Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Food Microbiology 110 (2006) 201–208
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Automated high-throughput immunomagnetic separation-PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk

Christoph Metzger-Boddien [a], Daryush Khaschabi [b], Michael Schönbauer [c], Sylvia Boddien [a], Thomas Schlederer [d], Johannes Kehle [a,*]

[a] *AnDiaTec GmbH & Co. KG, Leibnizstr. 11, 70806 Kornwestheim, Germany*
[b] *Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Institut für Veterinärmedizinische Untersuchungen, Langer Weg 27, 6020 Innsbruck, Austria*
[c] *Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Institut für Veterinärmedizinische Untersuchungen, Robert Koch-Gasse 17, 2340 Mödling, Austria*
[d] *Aureon Biosystems GmbH, Simmeringer Hauptstr. 24, 1110 Wien, Austria*

Received 1 August 2005; received in revised form 11 November 2005; accepted 31 January 2006

Abstract

Two monoclonal antibody-mediated immunomagnetic separation PCR kits (AnDiaTec *ParaTub-S*® IMS-PCR-ELISA and *ParaTub-SL*® IMS-real time PCR) were developed and evaluated for the automated high-throughput detection of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) in bulk milk of naturally infected dairy herds and made commercially available. *M. paratuberculosis* are first isolated from milk by high-throughput immunomagnetic bead separation using a completely automated magnetic particle pipetting robot within 45 min and released subsequently for analysis directly into PCR amplification mixtures for *real time* PCR or for PCR-ELISA. The threshold detection level and specificity of the tests were evaluated first with different *M. paratuberculosis* pure cultures and artificially contaminated (spiked) bulk milk samples. Both experiments proved a good detection limit, specificity and reliability of the tests that consistently detected 20 or less *M. paratuberculosis* organisms from cattle, deer and mufflon in 1 ml milk. Experiments with more than 200 bulk milk samples that were tested in parallel with the PCR methods and with the cultural method in a second evaluation study demonstrated that both PCR tests are superior to culture and sufficiently sensitive to detect single shedders in pooled milk samples. The experiments proved that the newly developed tests are sensitive, specific and fast, and thus for the first time allow the standardized large-scale routine *M. paratuberculosis* screening of bulk milk samples at acceptable costs.

Keywords: *M. paratuberculosis*; Raw milk; Automated; High-throughput; IMS-PCR; PCR-ELISA; Real time PCR

1. Introduction

Mycobacterium avium subsp. *paratuberculosis*, an acid-fast mycobactin-dependent bacterium, is the etiologic agent of paratuberculosis, also known as Johne's disease, a chronic and incurable granulomatous enteric disease affecting cattle, sheep, goats and other ruminants (Cocito et al., 1994). The disease occurs throughout the world and the economic losses to dairy and beef cattle producers are severe because of reduced milk production and poor reproductive performance (Ott et al., 1999). Animals mostly get infected in the first weeks of life via the colostrum, contaminated milk or feces. After a prolonged incubation period the typical diarrhea, progressive emaciation and decreased milk production may not develop until the age of 3 to 5 years. During the incubation period, *M. paratuberculosis* is shed intermittently in low numbers in feces and milk; in contrast, clinically infected animals can shed as many as 5×10^{12} *M. paratuberculosis* organisms day^{-1} in feces (Cocito et al., 1994; Clarke, 1997). How many organisms are present in naturally infected milk emanating from a diary herd with Johne's disease cannot be accurately determined due to deficiencies in current methodology for the isolation of *M. paratuberculosis* from milk (Dundee et al., 2001; Grant and Rowe, 2001). Clinically infected animals maintain a regular appetite and

* Corresponding author. Tel.: +49 7154 807195; fax: +49 7154 807197.
E-mail address: jk@andiatec.com (J. Kehle).

0168-1605/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2006.01.042

normal body temperature at the onset of disease, so a farmer may not be aware that a Johne's disease problem exists in his herd to isolate these animals immediately. Consequently, infected animals introduce *M. paratuberculosis* into the environment via feces and into the food chain via milk and meat.

Public health concerns about the presence of *M. paratuberculosis* in milk supplies are presently increasing due to accumulating data that link *M. paratuberculosis* to human Crohn's disease (CD), a chronic, incurable, low-grade inflammation of the terminal ileum. The reason for this assumption is that the pathogen has been identified in a substantial portion of intestinal biopsy tissues from patients with CD (Chamberlin et al., 2001; Sechi et al., 2001; Hermon-Taylor and Bull, 2002). Whether the presence of *M. paratuberculosis* in biopsy material indicates that this organism has a causative role in CD or is simply a complicating infection is still the subject of much debate. Although a definite association is still not proven, the suggestion has been made that milk could be the possible vehicle of transmission of *M. paratuberculosis* from clinically or subclinically infected cattle to humans (Hermon-Taylor, 1993; Thompson, 1994), as it has even been shown that the organism can survive to some extent the pasteurisation of milk (Grant et al., 1998a,b, 2002a,b). This explains the interest in testing milk supplies for the presence of this pathogen.

Determination of the incidence of *M. paratuberculosis* in milk supplies is fraught with difficulties. First, *M. paratuberculosis* is an extremely slow-growing organism which may take up to 20 weeks for primary isolation, thus making the cultural method inapplicable for testing milk supplies prior to direct consumption or further processing. Molecular techniques such as PCR could potentially provide a rapid means of detecting *M. paratuberculosis* in milk. However, due to inhibitory substances in milk (Rossen et al., 1992; Bickley et al., 1996; Wilson, 1997), appropriate sample preparation prior to PCR detection of organisms is crucial in order to ensure that the PCR reaction is able to proceed under optimal conditions to maximise sensitivity. Recently, immunomagnetic separation (IMS)-PCR methods were described for the isolation of *M. paratuberculosis* from milk (Grant et al., 1998a,b, 2000; Stratmann et al., 2002; Khare et al., 2004). The IMS procedure proved to be very effective in separating the desired organism from a heterogeneous suspension of microorganisms, such as are found in milk samples and from substances in milk which could inhibit the PCR reaction, additionally concurrently concentrating the bacteria from a larger sample volume. However, all of the so far described IMS-PCR methods are labor and time intensive, and thus not suitable as rapid routine diagnostic systems for a large number of bulk milk samples.

Our objective was to develop a commercially available monoclonal antibody-mediated immunomagnetic separation PCR system for the automated high-throughput detection of *M. paratuberculosis* that would be suitable as large-scale routine diagnostic test for bulk milk samples. Thereby, the most important aim would be establishing the IMS procedure for a magnetic particle pipetting robot enabling the non-labor intensive, fast and standardized detection of *M. paratuberculosis* from milk. The results of the evaluation studies for two automated IMS-PCR test formats (PCR-ELISA and *real time* PCR on the ABI PRISM System) that were developed as novel assays for screening milk samples for the presence of *M. paratuberculosis* are described.

2. Materials and methods

2.1. Bacteria

M. avium subsp. *paratuberculosis* were field strains originally isolated from cattle (strains 6095, 6165, 1305, 2034, 834, 442 and reference strain Arnsberg), deer (strains 735 and 44965) and mufflon (strain 626). The presence of the IS900 and the recently described IS*Mav2* (Strommenger et al., 2001) elements was confirmed by PCR as described previously (Moss et al., 1992; Strommenger et al., 2001). The strains were subcultured regularly on Herrold's egg yolk medium with Mycobactin J (Synbiotics, München, Germany). Cell suspensions were made by scraping off the bacteria of clearly visible colonies and resuspending them by vortexing in phosphate-buffered saline (PBS). Subsequently, the organisms were counted as described below and stored at −20 °C for short periods. *Mycobacteria* other than *M. paratuberculosis* (used for specificity testing) were: *M. avium* subsp. *avium* (strains ATCC 25291, ATCC 35714, ATCC 15769 and ATCC 16741), *M. smegmatis* (ATCC 19420), *M. bovis* (strains ATCC 19210 and ATCC 19211), *M. scrofulaceum* (ATCC 19981), *M. kansasii* (ATCC 14471) and *M. intracellulare* (strains ATCC 35767 and ATCC 35771), each grown as recommended by ATCC (LGC Promochem, Wesel, Germany).

2.2. Quantitation of cell number

For quantification of cell number, *M. paratuberculosis* organisms were harvested by centrifugation, diluted in PBS containing 0.05% Tween 80 after vigorous vortexing (5 min), loaded onto the platform of a Neubauer hematocytometer chamber and visually counted (Khare et al., 2004).

2.3. Preparation of spiked samples (PBS and raw milk)

For initial experiments, raw milk samples were obtained from healthy cattle belonging to four different farms with no known history of paratuberculosis that were tested for paratuberculosis by serologic ELISA and bacteriologic culture three times at 6-month intervals, with negative results each time. Further, we tested feces and bulk milk samples before starting the first experiments by PCR (Moss et al., 1992; Strommenger et al., 2001), again with negative results. Tenfold and twofold serial dilutions of intact *M. paratuberculosis* organisms were prepared from a stock suspension of 10^7 organisms. Dilutions from 10^6 to 1 organisms ml^{-1} were prepared in PBS and raw milk, respectively.

2.4. Spiking experiments with differently stored raw milk

Further spiking experiments were performed with differently stored raw milk samples. Bacterial dilutions from 10^6 to 1 organisms ml^{-1} were prepared in five different categories of raw milk (raw milk stored for 4 h at room temperature (RT), minor changes in the consistency of milk; raw milk stored for 8 h at RT,

visible ongoing creaming process; completely creamed raw milk after vigorous vortexing; raw milk frozen at −20 °C for 24 h; adulterated, sour raw milk (milk stored for 1 week at RT)).

2.5. Description of the AnDiaTec ParaTub-S® IMS-PCR-ELISA and ParaTub-SL® IMS-real time PCR kits

The *ParaTub-SL*® and *ParaTub-S*® kits (AnDiaTec GmbH and Co. KG, Kornwestheim, Germany) are divided into two and three modules, respectively. The first module includes the magnetic particles (coated with anti-*M. paratuberculosis* monoclonal antibody) and the magnetic particle wash buffer. The second module includes the ready-to-use amplification mixture and the positive and negative control. The third module (for the *ParaTub-S*® IMS-PCR-ELISA Kit) includes a micotiter plate, the denaturation solution, the hybridization probe solution, the wash buffer, the peroxidase-labelled conjugate, the tetramethylbenzidine (TMB) substrate and the stopping solution (sulphuric acid), to perform hybridization and detection of amplified products in microwells.

2.6. Principle of the analytical protocol

The following steps are necessary to perform the complete reaction: (i) automated IMS of the organisms from raw milk with the magnetic particle pipetting robot, (ii) PCR assay in a conventional thermocycler or with the ABI PRISM Real Time PCR System, and solely for PCR-ELISA (iii) hybridization and detection of amplified products in microwells.

2.6.1. Automated IMS of the organisms from raw milk with the magnetic particle pipetting robot

The magnetic particle pipetting robot was a Hamilton Microlab AT plus 2 liquid handling workstation (Hamilton, Bonaduz, Switzerland), specifically modified to be used for automated IMS (Aureon Biosystems, Wien, Austria; commercially available). The IMS is performed with 1-ml aliquots of raw milk samples, which have to be pipetted into, if necessary previously (bar code) labelled, 12 × 75 mm reaction tubes (Sarstedt, Karlsruhe, Germany) that are subsequently loaded onto the machine's sample rack. The separation program is started, after inserting the magnetic particle mixture and the magnetic particle wash buffer in designated vials into the robot. The robot pipets the required amount of magnetic particle mixture into the milk samples, captures the magnetic particles after a 30-min incubation, during which the sample–particle mixture is stirred three times, and pipets them for washing into microtiter plate wells previously filled by the robot with magnetic particle wash buffer. After washing, the particles are released directly into the ready-to-use PCR mixture (50 µl in case of the PCR-ELISA and the real time PCR) provided in microtiter plate wells (thawed and inserted into the designated workstation rack just shortly before). The complete IMS procedure is performed within 45 min.

2.6.2. PCR assay in a conventional thermocycler or with the ABI PRISM Real Time PCR System

Amplification in a conventional thermocycler (we used the Eppendorf Mastercycler; Eppendorf, Hamburg, Germany) is undertaken under the following conditions: 1 initial denaturation cycle at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 40 s; and a final extension at 72 °C for 5 min. Real time (TaqMan) PCR (we used the ABI PRISM 7000, 7500 and 7900 Real Time PCR Systems; Applied Biosystems, Foster City, USA) is carried out under the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. We defined the threshold cycle (C_T) as the cycle at which the fluorescence was significantly higher than the average standard deviation of the earlier cycles and the sequence detection application began to detect the increase in signal associated with an exponential growth of the PCR product.

2.6.3. Hybridization and detection of amplified products in microwells (PCR-ELISA)

After amplification with the *ParaTub-S*® IMS-PCR-ELISA amplification mixture, 30 µl of PCR product is incubated with 20 µl of denaturation solution in a 96-well microtiter plate for 15 min at room temperature to denature amplified DNA into single-strand DNA. Subsequently, hybridization and detection of amplified DNA is performed on the *ParaTub-S*® microtiter plate. For hybridization, 50-µl aliquots of denatured amplified DNA plus 200 µl hybridization buffer are pipetted in each microwell. The microtiter plate is then incubated with agitation for 1 h at 37 °C. After washing the plate five times with wash buffer, 100 µl peroxidase-labelled conjugate is added to each well and the plate incubated with agitation for 30 min at 37 °C. After final washing, 100 µl of chromogen reagent is added and the color reaction is stopped by adding 100 µl stopping solution. The plates are read in a spectrophotometer at 450/620 nm.

2.7. Manually performed IMS of M. paratuberculosis from raw milk

To control the results of the automated IMS with the magnetic particle pipetting robot in initial experiments, we performed in parallel the IMS procedure manually. For this, we pipetted 10 µl of homogeneously resuspended magnetic particle mixture to 1 ml of each milk sample in 1.5 ml reaction tubes (Eppendorf) and captured the magnetic particles after a 30-min incubation, during which the reaction tubes were inverted three times, with a magnetic reaction tube rack (Aureon Biosystems). After washing the beads three times with 0.75 ml magnetic particle wash buffer, the reaction tubes were centrifuged shortly (15 s at 8000×*g*) and residual liquid removed. The magnetic particles were resuspended with 50 µl of amplification mixture and transferred into PCR tubes. The PCR was carried out as described above.

2.8. DNA extraction from milk samples with the High Pure Nucleic Acid Kit

To evaluate the threshold detection level and bacteria isolation capability of the IMS procedure on raw milk samples, we performed in parallel a DNA extraction from 1-ml aliquots

of spiked samples with the High Pure Nucleic Acid Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The DNA was eluted in 50 µl elution buffer and following, 5 µl of eluted DNA were used in the *ParaTub-SL*® IMS-real time PCR and *ParaTub-S*® IMS-PCR-ELISA tests.

2.9. Isolation of *M. avium* subsp. *paratuberculosis* from raw milk samples by the bacteriological culture method

To evaluate the sensitivity and specificity of our PCR tests, we performed a parallel blind study of detection of *M. paratuberculosis* in 243 raw milk samples (obtained from three national veterinary and food research institutes and one practitioner paying attention to a farm with known Johne's disease history. In parts, milk from fecal culture-positive cows (with symptomatic paratuberculosis) or bulk milk from herds (with up to 30 cows) with one animal tested positive by fecal culture) by the bacteriological culture method. The samples were blind coded and tested during 2 days with the *ParaTub-SL*® IMS-real time PCR and the *ParaTub-S*® IMS-PCR-ELISA tests in our laboratory and at the national veterinary and food research institutes, and subsequently used for culture as follows. A 10-ml aliquot of each milk sample was centrifuged (15 min at 2500×*g*) and the pellet was resuspended in 10 ml of freshly prepared 0.75% (wt/vol) cetylpyridinium chloride (HPC; Sigma, München, Germany). Following incubation at room temperature for 5 h and further centrifugation (15 min at 2500×*g*), the pellet was resuspended in 1 ml of Middlebrook 7H9 broth (Sigma). Two slopes of Herrold's egg yolk medium containing 2 µg Mycobactin J (Synbiotics, München, Germany) ml^{-1} (HEYM) were inoculated each with 200 µl of the resuspended pellet and incubated at 37 °C for up to 20 weeks. Slopes were examined periodically for the presence of colonies. When growth was observed, IS900 PCR (Moss et al., 1992) was used to confirm the presence of *M. paratuberculosis*. To exclude false-positive results for samples tested positive with the *ParaTub-SL*® IMS-real time PCR and *ParaTub-S*® IMS-PCR-ELISA, and tested negative with the bacteriological culture method, we cloned the amplified PCR fragments from eight of these samples for sequencing, as described below.

2.10. Cloning and sequence analysis

For cloning, we used forward and reverse primers in the PCR reaction that contained the attachment sites attB1 and attB2 for site-directed recombination of the insert into the vector pDONR221 using the GATEWAY cloning system (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. The ligation mixture was used to transform *Escherichia coli* DH5α chemically competent cells (Invitrogen) with a standard transformation protocol (Inoue et al., 1990). Resulting clones were screened by PCR with above mentioned primers and plasmid DNA was extracted from positive clones using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sequencing was performed on an automated sequencer (Amersham Pharmacia model ALFexpress II) by cycle sequencing with the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia, Freiburg, Germany). Homology searches were carried out using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results

3.1. Specificity of AnDiaTec ParaTub-S® IMS-PCR-ELISA and ParaTub-SL® IMS-real time PCR tests

First, the specificity of the tests was determined with 10 different *M. paratuberculosis* strains originally isolated from cattle, deer and mufflon, and 11 other *Mycobacteria* strains (see Materials and methods). Bacterial suspensions with 10^2 *M. paratuberculosis* or 10^6 non-*M. paratuberculosis* organisms were set up in 1 ml raw, unprocessed milk and tested with both test formats in triplicate. All 10 *M. paratuberculosis* strains

Table 1
Threshold sensitivity of *ParaTub-S*® IMS-PCR-ELISA and *ParaTub-SL*® IMS-real time PCR tests

Strain	Test	Execution	Analytical sensitivity (bacteria ml^{-1})[a]	
			in raw milk	in PBS
6095 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	10
	ParaTub-SL	Automated	5	2
		Manual	20	5
6165 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	10
	ParaTub-SL	Automated	5	2
		Manual	20	5
1305 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	10
	ParaTub-SL	Automated	5	2
		Manual	20	10
2034 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	5
	ParaTub-SL	Automated	5	2
		Manual	20	5
834 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	100	20
	ParaTub-SL	Automated	10	2
		Manual	100	10
442 (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	5
	ParaTub-SL	Automated	5	2
		Manual	20	10
Arnsberg (cattle)	*ParaTub-S*	Automated	10	2
		Manual	20	5
	ParaTub-SL	Automated	5	2
		Manual	20	10
735 (deer)	*ParaTub-S*	Automated	20	5
		Manual	20	10
	ParaTub-SL	Automated	10	2
		Manual	50	10
44965 (deer)	*ParaTub-S*	Automated	20	10
		Manual	50	20
	ParaTub-SL	Automated	10	5
		Manual	50	10
626 (mufflon)	*ParaTub-S*	Automated	10	10
		Manual	20	10
	ParaTub-SL	Automated	5	2
		Manual	20	5

[a] Results are the average of those from three reactions.

tested positive with OD_{450} absorbance values higher than 1.000 (PCR-ELISA) and C_T values lower than 30 (real time PCR), respectively. The 11 non-*M. paratuberculosis* strains consistently tested negative.

3.2. Threshold sensitivity of the PCR-ELISA and the real time PCR

This study should determine the lower level of *M. paratuberculosis* organisms that could be detected with the kits in 1 ml raw milk samples. The study was carried out on all 10 individual *M. paratuberculosis* strains. Bacteria were suspended and dilutions from 10^6 to 1 *M. paratuberculosis* organisms were set up in 1 ml raw milk and PBS, respectively. The tests were performed in triplicate with the automatic magnetic particle pipetting robot and in parallel for comparison, manually. The results for the automated IMS-PCR-ELISA gave a detection limit of approximately 10–20 cells in 1 ml raw milk and 2–10 cells in PBS (depending on the strain that was used) (Table 1). This amount of bacteria resulted in an OD_{450} absorbance value of approximately 0.3. The detection limit of the automated IMS-real time PCR was approximately 5–10 organisms in raw milk (see Fig. 1) and 2–5 bacteria in PBS (depending on the strain that was used) (Table 1) (with C_T values of approximately 32). The results for the manually performed IMS-PCR methods (both PCR-ELISA and real time PCR) gave a detection limit of ~20–100 organisms in raw milk and 5–20 *M. paratuberculosis* cells in PBS (depending on the strain that was used) (Table 1).

Further, we compared the efficiency of our IMS procedure with the DNA extraction using the High Pure Nucleic Acid Kit (Roche) by isolating bacteria and bacterial DNA, respectively, from 1 ml spiked raw milk samples for PCR-ELISA and real time PCR. The results showed that our automated IMS procedure was more efficient than the DNA extraction method, leading to higher amounts of amplification product in PCR (data not shown). The results were confirmed by three independent experiments.

Table 2
ParaTub-S® and *ParaTub-SL*® threshold detection level in differently stored raw milk samples

Category of raw milk	Strain	Detection limit (bacterial ml^{-1})	
		ParaTub-S®	*ParaTub-SL*®
		Test 1/Test 2/Test 3	Test 1/Test 2/Test 3
Raw milk stored for 4 h at RT	Arnsberg	20/20/20	20/20/20
	735	20/100/20	20/20/20
	626	20/20/20	20/20/20
Raw milk stored for 8 h at RT	Arnsberg	20/20/20	20/20/20
	735	20/20/20	20/20/20
	626	20/20/20	20/20/20
Completely creamed milk	Arnsberg	20/20/20	20/20/20
	735	20/20/20	20/20/20
	626	20/20/20	20/20/100
Raw milk frozen at −20 °C	Arnsberg	20/20/20	20/20/20
	735	20/20/20	20/20/20
	626	20/20/20	20/20/20
Sour raw milk	Arnsberg	–[a]	–[a]
	735	–[a]	–[a]
	626	–[a]	–[a]

[a] Negative results due to inhibition.

3.3. Limit of detection in differently stored raw milk samples

This experimental set-up determined the lower limit of *M. paratuberculosis* detection using the AnDiaTec tests in differently stored raw milk samples, especially with regard to a possible PCR inhibition by different fractions of the milk (after creaming of milk has started), or loss of intact bacteria due to freeze-thawing. The study was carried out on five different categories of raw milk (see Materials and methods), artificially inoculated with three different *M. paratuberculosis* strains (strains Arnsberg, 735 and 626) at six levels of contamination (0, 20, 10^2, 10^3, 10^4, 10^5 cells in 1 ml milk sample). Milk sample testing by automated IMS-PCR-ELISA and IMS-real time PCR was performed in triplicate. The results obtained with raw milk stored for 4 or 8 h at RT, completely creamed raw milk

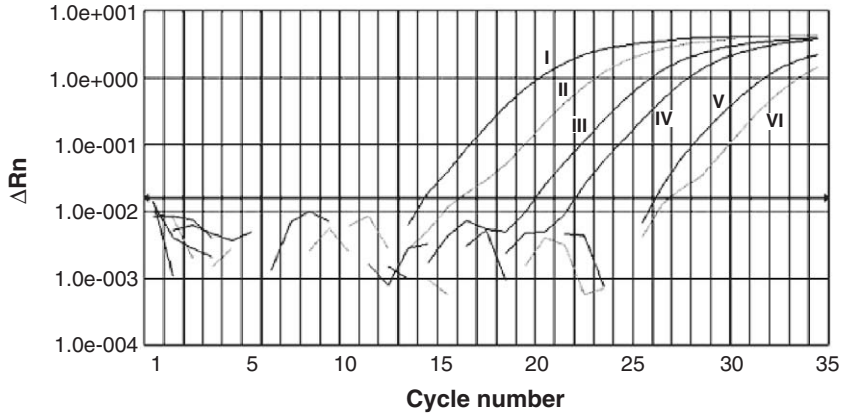

Fig. 1. Automated IMS-real time PCR testing of serial bacterial dilutions with the ParaTub-SL® kit. Amplification plots of fluorescence intensities (ΔRn) versus PCR cycle numbers are displayed for serial 10-fold bacterial dilutions (10^6 to 10 *M. paratuberculosis* organisms; strain Arnsberg) that were set up in 1 ml raw milk and tested with the ParaTub-SL® IMS-TaqMan PCR on the ABI PRISM 7000. Each plot corresponds to a particular input quantity as marked by above numbers (I: 10^6 *M. paratuberculosis* organisms in 1 ml milk; II: 10^5 bacteria/ml; III: 10^4 bacteria/ml; IV: 10^3 bacteria/ml; V: 10^2 bacteria/ml; VI: 10 bacteria/1 ml milk). Results are the average of those from three reactions.

Table 3
Evaluation study: screening for *M. paratuberculosis* in 243 raw milk samples

Method	Specimens tested	Positive	Confirmed by *ParaTub-S®*	Confirmed by *ParaTub-SL®*	Sensitivity
ParaTub-SL®	243	47	45		100%
ParaTub-S®	243	45		45	96%
Culture	243	38	38	38	81%

and raw milk frozen at −20 °C for 24 h, showed that the PCR methods could consistently detect at least 20 bacteria in all different raw milk samples (Table 2). With artificially spiked adulterated, sour raw milk, we obtained a strong inhibition of PCR and thus a negative result even with the highest level of contamination (10^5 cells/1 ml sample) (Table 2).

3.4. Evaluation study

This parameter determined the degree of correspondence of the results of AnDiaTec *ParaTub-S®* IMS-PCR-ELISA and *ParaTub-SL®* IMS-real time PCR tests to those obtained using the bacteriological culture method. The study was performed as blind study and was carried out on 243 non-contaminated or naturally contaminated bulk raw milk samples. The samples were analyzed in duplicate by both PCR tests and the cultural method in parallel in our laboratory and at national veterinary and food research institutes, as described above. Results are reported in Table 3, which gives the number of samples tested and the number of positive samples provided with each method. Of the 243 potentially contaminated milk samples tested, both AnDiaTec systems and the bacteriological method detected *M. paratuberculosis* in 38 samples, while the *ParaTub-S®* IMS-PCR-ELISA tested repeatedly 7 and the *ParaTub-SL®* IMS-real time PCR 9 additional samples positive (Table 3).

To exclude false-positive results for these samples tested positive with the AnDiaTec tests and negative with the cultural method, we cloned the amplified PCR fragment from eight of these samples for sequencing into the pDONR221 vector. Indeed, the nucleotide sequence unambiguously revealed a true-positive PCR result for *M. paratuberculosis* in all eight cases.

25 out of the 243 samples tested in the evaluation study were samples from fecal culture-positive cows (with symptomatic paratuberculosis) or bulk milk samples from herds (with up to 30 cows) with one animal tested positive by fecal culture. All 25 samples revealed positive results by PCR (*ParaTub-S®* IMS-PCR-ELISA and *ParaTub-SL®* IMS-real time PCR) (data not shown).

4. Discussion

Since the prevalence of *M. paratuberculosis* in dairy cattle is increasing and since there are rising concerns about the putative zoonotic potential of the organism (Hermon-Taylor and Bull, 2002), a simple, rapid, high-throughput, automatable and standardized test is needed for routine application in diagnostic laboratories to allow the implementation of effective control measures. Voluntary eradication programs in several European countries and in the United States (Paisly, 2001; http://europa.cu.int/comm/food/sc/scah/out38_en.pdf) have not improved the general situation (Wells and Wagner, 2000); thus, mandatory control programs need to be devised. For dairy cattle, the most economic and efficient control regime would involve regular monitoring of herd milk samples for the presence of *M. paratuberculosis*. Present techniques for the milk-based diagnosis of paratuberculosis mainly rely on the detection of antibodies in milk (Nielsen et al., 2002). However, due to the poor specificity of these tests and the fact that a positive antibody response in a herd does not necessarily indicate shedding of the organism, mandatory control measures should be based on the direct detection of the organism.

Recently, several IMS-PCR methods have been described for the direct detection of *M. paratuberculosis* in milk (Grant et al., 1998a,b, 2000; Stratmann et al., 2002; Khare et al., 2004). However, all of these methods are time and labor intensive, as the IMS procedure has to be performed manually and a DNA extraction step is needed to get high quality template DNA for PCR (Stratmann et al., 2002; Khare et al., 2004). As a consequence, these disadvantages make all the so far described antigen detection tests less suitable for a large-scale testing of bulk milk that would be needed for the implementation of effective control measures.

In the present work, we described the evaluation of two monoclonal antibody-mediated capture PCR assays which utilize two different PCR formats (PCR-ELISA and real time PCR) for the specific detection of *M. paratuberculosis* in milk. The procedures are very simple, rapid (90 samples in 3 h by IMS-real time PCR or 5 h by IMS-PCR-ELISA), reducing the "hands-on-time" to a minimum as performed fully automated, and standardized, thus fulfilling the needs for a routine, large-scale application as urgently required in diagnostic laboratories.

Both assays proved to be very specific, detecting different *M. paratuberculosis* strains from different hosts (cattle, deer and mufflon), with no cross-reactivity to other mycobacteria. Further, the lower limit of detection that we obtained with artificially inoculated bulk milk was comparable to the IMS results obtained with previously published procedures (Grant et al., 1998a,b, 2000; Stratmann et al., 2002; Khare et al., 2004). In particular, this meant that we could detect 5–10 bacteria ml^{-1} milk with the automated IMS-real time PCR and 10–20 organisms with the automated IMS-PCR-ELISA test. The detection limit was lower when the IMS was performed with the magnetic particle pipetting robot than when performed manually, most probably due to the more standardized execution of each washing and recovery step. This result showed that our automatable tests are superior to previously published procedures not only due to the fact that they are much faster, but also with regard to the overall reliability and standardization in detecting *M. paratuberculosis* in milk. It is also noteworthy that the IMS efficiency was reduced by factor up to 10 when unprocessed milk was compared with PBS, however better than the Roche DNA extraction method in terms of threshold

detection level, thereby additionally much less labor and time intensive.

Important logistic questions of future control and eradication programs for *M. paratuberculosis* will include aspects such as sample transport, storage of milk samples and/or further processing before testing. Thus, in further experiments, we determined the detection limit of the assays in differently stored artificially inoculated raw milk samples, also to find out which conditions would be suitable to retain intact bacterial cells in sample materials. We included partially and completely creamed milk, frozen milk and sour milk in our experiments and determined the lower level of *M. paratuberculosis* that could still be detected with our tests. Our experiments unambiguously revealed a very good reliability of the test with all different milk samples except for the sour milk. The sour milk was the only sample type artificially contaminated that could not be tested positive, possibly due to pH levels that inhibited the antibody-antigen binding (pH of ~5).

In a further evaluation study, we tested 243 non-contaminated or naturally contaminated bulk milk samples from Germany and Austria with both PCR assays in comparison to the bacteriological culture method. 38 samples were tested positive with all three methods. Seven samples were tested positive with both IMS-PCR methods and negative with culture, while two more samples were positive only with the IMS-real time PCR. To exclude a false-positive result for these samples, we cloned and sequenced the PCR amplicons from eight of these. Sequencing unambiguously revealed a true-positive result for *M. paratuberculosis* in all eight cases. The nucleotide sequences further demonstrated that these samples contained eight different strains. Thus, the negative results in culture were false negative, possibly due to that either non-culturable or, more probably, too few *M. paratuberculosis* organisms were included in these samples. Thus, for the determination of sensitivity, 47 samples were calculated as true positive and 196 as true negative. The sensitivity of the cultural method was 81% (38/47) and of the *ParaTub-S*® test 96% (45/47) (Table 3). In addition, we showed that our PCR tests could be used successfully to identify cows with acute paratuberculosis and were sufficiently sensitive to detect single shedders in pooled milk samples. Ongoing studies will possibly show when and to what extent *M. paratuberculosis* is shed intermittently in milk by asymptomatic cows. This information will possibly allow the establishment of more clear testing regiments in future control and eradication programs.

In this study, we have demonstrated the potential of two novel IMS-PCR assays for detecting *M. paratuberculosis* in bulk milk samples. The IMS-PCR assays are very simple, rapid, fully automated and standardized, allowing thus the large-scale testing of samples with minimal labor in 3–5 h (depending on the test format) and with low costs (reagent costs amount less than 10 euros/reaction). It was proven that the tests are not only very specific and sensitive in detecting minimal amounts of artificially spiked *M. paratuberculosis* organisms, but also superior in the reliability, sensitivity and overall effectiveness compared to culture. Our results show that both methods are very practical and can be applied for the diagnosis of *M. paratuberculosis* in future control and eradication programs.

Acknowledgement

We are especially grateful to Dr. Ralf Hess for comments and critical reading of the manuscript. Further, we thank Dr. R. Sting (CVUA Fellbach), Dr. J. Mandl (Tierseuchenkasse Baden-Württemberg) and Dr. G. Isa (STUA Aulendorf) for providing raw milk samples for evaluation studies.

References

Bickley, J., Short, J.K., McDowell, D.G., Parkes, H.C., 1996. Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett. Appl. Microbiol. 22, 153–158.

Chamberlin, W., Graham, D.Y., Hulten, K., El-Zimaity, H.M., Schwartz, M.R., Naser, S., Shafran, I., El-Zaatari, F.A., 2001. Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Aliment. Pharmacol. Ther. 15, 337–346.

Clarke, C.J., 1997. The pathology and pathogenesis of paratuberculosis in ruminants and other species. J. Comp. Pathol. 116, 217–261.

Cocito, C., Gilot, P., Coene, M., de Kesel, M., Poupart, P., Vannuffel, P., 1994. Paratuberculosis. Clin. Microbiol. Rev. 7, 328–345.

Dundee, L., Grant, I.R., Ball, H.J., Rowe, M.T., 2001. Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. Lett. Appl. Microbiol. 33, 173–177.

Grant, I.R., Rowe, M.T., 2001. Methods for the detection and enumeration of viable *Mycobacterium paratuberculosis* from milk and milk products. Bull. Int. Dairy Fed. 362, 41–52.

Grant, I.R., Ball, H.J., Rowe, M.T., 1998a. Effect of high-temperature, short-time (HTST) pasteurization on milk containing low numbers of *Mycobacterium paratuberculosis*. Lett. Appl. Microbiol. 26, 166–170.

Grant, I.R., Ball, H.J., Rowe, M.T., 1998b. Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. Appl. Environ. Microbiol. 64, 3153–3158.

Grant, I.R., Pope, C.M., O'Riordan, L.M., Ball, H.J., Rowe, M.T., 2000. Improved detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by immunomagnetic PCR. Vet. Microbiol. 77, 369–378.

Grant, I.R., Hitchings, E.I., McCartney, A., Ferguson, F., Rowe, M.T., 2002a. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Appl. Environ. Microbiol. 68, 602–607.

Grant, I.R., Ball, H.J., Rowe, M.T., 2002b. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl. Environ. Microbiol. 68, 2428–2435.

Hermon-Taylor, J., 1993. Causation of Crohn's disease: the impact of clusters. Gastroenterology 104, 643–646.

Hermon-Taylor, J., Bull, T., 2002. Crohn's disease caused by *Mycobacterium avium* subspecies *paratuberculosis*: a public health tragedy whose resolution is long overdue. J. Med. Microbiol. 51, 3–6.

Inoue, H., Nojima, H., Okayama, H., 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96, 23–28.

Khare, S., Ficht, T.A., Santos, R.L., Romano, J., Ficht, A.R., Zhang, S., Grant, I.R., Libal, M., Hunter, D., Adams, L.G., 2004. Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-time PCR. J. Clin. Microbiol. 42, 1075–1081.

Moss, M.T., Sanderson, J.D., Tizard, M.L., Hermon-Taylor, J., El-Zaatari, F.A., Markesich, D.C., Graham, D.Y., 1992. Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp *silvaticum* in long term cultures from Crohn's disease and control tissues. Gut 33, 1209–1213.

Nielsen, S.S., Gronbaek, C., Agger, J.F., Houe, H., 2002. Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. Prev. Vet. Med. 53, 191–204.

Ott, S.L., Wells, S.J., Wagner, B.A., 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40, 179–192.

Paisly, L.G., 2001. Economic aspects of disease monitoring with special reference to bovine paratuberculosis. Acta Vet. Scand. Suppl. 94, 17–25.

Rossen, L., Norskov, P., Holmstrom, K., Rasmussen, O.F., 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. 17, 37–45.

Sechi, L.A., Mura, M., Tanda, F., Lissia, A., Solinas, A., Fadda, G., Zanetti, S., 2001. Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. J. Clin. Microbiol. 39, 4514–4517.

Stratmann, J., Strommenger, B., Stevenson, K., Gerlach, G.F., 2002. Development of a peptide-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Clin. Microbiol. 40, 4244–4250.

Strommenger, B., Stevenson, K., Gerlach, G.F., 2001. Isolation and diagnostic potential of IS*Mav2*, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. FEMS Microbiol. Lett. 196, 31–37.

Thompson, D.E., 1994. The role of mycobacteria in Crohn's disease. J. Med. Microbiol. 41, 74–94.

Wells, S.J., Wagner, B.A., 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216, 1450–1457.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Further reading

Scientific Committee on Animal Health and Animal Welfare, European Commission, 2000. Possible Links Between Crohn's Disease and Paratuberculosis. [Online] http://europa.cu.int/comm/food/sc/scah/out38_en.pdf.