

A molecular beacon-based real-time NASBA assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk

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Abstract

A molecular beacon-based real-time NASBA assay for detection and identification of *Mycobacterium avium* subsp. *paratuberculosis* has been developed. It targets and amplifies sequences from the *dnaA* gene which are specific for this bacterium. The assay includes an internal amplification control, to allow identification of inhibited reactions. The assay was tested against 18 isolates of *M. avium* subsp. *paratuberculosis*, 17 other mycobacterial strains and 25 non-mycobacterial strains, and was fully selective in that it detected all the targets but none of the non-targets. The lowest number of cells which the assay can detect with 99% probability is 150–200 cells per reaction (as determined using pure culture suspensions). Using centrifugation and nucleic acid extraction as sample treatment, the assay was able to consistently detect 10^3 *M. avium* subsp. *paratuberculosis* cells in 20 ml artificially contaminated drinking water. With a simple detergent and enzymatic sample pretreatment before centrifugation and nucleic acid extraction, the assay was able to consistently detect 10^4 *M. avium* subsp. *paratuberculosis* cells in 20 ml artificially contaminated semi-skimmed milk. The assay will be a useful addition to the range of diagnostic tools available for the study of *M. avium* subsp. *paratuberculosis*. Crown Copyright © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Real-time NASBA; Internal amplification control (IAC); Food products; Milk

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis [1], an endemic disease of ruminants which is usually manifested by diarrhea, reduced feed intake, weight loss and eventual death [2]. In addition, this pathogen has been postulated as playing an important role in the etiology of Crohn's disease [3,4], a chronic granulomatous ileocolitis which presents several common features to ruminant paratuberculosis, and that affects human patients during the prime of life [5]. Drinking water and milk

have been postulated as important transmission vehicles of MAP to animals and potentially to humans [6–11].

The classical approach for detection of MAP is culturing; however, at least seven weeks is necessary in order to obtain visible growth on culture media [12]. Currently, rapid molecular-based methods are being developed [9–13]. Nucleic acid sequence-based amplification (NASBA) is an isothermal amplification technique [14–16] which uses three enzymes – RNase H, AMV reverse transcriptase and T7 RNA polymerase – working in concert at a low isothermal temperature (generally 41 °C). The product of a NASBA reaction is mainly single-stranded RNA, which can be detected by gel electrophoresis [17], enzyme-linked gel assay (ELGA) [18] or electrochemiluminescent detection (ECL) [19]. Alternatively, NASBA products can be

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detected in real time using molecular beacons [20] included in the reaction [21]. Molecular beacons are single-stranded nucleic acid sequence molecules that possess a stem-and-loop-structure which is double-labelled with a fluorescent dye and an universal quencher at the 5' and 3' end, respectively [20]. The loop region is a sequence complementary to a target sequence in the nucleic acid to be detected, and the stem is formed by the annealing of complementary arm sequences on the ends of the probe. When this structure is closed, the probe does not produce fluorescence because the energy is transferred to the quencher and released as heat [22]. However, during the NASBA reaction, the molecular beacon hybridizes to the target RNA, separating the reporter dye and the quencher, yielding a real-time measurable fluorescence emission directly proportional to the concentration of the target sequence [21]. The use of diverse molecular beacons labelled with different fluorophores allows multiplex detection in a single reaction [23]. This approach provides the possibility of signal detection in real time. In addition, everything is performed in a closed tube; this reduces the risk of cross-contamination and can allow high throughput and automation.

Although several PCRs for MAP have been published (e.g. [24–28]), there have been no reported NASBA assays for this bacterium. An advantageous feature of NASBA is that it abundantly produces single-stranded nucleic acid product, which is ideal for the use of hybridization-based assays [16] that can provide confirmation of the detection of the desired target. A NASBA assay could provide a useful additional analytical tool for analysis of clinical, veterinary and environmental samples. Such an assay could be used to confirm and complement results of analyses performed with PCR- and culture-based methods, such as those on Crohn's patients' samples [24]. In this paper, we describe a real-time NASBA (RTi-NASBA) assay for selective and sensitive detection of MAP. The assay contains an internal amplification control (IAC). An IAC is a non-target nucleic acid sequence present in the reaction, which is co-amplified simultaneously with the target sequence [29]. It can reveal any reaction which has failed due to malfunction of equipment, operator error, or the presence of inhibitory substances in the original sample matrix [30–32]. Finally, we show the suitability of RTi-NASBA-based methods for the detection of MAP in drinking water and pasteurized semi-skimmed milk.

2. Materials and methods

2.1. Bacterial strains, culture media and growth conditions

Thirty five *Mycobacterium* isolates (18 MAP and 17 *Mycobacterium* spp. isolates) and 25 non-*Mycobacte-*

rium spp. strains were used in this study (Table 1). The 18 MAP isolates were obtained from different countries and were of different RFLP types, as indicated in Table 1. The *M. avium* complex isolates, *M. avium* subsp. *silvaticum* and *M. intracellulare*, were chosen because they are very closely related to MAP. Unless stated, MAP strain ATCC 19698 was used to develop the RTi-NASBA assay. Mycobacteria were cultured in BBL™ Herrold's Egg Yolk Agar Slant with Mycobactin J and ANV (Becton, Dickinson and Co., Sparks, MD) and incubated at 37 °C without shaking. Non-*Mycobacterium* spp. strains were grown in brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) at 37 °C.

2.2. Nucleic acid extraction

This was carried out as previously described [33]. Mycobacterial nucleic acid was isolated from eight-week old cultures using the Nuclisens® Isolation Kit (bioMérieux bv, The Netherlands) according to the manufacturer's recommendations. Non-mycobacterial nucleic acid was isolated from liquid overnight cultures using the same kit. Nucleic acid concentration was determined using spectrophotometry at 260 and 280 nm [34].

2.3. Oligonucleotide primers and probes

The oligonucleotides used in this study are shown in Table 2. Primers MAP57F and MAP57R and molecular beacon MAP57MB were designed to target sequences from the MAP *dnaA* gene (GenBank GI41397153) which have been shown to be unique for MAP [35]. The primers amplify a 239-bp region of the *dnaA* gene. The BLAST-nv. 2.2.6 tool (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) was used to identify sequence areas suitable for the design of the MAP-specific RTi-NASBA system and to confirm that none of the selected oligonucleotides recognized any registered sequence other than from the target bacterium. All oligonucleotides were purchased from MWG Biotech AG (Germany). The target molecular beacon was labelled at the 5'-end with the fluorophore FAM, and the IAC molecular beacon was labelled with HEX. These fluorophores were selected as their fluorescent emission peaks do not overlap. Both molecular beacons used DABCYL as the quencher at the 3'-end.

2.4. IAC

The NASBA IAC was an in vitro synthesized chimeric RNA molecule containing an internal non-target sequence flanked by sequences complementary to the target NASBA primers MAP57F and MAP57R [36]. The non-target sequence was derived from the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), and contained sequences

Table 1
Bacterial strains used in this study

Bacterial strains	RFLP type	Origin	NASBA ^a	
			MAP57	IAC
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM ^b 97-1863	R01	Cow (NL)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-1855	R32	Cow (NL)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-1090	R01	Human (NL)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-1779	R09	Rabbit (UK)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-1516	R10	Human (USA)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-2186	R10	Human (NL)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 97-1796	R05	Cow (CZ)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-0016	R26	Cow (Arg)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-1876	R33	Deer (Arg)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-1574	R27	Cow (NL)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-1938	R34	Deer (CZ)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 01-0029	R09	Cow (Arg)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-2210	R04	Cow (Ven)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 01-0035	R01	Cow (Arg)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 99-2212	R04	Cow (Ven)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 01-2231		Cow (It)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> RIVM 01-2226	R01	Cow (It)	+	+
<i>M. avium</i> subsp. <i>paratuberculosis</i> ATCC ^c 19698	R01	Collection	+	+
<i>M. avium</i> subsp. <i>silvaticum</i> RIVM 98-0851			–	+
<i>M. avium</i> subsp. <i>silvaticum</i> RIVM 98-1574			–	+
<i>M. avium</i> complex RIVM 96-1246			–	+
<i>M. avium</i> complex RIVM 96-1238			–	+
<i>M. avium</i> complex RIVM 97-0615			–	+
<i>M. avium</i> complex RIVM 97-0622			–	+
<i>M. avium</i> complex RIVM 97-1643			–	+
<i>M. avium</i> complex RIVM 98-1017			–	+
<i>M. avium</i> complex RIVM 96-1127			–	+
<i>M. bovis</i> RIVM BCG P3			–	+
<i>M. bovis</i> RIVM 97-2262			–	+
<i>M. intracellulare</i> RIVM 97-1388			–	+
<i>M. kansasii</i> CSL ^d 10001			–	+
<i>M. kansasii</i> RIVM 97-2355			–	+
<i>M. malmoense</i> RIVM 98-1168			–	+
<i>M. marinum</i> RIVM 98-1853			–	+
<i>M. tuberculosis</i> RIVM 96-1604			–	+
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i> NCTC ^e 8049			–	+
<i>Acinetobacter calcoaceticus</i> NCTC 7844			–	+
<i>Bacillus cereus</i> NCTC 11145			–	+
<i>Brochothrix thermosphacta</i> NCTC 10822			–	+
<i>Citrobacter freundii</i> CSL 1516			–	+
<i>Corynebacterium bovis</i> NCTC 3224			–	+
<i>Escherichia coli</i> CSL 25922			–	+
<i>Klebsella pneumoniae</i> CSL 10T			–	+
<i>Kurthia gibsonii</i> NCIMB ^f 9758			–	+
<i>Listeria innocua</i> ATCC 5578			–	+
<i>Listeria ivanovii</i> ATCC 19119 ^g			–	+
<i>Listeria monocytogenes</i> NCTC 5214			–	+
<i>Listeria monocytogenes</i> ATCC 19114			–	+
<i>Listeria seeligeri</i> CSL 2001			–	+
<i>Micrococcus luteus</i> NCTC 8340			–	+
<i>Nocardia</i> spp. NCTC 434			–	+
<i>Propionibacterium acnes</i> NCTC 737			–	+
<i>Proteus vulgaris</i> CSL 4175			–	+
<i>Pseudomonas fluorescens</i> NCTC 10038			–	+
<i>Rhodococcus equi</i> NCTC 1621			–	+
<i>Staphylococcus aureus</i> ATCC 25923			–	+
<i>Lactococcus lactis</i> subsp. <i>lactis</i> NCTC 6681			–	+
<i>Salmonella enterica enterica</i> CSL 1015			–	+
<i>Salmonella enterica enteritidis</i> CSL E2187			–	+
<i>Salmonella enterica</i> Typhimurium CSL FMG2			–	+

Table 1 (continued)

Arg, Argentina; CZ, Czech Republic; It, Italy; NL, The Netherlands; Ven, Venezuela.

^a Qualitative results of RTi-NASBA.

^b Collection of National Institute of Public Health and the Environment (RIVM), The Netherlands.

^c American Type Culture Collection (ATCC), USA.

^d Collection of Food Microbiology Group, Central Science Laboratory, UK.

^e National Collection of Type Cultures (NCTC), UK.

^f National Collections of Industrial, Food and Marine Bacteria (NCIMB), UK.

^g Type strain.

Table 2

Oligonucleotides used in the RTi-NASBA assays for MAP and IAC construction

Use	Target sequence	Name	Type	Sequence
NASBA reaction	MAP <i>Dna A</i>	MAP57F	Forward primer	5'-CAA CGA CGA CCA AGA CGA-3'
		MAP57R	Reverse primer	5'-aat tct aat acg act cac tat agg gag aag gAG CAA ACC GAT CAC GAC A-3'
		MAP57MB	Molecular beacon	5'-FAM – CGA TCG CTG ATG AAA CCG AGC TCG TCG ATC G-DABCYL-3'
	Cms 16S rRNA	MB1	Molecular beacon	5' HEX CGC AGG AAC GTG CAG AGA TGT GCG CCC CTG CG DABCYL 3'
NASBA IAC construction	Chimeric RNA	IACF	Forward primer	5' aat tct aat acg act cac tat agg gag aag gCA ACG ACG ACC AAG ACG ACG ATG CAA CGC G 3'
		IACR	Reverse primer	5' AGC AAA CCG ATC ACG ACA GGT TGG CCC CGG CAG TCT 3'

complementary to a previously published molecular beacon [37]. The sequence of the IAC did not show any significant homology to any sequence deposited in public databases when analyzed using the BLAST-NV. 2.2.6 software. *Cms* is a quarantine plant pathogen in many countries and holding it may require an appropriate Plant Health license. *Cms* DNA is available through the authors.

2.5. RTi-NASBA

Reactions were carried out using reagents and protocols included in the NucliSens[®] Basic Kit (bioMérieux bv, The Netherlands). The assay was optimized prior to application (data not shown). The parameters which were optimized were those recommended in real-time PCR, i.e. the primers and probe concentrations giving the lowest time to positivity [38] (T_p , equivalent to threshold cycle (C_T) in real-time PCR assays) and the highest fluorescence intensity by a normalized reporter value (ΔR_n) (Perkin–Elmer Applied Biosystems User Bulletin 2 [ABI PRISM 7700 Sequence Detection System], 1997). In the optimised assay, 2.5 μ l of target template solution was added to 12.5 μ l NASBA pre-mix containing 80 mM Tris–HCl (pH 8.5), 24 mM MgCl₂, 140 mM KCl, 30% v/v DMSO, 2 mM each dNTP, 4 mM each NTP, 200 nM MAP57F and MAP57R primers and 1 fg (3600 copies) IAC. Two molecular beacons (200 nM) labelled with different reporters were used to simultaneously detect the template and the IAC (Table 2). The reaction mixture was incubated at 65 °C for 5 min

in order to destabilize the secondary structure of RNA. After being cooled to 41 °C for 5 min to allow primer annealing, 5 μ l of enzyme mixture was added. The enzyme mixture contained (per reaction) 0.08 U/ μ l RNase H, 32 U/ μ l T7 RNA polymerase, 6.4 U/ μ l AMV retrotranscriptase. Reactions were incubated at 42 °C for 100 min in an ABI Prism[®] 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) which allowed real-time monitoring of fluorescence as molecular beacon probe was bound to newly generated amplicons. The results for the samples were interpreted as positive if they met the two criteria described in the interpretation of the TaqMan assay results (observable ΔR_n and C_T values), with the exception that time to positivity (T_p ; in minutes) was used in the interpretation instead of C_T values [38].

2.6. Target specificity of the MAP RTi-NASBA assay

The capacity of the RTi-NASBA assay to discriminate between target and non-target bacteria was evaluated, using nucleic acids (1 ng in each reaction) from 35 *Mycobacterium* strains and 25 non-*Mycobacterium* strains.

2.7. Detection probability

We established the detection probability according to the method of Knuttson et al. [39]. Several loops of MAP strain ATCC 19698 were taken from a slope culture and added to 1 ml of phosphate-buffered saline

(PBS, Dulbecco's, Gibco, Paisley, Scotland). The number of cells in the suspension was determined by haemocytometer and a 10-fold dilution series was prepared in PBS. Nucleic acids were extracted from each dilution. Five RTi-NASBA reactions were performed on each extract. The number of positive signals obtained were expressed as a percentage, and compared with the number of cells in each dilution.

2.8. Artificial contamination of drinking water with MAP, and sample treatment prior to RTi-NASBA

In two replicate experiments, serially diluted samples of 100 μ l of PBS containing approximately 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 10, and 1, MAP cells quantified using a haemocytometer [33] were added to 50 ml centrifuge tubes containing 20 ml of drinking water. The tubes were centrifuged at 2000g for 10 min. The supernatant of each tube was discarded and the pellet resuspended in 1 ml PBS. The suspension was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13,000g for 5 min. The supernatant was discarded, and the pellet was resuspended in 100 μ l of PBS. Subsequently, the NucliSens[®] Basic Kit was used to extract nucleic acid, and 2.5 μ l of this suspension was used in an RTi-NASBA assay.

2.9. Artificial contamination of pasteurized semi-skimmed milk with MAP, and sample treatment prior to RTi-NASBA

In two replicate experiments, 100 μ l of PBS containing approximately 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 10, and 1, MAP cells quantified using a haemocytometer were added to 50 ml centrifuge tubes containing 20 ml of commercially available semi-skimmed milk. Triton X-100 (2.4 ml, Sigma, Saint Louis, MI, USA) and 1% w/v powdered Trypsin (Sigma, Saint

Louis, MI, USA) were added and the mixture was vortexed vigorously. The sample was incubated at 37 °C for 30 min. The tubes were centrifuged at 2000g for 30 min. The supernatant of each tube was carefully removed and the pellet resuspended in 1 ml of PBS. The suspension was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13,000g for 5 min. Subsequently, the NucliSens[®] Basic Kit was used to extract nucleic acid, and 2.5 μ l of this suspension was used in an RTi-NASBA assay.

3. Results

3.1. Target specificity of the MAP RTi-NASBA assay

Table 1 shows the results obtained. The assay detected only the MAP isolates (Table 1). Negative reactions had not been inhibited, as shown by a positive IAC signal. Fig. 1 shows the results obtained with one of the MAP strains, to give an example of the plot of positive amplification of the target sequence, and of amplification of the IAC.

3.2. Sensitivity of the MAP-specific duplex RTi-NASBA assay

Fig. 2 shows the plot of detection probability against cell number. The number of MAP cells which could be detected per reaction with 99% probability was $10^{3.754} = 5671$.

3.3. Detection of MAP in artificially contaminated drinking water using a MAP RTi-NASBA based method

Table 3(a) shows the results obtained when 20 ml samples of drinking water containing approximately

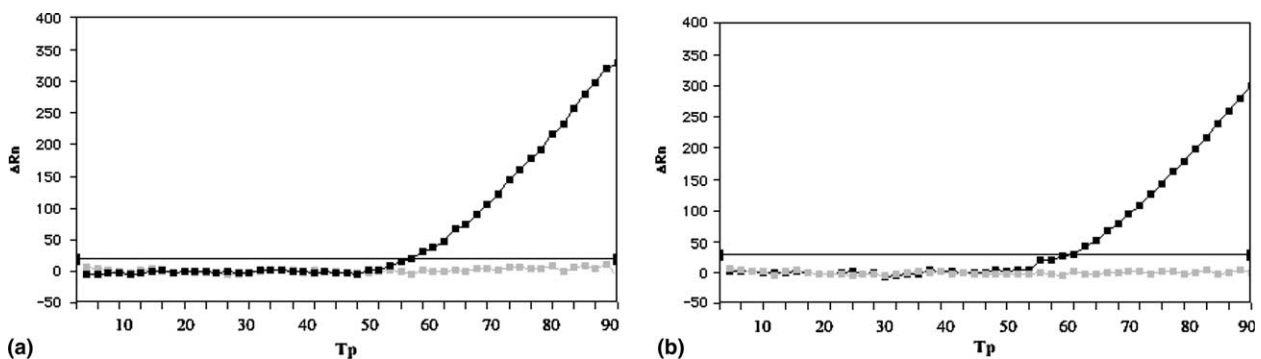


Fig. 1. RTi-NASBA detection and amplification of the sequences from the *DnaA* gene in the MAP strain ATCC 19698 (type strain). Representative amplification plots are shown (a) with MAP 57MB (FAM labeled) and (b) with MB1 (HEX labeled). In (a) the target is the *DnaA* gene (black) and in (b) the target is the IAC (black). Reactions performed without any target sequence (non-template control) are shown in grey. Y-axis: increment of fluorescence, and X-axis: time (Tp: time to positivity).

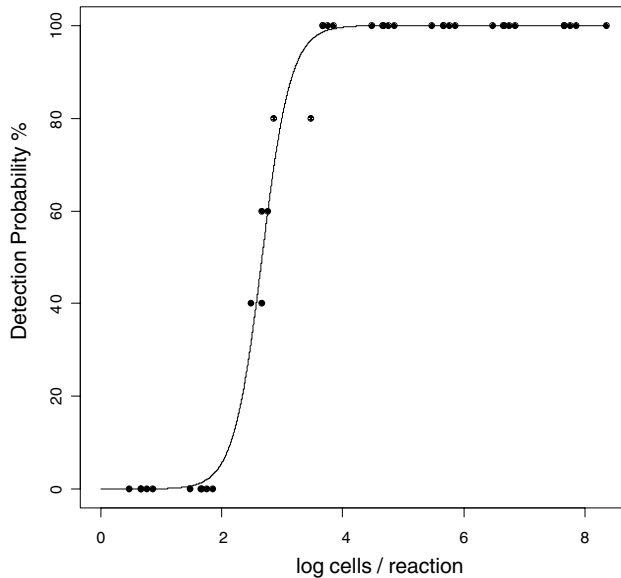


Fig. 2. Detection probability of the MAP RTi-NASBA assay.

1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 10^1 , or 10^0 MAP cells were analysed. Consistent detection (6/6) was achieved in samples containing 1×10^3 cells or more. Samples containing 1×10^2 cfu were positive in 50% (3/6) of analyses.

3.4. Detection of MAP in artificially contaminated pasteurized semi-skimmed milk using a MAP RTi-NASBA based method

Table 3(b) shows the results obtained when 20 ml samples of semi-skimmed milk containing approximately 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 10^1 , or 10^0 MAP cells were analysed. Consistent detection (6/6) was achieved in samples containing 1×10^4 cells or more. Samples containing 1×10^3 cfu were positive in approximately 33% of analyses (2/6).

Table 3
RTi-NASBA assay of artificially contaminated drinking water and semi-skimmed milk

	Log cells						
	1×10^6	1×10^5	1×10^4	1×10^3	1×10^2	10	1
<i>(a) Water</i>							
Experiment A	3/3	3/3	3/3	3/3	2/3	0/3	0/3
Experiment B	3/3	3/3	3/3	3/3	1/3	0/3	0/3
Overall	6/6	6/6	6/6	6/6	3/6	0/6	0/6
<i>(b) Milk</i>							
Experiment A	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Experiment B	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Overall	6/6	6/6	6/6	2/6	0/6	0/6	0/6

In two replicate experiments, MAP suspensions were quantified by haemocytometry and used to spike 20 ml of drinking water and pasteurized semi-skimmed milk samples which were then analyzed by RTi-NASBA based methods.

“Log cells” refers to the logarithmic unit of cells (as calculated by haemocytometer) used to artificially contaminated 20 ml of milk and water.

“Overall” refers to positive signal/total reactions from the two replicate experiments.

4. Discussion

NASBA is generally used to selectively amplify RNA [16], and this feature has been used to mediate the selective detection of viable microbial cells [34]. However, it was found that NASBA detects DNA in MAP [40], although the reason for this is unclear. Therefore, although unfortunately the NASBA assay cannot be used for selective detection of viable MAP, it could be used to confirm and complement results of analyses performed with PCR- and culture-based methods.

The routine use of a molecular-based method in the analysis of any sample type, will require controls to be applied at critical stages in the procedure. These would include a processing-positive control (a sample artificially contaminated with the target), and a processing-negative control (a sample which is known to be target-free). With regard to PCR-based approaches, the MicroVal protocol suggests that a method will not be given diagnostic status until it includes these controls and also an IAC [41,42]. This stricture should also apply to NASBA-based approaches for detection of microorganisms. In an amplification reaction with an IAC, a control signal should always be produced when there is no target sequence present [43]. When this control signal is not observed, this means that the reaction has failed, and the sample must be reanalyzed. The RTi-NASBA assay presented here, since it incorporates an IAC, provides a realistic foundation for reliable routine detection methods for MAP in several sample types.

NASBA-based methods should be useful for analyzing food and environmental samples. With such samples comes the necessity of having effective sample treatment prior to application of molecular-based detection, since they generally contain substances which can inhibit NASBA [44]. Drinking water (chlorinated)

and semi-skimmed milk were used to demonstrate that the combination of sample pretreatment, nucleic acid extraction and the RTi-NASBA assay could be a promising strategy for rapid and sensitive detection of MAP in water and milk samples, complementing those based on PCR [45,46]. Samples of matrices such as raw or full fat milk, or environmental waters, may contain more inhibitory substances, and the extraction methods might require modification to allow their effective analysis. The internal amplification control will reveal whether inhibition is occurring with any sample type, and indicate whether modification of pre-NASBA treatment is necessary.

In conclusion, we show in this paper a novel, selective and sensitive assay for detection of MAP, which should be a useful addition to the range of diagnostic tools available for the study of this pathogen.

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