

## Application of IS900 PCR for Detection of *Mycobacterium avium* subsp. *paratuberculosis* Directly from Raw Milk

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### ABSTRACT

A polymerase chain reaction (PCR)-based assay was developed for detection of insertion sequence 900 (IS900) of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk. This IS900 PCR assay included DNA extraction and PCR assay using commercially available kits. The DNA extraction and PCR assay were optimized to detect the IS900 sequence directly from raw milk. The IS900 PCR assay was evaluated by inoculating raw bulk milk and Middlebrook's 7H9 broth with 0 to 10<sup>8</sup> cfu/ml of each of four American Type Culture Collection strains of *M. paratuberculosis*. Under experimental conditions, both milk culture on Herrold's egg yolk medium slants, and IS900 PCR could detect 10 to 100 cfu/ml of *M. paratuberculosis*. Detection of *M. paratuberculosis* by IS900 PCR was consistent (24/24 PCR assays) when about 100 cfu/ml were present, whereas detection was variable (12/24 PCR assays) at concentrations as low as 10 cfu/ml. Based on the findings of the experimental study, IS900 PCR was further evaluated with pooled quarter milk samples from 211 cows from five herds with known history of Johne's disease. Out of 211 animals examined, nine (4%) and 69 (33%) were positive for *M. paratuberculosis* by milk culture and IS900 PCR from milk, respectively. A total of 20 bulk tank milk sample aliquots (one sample, four aliquots from each herd) were also examined, of which 10 (50%) were positive for *M. paratuberculosis* by IS900 PCR. By contrast, only one out of 20 (5%) bulk tank milk sample aliquots was positive by culture. The IS900 PCR amplified product of 229-bp obtained on testing of quarter milk and bulk tank milk samples was confirmed to be the IS900 of *M. paratuberculosis* by DNA sequence analysis. The results of this study suggest that *M. paratuberculosis* can be detected directly from quarter milk and bulk tank milk by IS900 PCR.

**(Key words:** IS900 polymerase chain reaction, *M. paratuberculosis*, bulk tank milk, milk culture)

**Abbreviation key:** ATCC = American Type Culture Collection, HEYM = Herrold's egg yolk medium, HPC = hexacetylpyridinium chloride, IS900 = insertion sequence 900, M7H9 broth = Middlebrook's 7H9 broth.

### INTRODUCTION

Johne's disease is a chronic progressive granulomatous enteritis of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (hereafter referred to as *M. paratuberculosis*; (Stabel, 1998). It is estimated that nearly 40% of US dairy herds are infected with *M. paratuberculosis* and that losses to the dairy industry may exceed \$1.5 billion/year (Jones, 1989; Stabel, 1998).

The control of Johne's disease has been hampered due to a lack of sensitive techniques for detection of asymptomatic paratuberculosis (Van der Giessen et al., 1992; Stabel, 1998). Fecal culture for *M. paratuberculosis* is considered the gold standard for diagnosis (Whitlock et al., 1985), but the fastidious nature of the organism along with its slow growth characteristics make isolation of *M. paratuberculosis* a time-consuming process (Hermon-Taylor et al., 2000; Stabel, 1998). Another drawback of fecal culture is that it detects only 38 to 50% of cows infected with *M. paratuberculosis* (Sanftleben, 1990; Stabel, 1998; Whitlock et al., 2000).

Serologic tests such as agar gel immunodiffusion test, complement fixation test, and ELISA have been developed to detect *M. paratuberculosis*. However, these tests are even less sensitive than fecal culture for detection of *M. paratuberculosis* (Sherman et al., 1984; Colgrove et al., 1989; Sanftleben, 1990; Sweeney et al., 1994; Stabel, 1998; Sweeney et al., 1995).

Several researchers have developed and evaluated DNA probes based on insertion sequence 900 (IS900), a species-specific insertion element in *M. paratuberculosis*, for early detection of Johne's disease (Vary et al., 1990; Whipple et al., 1992; Van der Giessen et al., 1992; Collins et al., 1993; Millar et al., 1996; Thoen and Haagsma, 1996; Englund et al., 1999). These techniques allow reporting of results as early as 3 to 4 d and could have practical value for purchasing replacement cattle

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or limiting the spread of infection within a herd (Thoen and Haagsma, 1996).

Cows presenting symptoms of Johne's disease are known to shed *M. paratuberculosis* in their milk (Taylor et al., 1981; Sweeney et al., 1992; Sweeney et al., 1994; Streeter et al., 1995), and IS900 PCR has been used successfully to detect *M. paratuberculosis* in raw and pasteurized milk (Millar et al., 1996; Giese and Ahrens, 2000). These findings suggest that IS900 PCR could be applied as a diagnostic tool for Johne's disease using quarter milk or bulk tank milk samples.

In addition to diagnostic significance, the detection of *M. paratuberculosis* in pooled quarter milk and bulk tank milk is also important from a public health standpoint. There are growing concerns that *M. paratuberculosis* may be an etiological agent of Crohn's disease in humans (Chiodini and Hermon-Taylor, 1993; Hermon-Taylor et al., 2000; Stabel, 2000), and *M. paratuberculosis* has been shown to survive pasteurization (Grant et al., 1996).

The objectives of this study were: 1) to evaluate detection of *M. paratuberculosis* by IS900 PCR and culture from experimentally inoculated raw bulk tank milk, and 2) to evaluate detection of *M. paratuberculosis* by IS900 PCR and culture from pooled quarter milk and bulk tank milk samples from commercial dairy herds with a known history of Johne's disease.

## MATERIALS AND METHODS

### Enumeration of *M. paratuberculosis* from Experimentally Inoculated Raw Bulk Tank Milk

Enumeration of *M. paratuberculosis* from experimentally inoculated raw bulk tank milk was done as described by Streeter et al. (1995). Bulk tank milk used for experimental inoculation with *M. paratuberculosis* was procured from a herd known to be free of Johne's disease. Raw bulk tank milk samples (50 ml) were experimentally inoculated with *M. paratuberculosis* to achieve final concentrations of  $10^8$ ,  $10^6$ ,  $10^4$ ,  $10^2$ , 10, 1, and 0 cfu/ml of each of four American Type Culture Collection (ATCC) strains. The strains of *M. paratuberculosis* included one bovine isolate—ATCC 19698 and three human isolates: ATCC 43544 (Ben), ATCC 43545 (Dominic), and ATCC 43015 (Linda). Samples were inoculated with Middlebrook's 7H9 (M7H9) broth (BD Biosciences, Sparks, MD) in the same concentrations and strains of *M. paratuberculosis* and used as positive controls. Milk and M7H9 broth samples were centrifuged (Mistral 3000 i, Curtin Matheson Scientific, Inc., Houston, TX) at  $1950 \times g$  for 30 min at 4°C. The supernatant was discarded and the pellets were washed twice in PBS (pH 7.2). After resuspension in 500  $\mu$ l of PBS, half the volume (250  $\mu$ l) was used for DNA extraction

and IS900 PCR. The other half was treated overnight at room temperature in 15 ml of 0.75% hexadecylpyridinium chloride (HPC [Sigma, St. Louis, MO]). The suspensions were centrifuged (Mistral 3000 i, Curtin Matheson Scientific, Inc., Houston, TX) at  $1600 \times g$  for 10 min, supernatants were discarded, and pellets were resuspended in 300  $\mu$ l of 0.75% HPC (containing 0.05% Tween 80 [Sigma, St. Louis, MO]). Six drops (150  $\mu$ l) of the suspensions were inoculated on two slants each of Herrold's egg yolk medium (HEYM) with Mycobactin J (BD Biosciences, Sparks, MD). The slants were incubated at 37°C for 16 wk, and typical colonies of *M. paratuberculosis* were counted. The colonies were stained by the Ziehl-Neelsen procedure (Zimmer et al., 1999) to confirm the presence of acid fast bacilli. To assess reproducibility, all recovery and detection experiments were replicated six times for each strain of *M. paratuberculosis*.

### IS900 PCR Assay from Milk

DNA was extracted with the Instagene DNA purification matrix (Bio-Rad Laboratories, Hercules, CA), as described previously (Nishimori et al., 1995). Milk and M7H9 broth suspensions (250  $\mu$ l of each concentration) were mixed with 300  $\mu$ l of the matrix and incubated in a water bath at 56°C for 30 min. The suspensions were vortexed for 10 s and boiled at 100°C for 15 min in a heat block followed by centrifugation at  $20,800 \times g$  in an Eppendorf type centrifuge (model 5417 C, Brinkmann Instruments Inc., NY) for 5 min. The resultant supernatants were used for PCR. The DNA extracts were stored at -20°C and recentrifuged before use. DNA was quantified from the supernatants using a DU 640 spectrophotometer (Beckman, Fullerton, CA). Absorbance of a 1:50 dilution of DNA in sterile water was measured at 260 and 280 nm.

IS900 PCR was performed in a total volume of 25  $\mu$ l with specific primers for IS900 as described previously (Vary et al., 1990) except that Ready-To-Go PCR beads (Amersham Pharmacia Biotech, www.apbiotech.com) were used. Briefly, 5  $\mu$ l of DNA extract containing approximately 100 ng of bacterial DNA was added to Ready-To-Go PCR beads. The reaction mixture had a final concentration of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 mM of each dNTP, stabilizers including BSA, and 200 pM of each primer. The 5' primer sequence was 5'-CCGCTAATTGAGAGATGC-GATTGG-3' and the 3' primer sequence was 5'-AAT-CAACTCCAGCAGCAGCGCGGCTCG-3'. IS900 PCR amplification was performed in a thermocycler (MJ Research, Waterstown, MA) with initial denaturation at 94°C for 2 min, then 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 66°C for 1 min and

extension at 72°C for 1 min. The resulting PCR-amplified products were electrophoresed in 2.5% agarose using Tris-borate-EDTA buffer (Sigma, St. Louis, MO) in gels at 150 V for 3 h. Gels were stained with ethidium bromide (1 mg/ml). DNA was visualized by UV transillumination (UVP Inc., San Gabriel, CA) and photographed with type 55 film (Polaroid Corp., Cambridge, MA). The negative of the Polaroid film was scanned with a computer-integrated scanner (model HPScanjet IIP, Hewlett Packard, Palo Alto, CA). The presence or absence of a 229-bp fragment was recorded.

To confirm the identity of the 229-bp amplified fragment, the fragment from 12 randomly chosen samples and a positive control sample (ATCC 19698) were digested using the GELase agarose gel-digesting preparation (Epicentre Technologies, Madison, WI). Purified DNA were sent to the nucleic acid facility of The Pennsylvania State University for sequence analysis. Cycle sequencing reactions were performed at the Nucleic Acid Facility using protocol # 4303237 from Applied Biosystems (Foster City, CA). Sequencing reactions were performed using 3' BigDye-labeled dideoxynucleotide triphosphates (dye terminators) and run on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Data was analyzed using the ABI PRISM XL Sequencing Analysis 3.3 Program. Oligonucleotide sequence data of amplified 229-bp fragments were compared to known bacterial insertion sequences by BLAST analysis from the National Library of Medicine database (Altschul et al., 1997).

### Evaluation of Johne's Positive Herds

A total of 211 cows from five herds with a known history of Johne's disease were included in the study. The five herds that participated in the study had a history of one or more lactating cows positive for *M. paratuberculosis* on fecal culture. Approximately 13 to 15 ml of milk was collected from each of the four quarters of individual cows in milk and pooled to make one composite sample per cow. Raw bulk tank milk samples (4 L) were collected from each herd after all the individual samples had been collected. The samples were transported to the laboratory on ice. Bulk tank samples were mixed thoroughly, and four aliquots of 50 ml each were evaluated from each herd. Raw bulk tank milk and pooled quarter milk samples were evaluated for the presence of *M. paratuberculosis* by culture and IS900 PCR as described earlier for experimentally inoculated samples.

## RESULTS AND DISCUSSION

The results of this study indicated that both milk culture on HEYM slants, and IS900 PCR detected about

**Table 1.** Comparison of milk and broth cultures for the detection of *Mycobacterium paratuberculosis* with IS900 PCR.

Milk <sup>1</sup>			M7H9 Broth <sup>1</sup>		
cfu/ml	PCR +	Counts <sup>2</sup> (mean cfu/ml)	cfu/ml	PCR +	Counts <sup>2</sup> (mean cfu/ml)
10 <sup>8</sup>	24/24	TNTC <sup>3</sup>	10 <sup>8</sup>	24/24	TNTC
10 <sup>6</sup>	24/24	TNTC	10 <sup>6</sup>	24/24	TNTC
10 <sup>4</sup>	24/24	TNTC	10 <sup>4</sup>	24/24	TNTC
10 <sup>2</sup>	24/24	53	10 <sup>2</sup>	24/24	75
10	12/24 <sup>4</sup>	2	10	24/24	7
1	0/24	0	1	0/24	0
0	0/24	0	0	0/24	0

<sup>1</sup>Six replicates each of four ATCC reference strains (total 24 assays) of *M. paratuberculosis*: ATCC19698 (Bovine), ATCC 43544 (Ben), ATCC 43545 (Dominic), ATCC 43015 (Linda).

<sup>2</sup>Mean of six replicates of four ATCC reference strains of *M. paratuberculosis*.

<sup>3</sup>Too numerous to count.

<sup>4</sup>PCR failed to detect ATCC 19698 (2/6), ATCC 43544 (3/6), ATCC 43545 (3/6), ATCC 43015 (4/6).

10 to 100 cfu/ml of *M. paratuberculosis* directly from raw bulk tank milk (Table 1). Detection of *M. paratuberculosis* from milk and M7H9 broth by IS900 PCR was consistent (24/24 assays, 100%) when about 100 cfu/ml were present. However, at lower levels of about 10 cfu/ml, detection of *M. paratuberculosis* by IS900 PCR from milk was variable (12/24 assays, 50% [Table 1]).

These findings are in close agreement with the results of a recent study (Giese and Ahrens, 2000) that reported the detection of about 100 cfu/ml of *M. paratuberculosis* from the milk of cows showing clinical signs of Johne's disease. Other investigators have reported that milk culture on HEYM slants could detect between 5 to 8 cfu/50 ml of *M. paratuberculosis* in the milk of cows with clinical Johne's disease (Taylor et al., 1981; Sweeney et al., 1992). The findings of the present study are also close to previously reported detection limits of 200 to 300 cfu/ml of *M. paratuberculosis* from pasteurized milk (Millar et al., 1996). Another study using immunomagnetic beads coated with polyclonal antibodies to *M. paratuberculosis* followed by IS900 PCR has reported detection limits of 10 cfu/ml of milk (Grant et al., 1998).

In contrast to the results of this study, a recent study by Giese and Ahrens (2000) reported that IS900 PCR could detect *M. paratuberculosis* from milk only when 1000 cfu/ml were present. However, in the same study, a culture could detect 100 cfu/ml of *M. paratuberculosis*, and the investigators felt that the lower sensitivity of the PCR assay compared with the culture could have been due to insufficient DNA preparation methods, or due to the small volume of milk (200 µl) used for DNA extraction. By comparison, in the present study, 50 ml of milk was spun down, and DNA was extracted with the Instagene DNA purification matrix (Bio Rad).

**Table 2.** Detection of *Mycobacterium paratuberculosis* in herds with a known history of Johne's disease.

Individual cow milk	Herd A n = 44	Herd B n = 32	Herd C n = 29	Herd D n = 37	Herd E n = 69	Total n = 211
Culture +	9 (4%)	0	0	0	0	9 (4%)
PCR +	30 (68%)	8 (25%)	7 (24%)	6 (16%)	18 (26%)	69 (33%)
Bulk tank milk n = 4	n = 4	n = 4	n = 4	n = 4	n = 4	n = 20
Culture +	1 (25%)	0	0	0	0	1 (5%)
PCR +	4 (100%)	2 (50%)	1 (25%)	1 (25%)	2 (50%)	10 (50%)

<sup>1</sup>+, positive.

The variability seen in the detection of *M. paratuberculosis* by IS900 PCR at lower concentrations of 10 cfu/ml could be due to loss of some organisms in the cream fraction after centrifugation of milk. In this study, the cream fraction of the milk sample was not tested for *M. paratuberculosis*. This could have resulted in lower recovery rates for *M. paratuberculosis* from milk (53/100 and 2/10 cfu/ml) than recovery rates for *M. paratuberculosis* from M7H9 broth (75/100, and 7/10 cfu/ml) at concentrations of 10 to 100 cfu/ml (Table 1). Additionally, other investigators (Giese and Ahrens, 2000) have recovered *M. paratuberculosis* from both pellet and cream fractions of milk, and yet another study (Millar et al., 1996) showed that the IS900 PCR signal was obtained from both the pellet and cream fraction after centrifugation of milk spiked with *M. paratuberculosis*.

A total of 211 cows from five Johne's positive herds (A through E) were evaluated for the presence of *M. paratuberculosis* in pooled quarter milk by culture and IS900 PCR (Table 2). *Mycobacterium paratuberculosis* was detected by culture from 9/211 (4%) samples, whereas *M. paratuberculosis* was detected by IS900 PCR from 69/211 (33%) samples. In herd A, *M. paratuberculosis* was detected by culture in 9/44 (20%) samples. In the same herd, *M. paratuberculosis* was detected by IS900 PCR in 30/44 (68%) samples (Table 2). In the remaining four herds (B through E, 167 samples), *M. paratuberculosis* was not detected by culture. In these herds *M. paratuberculosis* was detected by IS900 PCR in 29/167 (23%) samples (Table 2).

There are few studies on detection of *M. paratuberculosis* by IS900 PCR from quarter milk. In one study (Millar et al., 1996), *M. paratuberculosis* was detected by IS900 PCR in 22/312 (7%) pasteurized retail milk samples in Great Britain. Giese and Ahrens (2000) were able to detect *M. paratuberculosis* by IS900 PCR in quarter milk samples of 2/11 (18%) cows clinically affected with Johne's disease.

In this study, 20 bulk tank milk sample aliquots (four aliquots of 50 ml each from herds A through E) were analyzed by IS900 PCR and culture for *M. paratuberculosis*. Ten out of the 20 (50%) aliquots examined were positive for *M. paratuberculosis* by IS900 PCR. By con-

trast, only one out of 20 (5%) aliquots was positive by culture (Table 2). All four bulk tank milk aliquots from herd A were positive for *M. paratuberculosis* by IS900 PCR, and one of the four aliquots was also positive for *M. paratuberculosis* by culture (Table 2). The other four herds (B through E) had either one or two aliquots positive for *M. paratuberculosis* by IS900 PCR, but none of these herds was positive for *M. paratuberculosis* by culture (Table 2). Only one report, by Stabel (2000), was available for comparison of the bulk tank milk findings. In that report, *M. paratuberculosis* was not detected by IS900 PCR or culture from 69 raw bulk tank milk samples.

The higher number of positive bulk tank milk aliquots from herd A could be due to the presence of more animals shedding viable *M. paratuberculosis* in the milk. Herd A had the largest number of quarter milk samples positive by IS900 PCR, and herd A was the only herd from which *M. paratuberculosis* was recovered by culture. In this study, *M. paratuberculosis* was detected by IS900 PCR in 33% of quarter milk samples and 50% of bulk tank milk aliquots examined (Table 2). By contrast, only 4% of quarter milk samples and 5% of bulk tank milk aliquots were positive for *M. paratuberculosis* on culture. The lower detection rate of *M. paratuberculosis* by milk culture could be attributed to the following causes: 1) the isolation procedure for *M. paratuberculosis* from milk used by most researchers has been adapted from the protocol developed for fecal samples; 2) the number of organisms shed in milk may be few in number (compared with feces) due to intermittent shedding milk or the dilution factor occurring as a result of comingling of milk in bulk tank; and 3) *M. paratuberculosis* is very exacting in its need for growth requirements. These listed factors could have influenced the lowered detection rate by milk culture (Stabel, 1998; van der Giessen et al., 1992; and Whipple et al., 1992).

The reported isolation rates for *M. paratuberculosis* by milk culture vary widely. In one study from Pennsylvania (Sweeney et al., 1992), *M. paratuberculosis* was detected from the milk of 9/77 (11.6%) apparently healthy cows without any clinical signs of Johne's dis-

ease. In another study, the same group (Sweeney et al., 1994) reported the isolation of *M. paratuberculosis* from the milk of 7 of 16 (44%) clinically normal cows. By contrast, Streeter et al. (1995) from Ohio reported the isolation of *M. paratuberculosis* from the milk of only 3/126 (2.4%) clinically normal cows. Interestingly, in the same study, *M. paratuberculosis* was isolated from the colostrum of 10/126 (7.9%) cows. An important factor that could affect isolation rates for *M. paratuberculosis* could be the intermittent shedding of *M. paratuberculosis* by subclinically infected animals (Sweeney et al., 1992, 1994; Van der Giessen et al., 1992; Whipple et al., 1992; Kalis et al., 1999). Culturing *M. paratuberculosis* from feces of subclinically infected animals has also met with poor success. Reports suggest that fecal culture may detect only 15 to 25% of subclinically infected animals (Sanftleben, 1990; Stabel, 1998).

Recent studies suggest that improved methods of culture such as the BACTEC 12B radiometric culture may also result in faster and better recovery rates of *M. paratuberculosis* (Whittington et al., 1998; Eamens et al., 2000). It is also possible that chemical decontamination may affect the recovery of *M. paratuberculosis*. A recent study (Giese and Ahrens, 2000) has reported the isolation of *M. paratuberculosis* from the milk of 5/11 cows (45%) clinically positive for Johne's disease. In that study, milk was cultivated on Lowenstein-Jensen medium without any decontamination.

Although this investigation has shown that *M. paratuberculosis* was present in quarter milk samples of clinically normal cows as well as raw bulk tank milk samples, the source of *M. paratuberculosis* in milk needs to be considered. Subclinically infected animals do excrete *M. paratuberculosis* in their milk, but it is more likely that fecal contamination from infected cows could result in the contamination of quarter milk, as well as contribute to the presence of *M. paratuberculosis* in bulk tank milk (Giese and Ahrens, 2000; Stabel, 2000). Another factor to be considered is that the PCR does not discriminate between live and dead cells (Stabel, 2000).

To determine whether the 229-bp fragment amplified by the IS900 PCR assay was specific for *M. paratuberculosis*, the fragment obtained by amplification of the bovine ATCC reference strain 19698, as well as fragments obtained from 12 randomly selected milk samples, were sequenced and compared to the known IS900 of *M. paratuberculosis* by BLAST analysis. BLAST analysis revealed that the nucleotide sequence of the 229-bp fragment amplified by the IS900 PCR assay was identical to the known IS900 of *M. paratuberculosis*.

In summary, the results of this study indicate that IS900 PCR assay may be applied to detect *M. paratuberculosis* directly from pooled quarter milk and/or raw

bulk tank milk. The whole procedure, from centrifugation of milk until IS900 PCR analysis, can be completed within 12 to 16 h. The detection of *M. paratuberculosis* directly from bulk tank milk by IS900 PCR could become a valuable diagnostic or screening test for herds with Johne's disease.

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