Development of a Nested PCR Method for Detection of Mycoplasma Bovis in Preserved Milk Samples

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Summary and Implications

A nested PCR method was developed and validated for detection of *Mycoplasma bovis* in milk samples preserved with bronopol. A previously described, randomly cloned DNA fragment was sequenced. This sequence information was used to develop a set of internal primers for a nested PCR assay. Cationic surfactant purification was used to eliminate milk protein substances that inhibit amplification. Serial diluted seeded milk samples were used to determine a sensitivity of 5.1 CFU equivalents/ml of milk. Specificity of the assay was confirmed testing against 7 other mycoplasma species and 11 other mastitis organisms. A comparison of culture, with blind passage, to the nested PCR reaction was performed on clinical field milk samples from *M. bovis* affected and *M. bovis* free herds. The nested PCR method was more sensitive than culture (~5 CFU/ml), specific for *M. bovis*, effective with preserved milk, and could be completed in less than one day. This may provide a practical, rapid, cost effective procedure to screen for clinical and subclinical *M. bovis* carriers using routinely obtained preservative-treated milk samples.

Introduction

As the causative agent of mycoplasmal mastitis, *Mycoplasma bovis* causes considerable economic losses to the dairy industry. It has been reported that 100 cfu can colonize the udder and cause disease, and the incubation period for *M. bovis*-induced disease can last from 2 to 6 days during which time shedding may occur. Also, chronically infected cows can shed the organism for prolonged periods, long after clinical signs have ended. Since no vaccine is available, present methods for controlling *M. bovis* involve preventative measures to limit infection and the culling of shedders. The highly virulent and pathogenic nature of *M. bovis* creates a demand for the rapid identification of infected animals, since early identification results in lower overall impact on the herd.

The most commonly used method for diagnosis of *M. bovis* is detection by culture. This method is time-consuming and often problematic because of non-mycoplasmal bacterial overgrowth. Inoculated plates must be incubated for at least two days, and should not be considered negative until seven days have passed . As a result, significant efforts have been made for the development of new *M. bovis* detection techniques, including the use of DNA hybridization probes and PCR assays. Although PCR tests have been developed, none seem to solve all the problems with detecting *M. bovis* from milk. Either there have been problems with specificity), the issue of sensitivity was not addressed , or the method was not validated on field milk samples. Furthermore, in another protocol, the milk was cultured prior to PCR, thus eliminating the ability to use the method on preservative-treated milk.

Collecting individual cow milk samples to identify sub-clinical carriers may be time consuming and expensive. Currently, some producers obtain individual cow samples on a monthly basis for evaluation of somatic cell counts (DHI – programs). These samples cannot be used for culture because a preservative is added to the milk. They could, however, be used for PCR but only if extreme care and sanitation to prevent milk carryover between cow samples is achieved.

The present authors (Pinnow et al., 2001) described a protocol for the detection of M. bovis in preservative-treated milk using nested PCR. The procedure eliminates the need for culture before PCR, and uses a surfactant to remove milk proteins know to inhibit PCR reactions. Sensitivity and specificity were addressed and survival of *M. bovis* exposed to DHI milk-preservative was plotted. Finally, the procedure was validated on field milk samples.

Materials and Methods

Organisms and Culture Methods

The mycoplasmas and other bacteria used in this study are listed in Table 1. All organisms were grown to log phase prior to extraction of nucleic acids. For culture of *M. bovis* field strains, a 0.2 ml aliquot of each field milk sample was combined with 1.8 ml of modified Friis broth with bacterial inhibitors and incubated at 37°C for 48 hours. This broth culture was streaked onto Friis plates, incubated at 37°C for 48 hours, and examined for mycoplasma colony growth by microscopy. The mycoplasma species was identified using flourescent antibodies on colony impressions on glass slides.

Field Milk Samples

Fifty-three field milk samples from Iowa farms and bovine mastitis cases submitted to the ISU Veterinary Diagnostic Lab were used to validate the nested PCR procedure. Upon receipt, all field milk samples were immediately cultured by blind passage in Friis broth as described above. The samples were then frozen at -20°C for 2 years. After 2 years, samples were thawed, recultured, and prepared for the PCR assay. To simulate submission of a milk sample to the DHIA program, DHI milk preservative, which has an active ingredient of 18% 2-bromo-2-nitropropane-1, 3-diol (Bronopol-Boots, National DHIA, Columbus, OH), was added at the mandated 1.69 µl preservative per ml of milk sample. The samples were mixed until homogenous and then placed at 4°C for 20 hours.

Effect of DHI Milk Preservative

In order to determine the toxic effect of DHI milk preservative upon *M. bovis*, log phase *M. bovis* strain PG45 (American Type Culture Collection 25523) was used to seed 20 ml milk from a known negative cow. The seeded milk was vortexed until homogenous and divided into two samples. The DHI preservative was added at 1.69 µl/ml to one of the samples. The samples were placed in a 10°C water bath while aliquots were taken every hour for titration plating on Friis agar as described (Albers and Fletcher, 1982). A toxicity curve was plotted (Figure 1).

Nested PCR Development (milk, primers, techniques)
(Pinnow et al., J. Dairy Sci. 2001.)

Results and Discussion

Effect of DHI Milk Preservative

The toxicity curve of *M. bovis* exposed to DHI preservative is illustrated in Figure 1. The concentrations of mycoplasma remained relatively static in milk without preservative, while there was a steady decline in the number of viable *M. bovis* cells in the preservative-treated milk. By 10 hrs. post preservative, viable number of *M. bovis* were below most culture sensitivities.

Specificity

After preliminary work in optimization of the nested PCR conditions using purified DNA, the established protocol amplified the expected region of *M. bovis* type strain PG45 and reference strain M23 to produce a specific 442 bp product. Testing of the nested PCR protocol on isolated nucleic acids from numerous non-*M. bovis* microorganisms did not result in detectable amplification (Table 1). However the nested PCR assay was not strain-specific, as shown in direct testing of 26 known positive field milk samples. In all instances, the expected 442 bp amplicon was generated, showing the test specific for *Mycoplasma bovis* only.

Sensitivity

The use of ten-fold serial dilutions of *M. bovis* PG45 in preservative-treated milk resulted in amplification of the specific 442 bp product to 5.1 cfu equivalents per ml of milk. No detection was found at 0.51 cfu/ml.

Validation

Of the 53 milk samples used for culture and PCR comparison, 26 samples were positive and 27 were negative for *M. bovis* according to initial culture records in 1996. Only 7 of the 26 samples of the original culture-positive samples from 1996 recultured positive in 1998 after frozen storage. All 26 culture-positive samples were also positive by the nested PCR assay after frozen storage. Application of the nested PCR assay showed that 5 of the 27 initially culture-negative samples were positive by the nested PCR assay. The remaining 22 culture-negative samples were also negative by PCR. False positive results were ruled out by use of appropriate negative controls in each batch of PCR reactions run.

Conclusions

The nested PCR procedure has been shown to be a rapid, specific, and sensitive test that can aid in the detection of subclinical *M. bovis* carriers in dairy herds. The method can be used on preservative treated milk that is not acceptable for culture. If DHI preserved samples are utilized, prevention of milk carryover between individual cow samples must be prevented through proper sanitary procedures.

References

1. Pinnow, C., J. Butler, K. Sachse, H. Hotzel, L. Timms, and R. Rosenbusch. 2001. Detection of *Mycoplasma bovis* in preservative- treated field milk samples. J. Dairy Sci.84(7).

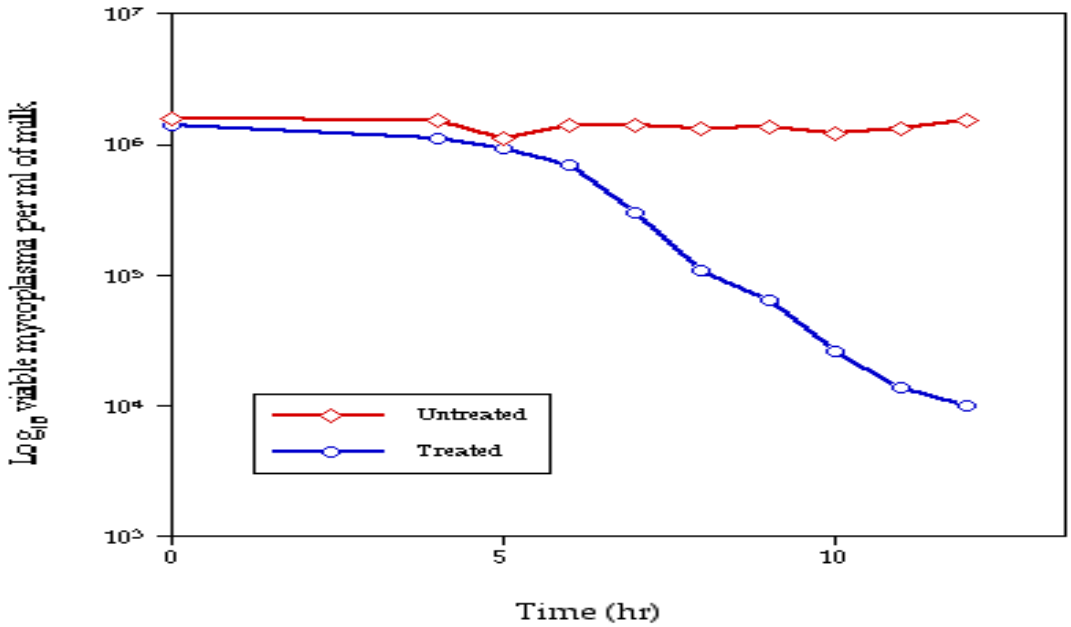

Figure 1. Time survival curve of *Mycoplasma bovis* exposed to DHIA milk preservative.

Table 1. Results of the nested polymerase chain reaction on reference strains using a commercial nucleic acid extraction or a cationic surfactant extraction from log phase broth culture.

Strain no.	Species	Origin	Nucleic Acid Template	Cationic Surfactant Template
	Reference strains			
PG45	*Mycoplasma bovis*	ATCC 25523	+	+
M23	*M. bovis*	R. Rosenbusch	+	+
PG51	*Mycoplasma alkalescens*	ATCC 29103	-	-
GM139	*Mycoplasma agalactiae*	ATCC 35890	-	-
PG11	*Mycoplasma bovigenitalium*	ATCC 14173	-	-
PG43	*Mycoplasma bovirhinis*	ATCC 27748	-	-
M165/69	*Mycoplasma bovoculi*	ATCC 29104	-	-
ST-6	*Mycoplasma californicum*	ATCC 33416	-	-
	Mycoplasma canadense	ATCC 29418	-	-
462/2	*Mycoplasma dispar*	ATCC 27140	-	-
PG31	*Mycoplasma gallisepticum*	ATCC 19610	-	-
PG21	*Mycoplasma hominis*	ATCC 23114	-	-
	Mycoplasma verecundum	ATCC 27862	-	-
PG50	*M. sp., bovine serogroup 7*	ATCC 27367	-	-
C492	*Acholeplasma laidlawii*	R. Rosenbusch	-	-
	Actinomyces pyogenes	R. Griffith, ISU	-	-
	Escherichia coli	R. Griffith	-	-
	Klebsiella pneumoniae	R. Griffith	-	-
	Pseudomonas aeruginosa	R. Griffith	-	-
	Serratia marcescens	R. Griffith	-	-
	Staphylococcus aureus	R. Griffith	-	-
	Streptococcus agalactiae	R. Griffith	-	-
	Streptococcus dysgalactiae	R. Griffith	-	-
	Streptococcus uberis	R. Griffith	-	-