APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3446–3452 Vol. 62, No. 9
0099-2240/96/$04.00+0

IS*900* PCR To Detect *Mycobacterium paratuberculosis* in Retail Supplies of Whole Pasteurized Cows' Milk in England and Wales

DOUGLAS MILLAR,† JON FORD, JEREMY SANDERSON,‡ SIMON WITHEY, MARK TIZARD, TIM DORAN, AND JOHN HERMON-TAYLOR*

Department of Surgery, St. George's Hospital Medical School, London SW17 0RE, United Kingdom

Received 1 March 1996/Accepted 3 June 1996

IS*900* PCR for *Mycobacterium paratuberculosis* was applied to cream, whey, and pellet fractions of centrifuged whole cows' milk. The test and simultaneous control reactions gave correct results for spiked milk and for native milk samples obtained directly from *M. paratuberculosis*-free, subclinically infected, and clinically infected cows. The test was then applied to units of whole pasteurized cows' milk widely obtained from retail outlets throughout central and southern England from September 1991 to March 1993. With peak periods in January to March and in September to November, when up to 25% of units were affected, an overall 22 of 312 samples (7%) tested positive for *M. paratuberculosis*. In 18 of the 22 positive samples (81%), the PCR signal segregated to the cream or pellet fractions or both, consistent with the presence of intact mycobacteria. Nine of 18 PCR-positive milk samples (50%) and 6 of 36 PCR-negative milk samples (16%) yielded long-term liquid cultures which tested positive for *M. paratuberculosis* after 13 to 40 months of incubation, despite overgrowth by other organisms. Taken together with data on the prevalence of *M. paratuberculosis* infection in herds in the United Kingdom, the known secretion of *M. paratuberculosis* in milk from subclinically infected animals, and the inability of laboratory conditions simulating pasteurization to ensure the killing of all these slowly growing or unculturable organisms, there is a high risk, particularly at peak times, that residual *M. paratuberculosis* will be present in retail pasteurized cows' milk in England.

Mycobacterium paratuberculosis is a chronic enteric pathogen which can affect many different species of animals including primates (8, 26). The organism was first identified 100 years ago as the cause of chronic inflammation of the intestine in a German cow (22, 23). Classically, Johne's disease or paratuberculosis in animals is characterized by the presence in the affected intestine of millions of bacillary-form acid-fast mycobacteria with macrophages but with little additional inflammatory cell infiltrate (1, 2). It is now becoming clear that there is a paucimicrobial form of this condition in animals in which abundant acid-fast *M. paratuberculosis* organisms cannot be seen but in which there is a florid chronic granulomatous inflammatory response (3, 11). *M. paratuberculosis* is rarely cultured in its bacillary form from these animals. This clinicopathological spectrum of pluribacillary-paucimicrobial paratuberculosis in animals is reminiscent of the extremes represented by the lepromatous and tuberculoid forms of leprosy in humans (33). Progress in our understanding of *M. paratuberculosis* and of the diseases it causes has been considerably retarded over the years by the sometimes great difficulty of identifying this agent by conventional culture in the laboratory (6).

Work carried out in our own laboratory in 1985 led to the discovery and characterization of IS*900*, an unusual DNA insertion element so far shown to be unique to *M. paratuberculosis*, with about 18 copies stably integrated into its genome (20, 27). IS*900* PCR applied to long-term in vitro cultures and to DNA extracts of intestinal tissues implicates *M. paratuberculosis* in chronic enteritis of the Crohn's disease type in humans (13, 15, 17, 25, 31, 35, 37, 42). This miserable condition has steadily increased in frequency since the 1940s, especially in Britain (16, 24, 34). Apparently healthy subclinically infected cows are known to secrete the organism in their milk (36, 39, 40). The present investigation was designed to determine whether *M. paratuberculosis* may be present in retail supplies of whole pasteurized cows' milk obtained over a period of 19 months throughout central and southern England and south Wales.

MATERIALS AND METHODS

Milk sample processing. A large number of preliminary experiments were carried out from January to June 1991 to identify suitable conditions for the experimental processing of milk samples prior to IS*900* PCR testing for *M. paratuberculosis*. The final procedure adopted was as follows. Milk samples were brought into a dedicated unit and handled within a class 2 safety cabinet. The outside of the carton or bottle was cleaned with 100% ethanol. After the sample had been shaken, 15 ml of milk was aspirated with a sterile syringe and needle without opening the container. The aspirated sample was immediately transferred to a Beckman ultraclear quickseal centrifuge tube, sealed, and centrifuged at 41,000 × *g* for 1 h at 4°C. This generated three discrete fractions, cream, almost clear whey, and pellet. Taking care not to disturb the fractions, a needle capped with a 0.2-μm-pore-size bacterial filter was inserted into the tube to admit air, and the whey fraction was gently aspirated with a fresh syringe and needle. The whey was transferred to a 20-ml Sterilin universal tube, which was then capped. The centrifuge tube containing the residual cream and pellet fractions was frozen at −20°C and cut in half, and the solidified fractions were each expressed directly into separate 20-ml Sterilin universal tubes. The cream and pellet fractions were then made up to the same volume as the whey fraction with 0.22-μm-pore-size filtered molecular biology grade water. A 500-μl aliquot of each centrifugal fraction was then transferred to a sterile 1.5-ml centrifuge tube, and the tubes were screw capped, boiled in a water bath for 20 min, and centrifuged in a microcentrifuge. Three 5-μl portions of the resulting supernatants were added to each of three PCR premixes in capped 0.5-ml tubes. Process

* Corresponding author. Mailing address: Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, United Kingdom. Phone: 181-767-7631. Fax: 181-725-3594. Electronic mail address: jhermon@sghms.ac.uk.

† Present address: Division of Biomolecular Engineering, CSIRO, Sydney Laboratory, North Ryde, New South Wales 2113, Australia.

‡ Present address: Guy's Hospital, London SE1 9RT, United Kingdom.

controls consisting of molecular biology grade water taken through all steps in the preparative procedure were included with each batch of milk samples.

IS*900* PCR. In a separate dedicated laboratory, PCR premixes were prepared containing the following components: primers $p90^+$ (5′-GAAGGGTGTTC GGGGCCGTCGCTTAGG-3′) and $P91^+$ (5′-GGCGTTGAGGTCGATCGCC CACGTGAC-3′), 6 ng of each per ml; *Taq* polymerase, 2 U (Promega); the four deoxynucleoside triphosphates, 200 μM each; and reaction buffer consisting of 67 mM Tris/HCl, 16.6 mM ammonium sulfate, 1.7 mg of bovine serum albumin per ml, and 10 mM β-mercaptoethanol in TE buffer (10 mM Tris/HCl [pH 8.8], 0.1 mM EDTA), to a final volume of 40 μl, subsequently overlaid with 55 μl of light mineral oil. Capped tubes containing the PCR premixes were stored at −20°C in the dedicated premix laboratory. In the test, the required number of premixes was transferred to the sample-processing laboratory. To each was then added 5 μl of 15 mM $MgCl_2$ and 5 μl of the milk sample processed as described above, and the cap was replaced. The reaction mixes were then centrifuged briefly and placed in a Perkin-Elmer 480 thermal cycler programmed for 94°C for 5 min and then 93°C for 1 min, 58°C for 1 min, and 72°C for 3 min, with the last three steps being repeated for 40 cycles. Cream, whey, and pellet fractions from each milk sample were each tested in triplicate with a simultaneous no-template PCR negative control and process controls.

Southern blot hybridization. The identity of IS*900* PCR amplification products was confirmed by hybridization with a 229-bp internal probe generated by PCR with the primers p25 (5′-CCAGGGACGTCGGGTATGGC-3′) and p26 (5′-GGTCGGCCTTACCGGCGTCC-3′). The 229-bp probe was radiolabelled by a technique based on random hexamer priming with the Multiprime DNA-labelling system (Amersham International, Little Chalfont, United Kingdom) as specified by the manufacturer. For the screening of large numbers of samples, the Bio-Rad Biodot system was used. Then, 400 μl of transfer buffer (0.4 M NaOH, 0.6 M NaCl) and 15 μl of PCR product were added to each well, and the apparatus was left for 20 min at room temperature. The liquid was sucked through the membrane under vacuum, the wells were refilled with a further 400 μl of transfer buffer and left for a further 20 min, and the liquid was sucked through the membrane as before. The membrane (Amersham Hybond N^+) was then removed from the apparatus, rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), air dried, and UV (320 nm) cross-linked. The membranes were prehybridized for 1 h at 65°C in an orbital shaker with 3× SSC containing 0.1% bovine serum albumin, 0.1% Ficoll (Pharmacia), 0.1% polyvinylpyrrolidine, 0.5% sodium dodecyl sulfate (SDS), and 100 mg of sheared and denatured salmon sperm DNA per ml. The denatured, labelled 229-bp probe was then added, and hybridization was carried out overnight. The membranes were washed three times with 3× SSC–0.1% SDS at 65°C for 30 min, then with 1× SSC–0.1% SDS, and finally with 0.1× SSC–0.1% SDS. The membranes were then air dried and autoradiographed. To confirm the presence of the correct 413-bp IS*900* PCR amplification product in positive wells, a further 10 μl of the corresponding PCR product was analyzed by agarose gel electrophoresis (1.5% agarose). The DNA was transferred to nylon membranes with a Posiblot apparatus (Stratagene Ltd.), washed, hybridized as above, and autoradiographed.

Spiking experiments. Stock cultures of a bovine isolate of *M. paratuberculosis* were grown in Dubos broth (Difco) without albumin but supplemented with 20% newborn calf serum (Gibco BRL) and 2 mg of Mycobactin J (Rhone Merieux) per liter. After incubation for 8 to 10 weeks, the cells were harvested by centrifugation and washed in phosphate-buffered saline (PBS). To prepare stock *M. paratuberculosis* DNA, cells were lysed overnight by incubation at 37°C in 50 μM Tris/HCl (pH 8.0)–100 mM EDTA–150 mM NaCl containing 1% SDS and 0.2 mg of proteinase K (Sigma, Poole, United Kingdom) per ml. After one cycle of phenol-chloroform extraction, the DNA was precipitated in 2 M ammonium acetate with an excess of 100% ethanol. After centrifugation and aspiration of the supernatant, the DNA pellet was taken up in TE buffer and stored at −20°C in 100-μl aliquots. The concentration of DNA was determined spectrophotometrically.

In spiking experiments with free *M. paratuberculosis* DNA, 30 pg of DNA was added to 15 ml of IS*900*-negative whole pasteurized cows' milk, which was then subjected to processing and subsequent IS*900* PCR as described above. *M. paratuberculosis*, cultured in liquid media by the method used, grows in tight clumps. Clumps of *M. paratuberculosis* harvested from these cultures were therefore bead beaten for 1 min with 0.4-mm-diameter glass beads (BDH) and a minibead beater (Biospec Products Inc. Bartlesville, Okla). Microscopic examination showed that most of the clumps were disaggregated by this procedure, and serial dilutions of a suspension of washed *M. paratuberculosis* could be counted with reasonable accuracy. In spiking experiments with intact *M. paratuberculosis*, 100-μl aliquots of a cell suspension were added to 15-ml samples of milk to give final concentrations of 50 to 2,000 *M. paratuberculosis* cells per ml. The spiked milk samples were then processed as described above.

Raw milk. Samples of milk (20 ml) were expressed directly into screw-cap Sterilin sample tubes from the washed teats of 24 healthy Fresian cows in the University of London Wye College herd, using fresh disposable gloves for each sampling. This inbred herd has been free of Johne's disease for many years. Six 20-ml coded samples of raw milk taken in a similar manner were obtained from the Institute of Animal Science and Health, Lelystad, The Netherlands (gift of Douwe Bakker). These were centrifuged, processed, and subjected to IS*900* PCR as described above. Finally, samples of raw milk were obtained from two cows with clinical Johne's disease (confirmed by fecal culture) and from four apparently healthy animals from the same herd on a small farm in Hampshire, England. After the udders were cleansed, washings from the outside of the teats of the animals with Johne's disease were also retained. In this case, total DNA extracts were prepared from these samples with 6 M guanidinium thiocyanate, and crude DNA was extracted on Promega Wizard resin as specified by the manufacturer. Aliquots (5 μl) of purified DNA were tested by IS*900* PCR in triplicate.

Collection of retail milk samples. Cartons and bottles of whole pasteurized cows' milk were obtained from retail outlets distributed widely throughout central and southern England continuously week by week from 1 September 1991 to 31 March 1993. In general, samples were obtained on Saturday or Sunday, kept in a domestic refrigerator at 4°C, and brought directly into the dedicated unit in the department on Monday morning, where they were processed and tested as described above.

Long-term culture. From the outset, we recognized that a relationship between a positive IS*900* PCR signal and the presence or absence of viable *M. paratuberculosis* in a retail milk sample would be difficult to establish for a species of mycobacterium which historically is slowly growing or impossible to culture. Although other studies in our laboratory demonstrated the ability of reverse transcriptase (RT) PCR to identify IS*900* mRNA in cultured *M. paratuberculosis* cells (28), the overall sensitivity of that approach fell short by several orders of magnitude from that required to be practically applicable to this problem. Reliance had therefore to be placed on the traditional but uncertain methods of sample decontamination followed by long-term culture.

Aliquots of centrifugal pellets and in some cases cream fractions from 18 IS*900* PCR-positive and 36 IS*900* PCR-negative retail milk samples were decontaminated by incubation overnight at room temperature with 0.1% benzalkonium chloride or 0.1 or 0.75% cetyl pyridinium chloride in 10 ml of PBS. After centrifugation and washing, the samples were resuspended in a small volume of sterile PBS, and 100-μl aliquots were inoculated into 10 ml of Dubos broth supplemented with Mycobactin J, as described above, in 25-cm^2 tissue culture flasks. The flasks were sealed and incubated at 37°C. Groups of at least 6 and in a few cases up to 27 culture flasks were established for each milk sample, together with 8 medium-only and 6 buffer controls. With great care not to introduce contamination, a 100-μl sample of culture fluid was withdrawn from an individual flask within the group of cultures derived from each of four of the IS*900* PCR-positive milk samples at intervals throughout the period of incubation. These specimens were examined by microscopy and IS*900* PCR.

Overall, 575 liquid culture flasks were incubated for between 13 and 40 months. At the end of the incubation period, the macroscopic appearance of each culture was recorded, the flasks were opened, and the contents were transferred to a Quickseal centrifuge tube made up with sterile PBS and centrifuged at 41,000 × *g* for 30 min. The resulting culture pellets were resuspended in 1 ml of residual supernatant. A 5-μl aliquot of this was placed on a microscope slide with 5 μl of formol milk (10% skim milk, 1.5% Formalin), air dried, fixed with formaldehyde vapor, stained by the Ziehl-Neelsen method, and examined microscopically. A further 850-μl portion of resuspended culture pellet in a screw-cap tube was made 6 M by the addition of solid guanidinium thiocyanate. To this was added 0.1% Tween 20, 10 mM EDTA, and 1 mM β-mercaptoethanol, and the mixture was incubated at 37°C overnight. The sample was then boiled in a water bath for 20 min and microcentrifuged to precipitate debris, and DNA was extracted from 500 μl of the supernatant by the Promega Wizard system as specified by the manufacturer. Purified DNA was eluted from the resin with 50 μl of preheated 80°C molecular biology grade water. Two 5-μl aliquots of the eluted material were subjected to IS*900* PCR as described above.

PCR contamination precautions. Stringent precautions were continuously applied to exclude and monitor the exclusion of contamination artifact from the PCR. These included the use of one dedicated laboratory located in a remote part of the building for the preparation of PCR premixes. This facility was kept locked and entered by one person only (D.M.). Laboratory coat, overshoes, and gloves were worn and changed regularly. All manipulations were performed in a class 2 safety cabinet that was UV irradiated between uses. All surfaces were cleaned with 1 M HCl at the end of each work period. All consumables for the preparation of premixes were taken directly to the premix laboratory on delivery. Premixes were stored frozen in capped centrifuge tubes, which were opened on one occasion only for collection of the 5-μl sample and addition of Mg^{2+}.

The second dedicated laboratory for milk sample processing also contained a class 2 cabinet, and all surfaces were regularly cleaned as described above. Protective clothing only was also worn in this laboratory. All pipettes were dismantled and washed after use, and the barrel was soaked overnight in 1 M HCl. Plugged tips only were used. PCR amplification and product analysis were carried out in a third separate dedicated laboratory. A molecular biology grade water sample was simultaneously brought through all processing steps throughout the preparative procedure and tested by IS*900* PCR in triplicate. An additional PCR no-template control was included with each run.

RESULTS

Tests on spiked and raw cows' milk. IS*900* PCR showed that disaggregated intact *M. paratuberculosis* spiked into whole pasteurized cows' milk gave a signal which segregated into the

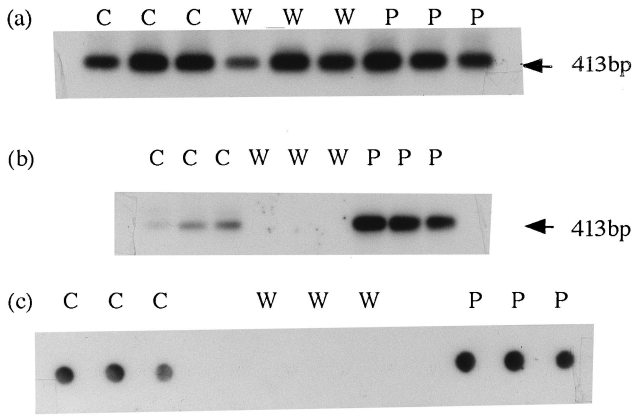

FIG. 1. Autoradiograph of IS*900* PCR specific amplification products in cream (C), whey (W), and pellet (P) fractions of whole pasteurized cows' milk after centrifugation at 41,000 × *g* for 1 h and processing as described in the text. (a) Milk sample spiked with 2 pg of free *M. paratuberculosis* DNA per ml (10 fg/5 μl); PCR products were run on agarose gel electrophoresis. (b) Milk sample spiked with 500 disaggregated *M. paratuberculosis* organisms per ml; PCR products were run on agarose gel electrophoresis. (c) Milk sample spiked with intact *M. paratuberculosis* as in panel b followed by analysis of PCR products in the dot blot format.

cream and pellet fractions after centrifugation and processing as described above (Fig. 1). The overall sensitivity of detection in spiking experiments was estimated to lie in the range of 200 to 300 *M. paratuberculosis* bacilli per ml of milk. Spiking with free *M. paratuberculosis* DNA at 10 fg/5 μl of milk (2 pg/ml) resulted in an even distribution of the IS*900* PCR signal across the centrifugal fractions.

Application of these methods to raw milk from 24 individual healthy cows in the University of London herd demonstrated the absence of detectable *M. paratuberculosis* in all milk samples in the presence of correctly positive internal controls. Six representative results are shown in Fig. 2a. Positive IS*900* PCR signals segregating principally to the cream and pellet fractions were observed in four of the six raw-milk samples from the Netherlands tested blind (Fig. 2b). When the origin of these positive samples was decoded, one (from cow 3) was from an animal with heavy fecal shedding of *M. paratuberculosis* (by culture) which also showed early clinical signs of Johne's disease and whose milk tested positive in all three fractions. Of the other three positive milk samples, two (from cows 1 and 5) were from clinically quite healthy animals, nonetheless shown by culture to be light fecal shedders of *M. paratuberculosis*. The last positive milk sample in this group, which was weakly positive by IS*900* PCR with signals in the cream and pellet fractions, came from an animal (cow 6) which was clinically quite healthy and fecal culture negative but was in a herd which also contained infected animals. The remaining two negative milk samples were from clinically healthy, fecal culture-negative animals.

The total DNA samples extracted from the raw milk of both animals with Johne's disease tested in the Hampshire herd were strongly positive by IS*900* PCR (Fig. 3). Washings from the outside of the teats were negative. All process and PCR controls were correctly negative. Milk from one of the four apparently healthy animals tested in the same Hampshire herd was also strongly positive for *M. paratuberculosis*. This milk enters the pool submitted to the pasteurization process prior to retail distribution.

Tests on retail cows' milk. Figure 4 shows the regions of central and southern England and south Wales included in the study and the sites where the 312 samples of whole pasteurized cows' milk were obtained from retail outlets. Examples of the results of IS*900* PCR tests on individual cartons and bottles are given in Fig. 5. Five of the milk samples shown in the figure exhibited strongly positive tests. In three of these, a signal was seen in cream or pellet fractions. All simultaneous process controls and no-template PCR controls were correctly negative. All PCR-positive controls were correctly positive.

Figure 6 shows the percentage of retail milk samples testing positive by IS*900* PCR, month by month from September 1991 to March 1993. Coming in surges especially in January, February, and March and in September, October, and November, when up to 25% of retail milk samples tested positive, an overall 22 of 312 samples (7.05%) contained *M. paratuberculosis* with an abundance equal to or greater than the detection limit of the system. In 18 of the 22 positive milk samples (81%), the PCR signal segregated to the cream or pellet fractions or both, consistent with the presence of intact organisms.

Figure 7A shows the results obtained with a 1-liter milk carton from a supermarket in Surrey, United Kingdom, for which the PCR amplification products were analyzed by aga-

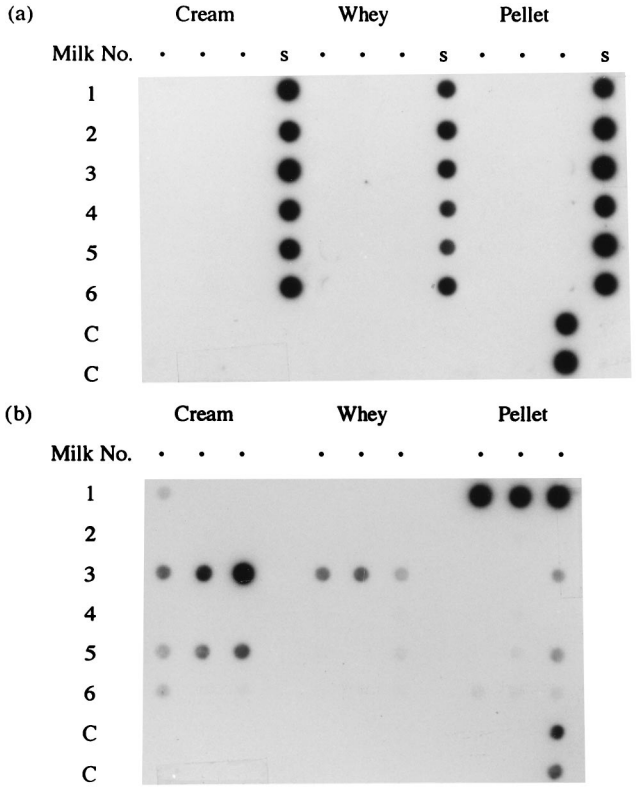

FIG. 2. IS*900* PCR for *M. paratuberculosis* with products analyzed in the dot blot format (see the text) (a) PCR-negative cream, whey, and pellet fractions from the milk of six representative cows in the healthy University of London herd in the presence of correctly positive control reactions (s) with 10 fg of *M. paratuberculosis* DNA per 5 μl of milk. C, negative simultaneous process controls with two additional PCR-positive controls. (b) IS*900* PCR for *M. paratuberculosis* in milk samples (obtained from the Institute of Animal Science and Health, The Netherlands) from the following sources: 1, a clinically healthy cow shedding *M. paratuberculosis* at 50 to 100 organisms per g of feces; 2, a healthy animal not shedding *M. paratuberculosis*; 3, a cow with Johne's disease shedding abundant *M. paratuberculosis*; 4, a healthy animal not shedding *M. paratuberculosis*; 5, a healthy animal shedding *M. paratuberculosis* at 50 to 100 organisms per g of feces; 6, an apparently healthy animal not shedding *M. paratuberculosis* by fecal culture but showing weakly positive PCR for *M. paratuberculosis* in its milk. C, negative PCR process controls and PCR-positive controls as in panel a.

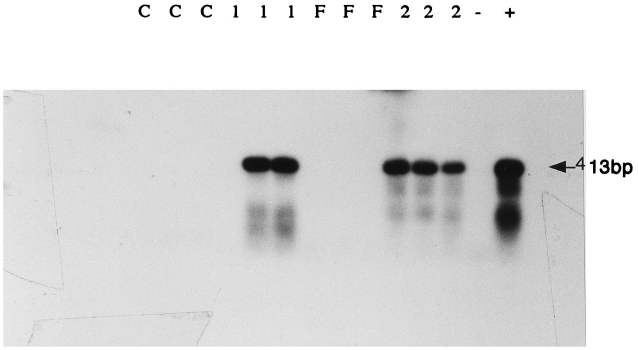

FIG. 3. IS*900* PCR applied to 5-μl portions of total DNA extract of raw-milk samples from two cows with Johne's disease (1 and 2) in a Hampshire herd together with washings from the outside of their teats (F). C, simultaneous process controls; − and +, PCR-negative and -positive controls, respectively. The milk from both animals was strongly positive for *M. paratuberculosis*.

rose gel electrophoresis and autoradiography. Strongly positive signals are seen in the cream and pellet fractions, typical of the presence of intact mycobacterial cells. Figure 7B and C shows the appearance of a representative liquid culture from this milk sample after 4 weeks of incubation. Typical acid-fast organisms were seen in clumps and clusters, characteristic of the morphology of *M. paratuberculosis* growing in liquid media. This flask tested strongly positive by IS*900* PCR (data not shown). Despite decontamination of the milk samples as described above, florid overgrowth by other organisms occurred in a substantial majority of the long-term liquid culture flasks. Although isolated clumps of acid-fast organisms were observed, none of the liquid cultures demonstrated an obvious abundant growth of clumped acid-fast mycobacteria when examined microscopically after 13 to 40 months of incubation. The centrifugal pellets from these cultures were, however, IS*900* PCR positive for *M. paratuberculosis* from 9 of the 18 PCR-positive milk samples (50%) and from 6 of the 36 PCR-negative samples (16%). Strongly positive PCR results were observed in the centrifugal pellets of multiple culture flasks derived from four of the PCR-positive milk samples and one of the PCR-negative samples. All the simultaneous medium-only and buffer controls incubated over the same period were IS*900* PCR negative.

DISCUSSION

Although the inclusion of a centrifugation step in milk sample processing followed by PCR testing of each of the cream, whey, and pellet fractions in the present study was laborious, it did permit some distinction to be drawn between the distribution of the PCR signal due to free target DNA and that due to intact lipophilic bacillary-form *M. paratuberculosis*. With free DNA, the PCR signal first occurred either in the whey or across all three centrifugal fractions. With intact *M. paratuberculosis*, the PCR signal characteristically segregated to the cream and pellet fractions. Some care must, however, be taken in extrapolating these results obtained by spiking with in vitro cultured bacillary-form *M. paratuberculosis* to those seen with raw or pasteurized cows' milk, since the range of phenotypes exhibited by *M. paratuberculosis* in native cows' milk has not yet been precisely defined but is likely to include intracellular forms present within macrophages.

The methods of sample processing and IS*900* PCR worked well when tested on raw milk samples obtained directly from known *M. paratuberculosis*-free and -infected cattle and gave results in close agreement with the predicted results. Twenty-four cows from the University of London Wye College herd known to be free of *M. paratuberculosis* over many generations all gave uniformly negative IS*900* PCR tests in all three centrifugal milk fractions in the presence of correctly reporting positive and negative PCR controls and simultaneous process

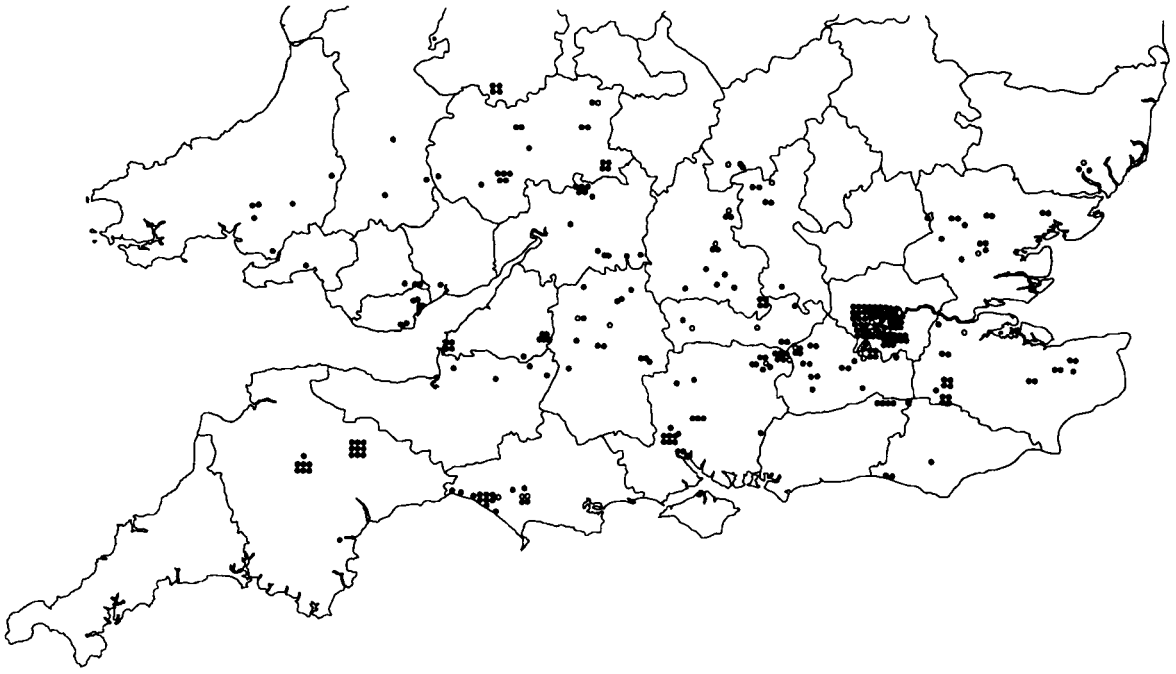

FIG. 4. England and Wales south of Birmingham, showing the sites from which 312 samples of whole pasteurized cows' milk were obtained from retail outlets week by week over the period 1 September 1991 to 31 March 1993. The sites are indicated in terms of negative (●) or positive (○) samples.

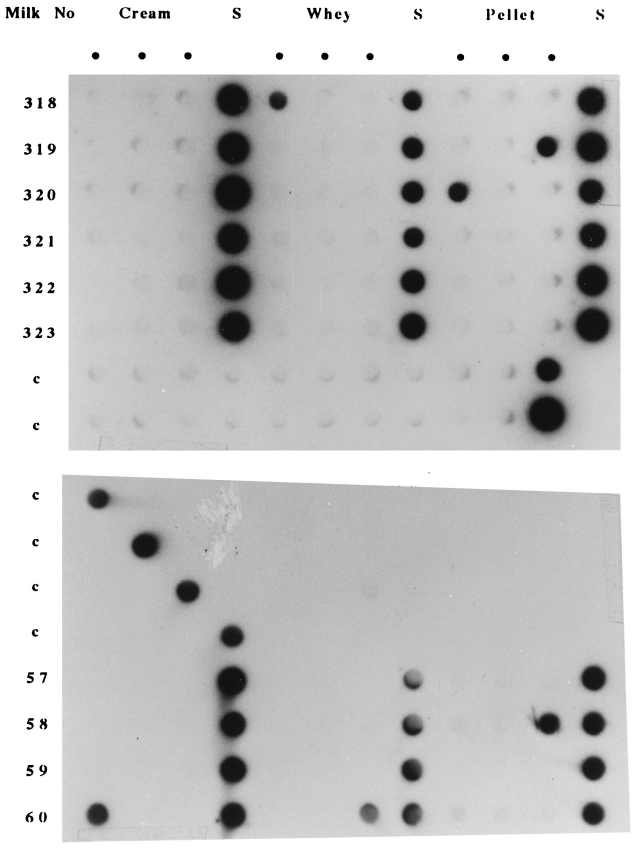

FIG. 5. IS*900* PCR tests for *M. paratuberculosis* in dot blot format together with negative and positive controls and process controls (c and S) applied to cream, whey, and pellet fractions from 10 samples of whole pasteurized cows' milk obtained from retail outlets in central and southern England. Samples 318 to 323 were obtained from the Oxfordshire and Buckinghamshire region, and samples 57 to 60 were obtained from Dorset. Samples 318, 319, 320, 58, and 60 are all positive for *M. paratuberculosis*. Three of the five *M. paratuberculosis*-positive milk samples had PCR signals in the cream or pellet fractions or both but not in the whey fraction, consistent with the presence of intact organisms.

controls. Strongly positive IS*900* PCR signals, particularly in the cream or pellet fractions, or both, of raw milk, correctly identified both clinically diseased and subclinically diseased cows shedding *M. paratuberculosis* in their feces. One healthy, fecal culture-negative cow from a herd with Johne's disease was weakly positive by IS*900* PCR testing of cream and pellet fractions, suggesting that milk may be an appropriate test sample to detect latent *M. paratuberculosis* infections. PCR-positive and -negative controls and simultaneous process controls all gave the correct results. Results obtained with external washings showed that positive PCR tests on milk were not due to contamination from the outside of the udder.

In a recent telephone survey of veterinary practices and farms in southwestern England, 1% of farms were reported as having cattle with Johne's disease, with about 2% of the animals in the herds being clinically affected (4). Studies carried out in the 1950s showed that in herds containing clinically diseased animals, a substantial proportion of apparently healthy animals in the same herd were subclinically infected with *M. paratuberculosis* (14). A recent study in which IS*900* PCR was performed on intestinal tissues and mesenteric lymph nodes obtained at slaughter, showed that 3.5% of cows throughout southwestern England tested positive for *M. paratuberculosis* in the absence of clinical or gross pathological signs of Johne's disease (5). Subclinically infected cows may shed *M. paratuberculosis* in their feces and milk (36, 39), as we also show here. Taken together, these data indicate that milk containing *M. paratuberculosis* shed by subclinically infected cows must be entering the pool presented to pasteurization processes in the United Kingdom and, indeed, elsewhere in Europe.

The present study found that an overall 7% of cartons or bottles of whole pasteurized cows' milk, obtained at random from retail outlets throughout central and southern England over a 19-month period, tested positive for *M. paratuberculosis* by IS*900* PCR. Positive test results clustered in the peak periods from January to March and September to November, with up to 5 months of negative testing in between. At peak periods, up to 25% of retail cartons or bottles were affected. In most of the positive samples, the distribution of the PCR signal in centrifugal milk fractions was consistent with the presence of intact organisms. However, these could have been due to the presence of intact dead mycobacterial cells. Viability is difficult to determine for *M. paratuberculosis* in conventional culture, because of the requirement for sample decontamination and the very slow growth or unculturable nature of the organism, particularly of human isolates (9, 10, 12, 18, 21, 32, 41). Further investigations of retail pasteurized milk, taking advantage of the advent of accurate quantitative PCR and recently developed processing methods (30), are suggested.

The methods used here for the sample processing and detection of *M. paratuberculosis* in cows' milk by IS*900* PCR were developed and optimized in the first half of 1991. They were applied throughout this study so that the results would be comparable. The overall lower limit of detection of these methods of 200 to 300 *M. paratuberculosis* organisms per ml of milk, established with reasonable accuracy by spiking with carefully counted disaggregated cultures, was not very sensitive but was more sensitive than an earlier estimate (29). Simpler, faster, and much more sensitive DNA-based tests for *M. paratuberculosis* in milk and other samples could undoubtedly be developed from the solid-phase hybridization capture methods for sample processing which have since become available (30).

Two studies have shown that laboratory conditions simulating pasteurization which ensure the destruction of *M. bovis* do not cause complete inactivation of *M. paratuberculosis* (7, 19). In the most recent of these, residual viable *M. paratuberculosis*

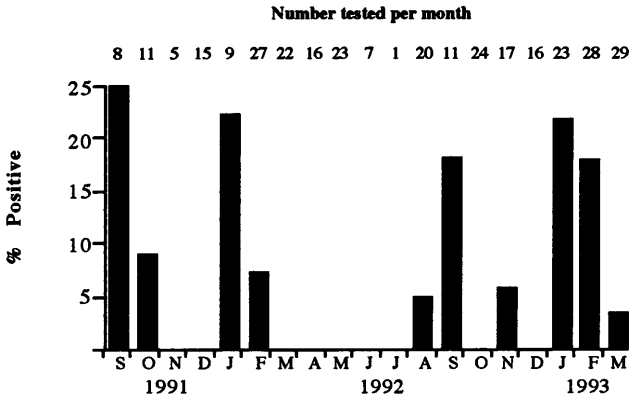

FIG. 6. Percentage of cartons and bottles of retail supplies of whole pasteurized cows' milk which tested positive by IS*900* PCR for *M. paratuberculosis* month by month from 1 September (S) 1991 to 31 March (M) 1993. Positive tests occur particularly frequently in January to March and in September to November. A total of 312 units of milk were tested; 22 (7.05%) were positive for *M. paratuberculosis*.

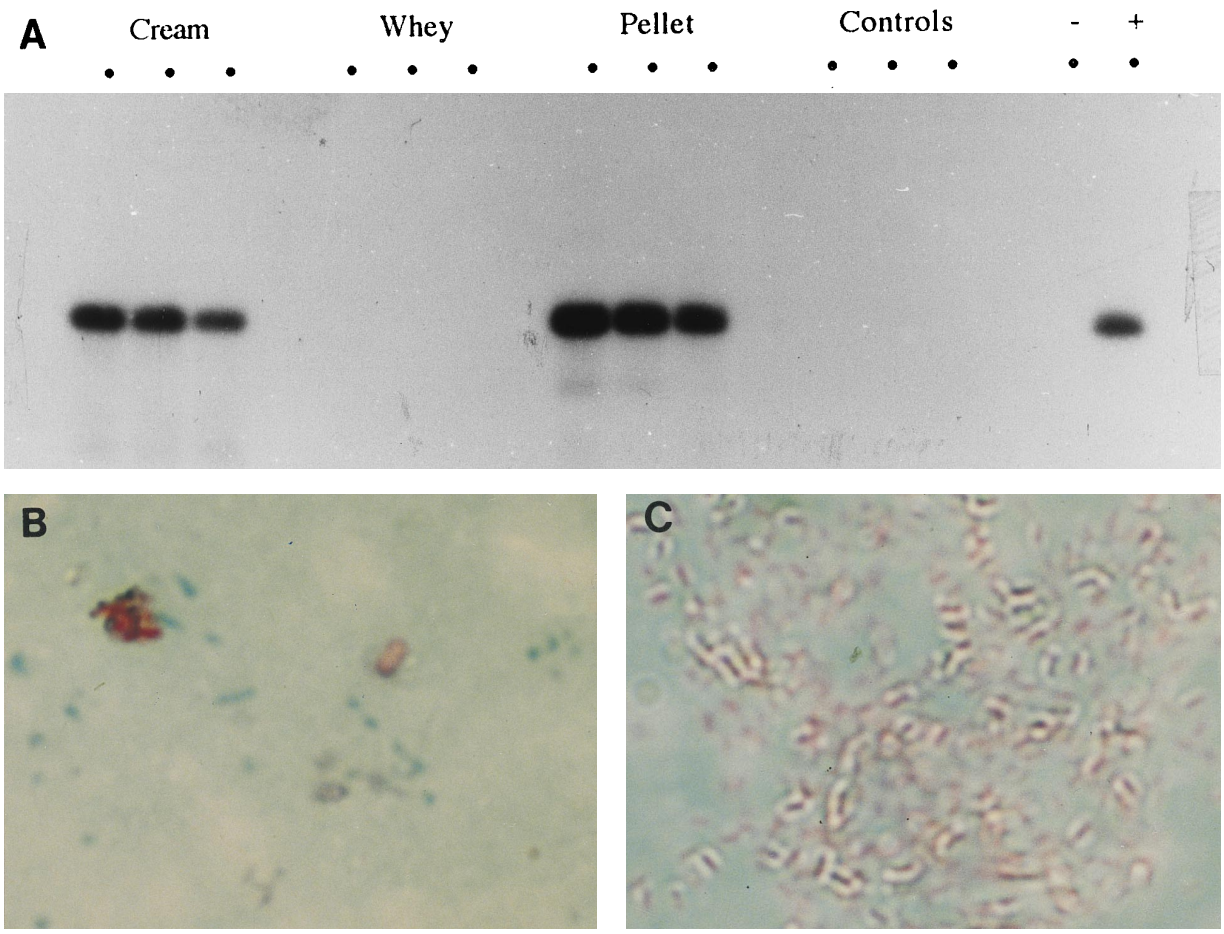

FIG. 7. (A) Agarose gel electrophoresis, Southern blotting, and autoradiography of the products of IS*900* PCR for *M. paratuberculosis* performed on cream, whey, and pellet fractions of a 1-liter carton of retail milk from a supermarket in Surrey, England. PCR is strongly positive for *M. paratuberculosis*, with signals in cream and pellet fractions only, consistent with the presence of intact organisms. (B and C) Microscopic appearances (magnification, ×3,000) of a liquid culture from this milk carton after 4 weeks of incubation showing clumps and groups of small acid-fast bacilli. This liquid culture was also strongly positive for *M. paratuberculosis* by IS*900* PCR.

organisms were found in 55% of milk samples spiked with 10^4 CFU/ml followed by exposure to 71.7°C for 15 s, the conditions of the high-temperature short-time commercial pasteurization method. In the present study, 50% of the liquid cultures from IS*900*-positive milk samples and 16% of cultures from PCR-negative milk samples were IS*900* positive in their centrifugal pellets after up to 40 months of incubation, despite florid overgrowth by other organisms. The subsequent occurrence of PCR-positive liquid cultures derived from milk samples which were originally PCR negative for *M. paratuberculosis* suggests that the PCR test, when performed by the methods used, underreported the true incidence of contaminated retail milk supplies in this study. The presence of a very slowly growing residual population of *M. paratuberculosis* cells in retail milk in England, particularly during peak periods, cannot be excluded.

Studies at the University of Pennsylvania have shown that administration of *M. paratuberculosis* as an oral bolus to healthy Guernsey heifers resulted in a period of fecal excretion which peaked at days 3 to 4 and was over by day 7 (38). At autopsy of the clinically unaffected animals 28 days later, the mesenteric lymph nodes were enlarged, with reactive follicles. However, *M. paratuberculosis* was not identified in these nodes by microscopy or culture. Although the gut itself appeared macroscopically and microscopically normal, *M. paratuberculosis* was cultured from the normal ileal mucosa of all the animals, suggesting that this enteric pathogen was retained by this region of the intestine. If a similar mechanism operates in other species, repeated exposure may result in the cumulative acquisition of a resident population of *M. paratuberculosis* in the human intestine. After months or years, this may lead to the emergence of a paucimicrobial chronic enteritis and clinical disease in people with an inherited or acquired susceptibility.

ACKNOWLEDGMENTS

This research carried out over the period from 1990 to 1995 was funded largely by the charity Action Research and the Ministry of Agriculture Fisheries and Food, to which we express our appreciation. Additional support, for which we are most grateful, was also provided at critical times by The Dinwoodie Trust and The Ileostomy Association.

We thank Carole Snalune, Jill Lake, and Duncan McGlashen for all they contributed over the period; we also thank the many people who helped to obtain milk samples. We are grateful to Katie Snalune and to Douwe Bakker and Jan Haagsma, Institute for Animal Science and Health, Lelystad, The Netherlands, for sending samples and to Rod Chiodini and Robin Offord for useful discussion.

REFERENCES

1. **Brotherston, J. G., N. J. L. Gilmour, and J. McSamuel.** 1961. Quantitative studies of *Mycobacterium johnei* in the tissues in sheep. I. Routes of infection and assay of viable *M. johnei*. J. Comp. Pathol. **71:**268–299.
2. **Buergelt, C. D., C. Hall, K. McEntee, and J. R. Duncan.** 1978. Pathological evaluation of paratuberculosis in naturally infected cattle. Vet. Pathol. **15:** 196–207.
3. **Carrigan, M. J., and J. T. Seaman.** 1990. The pathology of Johne's disease in sheep. Aust. Vet. J. **67:**47–50.
4. **Çetinkaya, B., K. Egan, and K. L. Morgan.** 1994. A practice-based survey of the frequency of Johne's disease in south west England. Vet. Rec. **134:**494–497.
5. **Çetinkaya, B., K. Egan, and K. L. Morgan.** 1996. An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England. Epidemiol. Infect. **116:**373–379.
6. **Chiodini, R. J.** 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. Clin. Microbiol. Rev. **2:**90–117.
7. **Chiodini, R. J., and J. Hermon-Taylor.** 1993. The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurisation. J. Vet. Diagn. Invest. **5:**629–631.
8. **Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal.** 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. **74:**218–262.
9. **Chiodini, R. J., H. J. Van Kruiningen, R. S. Merkal, W. R. Thayer, and J. A. Coutu.** 1984. Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. J. Clin. Microbiol. **20:**966–971.
10. **Chiodini, R. J., H. J. Van Kruiningen, W. R. Thayer, R. S. Merkal, and J. A. Coutu.** 1984. Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified *Mycobacterium* species isolated from patients with Crohn's disease. Dig. Dis. Sci. **29:**1073–1079.
11. **Clarke, C. J., and D. Little.** 1996. The pathology of ovine paratuberculosis: histological and morphometric changes and correlations in the intestine and other tissues. J. Comp. Pathol. **114:**419–437.
12. **Coloe, P., C. R. Wilkes, D. Lightfoot, and F. A. Tosolini.** 1986. Isolation of *Mycobacterium paratuberculosis* in Crohn's disease. Aust. Microbiol. **7:**188A.
13. **Dell'Isola, B., C. Poyart, O. Goulet, J.-F. Mougenot, E. Sadoun-Journo, N. Brousse, J. Schmitz, C. Ricour, and P. Berche.** 1994. Detection of *Mycobacterium paratuberculosis* by polymerase chain reaction in children with Crohn's disease. J. Infect. Dis. **169:**449–51.
14. **Doyle, T. M.** 1956. Johne's disease. Vet. Rec. **68:**869–886.
15. **Erasmus, D. L., T. C. Victor, P. J. Van Eeden, V. Falck, and P. Van Helden.** 1995. *Mycobacterium paratuberculosis* and Crohn's disease. Gut **36:**942.
16. **Fellows, I. W., J. G. Freeman, and G. K. T. Holmes.** 1990. Crohn's disease in the city of Derby, 1951–85. Gut **31:**1262–1265.
17. **Fidler, H. M., W. Thurrell, N. M. Johnson, G. A. W. Rook, and J. J. McFadden.** 1994. Specific detection of *Mycobacterium paratuberculosis* DNA associated with granulomatous tissue in Crohn's disease. Gut **35:**506–510.
18. **Gitnick, G., J. Collins, B. Beaman, D. Brooks, M. Arthur, T. Imaeda, and M. Palieschesky.** 1989. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. Dig. Dis. Sci. **34:**925–932.
19. **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.
20. **Green, E. P., M. Tizard, M. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor.** 1989. Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:**9063–9073.
21. **Haagsma, J., C. J. J. Mulder, A. Eger, and G. N. J. Tytgat.** 1991. *Mycobacterium paratuberculosis* isole chez des patients atteints de maladie de Crohn. Resultats preliminaires. Acta Endosc. **21:**255–260.
22. **Hermon-Taylor, J., M. Moss, M. Tizard, Z. Malik, and J. Sanderson.** 1990. Molecular biology of Crohn's disease mycobacteria. Bailliere's Clin. Gastroenterol. **4:**23–42.
23. **Johne, H. A., and L. Frothingham.** 1895. Ein eigenthuemlicher fall von tuberkulose beim rind. Dtsch Z. Tiermed. Pathol. **21:**438–454.
24. **Kyle, J.** 1992. Crohn's disease in the northeastern and northern isles of Scotland: an epidemiological review. Gastroenterology **103:**392–399.
25. **Lisby, G., J. Andersen, K. Engbaek, and V. Binder.** 1994. *Mycobacterium paratuberculosis* in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. Scand. J. Gastroenterol. **29:**923–929.
26. **McClure, H. M., R. J. Chiodini, D. C. Anderson, R. B. Swenson, W. R. Thayer, and J. A. Coutu.** 1987. *Mycobacterium paratuberculosis* (Johne's disease) in a colony of stump-tail macaques (*Macaca arctoides*). J. Infect. Dis. **155:**1011–1019.
27. **McFadden, J. J., P. D. Butcher, R. Chiodini, and J. Hermon-Taylor.** 1987. Crohn's disease isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. J. Clin. Microbiol. **25:**796–801.
28. **Millar, D. S.** 1995. *Mycobacterium paratuberculosis*, mycobacteria and chronic enteritis in humans and animals, p. 151–167. Ph.D. thesis. University of London, London, United Kingdom.
29. **Millar, D. S., J. Ford, J. D. Sanderson, M. Tizard, K. Kempsell, R. J. Lake, and J. Hermon-Taylor.** IS*900* PCR testing for *Mycobacterium paratuberculosis* (*Mptb*) in units of whole pasteurized cows milk widely obtained from retail outlets in England and Wales, p. 320. *In* R. J. Chiodini, M. T. Collins, and E. Bassey (ed.), Proceedings of the Fourth International Colloquium on Paratuberculosis 1994. International Association for Paratuberculosis Inc., Rehoboth, Mass.
30. **Millar, D. S., S. Withey, M. Tizard, J. Ford, and J. Hermon-Taylor.** 1995. Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*. Anal. Biochem. **226:**325–330.
31. **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.
32. **Pavlic, I., L. Bejckova, S. Koskova, B. Fixa, O. Komarkova, and J. Bedrna.** DNA fingerprinting as a tool for epidemiological studies of paratuberculosis in ruminants and Crohn's disease, p. 279–289. *In* R. J. Chiodini, M. T. Collins, and E. Bassey (ed.), Proceedings of the Fourth International Colloquium on Paratuberculosis 1994. International Association for Paratuberculosis Inc., Rehoboth, Mass.
33. **Ridley, D. S.** 1974. Histological classification and the immunological spectrum of leprosy. Bull. W. H. O. **51:**451–465.
34. **Rose, J. D. R., G. M. Roberts, G. Williams, J. F. Mayberry, and J. Rhodes.** 1988. Cardiff Crohn's disease jubilee: the incidence over 50 years. Gut **29:**346–351.
35. **Sanderson, J. D., M. Moss, M. Tizard, and J. Hermon-Taylor.** 1992. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Gut **33:**890–896.
36. **Streeter, R. N., G. F. Hoffsis, S. Bech-Nielson, W. P. Shulaw, and D. M. Rings.** 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. **56:**1322–1324.
37. **Suenaga, K., Y. Yokoyama, K. Okazaki, and Y. Yamamoto.** 1995. Mycobacteria in the intestine of Japanese patients with inflammatory bowel disease. Am. J. Gastroenterol. **90:**76–80.
38. **Sweeney, R. W., R. H. Whitlock, A. N. Hamir, and A. E. Rosenberger.** 1992. Isolation of *Mycobacterium paratuberculosis* after oral innoculation in uninfected cattle. Am. J. Vet. Res. **53:**1312–1314.
39. **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.
40. **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.
41. **Thorel, M.-F.** 1989. Relationship between *Mycobacterium avium, M. paratuberculosis* and mycobacteria associated with Crohn's disease. Ann. Rech. Vet. **20:**417–429.
42. **Wall, S., Z. M. Kunze, S. Saboor, J. Soufleri, P. Seechurn, R. Chiodini, and J. J. McFadden.** 1993. Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. J. Clin. Microbiol. **31:**1241–1245.