Applied and Environmental Microbiology, Sept. 1998, p. 3153–3158 Vol. 64, No. 9
0099-2240/98/$04.00+0

Isolation of *Mycobacterium paratuberculosis* from Milk by Immunomagnetic Separation

IRENE R. GRANT,[1]* HYWEL J. BALL,[2] and MICHAEL T. ROWE[1]

Department of Food Science (Food Microbiology), The Queen's University of Belfast, Belfast BT9 5PX,[1] and Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Belfast BT4 3SD,[2] United Kingdom

Received 3 November 1997/Accepted 10 June 1998

An immunomagnetic separation (IMS) technique was developed to facilitate selective isolation of *Mycobacterium paratuberculosis* cells from milk. Rabbit polyclonal antibodies against radiation-killed intact *M. paratuberculosis* cells were produced and used to coat sheep anti-rabbit immunoglobulin G (IgG) type M-280 Dynabeads. The rabbit anti-*M. paratuberculosis* IgG-coated beads (IMB) reacted strongly with laboratory strains of *M. paratuberculosis* as determined by slide agglutination, and microscopic examination confirmed that *M. paratuberculosis* cells attached to the IMB. The IMB were found to have a maximum binding capacity of 10^4 to 10^5 CFU of *M. paratuberculosis*. Studies showed that IMS selectively recovered *M. paratuberculosis* from inoculated milk containing as few as 10 CFU of *M. paratuberculosis* per ml when 10 μl of IMB (ca. 10^6 beads) was added to 1 ml of milk and the preparation was incubated for 30 min at room temperature with gentle agitation. Larger volumes of milk (10 and 50 ml) were centrifuged and resuspended in 1 ml of phosphate-buffered saline–0.05% Tween 20 prior to IMS in order to increase the sensitivity of the method. Currently, primary isolation of *M. paratuberculosis* from a milk sample relies on chemical decontamination, followed by culturing on Herrold's egg yolk medium, which must be incubated at 37°C for up to 18 weeks. The potential value of our IMS method is as an aid for rapid detection of *M. paratuberculosis* in milk when it is used in conjunction with end point detection methods, such as IS*900* PCR or an enzyme-linked immunosorbent assay.

Mycobacterium paratuberculosis causes paratuberculosis, commonly known as Johne's disease, in cattle, sheep, goats, and other ruminants (2). Although not currently classified as a zoonotic agent, *M. paratuberculosis* has been identified in intestinal biopsy tissues from some patients with Crohn's disease (CD) (1). CD is a chronic, incurable, low-grade inflammation of the terminal ileum, one of two similar diseases of the human gastrointestinal tract known as inflammatory bowel disease. Whether the presence of *M. paratuberculosis* in biopsy material indicates that this organism has a causative role in CD or is simply a complicating infection is still the subject of much debate. However, if *M. paratuberculosis* has a causative role in CD, then milk may be a possible vehicle of transmission of the organism from cattle to humans (7, 21). Detectable quantities of *M. paratuberculosis* have previously been found in the milk of both clinically infected (20) and subclinically infected (18, 19) cattle with Johne's disease. One theory put forward to explain the increasing incidence of CD in humans in certain parts of the world is that the human population may be repeatedly exposed to low levels of *M. paratuberculosis* in the milk supply (7). This explains the interest in determining whether *M. paratuberculosis* is present in the general supply of fluid milk, both raw and pasteurized. Only one such study has been published to date. Millar et al. (13) used IS*900* PCR to detect *M. paratuberculosis* in retail pasteurized cow's milk in England and Wales and reported that overall, 7% of 312 milk samples tested positive for the presence of *M. paratuberculosis* DNA over a 19-month period. At peak periods up to 25% of the milk samples were positive as determined by IS*900* PCR. However, the presence of viable cells was never confirmed by decontamination and culturing of PCR-positive milk samples, so the theory of repeated exposure of humans to viable *M. paratuberculosis* in milk was not substantiated by the results of this milk survey.

Determination of the incidence of *M. paratuberculosis* in milk supplies is fraught with difficulties. First, *M. paratuberculosis* is an extremely slow-growing organism which can take up to 20 weeks for primary isolation, whereas most other microorganisms in milk exhibit growth within 24 to 48 h. As no selective medium for *M. paratuberculosis* is available, successful isolation of *M. paratuberculosis* currently relies on selective suppression of nonmycobacterial contaminants in samples by chemical decontamination. The recommended decontamination procedure for *M. paratuberculosis* is treatment with 0.75% (final concentration) hexadecylpyridinium chloride (HPC) for several hours (23). A balance must be struck between adequate time for decontamination and the possibility of undue damage to the *M. paratuberculosis* cells if the decontamination period is too long. Unless adequate decontamination is achieved, any surviving undesirable microorganisms quickly overgrow the *M. paratuberculosis* colonies, thwarting isolation efforts. All of the milk surveys carried out to date (13, 18–20) have relied on chemical decontamination in some shape or form prior to culturing of *M. paratuberculosis* from milk. Second, *M. paratuberculosis* is likely to be present in low numbers in naturally infected milk samples. A titer of just 2 to 8 CFU of *M. paratuberculosis* per 50 ml of milk has been reported for milk obtained aseptically from asymptomatic cattle with Johne's disease (19). Consequently, the culture methods employed to isolate *M. paratuberculosis* must be extremely sensitive, or, alternatively, the sensitivity of the culture method employed must be improved by concentrating the *M. paratuberculosis* cells prior to culturing. In theory, immunomagnetic separation (IMS) could be used to resolve these difficulties.

IMS is a simple but powerful method for extracting a desired

* Corresponding author. Mailing address: Department of Food Science (Food Microbiology), The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, United Kingdom. Phone: 44 1232 255299. Fax: 44 1232 668376. E-mail: I.Grant@qub.ac.uk.

organism from heterogeneous bacterial suspensions, such as those that are encountered in food, clinical specimens, and feces (3, 15). It has previously been used successfully with several types of food samples, including milk (8, 14, 16, 17). IMS relies on the interaction between cell surface antigens and antibodies attached to paramagnetic beads. The desired cells are separated by placing a bead suspension in a strong magnetic field. The beads can be resuspended after IMS in a smaller volume of liquid, thereby concentrating the sample. If appropriate antibodies directed against surface antigens of *M. paratuberculosis* were obtained or produced, this organism could be selectively isolated from milk samples and concentrated by IMS, thereby improving the specificity and sensitivity of subsequent culture methods. During IMS the *M. paratuberculosis* cells would not be exposed to potentially damaging chemicals, as they are during traditional decontamination procedures, and, consequently, the physiological state of the cells would not be affected. Previous applications of IMS in mycobacteriology include detection of *Mycobacterium avium* in stool samples from AIDS patients (10) and detection of *Mycobacterium tuberculosis* in cerebrospinal fluid (11).

In this paper we describe the development, optimization, and evaluation of an IMS procedure to facilitate the isolation of *M. paratuberculosis* from milk samples.

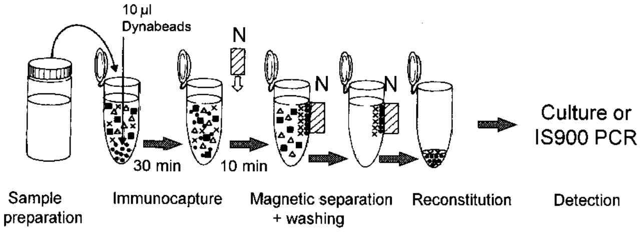

FIG. 1. Schematic diagram of the optimized IMS procedure for detection of *M. paratuberculosis* in milk. N, magnet.

MATERIALS AND METHODS

Production of rabbit anti-*M. paratuberculosis* antiserum. Colonies of *M. paratuberculosis* B4 (a bovine field strain isolated in Northern Ireland) grown on Herrold's egg yolk medium (HEYM) were suspended in phosphate-buffered saline (PBS), and the preparation was centrifuged, washed five times in PBS, and then irradiated (dose, 15 kGy) with a Gammabeam 650 instrument (Nordion International Inc., Kanata, Ontario, Canada) in order to kill the cells. A 0.5-ml portion of a dense suspension (concentration, approximately 10^8 CFU/ml) of irradiated cells was mixed with an equal volume of the adjuvant Quil A (125 μg per ml; Superfos, Vedbaek, Denmark) and used to inoculate a rabbit subcutaneously at multiple sites. This inoculation procedure was repeated after 5 weeks, followed (at 15- to 20-day intervals) by three intravenous inoculations of 0.5 ml. The rabbit was test bled 7 to 14 days after the second subcutaneous inoculation and after each intravenous inoculation. The antiserum obtained was tested by an enzyme-linked immunosorbent assay (ELISA) in which microtiter wells were coated with the cell suspension used to inoculate the rabbit; HEYM was used as a control. No ELISA reaction was obtained with the HEYM antigen. The rabbit was exsanguinated 14 days after a titer greater than 1:3,000 was recorded with the *M. paratuberculosis* antigen; the serum was separated and stored at −20°C.

Purification of the polyclonal rabbit anti-*M. paratuberculosis* serum. The rabbit anti-*M. paratuberculosis* serum was purified by precipitating albumin and other non-immunoglobulin G (IgG) proteins with caprylic acid (25 μg per ml) after 2 volumes of 0.06 M acetate buffer (pH 4.3) was added by the method of McKinney and Parkinson (12). After centrifugation at 10,000 × *g* for 15 min, the precipitate was discarded, and the IgG fraction was dialyzed overnight at 4 to 10°C against 0.01 M PBS (pH 7.2). The purified polyclonal IgG was divided into 1-ml aliquots and stored at −20°C.

Specificity of the polyclonal rabbit anti-*M. paratuberculosis* IgG. Slide agglutination was used to test for cross-reactions of the polyclonal IgG with other *Mycobacterium* spp. and bacterial isolates obtained from raw milk. Twenty microliters of undiluted polyclonal IgG was applied to a clean slide. A loopful of the test organism was mixed with the IgG, the slide was tilted several times, and agglutination was checked within 2 min. The slide agglutination test was repeated by using 20-μl portions of 1:10, 1:100, and 1:1,000 dilutions of the polyclonal IgG in PBS.

Coating of magnetic beads. The polyclonal IgG was used to coat sheep anti-rabbit IgG type M-280 Dynabeads (catalog no. 11203; Dynal UK Ltd., Wirral, United Kingdom) for 24 h at 2 to 4°C as recommended by the manufacturer. In all of the IMS trials 10-μl aliquots (ca. 10^6 beads) of the rabbit anti-*M. paratuberculosis* IgG-coated beads (IMB) were employed.

Strains studied. Three *M. paratuberculosis* strains were used in this study. These strains were type strain NCTC 8578 and field strains B2 and B4, which were previously isolated from cattle in Northern Ireland. The culture conditions used and the method used to prepare the inoculum have been described previously (6). In addition, *Mycobacterium intracellulare* NCTC 10425, *Mycobacterium kansasii* NCTC 10268, and a field strain of *M. avium* were used to test the specificity of the polyclonal IgG.

Milk samples. Milk samples with low levels of background microflora (either raw cow's milk aseptically obtained from a healthy Friesian cow as described previously [6] or pasteurized whole cow's milk routinely sent to the Food Microbiology Unit for testing), were used in this study.

Confirmation of *M. paratuberculosis* attachment to IMB. In order to visualize *M. paratuberculosis* cells bound to the IMB after IMS, 2 drops of a bead-cell suspension was transferred to a microscope slide, air dried, and then heat fixed. The smear was flooded with a 0.1% (wt/vol) phenolic auramine O solution for 15 min, decolorized with acid-alcohol for 2 min, counterstained with a 0.3% (wt/vol) methylene blue solution for 2 min, and then allowed to air dry prior to microscopic examination under blue light (9). Acid-fast cells fluoresced bright yellow against a dark background, whereas the IMB fluoresced only weakly.

Determination of the optimum immunocapture time and percentage of recovery. Seven 1-ml aliquots of milk and three 1-ml aliquots of PBS were each inoculated with 10^6 CFU of *M. paratuberculosis* NCTC 8578. One of the inoculated milk samples was immediately serially diluted and cultured on HEYM slopes in order to determine the number of *M. paratuberculosis* cells added to the samples prior to IMS. Another three inoculated milk samples were centrifuged (2,500 × *g* for 15 min) and resuspended in 1 ml of PBS before IMS; these samples were designated milk/PBS samples. Ten microliters of coated Dynabeads was added to each inoculated sample (three milk samples, three milk/PBS samples, and three PBS samples), and the tubes were incubated on a Dynal sample mixer at room temperature (21°C) for 15, 30, or 60 min. After a 15-min immunocapture, one milk sample, one milk/PBS sample, and one PBS sample were removed and processed by using the remainder of the IMS procedure. Following IMS, the IMB were resuspended in 1 ml of PBS. After 30- and 60-min immunocapture times, three additional tubes were removed and processed in the same way. All samples were then diluted as necessary, and the number of *M. paratuberculosis* cells recovered by IMS from each of the suspending media after each of the immunocapture times was determined by culturing on HEYM slopes. The percentage of recovery of *M. paratuberculosis* cells from each milk or PBS sample was calculated on the basis of the number of cells which attached to the IMB. The same experiment was performed with *M. paratuberculosis* B2.

A schematic diagram of the optimized IMS procedure used for the remainder of this study is shown in Fig. 1. Briefly, 10 μl of IMB (10^6 IMB) was added to 1 ml of a test sample, and the preparation was incubated at room temperature with gentle agitation on a Dynal sample mixer for 30 min. After incubation the IMB were separated from the cell suspension with a magnetic particle concentrator (model MPC-M; Dynal) for 10 min. The residual liquid was removed by aspiration, and three washes in PBS containing 0.05% Tween 20 (PBS-T), with separation with the model MPC-M concentrator for 2 min between washes were performed. The IMB were resuspended in 1 ml of sterile water prior to culturing.

Later, the percentage of recovery by IMS was determined by counting the *M. paratuberculosis* cells lost with the milk after the magnetic separation step (i.e., the cells that were not able to bind to the IMB during the immunocapture step). A 10-fold dilution series of milk samples (1-ml aliquots) containing 10^6 to 10^2 CFU of *M. paratuberculosis*/ml was prepared. Each milk sample was subjected to IMS as described above. Following immunocapture and magnetic separation for 10 min, each milk sample was carefully aspirated into sterile plastic tubes, taking care not to dislodge any of the IMB. Each aspirate was diluted as necessary and cultured on HEYM slopes (100 μl per slope) to obtain a colony count for each milk sample. A 1-ml milk sample that was inoculated with 10^6 CFU of *M. paratuberculosis* but not subjected to IMS was simply diluted and inoculated onto HEYM slopes to confirm the number of *M. paratuberculosis* cells present in the most concentrated sample before IMS. Each successive 10-fold dilution of this milk sample was assumed to contain 10 times fewer *M. paratuberculosis* CFU than the previous dilution. This experiment was carried out on three separate occasions. The number of cells recovered by IMS was estimated by subtracting the number of *M. paratuberculosis* cells lost in the aspirate from the number of cells present in the milk sample prior to IMS. The percentage of recovery from each milk sample was calculated accordingly.

Use of centrifugation prior to IMS to concentrate *M. paratuberculosis* cells. Different volumes of raw milk (5, 10, and 50 ml) were inoculated with a fixed number of *M. paratuberculosis* cells (approximately 10^6 CFU). Each milk sample was centrifuged at 2,500 × *g* for 15 min, and the pellet was resuspended in 1 ml of PBS-T prior to IMS. Following IMS, the IMB were resuspended in 1 ml of sterile water, and the number of *M. paratuberculosis* cells recovered from each milk sample volume was determined by serial dilution and inoculation of HEYM

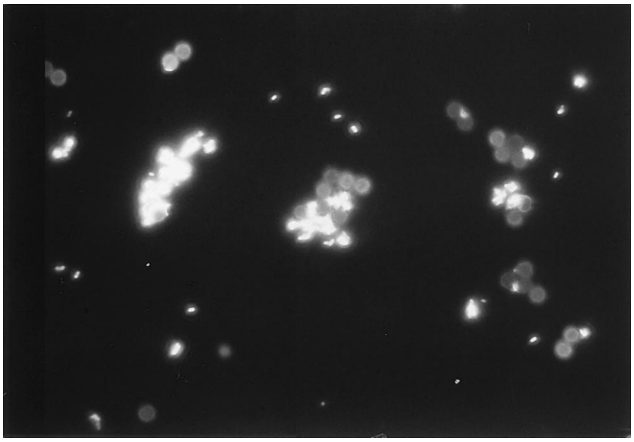

FIG. 2. *M. paratuberculosis* cells attached to Dynabeads coated with polyclonal rabbit anti-*M. paratuberculosis* IgG and stained by the auramine O fluorescent acid-fast stain (bead diameter, 2.8 μm).

slopes (100 μl per slope). Colony counts were obtained after incubation of the slopes for up to 12 weeks at 37°C. This experiment was repeated twice.

In order to assess whether the majority of the *M. paratuberculosis* cells present in a milk sample were located in the pellet after centrifugation, two 10-ml samples of raw milk were inoculated with a fixed number of *M. paratuberculosis* cells (approximately 10^6 CFU) and centrifuged at 2,500 × *g* for 15 min in order to obtain the following three milk fractions: cream, whey, and pellet. The cream and pellet fractions were resuspended in 1 ml of PBS to facilitate enumeration of the *M. paratuberculosis* cells present. The whey fraction was tested directly. Following dilution as necessary, the number of *M. paratuberculosis* cells in each fraction was determined by inoculating HEYM slopes (100 μl per slope) and counting the colonies after incubation for up to 12 weeks at 37°C. The percentage of *M. paratuberculosis* cells in each fraction was calculated by taking into account the dilution of the cream and pellet layers and the volume of each milk fraction.

Determination of the minimum detection limit of the IMS method. An inoculated milk sample containing 10^8 CFU of *M. paratuberculosis*/ml (the size of the inoculum was confirmed by dilution and plating on HEYM) was serially diluted in milk to obtain a set of eight milk samples containing between 10^1 and 10^8 CFU of *M. paratuberculosis*/ml. A 1-ml aliquot of each of the milk samples was then subjected to IMS and resuspended in 1 ml of PBS prior to inoculation onto HEYM slopes (100 μl per slope) without further dilution in order to determine the presence of any *M. paratuberculosis* cells recovered by IMS. This experiment was repeated four times.

Comparison of the recovery of a fixed number of *M. paratuberculosis* cells by culturing after centrifugation alone, centrifugation and IMS, and centrifugation and decontamination with 0.75% HPC. In previous *M. paratuberculosis* milk surveys (18, 19) the workers centrifuged and decontaminated the milk samples with 0.75% HPC before preparations were cultured on HEYM slopes. A study was carried out to compare the numbers of *M. paratuberculosis* cells recovered by culturing after centrifugation and IMS and by culturing after centrifugation and HPC decontamination from different volumes of milk (1, 5, 10, and 50 ml) inoculated with approximately 10^6 CFU of *M. paratuberculosis*/ml. Culturing after centrifugation alone was included as a control. Control milk samples were centrifuged at 2,500 × *g* for 15 min, and each pellet was resuspended in 1 ml of sterile water. IMS milk samples were centrifuged and resuspended in 1 ml of PBS prior to IMS as described above. IMB were resuspended after IMS in 1 ml of sterile water. For decontamination, milk samples were centrifuged, resuspended in 5 ml of 0.75% HPC, and incubated at room temperature for 4 h with occasional shaking. HPC-treated samples were then centrifuged again, and each pellet was resuspended in 1 ml of sterile water. All of the samples were then diluted as necessary and inoculated onto HEYM slopes (100 μl per slope). The slopes were incubated for up to 12 weeks, and colony counts were obtained.

RESULTS

Confirmation of attachment of *M. paratuberculosis* to coated IMB. Fluorescent microscopy revealed that *M. paratuberculosis* cells attached to the IMB coated with purified rabbit anti-*M. paratuberculosis* IgG. It was evident that large clumps, not just single cells, were bound to some IMB, and often more than one cell or clump of cells were attached to the same IMB or group of beads (Fig. 2).

TABLE 1. Specificity of the rabbit anti-*M. paratuberculosis* polyclonal IgG as determined by slide agglutination

Organism	Slide agglutination with the following dilutions of polyclonal IgG:			
	Undiluted	1:10	1:100	1:1,000
M. paratuberculosis NCTC 8578	++[a]	++	++	++
M. avium (field isolate)	+	+	+	±
M. intracellulare NCTC 10425	++	+	+	±
M. kansasii NCTC 10268	++	++	++	+
Pseudomonas sp.[b]	+	±	−	−
Staphylococcus sp.[b]	+	±	−	−
Hafnia alvei[b]	±	−	−	−

[a] ++, strongly positive; +, positive; ±, weakly positive; −, negative.
[b] Isolates obtained from raw milk.

Specificity of purified rabbit anti-*M. paratuberculosis* polyclonal IgG. The polyclonal IgG raised against *M. paratuberculosis* B4 produced strong agglutination reactions with all *M. paratuberculosis* strains available for testing in our laboratory (a total of 10 strains were tested, including 2 type strains and 8 field strains that originated from laboratories in the United States, Denmark, Northern Ireland, and Scotland). Moderate cross-reactions of the polyclonal IgG with three other *Mycobacterium* spp. and weak cross-reactions with bacterial isolates from raw milk were observed (Table 1). Table 1 also shows that a 1:1,000 dilution of the polyclonal IgG in PBS still reacted strongly with *M. paratuberculosis* NCTC 8578, whereas the levels of the cross-reactions with the other *Mycobacterium* spp. and the milk isolates diminished as the IgG was diluted.

Determination of optimum immunocapture time. Immunocapture times of 15, 30, and 60 min (with gentle agitation) after addition of 10 μl of IMB were investigated. Similar trends were observed for the two *M. paratuberculosis* strains tested (NCTC 8578 and B2). Figure 3 shows how the number of *M. paratuberculosis* cells recovered by IMS was affected by immunocapture time and suspending medium. Overall, the numbers of *M. paratuberculosis* cells recovered by IMS from milk and milk/PBS samples were not significantly different, but the number of cells recovered from PBS alone was significantly less for both strains ($P < 0.001$). The percentage of recovery of *M. paratuberculosis* from each of the suspending media, which was cal-

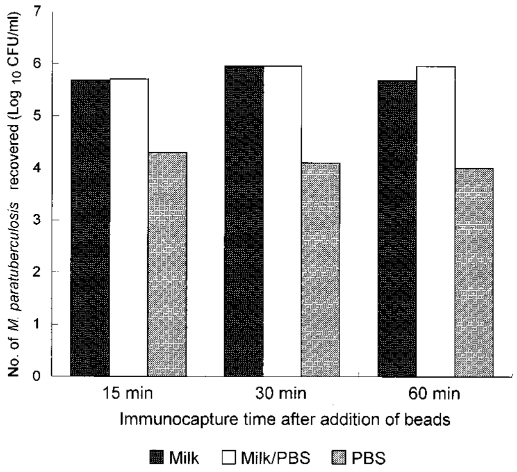

FIG. 3. Optimum immunocapture times after addition of rabbit anti-*M. paratuberculosis* IgG-coated Dynabeads to inoculated milk, inoculated milk that was centrifuged and resuspended in PBS, and inoculated PBS.

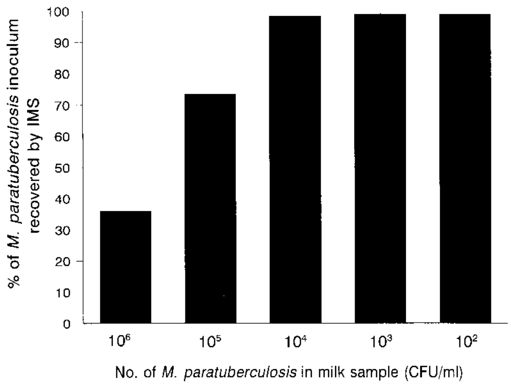

FIG. 4. Influence of the number of *M. paratuberculosis* cells present in milk on the percentage of recovery by IMS.

culated by determining the number of cells attached to the IMB, was surprisingly low. The highest percentage of recovery (37.1%) was obtained by incubating inoculated milk for 60 min with IMB, and the lowest percentage of recovery ($\leq$0.2%) was obtained with inoculated PBS. The optimum immunocapture times for *M. paratuberculosis* cells varied with the suspending medium, as follows: whole milk, 30 to 60 min; milk-PBS, 30 min; and PBS, 15 min (Fig. 3). Subsequently, when the numbers of *M. paratuberculosis* cells which did not bind to the IMB were determined for a range of milk samples containing 10^2 to 10^6 CFU/ml, we found that the IMB had a maximum binding capacity of between 10^4 and 10^5 CFU (Fig. 4). Consequently, significant proportions of the inoculated *M. paratuberculosis* population were effectively lost when milk samples containing $>10^4$ CFU were subjected to IMS. This explains why just 37% of *M. paratuberculosis* cells were recovered by IMS from milk initially inoculated with 10^6 CFU/ml.

Effect of centrifugation prior to IMS. *M. paratuberculosis* cells were present in all three milk fractions after centrifugation at 2,500 $\times$ *g* for 15 min. Overall, 13.0, 17.6, and 69.4% of the *M. paratuberculosis* cells present in a 10-ml milk sample segregated into the cream, whey, and pellet fractions, respectively, after centrifugation at 2,500 $\times$ *g* for 15 min. As the majority of the *M. paratuberculosis* cells were located in the pellet ($P < 0.05$), centrifugation was subsequently used to concentrate low numbers of *M. paratuberculosis* in larger volumes of milk prior to IMS. The numbers of *M. paratuberculosis* cells recovered by IMS after centrifugation of 5-, 10-, and 50-ml samples of milk inoculated with 10^6 CFU of *M. paratuberculosis* did not differ significantly ($P > 0.05$) (Fig. 5).

Detection limit of the IMS method. *M. paratuberculosis* was consistently isolated by the optimized IMS procedure from milk inoculated with 10 CFU/ml when milk samples inoculated with different concentrations of *M. paratuberculosis* (10^7 to 10 CFU/ml) were tested on four separate occasions. When centrifugation was used to concentrate the *M. paratuberculosis* cells in larger volumes of inoculated milk prior to IMS, the limit of detection by the culture method increased to 10 CFU per original volume (5, 10, or 50 ml) of milk tested.

Comparison of methods for the recovery of *M. paratuberculosis* from milk. Similar numbers of *M. paratuberculosis* cells were recovered from milk samples by culturing after centrifugation alone and after centrifugation and HPC decontamination irrespective of the initial milk volume (Fig. 5). In contrast, significantly lower numbers of *M. paratuberculosis* cells were recovered by culturing after centrifugation and IMS ($P < 0.001$), although similar numbers of cells were recovered by this method from different volumes of milk. The latter finding is explained by the limited binding capacity of the IMB.

DISCUSSION

In this paper we describe successful development and optimization of a novel IMS method for isolation of *M. paratuberculosis* from milk. It was clear from previous IMS studies (3–5, 16, 17, 22) that IMS was not intended for quantification purposes since it was generally used with preenriched samples. Our novel IMS method for *M. paratuberculosis* isolation should aid in detection, not quantification, of this organism in milk when it is used in conjunction with a suitable end point detection method. The IMS method consistently detected *M. paratuberculosis* in milk samples inoculated with 10 CFU/ml when it was used in association with culturing on HEYM slopes. This level of detection was possible in the absence of selective preenrichment, which is commonly performed prior to IMS of other food pathogens (4, 5, 22), and therefore indicates that the method is sensitive. The optimum immunocapture time after addition of IMB varied depending on the nature of the medium in which the *M. paratuberculosis* cells were suspended. The longest immunocapture time (60 min) was that required for whole milk, and the shortest immunocapture time (15 min) was that required for PBS. Our experiments showed that when 10-ml volumes of inoculated milk were centrifuged, the majority (69.4%) of *M. paratuberculosis* cells segregated in the pellet. Therefore, in future studies involving this IMS method, we will centrifuge 50-ml volumes of milk, resuspend each pellet in 1 ml of PBS-T prior to IMS, and use an immunocapture time of 30 min in order to maximize the sensitivity of the method.

Research groups reporting new IMS methods generally assess the performance of an IMS method in terms of the percentage of recovery obtained with an inoculated population (5, 22). A similar approach was taken in this study, although we readily acknowledge that interpretation of CFU data for *M. paratuberculosis* is difficult because of the probability that CFU arise from both single cells and clumps of cells. Counts obtained after IMS are semiquantitative at best (3). Examination of bead-cell complexes after IMS by using fluorescent acid-fast staining clearly illustrated that several cells or clumps can be bound to each IMB or cluster of IMB (Fig. 2). Nevertheless, an indication of the capabilities of the new IMS meth-

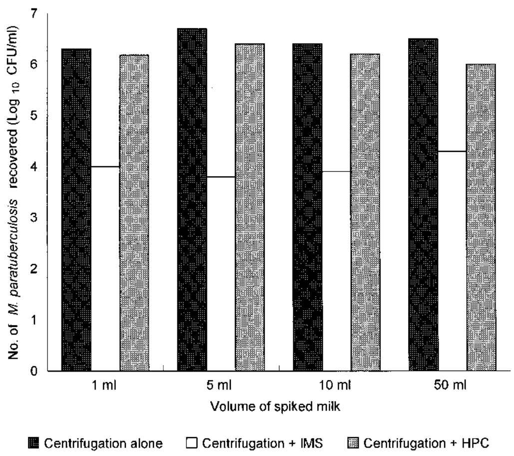

FIG. 5. Comparison of the levels of recovery of *M. paratuberculosis* from inoculated milk by culturing on HEYM after centrifugation alone, after centrifugation followed by IMS, and after centrifugation followed by decontamination with 0.75% HPC.

od was obtained. Initially, the percentages of recovery by IMS from milk, milk/PBS, and PBS samples inoculated with 10^6 CFU of *M. paratuberculosis*/ml were calculated by counting the *M. paratuberculosis* cells attached to the IMB. The values obtained were disappointingly low compared with the values given in previously reported IMS studies performed with other food pathogens (5, 22). However, an alternative approach for determining the percentages of recovery of cells by IMS is to enumerate the cells which do not bind to the beads but are lost with the milk after the magnetic separation step. Milk samples inoculated with a range of *M. paratuberculosis* concentrations (10^6 to 10^2 CFU/ml) were examined by taking this alternative approach. The results indicated that 10 μl of IMB (approximately 10^6 beads) had a maximum binding capacity of 10^4 to 10^5 CFU. This meant that when 10^2 to 10^4 CFU of *M. paratuberculosis* was present in a 1-ml milk sample, the percentage of recovery was close to 100% (Fig. 4), whereas when $>10^4$ CFU was present in a 1-ml milk sample, a maximum of only 10^4 CFU could be recovered. Additional evidence that the maximum binding capacity is limited was provided by the finding that the concentrations of *M. paratuberculosis* recovered after centrifugation and IMS from various volumes of milk that were initially inoculated with 10^6 to 10^7 CFU of *M. paratuberculosis* were consistently around 10^4 CFU/ml, approximately 100 times lower than the concentrations obtained by culturing after centrifugation and HPC decontamination (Fig. 4). During this study, some batch variation in the binding capacity of the IMB was observed. For example, data in Fig. 3 indicate that the binding capacity approached 10^6 CFU, whereas data in Fig. 5, which were obtained with a different batch of Dynabeads, indicate that the maximum level of recovery was just over 10^4 CFU of *M. paratuberculosis*. The limited binding capacity of the coated IMB is of no real consequence since an accurate determination of the number of *M. paratuberculosis* cells present in a milk sample is not possible after IMS; in any case, high numbers of *M. paratuberculosis* would never be encountered in naturally infected milk. The novel IMS method for *M. paratuberculosis* used in conjunction with culturing gives an indication of the presence or absence of viable *M. paratuberculosis* cells.

We used polyclonal antibodies raised against radiation-killed *M. paratuberculosis* to coat magnetic beads in this study. Polyclonal antibodies are directed against a number of surface antigens rather than against a single surface antigen, which avoids the problem of the high level of specificity which can sometimes occur with monoclonal antibodies. It also increases the likelihood of isolating the desired organism. Unfortunately, since some of the surface antigens may not be unique to *M. paratuberculosis*, there is a possibility of nonspecific cross-reactions with the polyclonal IgG. Slide agglutination confirmed that there were some cross-reactions between the polyclonal IgG and other mycobacteria and milk bacteria (Table 1). We used undiluted polyclonal IgG to coat the sheep anti-rabbit IgG-coated Dynabeads and were generally able to isolate *M. paratuberculosis* from milk containing 10 CFU/ml initially, so even though the IMS method was not completely specific, it was very sensitive. We found that cross-reactions of the polyclonal IgG with the milk isolates, in particular, were eliminated by diluting the IgG (Table 1). Therefore, it may be possible to improve the specificity of the IMS method by coating magnetic beads with a dilution of the polyclonal IgG (1:100 or 1:1,000). However, using a lower concentration of polyclonal IgG to coat magnetic beads may reduce the sensitivity of the IMS method, and this possibility should be investigated. It may be possible to circumvent this shortcoming in terms of specificity by using IMS in conjunction with BACTEC radiometric medium, to which PANTA antibiotic supplement could be added to combat the growth of contaminants, rather than HEYM. Alternatively, instead of culturing to confirm the presence of *M. paratuberculosis*, IMS could be used in conjunction with IS900 PCR, which is specific for this organism.

IMS is a relatively simple procedure to perform, although it is a little laborious. However, there is a potential risk that IMB and, therefore, *M. paratuberculosis* cells may be lost during the washing steps if care is not taken when the supernatant is removed by aspiration between washes. Milk is notorious as a suspending medium which can create problems for IMS applications due to its high fat content (3). Particular care is needed when the supernatant is removed after the first 10 min of magnetic separation. At this stage the IMB tend to slip down the side of the tube as the supernatant is being removed by aspiration rather than being tightly captured on the wall of the tube. However, with each subsequent washing step as the IMB are cleaned and milk components trapped among the IMB are removed, the beads tend to become more tightly captured on the wall of the tube, and there is less likelihood that they will be accidentally discarded.

In summary, we developed and evaluated an IMS procedure for isolating *M. paratuberculosis* from milk samples. This IMS procedure takes less than 1 h, compared to at least 5 h for HPC decontamination prior to culturing, and was found to consistently result in isolation of *M. paratuberculosis* cells from milk containing 10 CFU/ml. Centrifugation of larger volumes of milk and resuspension in 1 ml of PBS-T prior to IMS considerably improve the sensitivity of the method. The potential value of this novel IMS method is not for quantification of *M. paratuberculosis*; rather, it can be used for rapid detection of this organism in milk when it is combined with end point detection methods, such as IS*900* PCR or an ELISA (3). In future work we will concentrate on evaluating the use of this novel IMS method in conjunction with IS*900* PCR as a rapid technique for screening milk and feces samples for the presence of *M. paratuberculosis*.

ACKNOWLEDGMENTS

This work was supported by funds from the Ministry of Agriculture, Fisheries and Food, United Kingdom.

We thank Bill Graham for assistance during irradiation of the *M. paratuberculosis* antigen and Colin Bell, who carried out the ELISA work.

REFERENCES

1. **Chiodini, R. J.** 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. Clin. Microbiol. Rev. **2:**90–117.
2. **Cocito, C., P. Gilot, M. Coene, M. De Kesel, P. Poupart, and P. Vannuffel.** 1994. Paratuberculosis. Clin. Microbiol. Rev. **7:**328–345.
3. **Cudjoe, K. S., P. D. Patel, E. Olsen, E. Skjerve, and Ø. Olsvik.** 1993. Immunomagnetic separation techniques for the detection of pathogenic bacteria in foods, p. 17–29. *In* R. Kroll, A. Gilmour, and M. Sussman (ed.), New techniques in food and beverage microbiology. Blackwell Scientific Publications, Oxford, United Kingdom.
4. **Cudjoe, K. S., R. Krona, and E. Olsen.** 1994. IMS: a new selective enrichment technique for detection of *Salmonella* in foods. Int. J. Food Microbiol. **23:**159–165.
5. **Fratamico, P. M., F. J. Schultz, and R. L. Buchanan.** 1992. Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. Food Microbiol. **9:**105–113.
6. **Grant, I. R., H. J. Ball, S. D. Neill, and M. T. Rowe.** 1996. Inactivation of *Mycobacterium paratuberculosis* in cow's milk at pasteurization temperatures. Appl. Environ. Microbiol. **62:**631–636.
7. **Hermon-Taylor, J.** 1993. Causation of Crohn's disease: the impact of clusters. Gastroenterology **104:**643–646.
8. **Johne, B., J. Jarp, and L. R. Haaheim.** 1989. *Staphylococcus aureus* exopolysaccharide in vivo demonstrated by immunomagnetic separation and electron microscopy. J. Clin. Microbiol. **27:**1631–1635.
9. **Kubica, G. P.** 1984. Clinical microbiology, p. 133–175. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria: a sourcebook, part A. Marcel Dekker, Inc., New York, N.Y.

10. **Li, Z., G. Han Bai, C. F. Von Reyn, P. Marino, M. J. Brennan, N. Gine, and S. L. Morris.** 1996. Rapid detection of *Mycobacterium avium* in stool samples from AIDS patients by immunomagnetic PCR. J. Clin. Microbiol. **34:**1903–1907.
11. **Mazurek, G. H., V. Reddy, D. Murphy, and T. Ansari.** 1996. Detection of *Mycobacterium tuberculosis* in cerebrospinal fluid following immunomagnetic enrichment. J. Clin. Microbiol. **34:**450–453.
12. **McKinney, M. M., and A. Parkinson.** 1987. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J. Immunol. Methods **96:**271–278.
13. **Millar, D., J. Ford, J. Sanderson, S. Withey, M. Tizard, T. Doran, and J. Hermon-Taylor.** 1996. IS900 PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows' milk in England and Wales. Appl. Environ. Microbiol. **62:**3446–3452.
14. **Muramatsu, Y., M. Maruyama, T. Yanase, H. Ueno, and C. Morita.** 1996. Improved method for preparation of samples for polymerase chain reaction for detection of *Coxiella burnetii* in milk using immunomagnetic separation. Vet. Microbiol. **51:**179–185.
15. **Olsvik, Ø., T. Popovic, E. Skjerve, K. S. Cudjoe, E. Hornes, J. Ugelstad, and M. Uhlén.** 1994. Magnetic separation techniques in diagnostic microbiology. Clin. Microbiol. Rev. **7:**43–54.
16. **Skjerve, E., and Ø. Olsvik.** 1991. Immunomagnetic separation of *Salmonella* from foods. Int. J. Food Microbiol. **14:**11–18.
17. **Skjerve, E., L. M. Rorvik, and Ø. Olsvik.** 1990. Detection of *Listeria monocytogenes* in foods by immunomagnetic separation. Appl. Environ. Microbiol. **56:**3478–3481.
18. **Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, and M. Rings.** 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. **56:**1322–1324.
19. **Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger.** 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. **30:**166–171.
20. **Taylor, T. K., C. R. Wilks, and D. S. McQueen.** 1981. Isolation of *Mycobacterium paratuberculosis* from the milk of a cow with Johne's disease. Vet. Rec. **109:**532–533.
21. **Thompson, D. E.** 1994. The role of mycobacteria in Crohn's disease. J. Med. Microbiol. **41:**74–94.
22. **Vermunt, A. E. M., A. A. J. M. Franken, and R. R. Beumer.** 1992. Isolation of salmonellas by immunomagnetic separation. J. Appl. Bacteriol. **72:**112–118.
23. **Whitlock, R. H., A. E. Rosenberger, R. W. Sweeney, and L. J. Hutchinson.** 1992. Culture techniques and media constituents for the isolation of *Mycobacterium paratuberculosis* from bovine fecal samples, p. 94–111. *In* R. J. Chiodini and J. M. Kreeger (ed.), Proceedings of the Third International Colloquium on Paratuberculosis. International Association for Paratuberculosis, Providence, R. I.
24. **Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, B. H. I. Keller, and J. Verhoef.** 1991. Evaluation of the magnetic immuno PCR assay for rapid detection of *Salmonella*. Eur. J. Clin. Microbiol. Infect. Dis. **10:**935–938.
25. **Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, G. P. H. T. Verdonk, and J. Verhoef.** 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J. Clin. Microbiol. **30:**3195–3199.