

Incidence of *Mycobacterium paratuberculosis* in Bulk Raw and Commercially Pasteurized Cows' Milk from Approved Dairy Processing Establishments in the United Kingdom

Irene R. Grant,^{1*} Hywel J. Ball,² and Michael T. Rowe^{1,3}

Department of Food Science (Food Microbiology), Queen's University of Belfast,¹ and Food Science Division³ and Veterinary Sciences Division,² Department of Agriculture and Rural Development for Northern Ireland, Belfast, Northern Ireland, United Kingdom

Received 12 November 2001/Accepted 14 February 2002

Over a 17-month period (March 1999 to July 2000), a total of 814 cows' milk samples, 244 bulk raw and 567 commercially pasteurized (228 whole, 179 semiskim, and 160 skim), from 241 approved dairy processing establishments throughout the United Kingdom were tested for the presence of *Mycobacterium paratuberculosis* by immunomagnetic PCR (to detect all cells living and dead) and culture (to detect viable cells). Overall, *M. paratuberculosis* DNA was detected by immunomagnetic PCR in 19 (7.8%; 95% confidence interval, 4.3 to 10.8%) and 67 (11.8%; 95% confidence interval, 9.0 to 14.2%) of the raw and pasteurized milk samples, respectively. Confirmed *M. paratuberculosis* isolates were cultured from 4 (1.6%; 95% confidence interval, 0.04 to 3.1%) and 10 (1.8%; 95% confidence interval, 0.7 to 2.8%) of the raw and pasteurized milk samples, respectively, following chemical decontamination with 0.75% (wt/vol) cetylpyridinium chloride for 5 h. The 10 culture-positive pasteurized milk samples were from just 8 (3.3%) of the 241 dairy processing establishments that participated in the survey. Seven of the culture-positive pasteurized milk samples had been heat treated at 72 to 74°C for 15 s; the remainder had been treated at 72 to 75°C for the extended holding time of 25 s. When typed by restriction fragment length polymorphism and pulsed-field gel electrophoresis methods, some of the milk isolates were shown to be types distinct from those of laboratory strains in regular use within the testing laboratory. From information gathered at the time of milk sample collection, all indications were that pasteurization had been carried out effectively at all of the culture-positive dairies. That is, pasteurization time and temperature conditions complied with the legal minimum high-temperature, short-time process; all pasteurized milk samples tested phosphatase negative; and postprocess contamination was considered unlikely to have occurred. It was concluded that viable *M. paratuberculosis* is occasionally present at low levels in commercially pasteurized cows' milk in the United Kingdom.

Mycobacterium paratuberculosis has been of concern to the dairy industry worldwide for some years due to the as-yet-unresolved issue of its potential role in Crohn's disease in humans (European Commission, Report of the Scientific Committee on Animal Health and Animal Welfare, 2000, www.europa.eu.int/comm/food/fs/sc/sc/ah/out38_en.pdf; Food Safety Authority of Ireland, 2000, www.fsai.ie/research_index.htm) and published reports that it may not be effectively inactivated by high-temperature, short-time (HTST) pasteurization (72°C for 15 s) if present in raw milk (5, 8, 11, 18, 21, 28). The findings of previous United Kingdom government-funded research, carried out at Queen's University, Belfast, Northern Ireland, United Kingdom (QUB), on the heat sensitivity of *M. paratuberculosis* in milk at pasteurization temperatures demonstrated that under laboratory pasteurization conditions, *M. paratuberculosis* can survive heating at 72°C for 15 s. Survival was shown to occur when the organism was present in raw milk at >100 CFU/ml (8). Results of subsequent research suggested that an extended holding time of 25 s at 72°C might be more effective in inactivating high numbers of *M. paratuberculosis* spiked into

raw milk (9) than the current United Kingdom legal minimum holding time of 15 s. As a direct result of these findings, most dairy processing establishments in the United Kingdom voluntarily altered their pasteurizing equipment in mid-1998 to enable them to adopt a 25-s holding time for pasteurized liquid milk production. This action was taken in the hope of ensuring the complete inactivation of *M. paratuberculosis* that might be present in the raw milk supply (1).

General criticisms have been levelled against laboratory pasteurization studies, such as those described above, with regard to how closely the test pasteurization systems simulated the commercial process in terms of total process time, milk flow characteristics during heating, and volume and type of milk processed (2, 26). As a consequence, the reliability of the findings of such studies has frequently been called into question. Probably the biggest concern is that all pasteurization studies reported to date were done with milk spiked with laboratory-grown *M. paratuberculosis*. It is not known whether *M. paratuberculosis* present as a result of natural infection in the milk of cattle with Johne's disease (27, 29, 30) is likely to be more or less heat resistant than laboratory-grown cultures added to raw milk. It was recently asserted (2) that reliable results on the heat resistance of *M. paratuberculosis* would be obtained only by the use of an industrial-scale HTST pasteurizer with continuous-flow heating and naturally infected milk.

* Corresponding author. Mailing address: Department of Food Science (Food Microbiology), Queen's University of Belfast, Newforge La., Belfast BT9 5PX, Northern Ireland, United Kingdom. Phone: 00 44 28 9025 5299. Fax: 00 44 28 9025 5009. E-mail: ireneRgrant@hotmail.com.

To our knowledge, logistical and health and safety issues have prevented such a study from being undertaken by many groups.

An alternative means of assessing the efficacy of commercial HTST pasteurization is to test large numbers of commercially pasteurized milk samples for the presence of viable *M. paratuberculosis* based on the assumption that at least some of the raw milk supplying the dairy processing plants sampled during the survey will be naturally infected with *M. paratuberculosis*. In August 1998, the Food Hygiene Division of the United Kingdom Ministry of Agriculture, Fisheries and Food (which in April 2000 became part of the United Kingdom Food Standards Agency [FSA]) announced that a national cows' milk survey would be carried out in order to assess the microbiological quality of cows' milk in the United Kingdom before and after heat processing (United Kingdom Ministry of Agriculture, Fisheries and Food, News Release 334/98, 1998). *M. paratuberculosis* was just one of a number of pathogenic microorganisms for which samples would be tested. The objective of *M. paratuberculosis* testing was to confirm whether viable *M. paratuberculosis* existed in bulk raw and commercially pasteurized cows' milk in the United Kingdom. Johne's disease exists in the United Kingdom dairy herd (3, 4, 15) and retail pasteurized milk there has previously tested IS900 PCR positive (22), so it was considered possible that at least some of the dairy processing plants in the United Kingdom could be processing *M. paratuberculosis*-infected milk. Various chemical decontamination protocols and a novel immunomagnetic separation (IMS)-PCR method for *M. paratuberculosis* were thoroughly evaluated and optimized prior to commencement of the milk survey in order to be confident that sufficiently sensitive detection methods were chosen for large-scale milk testing (7, 10, 12). Details of the scope of *M. paratuberculosis* testing and the results obtained for this organism only are reported here. Results of the other microbiological analyses carried out during the national milk survey have been reported elsewhere (Advisory Committee on the Microbiological Safety of Food, paper ACM/499, 2000, www.foodstandards.gov.uk/pdf_files/papers/acm499.pdf).

MATERIALS AND METHODS

Scope of *M. paratuberculosis* milk testing. During the 17-month period from March 1999 to July 2000 (inclusive), a total of 814 bulk raw and commercially pasteurized cows' milk samples from 241 approved dairy processing establishments throughout England, Wales, Scotland, and Northern Ireland were tested for the presence of *M. paratuberculosis*. Participation in the United Kingdom milk survey was on an entirely voluntary basis, 755 approved dairy establishments which heat treat milk (both drinking milk and milk for the manufacture of other dairy products) having been approached in advance of its commencement and 258 having agreed to take part. Care was taken by the FSA to ensure that the companies who agreed to take part were as representative as possible of the United Kingdom dairy processing sector in terms of dairy size, annual throughput, and geographic location. For *M. paratuberculosis* testing purposes, dairy processing establishments were visited by sampling officers on a single occasion, and a minimum of one raw milk sample and a number of heat-treated milk samples were collected. In total, 244 bulk raw and 567 pasteurized (228 whole, 179 semiskim, and 160 skim) milk samples were examined for the presence of *M. paratuberculosis*.

Sample collection and transport. A sample of raw milk was collected from a bulk raw milk silo at each dairy processing establishment, and a carton or cartons of pasteurized product were collected while coming off the production line. Sampling officers were instructed that whenever possible, the raw and pasteurized milk samples were to be taken from the same batch of milk.

Milk samples collected from approved dairy establishments in England and Wales were taken to ADAS Laboratories, Wolverhampton, United Kingdom,

where the main microbiological analyses for milk samples from these regions were to be carried out. Each milk sample was assigned a numerical code, and a subsample was aseptically transferred into a sterile 150-ml plastic sample bottle for dispatch to QUB for *M. paratuberculosis* testing. Milk samples from dairy establishments throughout Scotland were taken to four testing laboratories, in Aberdeen, Glasgow, Edinburgh, and Dundee, where subsamples were taken for dispatch to QUB. All milk samples from the United Kingdom mainland were placed in insulated boxes containing ice packs to maintain the temperature of the samples at 4°C or below and were transported to the testing laboratory at QUB via overnight courier service. In Northern Ireland, Department of Agriculture and Rural Development sampling officers aseptically collected raw and pasteurized milk samples from local dairy processing establishments and delivered them directly to the testing laboratory at QUB.

M. paratuberculosis testing of all milk samples, irrespective of origin, commenced within 72 h of collection from the dairy processing establishment, and the temperature of the milk samples was maintained at or below 4°C at all times between collection and testing. No information regarding the origin of the milk samples accompanied the samples to QUB. Information on the origin of each milk sample, its corresponding sample code, and processing details for pasteurized milk samples (collected at the time of sampling) was held centrally by the FSA in London, United Kingdom, until the full results of *M. paratuberculosis* testing became available.

Milk testing. Each milk sample was subjected to two tests for *M. paratuberculosis*: IMS-PCR (to detect the presence of *M. paratuberculosis* cells, live or dead) and chemical decontamination and culture (to confirm the presence of viable *M. paratuberculosis*), as follows.

(i) **IMS-PCR.** A 50-ml portion of each raw milk sample was centrifuged (15 min at 2,500 × g), and the pellet was resuspended in 1 ml of phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T; Sigma Chemical Co. Ltd, Poole, Dorset, United Kingdom) prior to IMS, which was carried out as described previously (10). In an effort to maximize test sensitivity and produce consistently clear IMS-PCR results, samples were subjected to a modification of the previously described (12) DNA extraction and purification protocol after IMS and before PCR was carried out. This protocol involved overnight incubation of the resuspended beads after IMS at 37°C in 700 µl of TEN lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris [pH 8.0], 0.6% sodium dodecyl sulfate) containing 20 µg of proteinase K (Sigma); mechanical disruption of the sample in blue-cap tubes in a Hybaid Ribolyser (both from Hybaid Ltd., Middlesex, United Kingdom); and extraction, purification, and precipitation of the DNA with phenol, chloroform-isoamyl alcohol (24:1), and isopropanol (all from Sigma), respectively. Precipitated DNA was washed once in 70% ethanol and resuspended in 50 µl of Tris-EDTA buffer before PCR was performed. Each PCR mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.75 mM MgCl₂, 150 µM each deoxynucleoside triphosphate, 60 pmol each of primers P90 and P91 (23), and 1.25 U of Platinum Taq DNA polymerase (Life Technologies Ltd., Paisley, Scotland). The mixture was overlaid with 2 drops of light mineral oil. PCRs were carried out for 33 cycles of 94°C for 2 min, 58°C for 1 min, and 72°C for 1 min, with an initial denaturation step at 94°C for 10 min and a final annealing step at 72°C for 7 min. PCR products were visualized after 2% agarose gel electrophoresis by staining with ethidium bromide (0.5 µg/ml) and viewed on a UV transilluminator. The size of the amplified product was checked with molecular size markers (ϕX174 replicative-form DNA *Hae*III fragments; Sigma). IMS-PCR results were reported as positive if a band of the correct size (400 bp) was observed on the gel. The minimum detection limit of this modified IMS-PCR protocol was estimated to be in the region of 1 CFU/50 ml of milk (unpublished data), a value which represents a substantial improvement in sensitivity over that of the original IMS-PCR protocol (10³ CFU/50 ml) (12); the latter protocol simply used boiling at 100°C for 15 min to release DNA between IMS and PCR.

(ii) **Decontamination and culture.** A second 50-ml portion of each milk sample was centrifuged (15 min at 2,500 × g), and the pellet was resuspended in 10 ml of freshly prepared 0.75% (wt/vol) cetylpyridinium chloride (HPC; Sigma). Following incubation at room temperature (21°C) for 5 h and further centrifugation (as described above), the pellet was resuspended in 1 ml of PBS-T. Two slopes of Herrold's egg yolk medium containing 2 µg of mycobactin J/ml (HEYM) were each inoculated with 250 µl of the resuspended pellet. One vial of BACTEC 12B radiometric medium (Becton Dickinson, Cowley, Oxford, England) supplemented with 0.5 ml of Difco egg yolk emulsion, 2 µg of mycobactin J (Synbiotics Europe SAS, Lyon, France)/ml, and PANTA antibiotic supplement (Becton Dickinson) reconstituted in accordance with the manufacturer's instructions (100 µl for raw milk cultures and 50 µl for pasteurized milk cultures) was inoculated with 500 µl of the resuspended pellet. Both media were incubated at 37°C for up to 18 weeks. Slopes were examined periodically for the presence of colonies. BACTEC vials were read regularly on a BACTEC 460TB instrument (Becton

Dickinson). When growth was observed in either medium, acid-fast staining was carried out by the Ziehl-Neelsen method to confirm the presence of acid-fast organisms. Further confirmatory tests (14) to confirm slow growth rate, typical colony morphology, and mycobactin J dependence and IS 900 PCR of a sample from a colony were carried out on each suspect acid-fast isolate to determine whether it was *M. paratuberculosis* or some other *Mycobacterium* sp.

Statistical analysis of culture and IMS-PCR results. The number of confirmed *M. paratuberculosis* isolates or IMS-PCR-positive results obtained for each type of milk was expressed as a percentage of the total number of samples of that type of milk tested. The standard error of this value was calculated, and 95% confidence limits are presented.

Test controls. Negative (water only) and positive (DNA obtained from *M. paratuberculosis* strain B2) PCR controls and negative (PBS-T used to resuspend the milk pellet) and positive (*M. paratuberculosis* strain B2 broth culture) IMS controls were run alongside each batch of milk samples to confirm that both PCR and IMS were operating correctly. On three separate occasions during the survey, some raw cows' milk was obtained and spiked with *M. paratuberculosis* strain B2 (approximately 10^4 CFU added per 100 ml of milk) to act as an internal method control. This spiked milk sample was subjected to both tests to confirm that *M. paratuberculosis* was isolated after decontamination and culture and detected after IMS-PCR. Spiked positive controls yielded typical colonies after HPC decontamination and culture and tested IMS-PCR positive on all three occasions.

Phosphatase testing. All pasteurized milk samples received for *M. paratuberculosis* testing at QUB were subjected to phosphatase testing upon receipt. Initially, the long-established Aschaffenberg-Mullen method was applied, but part way through the survey (December 1999 onward), this method was replaced by the Fluorophos method, which is capable of detecting much lower levels of residual phosphatase activity (20).

General laboratory control measures. Control measures were in place throughout the survey in order to prevent possible accidental laboratory contamination of the milk samples with *M. paratuberculosis* strains in use in the testing laboratory at QUB over the same period. (i) Milk samples were processed in a part of the category 3 pathogen laboratory physically removed from the class I safety cabinet to which all *M. paratuberculosis* research involving live *M. paratuberculosis* cultures was confined. (ii) Separate sets of dedicated pipette aids were used for IMS-PCR and culturing, and disposable filter tips were used at all times. (iii) IMS-dedicated pipette aids were regularly subjected to UV treatment to render any contaminating DNA nonamplifiable by PCR. (iv) HPC decontaminant solution and PBS-T solution (used during IMS and for resuspension of the milk pellet after decontamination) were freshly made on each day of testing, filter-sterilized (0.2- μ m pore size), and divided into aliquots of the required volumes before use.

Molecular typing of confirmed *M. paratuberculosis* milk isolates. Fourteen confirmed *M. paratuberculosis* milk isolates were typed at QUB by IS 900 restriction fragment length polymorphism (RFLP) typing with restriction enzyme *BstEII* broadly as described in the standardized protocol for the RFLP typing of *M. paratuberculosis* strains (24). DNA from four laboratory strains of *M. paratuberculosis* (ATCC 19698, B2, DVL 943, and NCTC 8578) that had been in use in the laboratory at QUB during the period of the survey was also RFLP typed. Briefly, DNA was obtained from the pellet of cells resulting from centrifugation of a 10-ml sample of a Middlebrook 7H9 broth culture of each strain by overnight proteinase K treatment in TEN lysis buffer, ribolysation, and extraction as described earlier for IMS-PCR. DNA quantitation standards (Life Technologies Ltd.) were used to quantify the yield of DNA and to standardize the amount of DNA subjected to treatment with restriction enzyme *BstEII* (Life Technologies Ltd.). DNA was digested at 37°C for 3 h before being applied to a 1% agarose gel along with lambda DNA *HindIII* molecular weight markers (Sigma). DNA fragments were separated by electrophoresis at 30 V for 16 h. After depurination, denaturation, and neutralization, the digested DNA in the gel was transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech United Kingdom Ltd., Little Chalfont, Buckinghamshire, United Kingdom) by Southern blotting. The DNA was fixed to the membrane by UV cross-linking at 2 J/cm² with a Fluo-Link machine (Vilber Lourmat, Torcy, France). The membrane was hybridized overnight with an IS 900 probe generated in-house from DNA of type strain ATCC 19698 (as described by Pavlik et al. [24]) according to the instructions accompanying the ECL direct nucleic acid labeling kit (Amersham). RFLP profiles were visualized on Hyperfilm ECL (Amersham). RFLP profiles were visually compared to published profiles (24) and assigned to a particular RFLP type, whenever possible.

The 14 confirmed milk isolates and four laboratory strains were also independently typed by Karen Stevenson and Valerie Hughes at Moredun Research Institute near Edinburgh, Scotland, by using pulsed-field gel electrophoresis

TABLE 1. IMS-PCR and culture results for 244 bulk raw and 567 commercially pasteurized cows' milk samples from 241 United Kingdom dairy processing establishments

Type of milk	No. (%) of samples with the indicated result ^a :					
	IMS-PCR			Culture		
	+	-	No result	+	-	No result
Raw	19 (7.8)	211 (86.5)	14 (5.7)	4 (1.6)	217 (89.9)	23 (9.4)
Pasteurized	67 (11.8)	480 (84.7)	20 (3.5)	10 (1.8)	534 (94.2)	23 (4.0)

^a +, positive; -, negative; no result, insufficient sample was received, no clear result was obtained for IMS-PCR, or HEYM slopes and BACTEC medium were overgrown by contaminants for culture.

(PFGE) and three restriction enzymes, *SnaBI*, *NdeI*, and *SpeI*, in accordance with the method described recently by Hughes et al. (19).

RESULTS

Detection of *M. paratuberculosis* by IMS-PCR. The IMS-PCR results for raw and pasteurized milk samples tested during the survey are summarized in Table 1. Overall, *M. paratuberculosis* DNA was detected by IMS-PCR in 19 (7.8%; 95% confidence interval, 4.3 to 10.8%) of the raw and 67 (11.8%; 95% confidence interval, 9.0 to 14.2%) of the pasteurized (12.0% whole, 14.4% semiskim, and 8.1% skim) milk samples tested. The distribution of IMS-PCR-positive results by month of survey is shown in Fig. 1. In all except the final 2 months of the survey (June and July 2000), IMS-PCR-positive results were obtained. The percentage of IMS-PCR-positive samples ranged from 2 to 27.3% of samples tested per month (mean, 10.4%). The IMS-PCR findings suggest that 56 (23.5%) of the 241 United Kingdom dairy processing plants from which milk samples were submitted during the survey may have been processing milk contaminated by *M. paratuberculosis*. However, as not many of the IMS-PCR results were strongly positive, the organism is liable to have been present at low levels in the milk being processed.

Isolation of viable *M. paratuberculosis* by culture. The culture results for raw and pasteurized milk samples tested during the survey are also summarized in Table 1. A total of 14 confirmed *M. paratuberculosis* isolates were obtained during the course of the milk survey, 4 from bulk raw milk samples and 10 from commercially pasteurized milk samples, representing 1.6% (95% confidence interval, 0.04 to 3.1%) of raw and 1.8% (95% confidence interval, 0.7 to 2.8%) of pasteurized milk samples, respectively. Overall, 2.8% of pasteurized semiskim, 1.3% of pasteurized whole, and 1.2% of pasteurized skim milk samples tested culture positive. On only one occasion did the raw and two different pasteurized milk samples from the same dairy processing establishment all test culture positive for *M. paratuberculosis*. Otherwise, there was no correlation between culture results for raw and pasteurized milk samples from the same dairy processing plant. Overall, pasteurized products from 8 (3.3%) of 241 United Kingdom dairy processing plants tested culture positive for *M. paratuberculosis*.

All 14 confirmed *M. paratuberculosis* isolates were obtained from HEYM slope cultures, and only 1 pasteurized milk sample was culture positive in BACTEC medium as well. Typically, only a small number of colonies ($n = 1$ to 5) were observed on

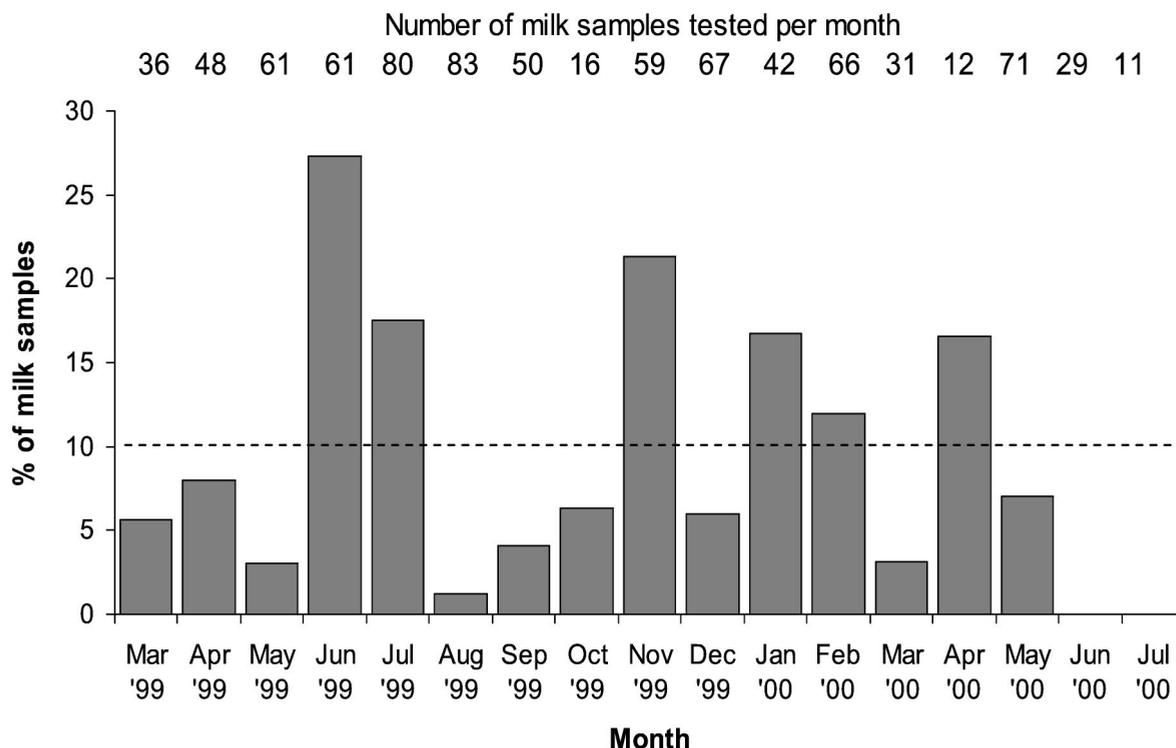


FIG. 1. Distribution of IMS-PCR-positive results for raw and commercially pasteurized cows' milk by month of survey. The dashed line indicates the overall mean percent IMS-PCR-positive level.

HEYM cultures from *M. paratuberculosis*-positive milk samples. This finding translates to an *M. paratuberculosis* count in the original milk sample of about 4 to 20 CFU/50 ml of milk. As chemical decontamination was applied prior to culturing, this count is likely to be an underestimate of the number of *M. paratuberculosis* organisms actually present (7, 13). The one pasteurized milk sample that was culture positive with both HEYM and BACTEC medium yielded a lawn of typical colonies on HEYM slope cultures, suggesting the presence of larger numbers of *M. paratuberculosis* organisms in that particular sample. In addition to the 14 milk samples confirmed to contain viable *M. paratuberculosis*, two further pasteurized semiskim milk samples gave rise to BACTEC cultures that tested strongly acid fast and IS900 PCR positive after incubation. However, colonies were never obtained from these liquid cultures upon subculturing to HEYM, and so the presence of viable *M. paratuberculosis* could not be confirmed in these two samples.

Contamination or overgrowth of HEYM and BACTEC cultures may have accounted for the failure to isolate more culture-positive samples from raw milk in particular. Table 2 compares the culture medium contamination rates for raw and pasteurized milk cultures during the survey. HEYM contamination was considerably greater for raw milk cultures than for pasteurized milk cultures (27.8 and 6.9%, respectively). Conversely, fewer BACTEC cultures from raw milk (11.2%) than from pasteurized milk (26.4%) were contaminated, probably due to the addition of twice as much PANTA antibiotic supplement to raw milk cultures as to pasteurized milk cultures. However, we also suspect that a more general problem existed

for much of the survey with the Difco egg yolk supplement inhibiting the growth of *M. paratuberculosis* in the BACTEC cultures, for some reason. This problem may explain the unexpectedly poor performance of the liquid BACTEC medium compared to the solid HEYM for the recovery of heat-injured *M. paratuberculosis* during this study.

Molecular typing of confirmed *M. paratuberculosis* milk isolates. RFLP profiles were obtained for the 14 milk isolates and four laboratory strains. Five different RFLP types were differentiated among the strains tested, and two distinct RFLP types were shown by two of the milk isolates (Fig. 2A). Twelve of the

TABLE 2. Rates of medium overgrowth with nontarget microorganisms in raw and pasteurized milk cultures

Type of milk	No. (%) of samples contaminated under the following conditions ^a :					Total no. (%) of samples affected
	HEYM only		BACTEC medium only	BACTEC medium + HEYM		
	One slope	Two slopes		One slope	Two slopes	
Raw	37	33 (28.3)	28 (11.3)	7	16 (9.3)	121 (48.9)
Pasteurized	27	13 (6.9)	152 (26.5)	17	6 (4.0)	215 (37.5)

^a For HEYM, contamination was overgrowth of slopes by fungi and/or other non-acid-fast bacteria or blue discoloration of the slopes, which is known to inhibit the growth of *M. paratuberculosis*. For BACTEC medium, contamination was a mixed culture (acid-fast and non-acid-fast bacteria present together) or only non-acid-fast bacteria observed upon Ziehl-Neelsen staining of growth-positive cultures. Percentages for HEYM data are averages of data for one slope and two slopes.

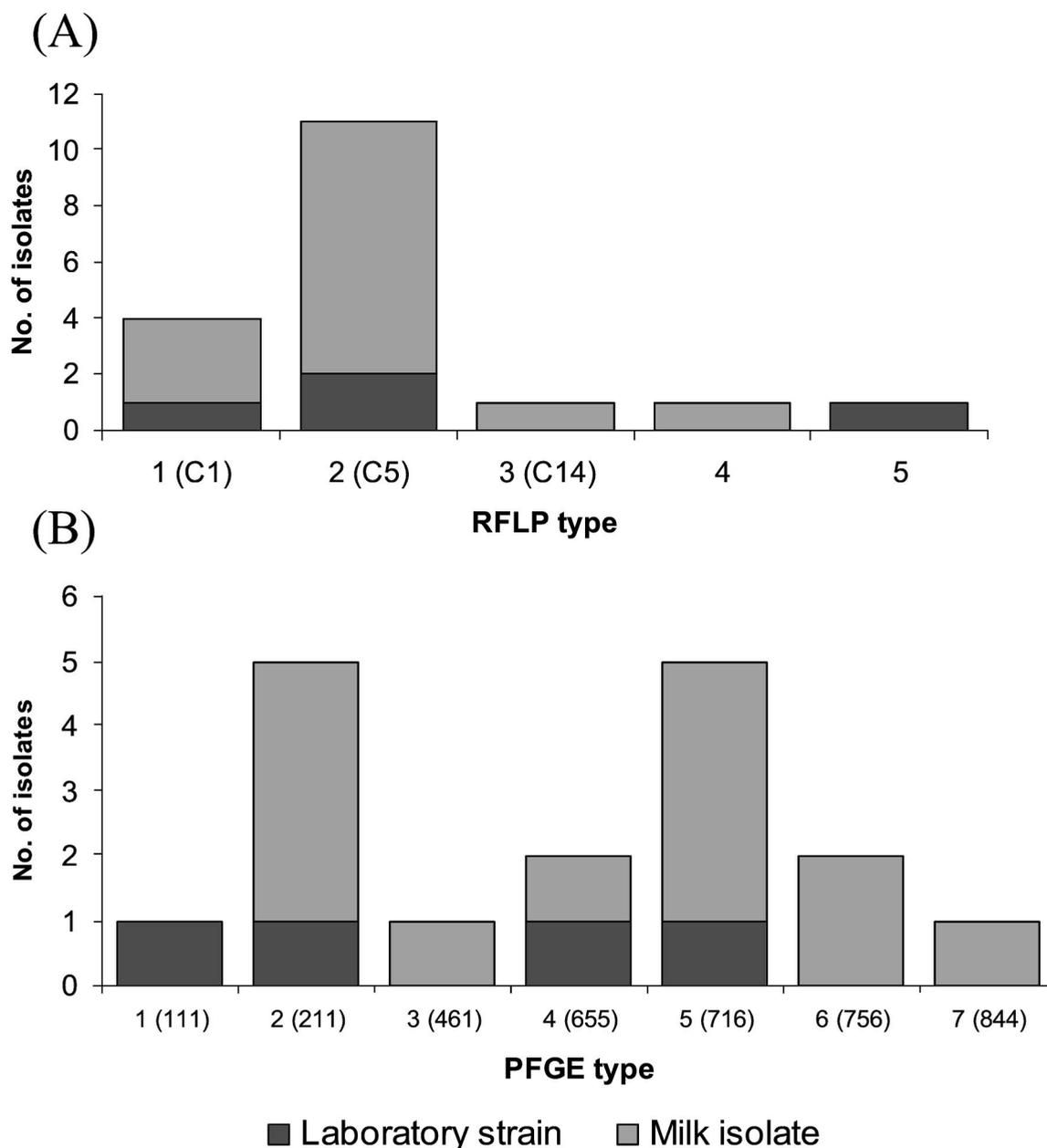


FIG. 2. Distributions of RFLP and PFGE types among the *M. paratuberculosis* milk isolates and laboratory strains. Strain designations, when they could be assigned, are indicated in parentheses.

RFLP profiles obtained for the milk isolates were easily assigned to particular RFLP types described previously by Pavlik et al. (24); the remaining two profiles were not.

PFGE profiles were obtained for the four laboratory strains and all except 1 of the 14 milk isolates. The 14th milk isolate did not yield sufficient DNA after culturing, despite several attempts, to permit PFGE analysis. Six different PFGE types were differentiated among the 13 milk isolates (Fig. 2B); three of these were distinct from the PFGE types exhibited by the four laboratory strains. Hence, the results of both RFLP typing and PFGE typing indicate that *M. paratuberculosis* types distinct from those of the laboratory strains in

use in the milk testing laboratory at QUB existed among the milk isolates.

Processing details for culture-positive pasteurized milk samples. The culture-positive pasteurized milk samples were from dairy processing establishments in all four regions of the United Kingdom, and the majority of the pasteurized milk tested was intended for retail sale as liquid milk. Information regarding pasteurization time and temperature conditions and other treatments applied to the milk during processing, collected at the time of milk sampling from the eight dairy processing establishments which yielded culture-positive pasteurized milk samples, is summarized in Table 3. Seven of the 10

TABLE 3. Processing details for the culture-positive pasteurized milk samples

Dairy	Vol of milk heat treated (10 ⁶ liter/yr)	Milk type ^a	Package type	Pasteurization temp (°C)	Holding time (s)	Homogenization	Pressure	Intended final use
1	<0.1	SS	Glass bottle	72	14			Retail
2	1–25	SS	Plastic	72	15	Two stage	10 MPa	Retail
3	<0.1	W	Plastic	74	15			Retail
4	0.5–1	W	Plastic	72	17			Not retail
		S	Not packaged	72	17			Not retail
5	0.5–1	SS	Plastic	72	15	One stage	2,000 lb/in ²	Retail
		S	Plastic	72	15	One stage	2,000 lb/in ²	Retail
6	0.1–0.5	SS	Plastic	75	25	One stage	150 lb/in ²	Retail
7	1–25	W	Plastic	72	28	Two stage	2,000 lb/in ²	Retail
8	>100	SS	Plastic	72	25			Retail

^a W, whole; SS, semiskim; S, skim.

culture-positive milk samples had received a pasteurization treatment of 72 to 74°C for a minimum of 15 s; the remaining 3 had been treated at 72 to 75°C for a minimum of 25 s. Five of the pasteurized milk samples had been subjected to homogenization during production. All pasteurized milk samples received for *M. paratuberculosis* testing at QUB were confirmed to be phosphatase negative, indicating that proper pasteurization had been achieved during their production.

DISCUSSION

The United Kingdom FSA considered the possibility that viable *M. paratuberculosis* may be present in commercially pasteurized milk worthy of investigation. *M. paratuberculosis* testing carried out as part of a much larger survey of the microbiological quality of raw and commercially pasteurized cows' milk in the United Kingdom demonstrated that 1.8% of pasteurized milk samples tested were culture positive for this organism. Hence, this study has provided firm evidence of the presence of viable *M. paratuberculosis* in commercially pasteurized cows' milk manufactured for retail sale. An expectation had been expressed (16, 25, 26) that by the time *M. paratuberculosis*-contaminated milk from individual cows in a Johne's infected herd was bulked at the farm, during road tanker collection, and then again at the dairy processing plant, any *M. paratuberculosis* present would be reduced to very low levels and pasteurization would effectively render any remaining cells nonviable. Given that viable *M. paratuberculosis* was cultured from 1.6% of bulk raw and 1.8% of pasteurized milk samples tested during this United Kingdom survey, this scenario does not appear to be the case. Colony counts obtained for culture-positive milk samples during this United Kingdom survey indicated that low levels of *M. paratuberculosis* (4 to 20 CFU/50 ml) were present in both types of milk. This contamination level is similar to that reported for the milk of asymptotically affected cows with Johne's disease (2 to 8 CFU/50 ml) (29). The data are directly comparable, since the same decontamination protocol was applied in both studies. However, the data reported by Sweeney et al. (29) have possibly misled many readers who have not noted that the figure of 2 to 8 CFU/50 ml relates only to direct infection of milk within the udder of an infected animal and does not take into account the potentially significant *M. paratuberculosis* contamination arising from fecal contamination of milk during the milking operation. Given that the feces of cows with Johne's disease may contain 10⁸

CFU of *M. paratuberculosis*/g (6), the real level of *M. paratuberculosis* infection of raw milk from an individual infected animal may in reality be much higher than 2 to 8 CFU/50 ml, depending on the hygiene practices in use in the milking parlor.

Overall, similar percentages of culture-positive samples were obtained from raw milk and pasteurized milk (1.6 and 1.8%, respectively). This was a surprising finding that may suggest that commercial pasteurization has no lethal effect on *M. paratuberculosis* viability in naturally infected milk. However, if it is assumed that the overall IMS-PCR result (which reports the presence of DNA from both living and dead cells) for pasteurized milk (11.6%) is a truer reflection of the proportion of milk originally contaminated with *M. paratuberculosis*, then the difference between IMS-PCR positivity (11.6%) and culture positivity (1.8%) could represent inactivation due to pasteurization, in which case commercial pasteurization does have a substantial effect on *M. paratuberculosis* viability. Of course, this assumption does not explain the smaller difference between the IMS-PCR (7.6%) and culture (1.6%) results for the raw milk samples tested; in theory, these results should have been identical because all cells present would have been assumed to be viable. Some of the difference between the IMS-PCR and culture results may be explained by the possibility that IMS-PCR is more sensitive than culturing. The minimum detection limit of the culture protocol involving decontamination with 0.75% HPC for 5 h has been estimated to be 10 CFU/50 ml (7). The minimum detection limit of the IMS-PCR method is believed to be lower than this value. In laboratory trials with a modified IMS-PCR protocol (as described in Materials and Methods), a PCR-positive result was regularly obtained when 5 µl of template DNA was tested from an available 50 µl of DNA derived from 50 ml of milk containing 10 CFU of *M. paratuberculosis*, which would equate to the detection of 1 CFU (unpublished observations).

The data on culture medium contamination rates presented in Table 2 may also help to explain the nonisolation of viable *M. paratuberculosis* from more samples, in particular, raw milk samples, during the survey. All of the confirmed *M. paratuberculosis* milk isolates were obtained from HEYM slopes rather than from BACTEC cultures. Almost four times as many raw milk HEYM cultures as pasteurized milk cultures were lost due to overgrowth by non-acid-fast milk microorganisms that had survived chemical decontamination (27.8 versus 6.9%, respectively). This finding is simply a reflection of the different

TABLE 4. Summary of the results of other microbiological analyses of raw and pasteurized milk samples carried out during the United Kingdom cows' milk survey

Organism(s)	% of samples found culture positive in the following type of milk ^a :	
	Raw (n = 611)	Pasteurized (n = 1,418)
Coliforms	85.9	9.7
<i>Escherichia coli</i>	52.0	0.1
<i>E. coli</i> O157	0.2	ND
Coagulase-positive staphylococci	18.8	0.1
<i>Listeria</i> spp.	37.0	0.4
<i>L. monocytogenes</i>	17.0	ND
<i>Salmonella</i> spp.	0.3	ND
<i>Campylobacter</i> spp.	0.8	ND

^a ND, not detectable.

microbial loads of raw milk and pasteurized milk. The growth of non-acid-fast contaminants on HEYM slopes may have masked the presence of *M. paratuberculosis* colonies and/or prevented the growth of *M. paratuberculosis* altogether in at least some instances. In BACTEC cultures, the growth of *M. paratuberculosis* may have been adversely affected by the addition of the recommended amount of PANTA antibiotic supplement to raw milk cultures, in an attempt to combat the growth of undesirable contaminants, but only half-strength antibiotics to the pasteurized milk cultures. These factors may also explain why the culture results for raw and pasteurized milk samples obtained from the same dairy and even on occasion the same initial batch of milk did not correlate better.

Since the preliminary findings of this milk survey were released in September 2000 (Advisory Committee on the Microbiological Safety of Food, paper ACM/485, 2000, www.foodstandards.gov.uk/pdf_files/papers/acm485.pdf), some representatives of the United Kingdom dairy industry have tried to argue that the presence of viable *M. paratuberculosis* in commercially pasteurized milk does not necessarily indicate that this organism is capable of surviving HTST pasteurization and that the possibility of postpasteurization contamination of the milk either at the processing plant during packaging or during subsampling or testing should not be excluded (United Kingdom Dairy Industry Federation, personal communication). Of course, these scenarios are possible, but information gathered regarding time and temperature of pasteurization at the dairy processing plants at the time of collection of the culture-positive milk samples (Table 3) and Fluorophos results verified that proper pasteurization had been achieved in all cases. Furthermore, laboratory personnel responsible for subsampling milk from retail cartons into sterile specimen containers and those responsible for ultimately testing the milk were experienced in aseptic techniques, so laboratory (i.e., post-process) cross contamination of the milk samples with *M. paratuberculosis* was considered by the FSA not likely to have occurred.

The results of the other microbiological analyses of raw milk and pasteurized milk (summarized in Table 4) also confirm that, in general, commercial HTST pasteurization was carried out effectively, causing a substantial reduction in the levels of all the microorganisms for which testing was done. Total viable

counts generally ranged between 10^3 and 10^6 CFU/ml for raw milk samples and between 10^2 and 10^4 CFU/ml for pasteurized milk samples. The pathogenic bacteria *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* O157 were not detected in commercially pasteurized milk but were detected in raw milk. The presence of low levels of coliforms in 9.7% of commercially pasteurized milk samples may indicate underpasteurization or postprocess contamination at some dairies. However, FSA records show that none of the *M. paratuberculosis* isolates from pasteurized milk were from dairies whose pasteurized milk samples tested culture positive for coliforms. Molecular typing subsequently provided additional support that the *M. paratuberculosis* isolates obtained from milk samples during the survey were genuine and were not likely to be the result of laboratory contamination.

Time and temperature information collected at the dairy processing plants at the time of milk sampling and held by the FSA until milk testing was complete indicated that the majority of the culture-positive pasteurized samples ($n = 7$) had been subjected to the legal minimum pasteurization holding time of 15 s and that the remainder ($n = 3$) had been subjected to the extended holding time of 25 s. This finding is in line with those of a previous laboratory pasteurization study carried out at QUB which indicated that a holding time longer than 15 s would be more effective in killing *M. paratuberculosis* (9). However, the laboratory study demonstrated no survival after heating milk containing 10^6 CFU of *M. paratuberculosis*/ml at 72°C for 25 s, whereas low levels of *M. paratuberculosis* survived in a very small number of commercially pasteurized milk samples heat treated for 25 s in the survey. Several explanations are possible. (i) Sublethally heat-injured *M. paratuberculosis* cells may have had sufficient time to recover viability in the period between commercial pasteurization and milk testing (up to 72 h) in survey samples but not in the laboratory study because the heated milk was tested immediately. (ii) The chances of recovering surviving *M. paratuberculosis* were increased during the survey because a larger volume of milk (50 ml) was tested in the survey than in the laboratory study (10 ml). (iii) *M. paratuberculosis* cells present in naturally infected milk were more heat resistant than the in vitro-grown strains used in the laboratory study. Some researchers have acknowledged the possibility of repair of sublethal heat injury by *M. paratuberculosis* (17) but, to date, no scientific evidence to substantiate this idea is available. Possible repair of sublethal heat injury by *M. paratuberculosis* cells definitely merits further investigation.

In conclusion, *M. paratuberculosis* testing of United Kingdom milk has provided evidence that commercially pasteurized milk may occasionally contain low levels of viable *M. paratuberculosis*. The potential public health impact of this situation is uncertain given that an association with Crohn's disease in humans remains unproven. However, the presence of *M. paratuberculosis*, a known animal pathogen with possible zoonotic potential, in pasteurized milk is probably undesirable.

ACKNOWLEDGMENTS

M. paratuberculosis testing of raw and pasteurized cows' milk in the United Kingdom was instigated and funded by the United Kingdom FSA.

Thanks are due to Alan Ashmore at ADAS Laboratories, who coordinated sample collection, coding, and aseptic subsampling of milk samples before dispatch to Belfast for testing, and to Karen Stevenson

and Valerie Hughes at Moredun Research Institute, who carried out PFGE typing of the milk isolates.

REFERENCES

1. Amor, D. 1998. Why milk needs reassessing. *Food Ind. News* **1998**:10.
2. Cerf, O., and M. W. Griffiths. 2000. Letter to the Editor: *Mycobacterium paratuberculosis* heat resistance. *Lett. Appl. Microbiol.* **30**:341–344.
3. Cetinkaya, B., K. Egan, D. A. Harbour, and K. L. Morgan. 1996. An abattoir-based study of the prevalence of sub-clinical Johne's disease in adult cattle in south west England. *Epidemiol. Infect.* **116**:373–379.
4. Cetinkaya, B., H. M. Erdogan, and K. L. Morgan. 1998. Prevalence, incidence and geographical distribution of Johne's disease in cattle in England and the Welsh borders. *Vet. Rec.* **143**:265–269.
5. Chiodini, R. J., and J. Hermon-Taylor. 1993. The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *J. Vet. Diagn. Investig.* **5**:629–631.
6. Cocito, C., P. Gilot, M. Coene, M. De Kesel, P. Poupard, and P. Vannuffel. 1994. Paratuberculosis. *Clin. Microbiol. Rev.* **7**:328–345.
7. Dundee, L., I. R. Grant, H. J. Ball, and M. T. Rowe. 2001. Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. *Lett. Appl. Microbiol.* **33**:173–177.
8. Grant, I. R., H. J. Ball, and M. T. Rowe. 1998. Effect of high temperature, short time (HTST) pasteurisation on milk containing low numbers of *Mycobacterium paratuberculosis*. *Lett. Appl. Microbiol.* **26**:166–170.
9. Grant, I. R., H. J. Ball, and M. T. Rowe. 1999. Effect of higher pasteurisation temperatures, and longer holding times at 72°C, on the inactivation of *Mycobacterium paratuberculosis* in milk. *Lett. Appl. Microbiol.* **28**:461–465.
10. Grant, I. R., H. J. Ball, and M. T. Rowe. 1998. Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Appl. Environ. Microbiol.* **64**:3153–3158.
11. Grant, I. R., H. J. Ball, S. D. Neill, and M. T. Rowe. 1996. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. *Appl. Environ. Microbiol.* **62**:631–636.
12. Grant, I. R., C. M. Pope, L. M. O'Riordan, H. J. Ball, and M. T. Rowe. 2000. Improved detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by immunomagnetic PCR. *Vet. Microbiol.* **77**:369–378.
13. Grant, I. R., and M. T. Rowe. 2001. Methods for the detection and enumeration of viable *Mycobacterium paratuberculosis* from milk and milk products. *Bull. Int. Dairy Fed.* **362**:41–52.
14. Grant, I. R., M. T. Rowe, L. Dundee, and E. Hitchings. 2001. *Mycobacterium avium* ssp. *paratuberculosis*: its incidence, heat resistance and detection in milk and milk products. *Int. J. Dairy Technol.* **54**:2–13.
15. Greig, A. 1998. Johne's disease or paratuberculosis—an increasing problem in Scottish livestock. *SCIEH Wkly. Rep.* **32**:248.
16. Hammer, P. 2000. *Mycobacterium paratuberculosis* in cattle and food hygiene. *Bull. Int. Dairy Fed.* **345**:19–22.
17. Hammer, P., and C. Kiesner. 2001. Heat resistance of *Mycobacterium paratuberculosis* in raw milk tested in a pilot plant pasteurizer. *Bull. Int. Dairy Fed.* **362**:59–60.
18. Hope, A. F., P. A. Tulk, and R. J. Condron. 1996. Pasteurization of *Mycobacterium paratuberculosis* in whole milk, p. 377–382. In R. J. Chiodini, M. E. Hines, and M. T. Collins (ed.), *Proceedings of the Fifth International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Rehoboth, Mass.
19. Hughes, V. M., K. Stevenson, and J. M. Sharp. 2001. Improved preparation of high molecular weight DNA for pulsed-field gel electrophoresis from mycobacteria. *J. Microbiol. Methods* **44**:209–215.
20. Langridge, E. 2000. Tests on trial. *Dairy Ind. Int.* **2000**:30–31.
21. Meylan, M., D. M. Rings, W. P. Shulaw, J. J. Kowalski, S. Bech-Nielsen, and G. F. Hoffsis. 1996. Survival of *Mycobacterium paratuberculosis* and preservation of immunoglobulin G in bovine colostrum under experimental conditions simulating pasteurisation. *Am. J. Vet. Res.* **57**:1580–1585.
22. 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.
23. Moss, M. T., J. Sanderson, M. Tizard, J. Hermon-Taylor, F. El-Zaatari, D. Markesich, and D. Graham. 1992. PCR detection of *Mycobacterium paratuberculosis* in long-term cultures from Crohn's disease tissues. *Gut* **33**:1209–1213.
24. Pavlik, I., A. Horvathova, L. Dvosdka, J. Bartl, P. Svastova, R. du Maine, and I. Rychlik. 1999. Standardisation of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies *paratuberculosis*. *J. Microbiol. Methods* **38**:155–167.
25. Stabel, J. 2000. Johne's disease and milk: do consumers need to worry? *J. Dairy Sci.* **83**:1659–1663.
26. Stabel, J., L. Pearce, R. Chandler, P. Hammer, N. Klijn, O. Cerf, M. T. Collins, C. Heggum, and P. Murphy. 2001. Destruction by heat of *Mycobacterium paratuberculosis* in milk and milk products. *Bull. Int. Dairy Fed.* **362**:53–61.
27. 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.
28. Sung, N., and M. T. Collins. 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. *Appl. Environ. Microbiol.* **64**:999–1005.
29. 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.
30. 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.