consistent with the simulation test results in which raw milk was used. This was probably due to significant changes in the electrolyte composition or enzyme activity in the mastitic milk of the affected cow, which caused rapid diminution of mycoplasma in the mastitic milk, thereby influencing the detection rate [23].

The current diagnosis by isolation and identification of mycoplasma requires one week or longer to diagnose mycoplasmal mastitis [5, 19]. In contrast, the diagnosis time was shortened to several hours in the our PCR method. Mastitis caused by any bacteria including mycoplasma causes latent mastitis due to latent or mild infection during the early stage of infection, which later shows marked clinical symptoms of clinical mastitis. During the early latent state, treatment is effective in many cases but the disease is not readily discovered [11]. The disease that is overlooked during this stage and progresses to clinical mastitis will not be markedly improved by therapy in many cases [11]. Therefore, early diagnosis and therapy is a basic measure against mastitis, and the reduction of time needed for a definitive diagnosis is clinically very significant.

In Australia, *M. bovis* was mainly detected from mastitic milk [6]. However, in this study, *M. bovis* were detected in only 7% of the cows with mastitis, whereas *M. bovirhinis* was detected in more than 70%. It seems that the difference in the detection rates of *M. bovis* between Australia and Japan originates in the difference of the degree of spread of *M. bovis* infection in cows. *M. bovirhinis*, which is often isolated from respiratory organs, was also detected with high frequency in clinical mastitic milk specimens, and that was probably due to infection caused by secretions of calves through nipples simultaneously contaminated by other organisms during lactation, as reported in the past [7, 9]. Therefore *M. bovirhinis* seems to be an important pathogen as a secondary infection agent in clinical mastitis as well as bovine pneumonia.

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