A simple, rapid, and effective method for the extraction of Mycobacterium paratuberculosis DNA from fecal samples for polymerase chain reaction

J. R. Stabel,¹ T. L. Bosworth, T. A. Kirkbride, R. L. Forde, R. H. Whitlock

Abstract. Diagnosis of paratuberculosis (Johne’s disease) is stymied by the lack of a diagnostic tool that can be used to detect both subclinically and clinically infected animals. At present, fecal culture remains the single diagnostic test that can detect infection in both disease states provided the animals actively shed Mycobacterium paratuberculosis in their feces. Yet, fecal culture has a disadvantage associated with the protracted incubation period of 8–16 weeks before results are available. Detection of nucleic acids specific to M. paratuberculosis in fecal samples is a technique that can circumvent the culture method. This study describes a rapid, simple, and effective method to extract DNA from fecal samples and modification of a polymerase chain reaction assay for optimal sensitivity of detection. An evaluation of 1,000 well-characterized fecal samples was performed by the Colorado Department of Agriculture (Denver, CO) and the National Animal Disease Center (Ames, IA) to determine the sensitivity, specificity, and reproducibility of the new method. Results from this study show that the sensitivity of detection was highly dependent on the load of bacteria in the fecal sample with 81% detection of samples containing >70 colony-forming units (cfu)/g of feces and a 45% detection rate for samples containing less than 1 cfu/g. Similarly, reproducibility of the technique between the 2 laboratories (n = 250 samples) was much higher (75%) for the fecal samples containing high levels of M. paratuberculosis and reduced to 25% for samples with less than 1 cfu/g. An overall specificity of 83% was obtained for known negative samples. The method described here is rapid, simple, and inexpensive compared with other techniques. In addition, this method can detect animals that are shedding less than 1 cfu/g.

Paratuberculosis is widely distributed both nationally and internationally in domestic ruminants, such as cattle, sheep, and goats, as well as wildlife, such as deer, antelope, and bison. The prevalence of the disease in the United States is difficult to ascertain because comprehensive studies have not been conducted to date. In 1996, the National Animal Health Monitoring System conducted a survey of dairy farms in the United States using serologic analysis and estimated that 20–40% of these herds have some level of paratuberculosis.¹ The accuracy of prevalence estimates from this and other studies is limited by the sensitivity of the diagnostic test used, the accurate recognition and reporting of the disease, and the number of animals sampled. It is estimated that annual losses in the United States from paratuberculosis in cattle herds may exceed $220 million. This figure is extrapolated from estimated prevalence values as well as computation of financial losses due to culling or death of clinically infected cows and reduced reproductive efficiency, feed efficiency, and milk production in subclinically infected animals. The significance of subclinical infection on economic losses to the producer is detailed in a recent review, with a 15–16% reduction in milk production accounting for the major portion of net monetary loss.¹,³ The need for adequate diagnostic tools to allay further spread of this disease within the United States is without question.

Diagnosis of paratuberculosis is difficult because of the fastidious growth pattern of the microorganism and the different host immune responses invoked during subclinical and clinical stages of infection.²,¹⁴ In the early subclinical phase of infection, cell-mediated immunity predominates with negligible humoral responses noted. As the animal progresses to other clinical stages of the disease, cell-mediated immunity wanes and humoral immune responses increase. This paradigm makes it difficult to recommend a serologic tool for the detection of Johne’s disease within a herd that can accurately diagnose animals in varying stages of infection. Bacteriologic culture provides a useful alternative to serologic tests because it can be used during both subclinical and clinical stages of disease. However, culture of Mycobacterium paratuberculosis from feces suffers from some major disadvantages. The fecal culture methods are time consuming, requiring up to 12–16 weeks of incubation, labor intensive, and more costly than serologic tests, such as enzyme-linked immunosorbent assay or interferon-γ.⁴

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Methods of detection of \textit{M. paratuberculosis} infection have also been developed using nucleic acid probes combined with the polymerase chain reaction (PCR). An early study that compared a commercial DNA probe test kit with 3 different fecal culture procedures indicated that only about 60\% of infected cattle were detected by the probe compared with fecal culture.\textsuperscript{17} Although highly specific for \textit{M. paratuberculosis}, the DNA probe was unable to detect infected cattle that were shedding low numbers of organisms. However, PCR tests for detection of \textit{M. paratuberculosis} in fecal samples have vastly improved in recent years, leading to an increased sensitivity of detection of low shedders, including 1 report of a detection level of 1 colony-forming unit(s) (cfu)/g feces.\textsuperscript{5,7,8,10,11} Yet, these methods are quite extensive and some require expensive equipment as well.

This article describes a modified method that is rapid, simple, and inexpensive for the extraction of \textit{M. paratuberculosis} DNA from fecal samples. In addition, this method can detect animals that are shedding less than 1 cfu/g of feces.

\textbf{Materials and methods}

\textit{Fecal samples}. Fecal samples ($n = 1,000$) were obtained from an archival collection stored frozen at $-70$ C at the University of Pennsylvania, New Bolton Center (NBC), Kennett Square, PA. Samples werebriefly thawed, aliquots were placed into fecal tins (10–20 g), and each tin was placed into a separate plastic bag to reduce cross-contamination between samples during shipping and processing. The samples were then shipped frozen on dry ice to the Colorado Department of Agriculture (CDA), Rocky Mountain Regional Animal Health Laboratory, Denver, CO, for processing. Samples were shipped in 4 separate batches of 250 samples each to allow each set to be processed before receipt of the next set. The fecal samples consisted of well-characterized samples obtained from herds in the Pennsylvania area and had been cultured during a 10-yr period. Samples represented a broad spectrum of cfu, ranging from $>75$ cfu/g (too numerous to count) on all 4 tubes to 1 cfu on 1 of 4 culture tubes (0, 0, 0, 1). Samples from known negative herds that had been tested frequently during a 5- to 10-yr period with no cases of paratuberculosis were also included in the sample set. After culture and PCR analyses at the CDA, 250 fecal samples were selected from the sample set for shipment to the National Animal Disease Center (NADC, Ames, IA). These fecal samples were extracted for \textit{M. paratuberculosis} DNA, and PCR analysis was performed to allow for an assessment of assay reproducibility between laboratories. At all times, samples run by CDA and NADC were double blinded to preclude any previous knowledge of sample status that might have biased the results.

\textit{Fecal culture of M. paratuberculosis}. Culture of \textit{M. paratuberculosis} was performed on all samples at the CDA by the double-centrifugation, double-decontamination procedure described previously.\textsuperscript{13} Each fecal sample was inoculated onto 4 culture tubes of Herrold egg yolk medium (HEYM) containing mycobactin J\textsuperscript{a} (2 mg/liter) and antibiotics\textsuperscript{a} (naladixic acid [50 $\mu$g/ml], vancomycin [50 $\mu$g/ml], amphotericin B [50 $\mu$g/ml]).\textsuperscript{13} Samples were read after 12 wk of incubation at 37 C. Positive samples were scored as $+4 = >70$ cfu/g; $+3 = 31–70$ cfu/g; $+2 = 8–30$ cfu/g; $+1 = 1–7$ cfu/g; and $< +1 = 1$ cfu on 1 tube of media (4 tubes in total). Samples were verified as \textit{M. paratuberculosis} by growth rate, colony morphology, and Ziehl–Neelsen stain for acid-fastness.

\textit{Mycobacterium paratuberculosis DNA extraction from fecal samples}. Fecal samples (1 g) were diluted in 9 ml of 1X Tris–ethylenediamine tetraacetic acid (EDTA) buffer (10 mM Tris–HCl, 1 mM EDTA; pH 7.6) in 15-ml polypropylene conical tubes.\textsuperscript{9} Samples were vortexed for 5 sec and allowed to settle for 2 min and then vortexed again. Samples were centrifuged at 200$ \times$ g for 30 sec, and the supernatants from each sample were transferred to new 15-ml conical tubes. The supernatants were then diluted 1:10, 1:100, or 1:1,000 in 1X TE buffer. One milliliter of each dilution was placed into 1.5 ml sterile, deoxyribonuclease (DNAase), ribonuclease (RNAase)-free Eppendorf tubes\textsuperscript{d} and centrifuged at 13,000 $\times$ g for 2 min. Supernatants were discarded and pellets washed 2 times with 1 ml of 1X TE buffer. Pellets were resuspended in 500 $\mu$l of 1X TE buffer and placed in a heating block at 100 C for 10 min. During some sample runs on different days a negative control containing 1X TE buffer was included in the heating block as a sentinel for DNA cross-contamination during sample processing. After cooling to room temperature, 4 $\mu$l of RNAase\textsuperscript{e} (500 $\mu$g/ml) was added to each sample. Samples were stored at $-20$ C until PCR analyses were performed.

\textit{Polymerase chain reaction analyses}. Reaction tubes contained a total volume of 50 $\mu$l consisting of Ultra-pure distilled water\textsuperscript{2} (DNAase, RNAase-free), GeneAmp 10X PCR buffer II,\textsuperscript{2} 2.5 mM MgCl\textsubscript{2}, 0.25 mM deoxyribonucleoside triphosphates,\textsuperscript{8} 0.25 mM primers, and 2 U of AmpliTaq gold.\textsuperscript{8} Primer sequences for the \textit{M. paratuberculosis}–specific genetic element, IS900, were used in the reaction mixture as follows: 5'-CCGCTAATGAGAGTGCGATTGG-3', forward primer, and 5'-AACGCTTCCAGCGCGCGCCCCGCTTG-3', backward primer, to amplify a 229-bp gene sequence as described previously.\textsuperscript{12} Controls consisted of reaction mixture alone, positive control contained 1 $\mu$l of genomic DNA from \textit{M. paratuberculosis}.
(strain 19698), and sample tubes contained 5 μl of *M. paratuberculosis* DNA extracted from fecal samples. The CDA ran PCR analyses on DNA from undiluted fecal supernatants and 1:10 and 1:100 dilutions. The NADC performed PCR on the 1:100 dilution of the subset of 250 fecal samples. The NADC also included a positive control from a DNA extraction performed on feces from a clinical cow shedding high numbers of *M. paratuberculosis*. This positive control was included during each round of PCR performed. Samples were run according to this protocol: 1 cycle at 94 C, 10 min; 50 cycles at 94 C, 59 sec, 60 C, 30 sec, and 72 C, 59 sec; followed by a final extension cycle at 72 C, 10 min. Polymerase chain reaction amplificates and a phiX174 molecular weight marker were then electrophoresed in a 4% NuSieve 3:1 Plus agarose gel in 1× Tris–borate–EDTA (1 M Tris–HCl, 0.9 M boric acid, 0.01 M EDTA) buffer at 65 V for 1 hr. Gels were then stained with ethidium bromide and observed on a UV transilluminator for bands.

**Polymerase chain reaction optimization.** For initial comparison when optimizing this technique, a titration of PCR cycle number on the intensity of the product was also run for some known positive samples at the NADC and compared with the cycle conditions for IS900, which had been described previously and used in the authors’ laboratory. Also, the effect of the addition of 2.5 μl of dimethyl sulfoxide (DMSO) to the PCR reaction (5% concentration) on the sensitivity of detection was evaluated. Using samples prepped for this study, the sensitivity of detection of the method used in this study was also compared using the IS900 gene and the hspX gene. The hspX gene is an *M. paratuberculosis*–specific gene that was identified previously. The PCR amplification conditions for the hspX gene were the same as for the IS900 gene except for the MgCl₂, (1.5 mM) with the following primers, 5′-GACCGGCTATCTGTGGAAC-3′, forward, and 5′-CTCGTCGGCTTGCACCTG-3′, backward, yielding a 211-bp product.

Evaluation of the sensitivity of detection of *M. paratuberculosis* DNA by PCR. The sensitivities of the DNA extraction method and the PCR assay were evaluated using a pure culture of *M. paratuberculosis* (strain 19698) harvested in the log phase of growth (Abs540nm = 0.2). The bacteria were pelleted by centrifugation at 7,500 × g, washed twice with phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS to a concentration of 10⁸ cfu/ml. The pure culture was then serially diluted to yield 10-fold dilutions ranging from 10⁸ to 1 cfu/ml in a volume of 5 ml of 1× TE buffer. In addition, 10-fold dilutions of *M. paratuberculosis* were used to spike feces from known negative cow to yield final concentrations ranging from 10⁷ to <1 cfu/g feces. Pure bacteria samples in buffer and spiked fecal samples were then processed by the DNA extraction and PCR protocols described previously.

**Results**

The effect of PCR cycle number on the sensitivity of the detection of *M. paratuberculosis*–specific DNA using IS900 primers is shown in Fig. 1. Increasing the cycle number from the standard 35 cycles incrementally to 50 cycles enhanced the sensitivity of detection of the PCR amplificate, particularly for the 1:100 dilution of fecal supernatant. However, inclusion of a hot-start for 10 minutes at 94 C along with a reduction of the annealing time from 1 minute to 30 seconds and an additional extension at the end at 72 C for 10 minutes further improved the sensitivity of the PCR detection. These modifications were used to establish the PCR cycle profile that was used for all the samples within this study. Addition of DMSO to the PCR reaction did not affect the results of the assay (data not shown). The sensitivity of detection for the different dilutions of fecal supernatant, 1:10, 1:100, and 1:1,000, with this protocol was best at the 1:100 dilution (Fig. 1).

The sensitivity of detection of *M. paratuberculosis* DNA using the extraction and PCR protocols is demonstrated in Fig. 2. Detection of amplification after extraction of the DNA from 10-fold dilutions of a pure culture of *M. paratuberculosis* in buffer indicated that the assay was sensitive to a level of 10 cfu/ml of buffer (Fig. 2A). Extraction of DNA from a fecal sample spiked with 10-fold dilutions of *M. paratuberculosis* resulted in a lowered sensitivity of detection at 10⁷ cfu/g of feces (Fig. 2B). Further modification of the extraction protocol to include a step to filter the initial fecal supernatant before dilution by gravity filtration through a variety of sieves and filter papers was not successful in improving the sensitivity of the assay (data not shown).

The archival set of fecal samples obtained from the NBC contained samples with varying amounts of *M. paratuberculosis* (Table 1). The stratification of the fecal sample set in the different categories was based on historical culture results from the NBC, some of which dated back to several years before this study. Interestingly, after culture at the CDA, fecal samples were distributed between the categories in a pattern similar to the NBC results except for a marked reduction noted for the +1 group. Of the 421 defined culture-positive samples obtained from the NBC, 298 were culture positive and 123 were culture negative at the CDA. Eighty-nine samples that were previously fecal culture positive at the NBC were negative for both culture and PCR assays at the CDA. Fungal contamination precluded the accurate identification of 65 samples after fecal culture. Some of these samples (9/65)
were PCR+, but it was unclear how to categorize them without clean culture results. Fifty-nine samples were classified as false positive as they were collected from known negative herds and showed positive in the PCR assay in at least 1 of the 2 laboratories (CDA or NADC). This resulted in an overall specificity of 83%. A category of unknown samples ($n = 161$) was created because some of these samples were categorized as negatives based on negative culture results at the NBC but were not collected from known negative herds. These samples showed positive on culture and PCR at the CDA. Additional samples assigned to this category had unclear historical fecal culture results from the NBC yet did not belong to the negative control group and were PCR+. In fact, several of these samples were obtained from cows with positive tissue cultures for *M. paratuberculosis*. Although an “unknown” category is a bit unusual, it is acceptable for this study for several reasons. The samples from NBC were cultured by a different method from that used at the CDA; therefore, detection sensitivities were not comparable. The samples were part of an archival set that had been subjected to freeze–thaw cycles so loss of sensitivity for culture and PCR was an issue, and PCR was never performed on the samples at NBC so a baseline PCR detection level was unavailable.

The PCR results for fecal culture–positive samples and the sensitivity of detection according to the level of *M. paratuberculosis* within the sample are shown in Table 2. Results from this study indicate that the efficiency of the extraction protocol and subsequent PCR analysis was positively correlated with the concentration of *M. paratuberculosis* in the fecal samples. With this technique, 81% of the culture-positive samples that were $\geq 4$ or had $>70$ cfu/g of feces could be accurately detected. Sensitivity of detection declined to 58% for $\leq 3$ culture-positive samples and stabilized to approximately 45% for samples in $+2$, $+1$, or $<+1$ groups. A dramatic loss in sensitivity of detection occurred in fecal samples containing between 1 and 7 cfu/g ($<1$ category), with only 46 of 110 samples detected by PCR. Samples that were $-C/+PC$ (culture negative, PCR positive; $n = 34$) were not included. Because PCR can detect nonviable microorganisms as well as viable organisms, it is quite possible to have PCR+ fecal samples that may be currently culture negative. Microorganisms may have become senescent for a variety of reasons including multiple freeze–thaw cycles imposed on the fecal samples during storage at the NBC.

Reproducibility of this technique between CDA and NADC laboratories was evaluated using the 250-sample subset randomly selected by CDA. An overview of the PCR results for the subset of fecal samples is

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**Figure 1.** Titration of cycle number for some known positive samples. Top of gel: Lane 1, phiX174, molecular weight marker; lane 2, negative control, PCR reaction mix only; lane 3, positive DNA control; lanes 4–12, PCR products from 1:10, 1:100, and 1:1,000 dilutions of fecal supernatant from a known positive cow after 35, 40, and 45 cycles, respectively, using previously published PCR conditions. Bottom of gel: Lane 1, phiX174, molecular weight marker; lanes 2 and 7, negative control, PCR reaction mix only; lanes 3 and 8, positive DNA control; lanes 4–6 and 9–11, PCR products from 1:10, 1:100, and 1:1,000 dilutions of fecal supernatant from a known positive cow after 50 cycles using previously published PCR conditions and 50 cycles using the new method.
Figure 2. An evaluation of the sensitivity of the DNA extraction method and the PCR assay using a pure culture of *M. paratuberculosis* (strain 19698) (2A) and negative feces spiked with known quantities of *M. paratuberculosis* (2B). 2A, top of gel: Lane 1, phiX174, molecular weight marker; lane 2, negative control, PCR reaction mix only; lane 3, positive DNA control; lanes 4–12, dilutions $10^0$ to $10^3$, in duplicate. 2A, bottom of gel: Lane 1, phiX174, molecular weight marker; lanes 2–12, dilutions $10^4$ to $10^8$ in duplicate. 2B, top of gel: Lane 1, phiX174, molecular weight marker; lane 2, negative control, PCR reaction mix only; lane 3, positive DNA control; lanes 4–5, feces only; lanes 6–12, $10^0$ to $10^2$ dilutions of spiked feces. 2B, bottom of gel: Lane 1, phiX174, molecular weight marker; lanes 2–12, $10^0$ to $10^2$ dilutions of spiked feces.
All fecal culture-positive samples, ranging from 1 to 4, were detected using the authors’ current protocol for DNA extraction and their PCR protocol using IS900 primers. In contrast, the hspX gene was able to accurately identify only culture-positive samples in the +4 category.

### Discussion

Other researchers have attempted modification of the fecal PCR method to enhance sensitivity for the detection of *M. paratuberculosis* DNA in fecal samples. A modification of the PCR test to include 2 consecutive amplification reactions using nested primers markedly increased the sensitivity of detection of fecal DNA in either sample set.

A comparison of the sensitivity of detection of the IS900 and hspX genes in *M. paratuberculosis* DNA extracted from the NBC fecal samples is shown in Fig. 3. All fecal culture-positive samples, ranging from +1 to +4, were detected using the authors’ current protocol for DNA extraction and their PCR protocol using IS900 primers. In contrast, the hspX gene was able to accurately identify only culture-positive samples in the +4 category.

<table>
<thead>
<tr>
<th>Sample stratification</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NBC</td>
</tr>
<tr>
<td>+4</td>
<td>79</td>
</tr>
<tr>
<td>+3</td>
<td>16</td>
</tr>
<tr>
<td>+2</td>
<td>15</td>
</tr>
<tr>
<td>+1</td>
<td>43</td>
</tr>
<tr>
<td>&lt;+1</td>
<td>12</td>
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</tbody>
</table>

Table 2. Sensitivity of the PCR method for detection of *Mycobacterium paratuberculosis* DNA extracted from fecal culture-positive samples according to colony-forming units per gram (cfu/g) of feces.*

<table>
<thead>
<tr>
<th>Sample status</th>
<th>+4</th>
<th>+3</th>
<th>+2</th>
<th>+1</th>
<th>&lt;+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>+C/+PCR (n = 165)</td>
<td>79</td>
<td>16</td>
<td>15</td>
<td>43</td>
<td>12</td>
</tr>
<tr>
<td>+C/-PCR (n = 145)</td>
<td>20</td>
<td>13</td>
<td>22</td>
<td>64</td>
<td>26</td>
</tr>
</tbody>
</table>

* The number of cfu/g of feces is categorized as follows: +4 = 70 cfu/g; +3 = 31–70 cfu/g; +2 = 8–30 cfu/g; +1 = 1–7 cfu/g; <+1 = 1 cfu on 1 tube of media.

The sensitivity of detection of samples that were categorized as +1 or <+1 culture status by the NADC (Table 4).

<table>
<thead>
<tr>
<th>Sample status</th>
<th>NADC</th>
<th>CDA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR−</td>
<td>142</td>
<td>181</td>
<td>249</td>
</tr>
<tr>
<td>PCR+</td>
<td>107</td>
<td>68</td>
<td>175</td>
</tr>
<tr>
<td>Reproducibility (%)</td>
<td>62</td>
<td></td>
<td>62</td>
</tr>
</tbody>
</table>

Table 3. Reproducibility of fecal PCR technique between the NADC and the CDA for the sample subset (n = 249).

<table>
<thead>
<tr>
<th>cfu/g feces</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADC</td>
</tr>
<tr>
<td></td>
<td>+4</td>
</tr>
<tr>
<td>NADC</td>
<td>20</td>
</tr>
<tr>
<td>CDA</td>
<td>17</td>
</tr>
<tr>
<td>Both</td>
<td>15</td>
</tr>
</tbody>
</table>

* The number of cfu/g of feces is categorized as follows: +4 = 70 cfu/g; +3 = 31–70 cfu/g; +2 = 8–30 cfu/g; +1 = 1–7 cfu/g; <+1 = 1 cfu on 1 tube of media.

Outlined in Table 3, of the 249 samples that were evaluated by both laboratories (1 sample was lost), 126 were identified as PCR− by both laboratories with an additional 16 samples identified by NADC. In contrast, only 29 samples yielded PCR+ results for both laboratories; yet, NADC reported 107 PCR+ samples in this subset. This resulted in an overall reproducibility of 62% for all samples. Stratification of assay reproducibility according to culture status of the fecal samples suggested that the low level of reproducibility between laboratories was a factor of the greater sensitivity of detection of samples that were categorized as either +1 or <+1 culture status by the NADC (Table 4).

Hybridization-capture PCR and immunomagnetic bead-capture PCR, which capture either the nucleic acids of *M. paratuberculosis* or the whole organism, respectively, were not as sensitive as direct PCR on fecal samples for the detection of paratuberculosis in sheep. After comparison of the 3 PCR techniques on both pooled fecal samples and individual fecal samples, the direct PCR was more effective than the other 2 methods for the detection of *M. paratuberculosis* DNA in either sample set.

In this study, 2 laboratories applied a simplified method of DNA extraction to a large volume of fecal samples with highly variable culture status. The procedure encompassed dilution of the fecal sample, rapid boiling for the extraction of the DNA, and use of the DNA in an optimized PCR. Because no further sample preparation after DNA extraction was necessary and no special equipment was required, the assay remained simple for the detection of paratuberculosis in feces.
cost-effective and simple to perform. Most diagnostic laboratories charge an average of $35.00 per fecal sample for PCR analysis. This cost is prohibitory for most producers in today’s market. The simplicity of this extraction/PCR technique results in a cost between $2.00 and $5.00 per sample depending on labor costs. Patent holders for the IS900 gene sequence recently implemented a system of charging diagnostic laboratories a fee for each sample analyzed by PCR with IS900 primers. This would inflate the cost of the assay described in this study; however, the cost of reagents to perform the DNA extraction and PCR assay described was less than $1.00 per sample, leaving room for labor charges and patent fees and still remaining a reasonable fee to the customer. In addition, up to 50 fecal samples could be processed in 1 day for DNA extraction using the method discussed in this study, signifying that many samples could be processed for DNA extraction and PCR in a relatively short 2-day period. Because culture of viable M. paratuberculosis from fecal samples requires 8–16 weeks, use of the fecal PCR method described in this study would be more efficient, resulting in a rapid assessment of fecal-shedding status of suspect animals.

The overall sensitivity of the method for the detection of M. paratuberculosis DNA in fecal samples was 56% with a higher degree of detection noted for samples from cows that were heavy shedders (81%). This sensitivity is higher than that previously reported for commercial methods but lower than the sensitivity recently reported for a direct PCR method on ovine fecal samples.\textsuperscript{10,17} Some of the reduced sensitivity reported

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Comparison of PCR products after amplification of DNA from fecal samples with IS900 primers (top) and hspX primers (bottom). Lane 1, phiX174, molecular weight marker; lane 2, negative control, PCR reaction mix only; lane 3, positive DNA control; lanes 4–12, samples 160 (negative fecal culture), 386 (+1 cfu/g), 604 (+1 cfu/g), 221 (+1 cfu/g), 126 (+2), 129 (+4), 198 (+4), 588 (+4), 323 (positive fecal control, +4).}
\end{figure}
for this method may be due to logistical problems associated with evaluating the method in 2 laboratories. Although adept at performing PCR analyses on fecal samples, the CDA had no previous experience working with this method. The NADC was more proficient in the detection of *M. paratuberculosis* DNA in fecal samples with very few colonies.

The specificity of detection observed in this study was also reduced compared with other reports but perhaps was not surprising. The elaborate method of packaging and shipping samples was intended to preclude sample cross-contamination; yet, the simple act of transfer of 1,000 samples in the manner described from the NBC to the CDA and then on to the NADC probably contributed to the contamination of samples. According to this protocol the samples had to be handled at least 3–4 times by 3 laboratories. It must also be acknowledged that the extensive collection of NBC fecal samples included samples that had been through multiple freeze–thaw cycles before this study and also contained samples that had varying levels of fungal contamination. Rather than being an asset, the large number of samples probably created a bigger obstacle in the overall evaluation of the methodology. Although completely subjective, this viewpoint is supported by the fact that results for the first 200 samples that were analyzed demonstrated a sensitivity of detection of 78% (61/78 culture-positive samples were PCR positive) and a specificity of 100% (all 59 known negative samples were PCR and culture negative).

With this in mind the true sensitivity and specificity are probably much higher than this sample set indicates. Previous work evaluating the efficacy of this technique with samples from a small herd of cows (*n* = 31) with Johne’s disease demonstrated that the PCR method was able to repeatedly detect cows that were shedding as low as 1 colony on 1 of 4 tubes of HEYM. In addition, the 9 noninfected control cows maintained on-site at the NADC were always negative in the PCR assay, suggesting that cross-contamination problems can be surmounted with vigilant technique.

Use of the IS900 primers to amplify the gene sequence resulted in a greater sensitivity of detection than the *hspX* primers. This is not surprising because the IS900 gene is represented between 15 and 20 times within the genome, and the *hspX* gene sequence is present as a single copy. At this time, amplifying the IS900 gene sequence is the most reliable way of detecting cows shedding low levels of *M. paratuberculosis* in their feces.

In summary, the method described in this study can identify infected animals actively shedding the microorganism in their feces at a level as low as 1 cfu/g of feces. Therefore, this method shows great promise in accurately detecting animals in the early stages of disease that are shedding very low levels of the bacterium. A diagnostic tool that can detect both subclinically and clinically affected animals is an essential element for a control program designed to allay the spread of this disease. In addition, because of its simplicity the method is both cost-effective, and results are rapid compared with fecal culture.

**Acknowledgements**

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**Sources and manufacturers**

a. Allied Monitor, Fayette, MO.

b. Sigma Chemical Co., St. Louis, MO.

c. Becton-Dickinson, Franklin Lakes, NJ.

d. Scientifics, Frederick, MD.

e. Roche, Indianapolis, IN.

f. Gibco-BRL, Grand Island, NY.

g. PE-Applied Biosystems, Branchburg, NJ.

h. Invitrogen, Carlsbad, CA.

i. FMC Bioproducts, Rockland, ME.

j. Gel-Doc, Bio-Rad, Hercules, CA.

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