Use of the bead beater for preparation of *Mycobacterium paratuberculosis* template DNA in milk

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Abstract

Mycobacterium paratuberculosis is a recognized chronic enteric pathogen that can affect many different species of animals, including primates. It has been suggested that this organism is associated with Crohn's disease in humans, and that milk is a potential source of human exposure to this organism. The limit of the detection of *M. paratuberculosis* in milk samples by direct PCR was 10^5 cfu/mL if the traditional boiling method was used for template DNA preparation. In this study, an improved method for template DNA preparation was examined. The method involves the use of a bead beater, which breaks up bacterial cell wall mechanically by vibrating bacteria with microbeads at high speed. The effectiveness of this method for lysing *M. paratuberculosis* cells was compared to that of the freeze-thaw method, and use of commercial kits such as the InstaGene Matrix and the QIAamp Tissue Kit. The bead beater procedure was tested in combination with various cell lysis and template DNA preparation procedures to determine which of these steps improved the limit of detection of PCR assay that amplifies a 413 bp fragment of the IS*900* gene. Results showed that the use of the bead beater, in combination with the use of lysis buffer, boiling, and isopropanol precipitation, decreased the limit of detection of *M. paratuberculosis* in milk by the PCR to 10^2 cfu/mL. The limit of detection was further decreased to 10 cfu/mL when 0.0037% bovine serum albumin was included in the PCR reaction mixtures. The improved assay was 10- to 10^4-fold more sensitive than the PCR assays using template DNA prepared by other lysis procedures including boiling alone, freeze-thaw plus boiling, or use of commercial kits for lysis.

Résumé

Mycobacterium paratuberculosis *est reconnu comme un pathogène entérique responsable d'une infection chronique pouvant affecter plusieurs espèces animales, incluant les primates. Il a été suggéré que ce micro-organisme est associé à la maladie de Crohn chez l'humain et que le lait constituerait une source potentielle d'exposition humaine à ce micro-organisme. La limite de détection de* M. paratuberculosis *par une méthode directe d'amplification en chaîne par la polymérase (ACP) dans des échantillons de lait est de 10^5 ufc/mL lorsque la méthode traditionnelle d'ébullition est utilisée pour la préparation de la matrice d'ADN. Une méthode améliorée de préparation de la matrice d'ADN utilisant un batteur à billes fut étudiée. Cet appareil brise la paroi cellulaire de façon mécanique en agitant à haute vitesse les bactéries avec des micro-billes. L'efficacité de cette méthode pour lyser* M. paratuberculosis *fut comparée à la méthode gel-dégel, ainsi qu'à l'utilisation de trousses commerciales telles InstaGene Matrix et QIAamp Tissue Kit. La procédure à l'aide du batteur à billes fut testée conjointement avec différentes méthodes de lyse cellulaire et de préparation de la matrice d'ADN afin de déterminer quelles étapes permettraient d'améliorer la limite de détection d'une épreuve ACP amplifiant un fragment de 413 paires de bases provenant du gène IS900. Les résultats montrent que l'utilisation du batteur à billes combinée à un tampon de lyse, à l'ébullition et à une précipitation à l'aide d'isopropanolol diminue la limite de détection de* M. paratuberculosis *par ACP dans le lait à 10^2 ufc/mL. La limite de détection est réduite à 10 ufc/mL lorsque 0,0037 % d'albumine sérique bovine est incorporée dans les mélanges de réactifs de l'ACP. L'épreuve améliorée est de 10 à 10^4 fois plus sensible que les épreuves ACP utilisant une matrice d'ADN préparée par les autres méthodes de lyse incluant l'ébullition seulement, le gel-dégel suivi de l'ébullition ou l'utilisation de trousses de lyse commerciales.*

(Traduit par Docteur Serge Messier)

Introduction

Mycobacterium paratuberculosis is a recognized chronic enteric pathogen that can affect many species of animals, including primates. It is the etiological agent of Johne's disease in cattle. There are reports that this organism is associated with Crohn's disease in humans, and milk is a potential source of human exposure to this organism (1–5). *Mycobacterium paratuberculosis* organisms shed in raw milk and feces by cows with clinical Johne's disease have also been isolated by direct culture methods (6,7). Several studies have provided evidence that *M. paratuberculosis* in milk may not be completely inactivated by pasteurization using the tube holding

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method (8–13). A report on the screening of *M. paratuberculosis* in retail pasteurized whole-milk samples in southern England and Wales stated that 7% of the 312 samples were positive for *M. paratuberculosis* by PCR and hybridization, with a limit of detection of 200 to 300 *M. paratuberculosis* organism per mL of milk (14). A study on the prevalence of *M. paratuberculosis* in raw milk by direct culture method showed no positive samples after 16 wk incubation among the 1200 bulk tank milk samples collected from farms in southwestern Ontario. In the study, the detection limit of the assay was about 10^4 cfu per 40-mL sample (15). Detection of *M. paratuberculosis* by direct culture methods requires long-term incubation of 3 mo or more, and without efficient decontamination, the agar slants are susceptible to overgrowth by fast-growing microorganisms. A rapid, sensitive and specific method to detect *M. paratuberculosis* contamination in milk or other dairy products is, therefore, important for monitoring purposes. Molecular techniques, such as DNA probe and polymerase chain reaction (PCR), have been extensively used for detection of *M. paratuberculosis*, taking the advantage of the rapid screening, high sensitivity and specificity of PCR assays. The most commonly used DNA probe and PCR target is insertion sequence (IS) *900*, which is specific for *M. paratuberculosis* (16). The use of this probe in diagnostic assays for *M. paratuberculosis* has been reported in a number of studies (14,17,18). In samples contaminated with low numbers of *M. paratuberculosis*, the sensitivity of bacterial DNA detection by direct PCR was low, and nested PCR or hybridization protocols were often employed (14,18–20). In our initial study on the detection of *M. paratuberculosis* in milk using the IS*900* PCR assay, the limit of detection was 10^5 cfu/mL when the template DNA was prepared using simple procedures, such as boiling, lysozyme, and proteinase K treatment of the sample (unpublished data). This level of sensitivity is not acceptable for screening of food samples in a food testing laboratory. *M. paratuberculosis* cells are difficult to lyse, DNA recovery is low, and residual components in the DNA preparations can inhibit PCR, compromising detection of the target DNA. In order to increase the sensitivity of the IS*900* PCR detection system, it is important to remove the inhibitors and optimize conditions for release and recovery of bacterial template DNA. A number of different methods have been employed for lysis of bacterial cells and preparation of template DNA. These include the use of freeze-thaw methods in extraction of template DNA from soil and sediment samples (21–23), the use of high velocity microbead homogenization of bacterial cells using the bead beater protocols to prepare DNA from soil organisms (24,25), and from various slow- and fast-growing species of mycobacteria tested in our laboratory (unpublished data). A recent report on the use of the bead beater for the preparation of *M. paratuberculosis* DNA from milk improved the limit of detection to 10^3 cfu/mL in PCR (26). *Mycobacterium paratuberculosis* cells in milk partitioned to the cream and pellet fractions after centrifugation, with few if any cells recovered from the whey fraction (14). Studies performed in our laboratory showed that while PCR inhibitors were present in the cream, whey, and pellet fractions of milk, there was a significantly higher concentration of these inhibitors in the whey fraction (unpublished data). We also found that *M. paratuberculosis* cells recovered from milk were highly resistant to lysis by boiling alone, and stringent cell lysis procedures were employed to achieve optimal release of bacterial DNA and enhance the sensitivity of detection by PCR methods. The objective of this study was to develop a PCR protocol for rapid and sensitive detection of *M. paratuberculosis* in milk by evaluating different DNA preparation procedures for improved PCR detection. In order to increase the sensitivity of detection of *M. paratuberculosis* in milk, the following steps were included in the PCR protocol: 1) removal of the whey fraction from milk samples to reduce the amount of inhibitors in the sample; 2) optimizing conditions for cell lysis and recovery of template DNA; 3) concentration of template DNA in order to increase the likelihood of detecting small numbers of *M. paratuberculosis* DNA copies in the milk sample; and 4) use of bovine serum albumin (BSA) to overcome the effect of PCR inhibitors.

Materials and methods

Preparation of *M. paratuberculosis* cell suspension

Mycobacterium paratuberculosis bacteria were harvested from 3-month-old culture on agar slants of 21 g/L mycobacteria 7H11 agar (Difco, Detroit, Michigan, USA) containing 2 mg/L mycobactin J (Allied Monitor, Fayette, Missouri, USA), egg yolk (6/L), 100 000 units/L penicillin G (Sigma Chemical Company, St. Louis, Missouri, USA), 50 mg/L chloramphenicol (Sigma Chemical Company), and 10% OADC enrichment. The OADC enrichment contains BSA (fraction V, 50 g/L; Fisher Scientific, Suwanee, Georgia, USA), oleic acid (0.5 g/L; Sigma Chemical Company), dextrose (20 g/L; Sigma Chemical Company), catalase (0.04 g/L; Sigma Chemical Company) and NaCl (8.5 g/L). The culture harvested was suspended in phosphate-buffered saline (pH 7.4). Clumps of cells were broken up by forcing them through a 21-gauge needle with a syringe to obtain a single-cell suspension. The mixture was allowed to stand at room temperature for 20 min and the top suspension carefully removed. A standard suspension of 10^9 cfu/mL *M. paratuberculosis* was prepared and cell numbers were estimated by comparison with McFarland standards and by plate colony counts. The colony counts for the standard suspension used for spiking were in the range of 7×10^8 to 1.2×10^9 cfu of *M. paratuberculosis*/mL. Ten-fold serial dilutions of the *M. paratuberculosis* cell suspension were prepared and used to spike milk samples.

Preparation of spiked milk samples

Pasteurized milk samples were spiked with *M. paratuberculosis* organisms to final concentrations of 10^1, 10^2, 10^3, 10^4 and 10^5 cfu/mL for each combination (Table I). Milk samples (1 mL) were centrifuged immediately after spiking using a Beckman J2-M1 centrifuge with JA 18.1 rotor at 42 000 × *g* (18 000 rpm) for 1.5 h. The whey fraction was removed and discarded. The pellet and cream fractions were pooled and used for preparation of *M. paratuberculosis* DNA.

Preparation of *M. paratuberculosis* template DNA from milk

Table I shows the combinations of procedures used for cell lysis and preparation of *M. paratuberculosis* template DNA from the pooled pellet and cream milk fractions as described above. The efficiency of *M. paratuberculosis* cell lysis and DNA recovery for each

Table I. Efficiency of procedures for preparation of *M. paratuberculosis* template DNA as evaluated by IS*900* based PCR

	Combined procedures used in preparation of template DNA							
Procedures	1	2	3	4	5	6	7[a]	8
Bead beating		✓	✓	✓	✓	✓	✓	
Freeze-thaw (5 cycles)								✓
Lysis buffer					✓	✓	✓	✓
Boiling (20 min)	✓	✓		✓		✓	✓	✓
Precipitation			✓	✓	✓	✓	✓	✓
BSA (0.0037%) in PCR							✓	
Sensitivity of detection (cfu/mL)	10^5	10^2 to 10^3	10^4 to 10^5	10^2 to 10^3	10^2	10^2	10 to 10^2	10^4

[a] Same DNA template as in procedure 6

method combination was assessed using the IS*900* PCR assay. The pooled pellet and cream fractions were suspended in water (combinations 1 to 4) or in lysis buffer (combinations 5, 6, and 8) prior to extraction of template DNA. Combination 7 shared the template DNA of combination 6, however, BSA (5 μL of 0.037%) was added to the PCR reaction mixture (50 μL) in combination 7.

For DNA preparation using the bead beater protocol, the pooled pellet and cream were transferred to a 2.0 mL screw cap tube containing 0.6 g of 0.1-mm zirconia/silica beads (BioSspec) and 0.8 mL of water for combinations 2 to 4, or 0.8 mL of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Tween-20, 0.5% Triton X-100, 1 M guanidinium thiocyanate, 0.3 M sodium acetate (or 0.5 M NaCl), pH 7.5) for combinations 5 and 6 (Table I). The sample suspensions were homogenized at 5000 rpm for 5 min using the Mini-Beadbeater (BioSpec, Oklahoma, USA). The samples were then boiled in a water bath for 20 min (combinations 2, 4, and 6), followed by cooling on ice water for at least 5 min. Then samples were centrifuged using a microcentrifuge (5 min, 14 000 rpm) and 0.65 mL of supernatant from each sample was transferred to either a new 1.5-mL Eppendorf tube (combination 2), or a tube containing 0.65 mL of 100% isopropanol (combinations 3 to 6). The latter were mixed by inverting the tubes several times and incubated at room temperature for 10 min. Following centrifugation (10 min, 14 000 rpm), the supernatant was carefully removed. The pellet was washed with 0.5 mL of 75% isopropanol, resuspended in 140 μL of water and incubated at 95°C for 3 min. The DNA was used for PCR immediately after preparation or stored at -20°C. When water instead of lysis buffer was used, 3 M sodium acetate was added before precipitation of the DNA at one tenth of the sample volume (combinations 3 and 4).

In combination 1, the pooled milk fractions were boiled in water, centrifuged and the supernatant directly used in PCR assays. In combination 8, the freeze-thaw method replaced cell lysis by the bead beater. The sample tubes were subjected to 5 cycles of 2 min incubation in liquid nitrogen followed by 2.5 min incubation in hot (90°C) water.

Commercial kits including the QIAamp Tissue Kit (QIAGEN, Santa Clarita, California, USA) and InstaGene (Bio-Rad, Hercules, California, USA) were also used for the preparation of template DNA according to manufacturer's protocols.

DNA amplification

Template DNA prepared as described in Table I was subjected to IS*900* PCR using a modification of the procedure as described by Millar et al (14). The PCR reaction mix (50 μL) contained 0.2 mM each of the 4 dNTPs, 0.2 μM each of the 2 primers, $P90^+$, $P91^+$, 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase (QIAGEN, Mississauga, Ontario), 0.0037% BSA (in combination 7 only, Table I), 20 μL of template DNA. The Q-solution was used in PCR after optimization in our laboratory as recommended by the manufacturer. The PCR reactions were conducted (GeneAmp PCR System 9700; Perkin Elmer, Norwalk, Connecticut, USA) under the following conditions: 94°C for 5 min; 40 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min. The PCR products were separated in 1.6% agarose gel at 5 V/cm for 30 min, stained with ethidium bromide, and the expected 413 bp PCR products were visualized (Gel Doc 1000; Bio- Rad).

Results

The template DNA extracted from *M. paratuberculosis* in milk using commercial kits, QIAamp Tissue Kit or InstaGene, yielded strong signal in direct PCR, with a detection limit of 10^5 cfu/mL of *M. paratuberculosis* (gel data not shown). In order to improve the sensitivity of detection by direct PCR of *M. paratuberculosis* in milk, a combination of cell lysis and DNA purification procedures were examined. The results are shown in Table I and Figure 1. Template DNA prepared by boiling alone showed a detection limit of 10^5 cfu/mL of *M. paratuberculosis* in milk (Figure 1, lanes 1 to 5). This detection limit decreased 10-fold to 10^4 cfu/mL when boiling was combined with freeze-thaw, lysis buffer, and isopropanol precipitation of the template DNA (lanes 36 to 40). When bead beating and boiling (lanes 6 to 10) or bead beating, boiling, and isopropanol precipitation were used (lanes 16 to 20), the detection limit decreased by 100- to 1000-fold to 10^2 to 10^3 cfu/mL *M. paratuberculosis*. Bead beating combined with isopropanol precipitation resulted in the limit of detection of 10^4 to 10^5 cfu/mL (lanes 11 to 15). The use of bead beating combined with lysis buffer and precipitation decreased the limit of detection by 1000-fold to 10^2 cfu/mL (lanes 21 to 25). Addition of the boiling step to these procedures did not improve the limit of detection further (lanes 26 to 30). However, the addition of 0.0037% BSA to the PCR reactions further improved the sensitivity of the assay by 10-fold to a limit of detection in the range of 10 to 10^2 cfu/mL *M. paratuberculosis* in milk (lanes 31 to 35). The PCR assays containing BSA yielded stronger signal in the 10^2 cfu/mL *M. paratuberculosis* sample and a very weak signal in 10 cfu/mL *M. paratuberculosis* sample (lanes 31 to 35), while similar reactions without BSA showed only a weak band in the 10^2 cfu/mL preparation (lanes 26 to 30).

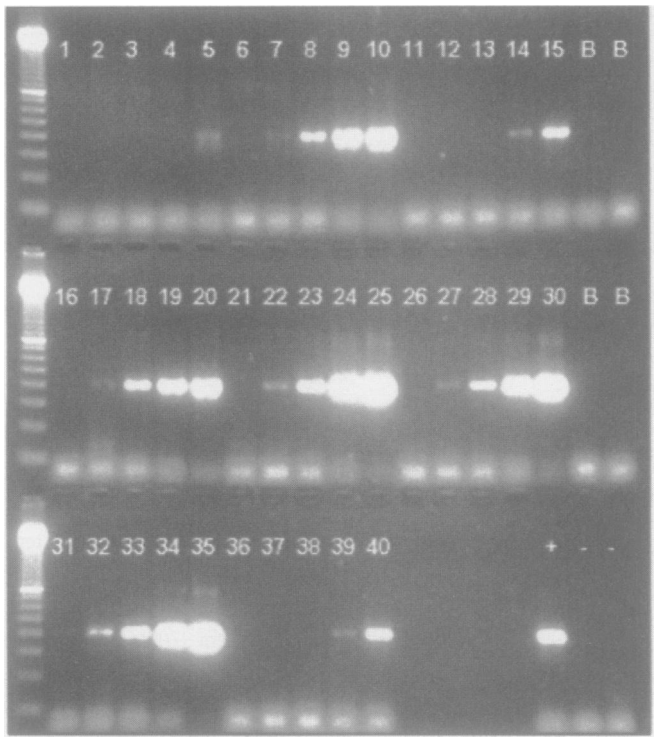

Figure 1. Agarose gel electrophoresis of the IS*900* PCR products for different combinations of *M. paratuberculosis* template DNA preparations in Table I. Marker, 100 bp ladder; lanes 1–5, combination 1 (milk samples spiked with 10 to 10^5 cfu/mL of *M. paratuberculosis*, respectively. Same order for all the other combinations); lanes 6–10, combination 2; lanes 11–15, combination 3; lanes 16–20, combination 4; lanes 21–25, combination 5; lanes 26–30, combination 6; lanes 31–35, combination 7; lanes 36–40, combination 8; B, blank sample control; +, PCR positive control (413 bp); −, PCR negative control.

Discussion

In this study, boiling alone or boiling in combination with freeze-thaw produced a detection limit by direct PCR in the range of 10^4 to 10^5 cfu/mL *M. paratuberculosis* organisms. This range of detection is not sensitive enough for detection of low levels of *M. paratuberculosis* contamination in milk. Enhanced release of DNA from *M. paratuberculosis* cells was obtained using the Hybaid Ribolyser, a process that involves high speed vibrations (45 s at 6.5 M/s) of bacterial cells suspended in a slurry of silica and ceramic particles (J. Hermon-Taylor, personal communication). In our laboratory, we have obtained efficient lysis of cultured *M. paratuberculosis* cells using the Biospec bead beater, a process using high velocity mechanical homogenization (3 × 30 s) of bacterial cells suspended in a slurry of zirconium/silica beads (unpublished data). The bead beating times used by Madiraju et al (27) were 2 min for lysis of *M. smegnatis*, a rapid grower, and 4 min for *M. bovis* BCG, a slow-growing mycobacterium. Giese and Ahrens (26) used 2 × 50 s cycles for lysis of *M. paratuberculosis* in milk. For this study, we found that 2 min was not adequate for optimal cell lysis, and that bead beating for 5 min at 5000 rpm, the maximum speed of the instrument, generated optimal release of *M. paratuberculosis* DNA template for the assays. These operation parameters were used in all experiments described.

The introduction of bead beating alone combined with isopropanol precipitation of DNA increased the sensitivity of detection of the IS*900* PCR assay 10-fold. When boiling was used in addition to bead beating, the sensitivity increased up to 1000-fold. The use of lysis buffer with bead beating also increased the sensitivity 1000-fold. However, addition of BSA to this combination did not improve the sensitivity further. On the other hand, addition of BSA to samples prepared by a combination of bead beating, lysis buffer, boiling, and isopropanol precipitation steps resulted in a further improvement in sensitivity. We observed that when high concentration of template DNA was obtained, there was a concomitant high concentration of inhibitors, which in turn reduced the sensitivity of the PCR (combination 3; Figure 1, lanes 11 to 15). When the samples were boiled before precipitation, the sensitivity of PCR was enhanced (combination 4). Boiling of the samples appeared to play a role in reducing the effect of inhibitors on PCR. Forbes and Hicks (28) working with sputum specimens reported that addition of 0.0026% BSA effectively reduced the effect of inhibitors of PCR reaction. Concentration of BSA in PCR was optimized from levels of 0.1% to 0.00014% (data not shown). We found that 0.0037% was the optimum concentration for our experiments. The use of BSA in reducing the effect of inhibitors of the PCR reaction appeared to be more effective when the samples were boiled prior to precipitation. Addition of BSA to non-boiled samples did not produce a PCR signal at the level of 10 cfu/mL *M. paratuberculosis*.

The template DNA prepared using QIAamp Tissue Kit and InstaGene Matrix yielded a limit of detection by direct PCR of 10^5 cfu/mL of *M. paratuberculosis* organisms in milk, the same level of sensitivity observed for boiling only or boiling combined with freeze-thaw procedures. Clearly, these methods were not efficient enough to be used for the preparation of *M. paratuberculosis* template DNA from milk. The most effective procedure identified in this study involves a combination of cell lysis procedures, including bead beating and boiling in lysis buffer, and DNA template concentration using isopropanol precipitation, and the use of BSA to reduce the effect of inhibitors. This procedure improved PCR detection of *M. paratuberculosis* to a level of 10 cfu/mL. The application of these procedures should significantly improve and enhance the detection of low levels of *M. paratuberculosis* contamination in raw and pasteurized milk.

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