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Direct detection of *Mycobacterium avium subsp. Paratuberculosis* in bovine milk by multiplex Real-time PCR

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The study aimed at direct detection of *Mycobacterium avium* subsp. *Paratuberculosis* (*MAP*) in milk by evaluating a multiplex real-time PCR assay targeting IS900 and ISMAV2 sequences including the amplification of PUC19-plasmid as internal control. The sensitivity of the assays was evaluated by testing *MAP* isolates in broad linear range of DNA (50 ng – 5 fg/µl). For the validation of the specificity, 6 *MAP* isolates and 22 isolates of genus *Mycobacteriaceae* were tested. Results revealed that reproducible detection limit for real-time PCR targeting IS900 and ISMAV2 was 5 fg/µl and 50 fg/µl respectively. By targeting ISMAV2 sequence, 100% specificity was detected. However, a cross reaction with 5 ng/µl of genome of 3 *M. avian* subspecies *avium* strains was detected by targeting IS900 and negative in lower genome quantity (5pg/µl). To maximize the assay's detection sensitivity, an efficient strategy for *MAP*-DNA extraction from spiked milk was assessed. Targeting of IS900 was sensitive and targeting ISMAV2 was very specific. Therefore, a multiplex real-time PCR assay targeting IS900 and ISMAV2 in combination with two commercial DNA extraction kits could be an ideal sensitive and specific protocol for routine large-scale analysis of milk samples and other clinical specimens from man and animals.

KEYWORDS: internal control / milk / multiplex real-time PCR / paratuberculosis

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Paratuberculosis or Johne´s disease is a chronic and incurable granulomatous enteric disease afflicting cattle, sheep, goats and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). Apart from great individual variability of subclinical and clinical forms, the infection is typically characterized by a long incubation period followed by chronic progressive diarrhoea, decrease in milk production, oedema, anaemia, loss of weight leading to cahexia and finally to death [Cocito *et al*. 1994, Harris and Barletta 2001]. Diseases caused by *MAP* have become a worldwide problem. Contaminated milk may be a source of exposure to the human organism need to be further investigated [Dalziel 1913]. Therefore, the essential precondition for eradication of paratuberculosis from herds is the identification and elimination of *MAP*-shedders. Moreover, a rapid, cost-effective and automated diagnostic assays of this pathogen are a high priority task not only for animal breeders but also for the food production industry and for public health concern.

In control programme ELISAs are widely used screening assays for the detection of antibodies against *MAP* in blood or milk because of their advantages like easy performance, high capacity and low costs. Due to the late formation of antibodies ELISAs are characterized by low sensitivity especially in animals without or with moderate shedding of *MAP* in their faeces [Kohler *et al*. 2008, Stabel 1998]. Moreover, the culture method for detection of *MAP* takes 8-16 weeks due to the slow growth of the organism. Although molecular methods offer the advantage of rapid turn around time for test results they suffer from low sensitivity of detection [Giese and Ahrens 2000]. Therefore, a sensitive and specific PCR assays detection of *MAP* in clinical samples including faeces should regularly be developed and evaluated. In addition, diagnostic procedures for *MAP* have to avoid co-isolation of inhibitors during DNA-preparation to prevent false-negative results.

The insertion element IS900 is the mostly used target for identification and also differentiation of *MAP* from other *Mycobacteria*. It is found in 15-20 copies in the *MAP* genome [Khare *et al*. 2004]. Some recent reports indicate that IS900-like sequences can be found in *M. avium* subsp. *avium* and in some isolates found in the *M. intracellular*-complex, which may affect the specificity of PCR targeting IS900 [Cousins *et al*. 1999]. Therefore, alternative genetic elements, including ISMAV2 sequence have been evaluated to improve the specificity and therefore the reliability of *MAP* detection by PCR. The *MAP*-specific insertion element ISMAV2 found in at least three copies in *MAP*-cells [Stratmann *et al*. 2006]. The objective of this study was to compare three extraction methods for *MAP*-DNA from bovine milk and followed by multiplex real-time PCR target IS900 and ISMAV2 sequences together with internal control (PUC19-plasmid).

Material and methods

Sampling

Milk samples were collected from aseptic teats of cows tested as negative for *MAP* infection by culture and stored at -20°C.

Tabela 1. *Mycobacteria* and *non-mycobacteria* strains analysed for the determination of the specificity of real-time *MAP*-PCR

Species, subspecies and designation	Type	Host species / Source	Target sequence			
			IS900		*ISMAV2*	
			template concentration			
			5ng/µl	5pg/µl	5ng/µl	5pg/µl
M. avium subsp. paratuberculosis						
(02A0267)	TS	cattle	17.9	31.1	21.8	31.6
(03A2388)	TS	cattle	21.4	31.1	20.9	30.6
(06A0159/b)	FI-J	cattle	17.1	28.5	19.9	29.1
(05A3268)	FI-J	cattle	18.1	26.2	18.1	31.5
(06A01262/6)	FI-J	cattle	23.8	33.9	21.0	31.0
(06A0987/f)	FI-J	sheep	26.9	36.9	31.0	38.5
M. avium subspecies avium						
(M128/2)	TS	cattle	37.7	–	–	–
(01A1077/2)	FI-J	cattle	–	–	–	–
(00A0720/2)	FI-J	pig	–	–	–	–
(03A0910/2)	FI-J	poultry	34.1	–	–	–
(03A2530/1)	FI-J	poultry	37.0	–	–	–
M. avium subspecies hominisus						
(00A0854)	FI-J	cattle	–	–	–	–
(01A0554/1)	FI-J	pig	–	–	–	–
(01A1054/1)	FI-J	human	–	–	–	–
(01A0255/1)	FI-J	dog	–	–	–	–
M. bovis (07A0151)	TS		–	–	–	–
M. bovis BCG (99A1119/1)		*a*	–	–	–	–
M. dierhoferi (M132/1)	TS	environ ment	–	–	–	–
M. fortuitum (M134/1)	TS	human	–	–	–	–
M. intracellulare (M136/1)	TS	*a*	–	–	–	–
M. nonchromogenicum (M433/1)	FI-J	environ ment	–	–	–	–
M. abuense (03A0262/3)	TS	human	–	–	–	–
M. palustre (04A0610/1)	TS	environ ment	–	–	–	–
M. phlei (M139/1)	TS	phage	–	–	–	–
M. scrofulaceum (M 140/3)	TS	human	–	–	–	–
M. smegmatis (M141/1)	TS	*a*	–	–	–	–
M. terrae (M142/B)		cattle	–	–	–	–
M. tuberculosis (05A3246)	FI-J	*a*	–	–	–	–

TS – reference strains of species or subspecies; FI-J – field isolates from Germany cultivated in FLI Jena; ATCC – designation of type strains by the American Type Culture Collection, Rockville, USA; DSM – designation of type and reference strains of the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
a –host species unknown.

Bacterial strains

For the determination of sensitivity of the multiplex real-time PCR protocols six *MAP* isolates and 22 non *MAP*-isolates representing 14 further *Mycobacterial* subspecies were included for evaluation of specificity (Tab. 1). The origin and detailed characterization of these *Mycobacteria* were described by [Mobius *et al*. 2008]. Genomic DNA of all *Mycobacterium* strains was prepared from bacterial strains by the Cetyl- trimethyl-ammonium-bromide (CTAB) method [Van Soolingen *et al.* 1991]. The DNA of other non-*Mycobacteria* avium subsp. paratuberculosis strains was extracted using the High Pure PCR Template Preparation Kit (ROCHE DIAGNOSTICS, Mannheim, Germany) according to the manufacturer's instructions. DNA concentration was measured by spectrophotometer (DU640 Photometer, BECKMAN COULTER GmbH, Krefeld, Germany) at 260 and 280 nm. Several *MAP* field strains were isolated from faecal samples on Herrlod's egg yolk medium and isolates were further identified using I900-PCR assay [Khare *et al.* 2004] and ISMAV2-PCR [Selim 2011]. Then, *MAP* colonies were suspended in PBS forming stock solution of *MAP*. The *MAP*-cell counts were calculated by measuring the optical density at 550 nm as described by Hughes *et al*. [2001]. An optical density of 0.25 at 550 nm was equivalent to about 10^8 organisms/ ml**.**

Target sequences for *MAP* detection using real-time PCR

To detection the *MAP* real-time PCR targeting two different genomic regions were evaluated (Tab. 2). The identification of the most frequently used IS900 was based on the procedure by Khare *et al*. [2004]. The detection of the ISMAV2 sequences was described by Selim [2011].

Table 2. Primers and probes for MAP-detection and for internal amplification control (PUC19)

Application	Target	Primer	Sequence	Acc-No	Position	Product size (bp)	Reference
Real time PCR	IS900	K-F	5′- CGG GCG GCC AAT CTC-3′	S74401	1343-1357	66	Khare *et al*. [2004]
		K-R	5′- CCA GGG ACG TCG GGT ATG-3′		1409-1392		
		Khare-TaqMan	FAM -TTC GGC CAT CCA ACA CAG CAA CC - BHQ1		1359-1381		
	ISMAV2	SM-F	5′- GGC CTA AAA CAC AAC CCA GA -3′	AF286339	2092-2111	60	Selim 2011
		SM-R	5′- GGT TGC TCG GTG ACA TTT CTA -3′		2152-2132		
		Mon-TaqMan	FAM- GTG TCA CCG ATC AAC CGA C - BHQ1		2112-2130		
Internal amplification control	PUC19	PUC18-F	5′- TGT CGT GCC AGC TGC ATT A-3′	L09137	620-638	82	Mäde *et al.* [2008]
		PUC18-R	5′- GAG CGA GGA AGC GGA AGA G-3′		702-684		
		PUC18-TaqMan	HEX - AAT CGG CCA ACG CGC GG - BHQ1		642-658		

Internal control for real-time PCR assay

For the monitoring of amplification conditions and detection of PCR-inhibitors especially in milk samples, commercially available PUC19-plasmid DNA (BIORON, Ludwigshafen, Germany) was used as non-competitive internal amplification control (IC). Multiplex-PCR was performed for co-amplification of the *MAP*-targets and IC using independent primer probe sets. In order to optimize the IC-concentration per reaction a serial dilution of PUC19-plasmid was prepared ranging from 10^0 to 10^{11} copies per ml. Based on primers and probe according to Mäde *et al.* [2008], the concentration of PUC19 plasmid was finally adjusted to 100 copies per reaction resulting in a Ct-value of about 35 when amplification of IC was combined with *MAP* detection in a multiplex-PCR.

Protocols for multiplex-PCR (*MAP* with IC)

Multiplex-PCR was carried out in 25 µl reaction volumes. PCR-mixtures contained 0.75 µl of both reverse and forward *MAP*-primer of IS900 and ISMAV2 sequences, 0.375 µl *MAP*-probe, 0.625 µl of both reverse and forward IC-primer, 0.25 µl IC-probe, 100 copies of PUC19-plasmid resolved in 1.0 µl 0.1xTE-buffer and 12.5 µl 2XQuantiTect Multiplex NoRox MasterMix (Qiagen). Finally *5* µl template was added. All primers were delivered by BIOTEZ (Berlin, Germany) and probes by EUROGENTEC (Belgium), respectively. They were added to the reaction mixtures in working solutions of 10 pmol/µl. The PCR reaction was performed on STRATAGENE MX3005 with the following programme: initial denaturation and activation of Taq-polymerase for 15 min at 95°C followed by 45 cycles of 1 min at 94°C and 1.5 min at 60°C.

Determination of analytical sensitivity by dilution of isolated *MAP*-DNA

Sensitivity of multiplex real-time PCR was determined by serial dilution of purified DNA from cultures of *MAP*-reference strain 03A3288 and field strains 05A3268, 06A0162 and 06A0159/b: DNA concentration of stock solutions was measured spectrophotometrically at 260 and 280 nm. Next, serial dilutions were freshly prepared from stock solutions with sterile water in broad range from 50 ng to 5 fg/µl. These serial dilutions were examined 3-fold with following calculation of mean values and standard deviation (SD) using Excel 2007.

Analytical specificity

For determination of the analytical specificity of real-time PCR for IS900, ISMAV2 sequences 22 *Mycobacteria* were analysed separately at DNA concentration of 5 ng/µl and 5 pg/µl as shown in Table 1.

Preparation of *MAP* spiked raw milk samples

Ten-fold serial dilutions of viable *MAP* cells were prepared from a stock suspension containing 10^8 per ml. Dilutions containing 10^8 to 10^1 *MAP* cells per ml were prepared in PBS. An aliquot from each dilution step was diluted 10-fold in raw milk to obtain

spiked samples containing from 10^8 to 10^1 *MAP* cells per ml of raw milk. As a negative control, a non-spiked milk aliquot from the same batch was included in each *MAP* detection run.

Evaluation of *MAP*-DNA isolation methods for *MAP*-spiked raw milk samples

To maximize the sensitivity of detection of *MAP* in milk using real-time PCR, tested were two commercial DNA template preparation protocols. Total genomic DNA templates were prepared from triplicated samples of milk that had been spiked with ten-fold serial dilution from *MAP*-cells ranging between 10^8 to 10 per ml. two commercial kits were evaluated. These spiked milk samples were examined 3-fold with following calculation of mean values and standard deviation (SD) using Excel 2007.

High pure template preparation kit (ROCHE)

One hundred microliters of Triton X 100 (CALBIOCHE, Germany) were added to 10 ml of spiked milk samples and the mixtures were centrifuged for 30 min at 4.500 rpm to obtain pellets. Most of each supernatant was discarded leaving about 0.5 ml. The pellets were resuspended in the remaining supernatant and transferred to Eppendorf tubes. A second centrifugation step was performed (10 min at 14.000 rpm), the supernatant was discarded. Milk pellet suspended in 240 µl lysis buffer [20 mM Tris-HCl (PH 8.0), 400 mM NaCl, 0.6% Sodium dodecyl sulfate, 2 mM EDTA] and 60 µl proteinase K were added, then incubated at 65°C until milk pellets were dissolved. Next 300 µl binding buffer of high pure PCR template preparation kit were added. The mixtures were transferred onto the ribolysing matrix in Ribolyser tubes, and mechanical lysis step ($6.5ms^{-1}$ for 45 sec) was performed using Ribolyser (HYBAID, ASHFORD, United Kingdom). The samples were incubated immediately at 70°C for 10 min. Then the mixtures were shortly centrifuged, add to the DNA binding columns, and processed as described in the kit protocol. Finally, the DNA templates were eluted in 100 µl of the elution buffer supplied in the kit and 5 µl aliquots were used as template in PCR-protocol.

Adiapure® PARATB milk

DNA was extracted from 10 ml of spiked milk following the manufacturer's instructions.

Results and discussion

Analytical sensitivity

Analytical sensitivity of real-time PCR based on serial dilution of isolated *MAP*-DNA differed according to the target gene. The detection limit of IS900 sequence was 5 fg, i.e. approximately one *MAP*-genome per reaction as shown in Table 3. For ISMAV2 the detection limit based on consistent positive results was 50 fg/µl due to one negative result in the 3-fold assay at the 5 fg-level. The internal control has been

Tabela 3. Analytical sensitivity recorded by Ct values for serial *MAP*-dilution of quantified *MAP*-DNA

MAP-DNA per PCR-reaction	IS900		ISMAV2	
	mean	SD	mean	SD
50 ng	16.6	0.35	17.9	0.07
5 ng	17.6	0.23	20.6	0.09
500 pg	20.8	0.18	24.1	0.09
50 pg	24.5	0.07	27.6	0.06
5 pg	28.0	0.18	31.3	0.07
500 fg	30.6	0.14	33.5	0.22
50 fg	33.8	0.33	36.4	0.18
5 fg	35.5	0.41	38.29*	0.29

*One run negative from the three fold.

positive in all assays at a level of Ct 34-36. The results of amplification of MAP-DNA in three consecutive DNA-concentrations confirmed also precision of the assays. As shown in Table 3, the standard deviations (SD) of the threshold cycles (Ct) values ranged between 0.07 and 0.41 for IS900-PCR and 0.07 and 0.29 for ISMAV2-PCR. Decrease of the amplification efficiency due to inadequate primer and probe design and non-optimized PCR reagents can therefore be excluded.

Analytical Specificity

All six *MAP*-strains analysed for the determination of the analytical sensitivity could be correctly detected by multiplex-PCR-protocol (Tab. 1). Examining *Mycobacterium avium* subsp. *avium* of bovine and avian origin with IS900-PCR weak positive results were obtained for highly concentrated DNA templates (5 ng/µl) isolated from 4 of 6 strains. For low concentrated DNA of *Mycobacterium avium* subsp. *avium* (5 pg/µl) and also for the remaining *Mycobacteria* and non-*Mycobacteria* species IS900-PCR showed the expected negative results. For ISMAV2-PCR amplification could be observed neither with *Mycobacteria* different from *MAP* nor with non-*Mycobacteria* revealing the specificity of this sequence for *MAP* detection.

Optimization of extraction of *MAP*-DNA from milk samples

As mentioned in Table 4, the most efficient DNA isolation protocols were based on DNA template quality and detection sensitivity based on sample crossing point means from multiplex real-time PCR targeting IS900 and ISMAV2 sequences analysis of spiked milk samples with the same *MAP* concentrations. The best performance was obtained with the High Pure template preparation kit (Roche) in combination with IS900, the detection limit was 100 *MAP*-cells per ml milk with mean Ct of 38.9 (SD, 0.7). In another hand, the detection level of the same kit but in combination with ISMAV2 sequence was 1000 *MAP*-cells/ml milk with mean Ct of 38.6 (SD, 0.7).

The minimum detection level of Adiapure® PARATB milk kit in combination with IS900 sequence was 100 *MAP*-cells/ml of milk with mean Ct of 39.16 (SD, 1) but has

Table 4. Comparative evaluation of different template isolation methods for isolation of *MAP*-DNA from spiked milk samples

MAP-Conc/ml	Methods for isolation *MAP*-DNA from milk							
	IS900				ISMAV2			
	Roche Kit		Adiapure Kit		Roche Kit		Adiapure Kit	
	mean	SD	mean	SD	mean	SD	mean	SD
10^8	18.6	1.5	17.4	0.4	21.2	0.9	19.4	0.7
10^7	22.0	1.1	20.0	0.5	25.0	1.1	21.6	1.6
10^6	25.5	1.1	23.3	1.0	27.8	1.4	24.9	1.6
10^5	28.8	1.1	27.8	0.6	30.8	1.2	29.2	1.9
10^4	31.7	0.9	32.5	0.1	33.2	1.8	32.9	1.7
10^3	34.0	0.8	34.1	0.7	36.2	1.4	36.8	0.8
10^2	37.0	1.0	35.9	0.9	38.6	0.7	38.3	1.0
10^1	38.9	0.7	39.2	1.0	No Ct	No Ct	No Ct	No Ct

one negative result from three replicates. The detection level of Adiapure® PARATB milk kit in combination with ISMAV2 sequence was 1000 *MAP*-cells/ml milk with mean Ct of 38.3 (SD, 1) as shown in details in Table 4. The internal control showed positive Ct values between 32 and 36 in all *MAP* concentration in case of High Pure template preparation kit (Roche) and Adiapure® PARATB milk kit, respectively.

For direct detection of *MAP* in several sample matrices former conventional and nested PCR-protocols are increasingly replaced by real-time PCR. The main advantages are simplified laboratory procedure due to elimination of electrophoresis, avoidance risk of contaminations during nested PCR and the shortening of analysis time. Several protocols for real-time PCR have been described for detection of *MAP* genome until now [Cousins *et al*. 1999, Mobius *et al*. 2008, Selim 2011]. Among these, IS900 represents the most widely applied target sequence. Alternative assays have been described for ISMAV2, the genetic element F57 and *locus* 251. Analytical sensitivity of real-time *MAP*-PCR differs according to both target sequence and sample matrix. In this study, results obtained for serial dilutions of genomic *MAP*-DNA were correlated with the frequency of the target sequence in *MAP*-cells. For IS900, the copy number varies between 12-20 per cell [Kim *et al*. 2002], thereby facilitate high sensitivity of IS900-based assays. ISMAV2 can only be found in three copies per *MAP*-cell resulting in a lower sensitivity than IS900-PCR [Stratmann *et al*. 2006].

For comparison with further data for sensitivity of PCR the detection limit based on DNA amount can be transformed into copy number. With respect to a *MAP*-genome of 5,867,714 bp, each *MAP* cell theoretically contains approximately 6.8 fg of DNA. A slightly lower DNA content of 5.1 fg *MAP*-DNA per *MAP* cell was calculated by Ravva *et al*. [2005]. In a similar range Rodriguez-Lazaro *et al.* [2005] reported 5.28 fg DNA per *MAP*-genome. Therefore, 5 fg DNA can be approximated to one *MAP*-genome or one cell equivalent.

The outcome of analytical sensitivity was highly influenced by the type of the matrix examined. Results of this study show a sensitivity of multiplex real-time PCR ranged from 5 fg to 50 fg (i.e. 1-10 MAP-genome) for diluted MAP-DNA for IS900

and ISMAV2 sequences respectively. In a similar scale Ravva *et al*. [2005] found a detection limit of 0.07 cells in their direct cell assay using SybrGreen-PCR targeting IS900. The detection limit of less than one *MAP*-cell was explained by the dilution of complete growth medium containing intact and broken cells as well. A considerably higher detection limit, of 100 fg DNA, was reported by Schonenbrucher *et al*. [2008] for a triplex PCR method.

Real-time PCR is considered more sensitive than conventional single step PCR for which detection limits were found to range between 1 pg and 100 fg [Mobius *et al*. 2008, Englund *et al*. 2001]. Reported detection limits for nested PCR were 1,2 fg [Englund *et al*. 2001] and 1 CFU (5 fg) [Vansnick *et al*. 2004]. By direct comparison of several PCR-assays analysing unique dilution series of *MAP*-DNA, Möbius *et al*. [2008] found detection limits of up to 1 fg for nested PCR. Furthermore, real-time PCR has been proven to be superior to nested PCR by the evidently reduced risk of contamination, the rapid and simple performance and finally the opportunity to verify the absence of false-negative results due to PCR-inhibitors.

In this study, the absence of positive PCR-results for different *Mycobacteria* different from *MAP* and non-Mycobacterial bacteria confirmed the selective specificity of the genomic element ISMAV2 only for *MAP* as described previously [Stratmann *et al*. 2006, Stabel and Bannantine 2005]. For IS900-PCR we observed false-positive amplification for *Mycobacterium avium* subsp. *avium* analysing highly concentrated DNA (5 fg/µl) of one strain isolated from cattle and two poultry strains. Similarly, Tasara *et al.* [2005] got also false-positive results of nested IS900-PCR for *M. chelonae, M. scofulaceum, M. terrae, M. xenopi* and *Mycobacterium* strain 2333. As far as we could examine the specificity of real-time PCR targeting IS900, we did not observe cross reactions with other *Mycobacteria*. Nevertheless, based on the above results, it was concluded that the combination of ISMAV2 and IS900 sequences in one assay besides internal control is preferred for higher sensitivity and specificity for detection of *MAP*.

Milk is considered a difficult medium for detection of organisms by PCR, due to the presence of large amounts of fat and calcium ions [Lantz *et al*. 1994]. Apart from these limitations, the cell walls of *Mycobacteria* are highly specialized with a wide diversity of lipids, which shield them from various stresses and make virtually impermeable to many chemicals. To address some of these problems, we designed an approach to improve sensitivity by concentrating *MAP* from clinical usually means human medicine, veterinary would be more appropriate for animal specimens. In a separate study by Odumeru *et al.* [2001], the use of bead beating in combination with the use of lysis buffer, boiling, and isopropanol precipitation was found to affect the efficiency of detection of *MAP* in milk by PCR to 10 to 10^2 CFU/ml. However, in the immunomagnetic bead capture-PCR based-diagnostic test described here, 100% of culture-positive samples were detected. The greater sensitivity of the detection was likely due to the DNA preparation procedure and detection methods. Bead beating, in combination with digestion and extraction steps, significantly enhanced the quality

and quantity of DNA yield. The lower detection limits of earlier studies were 10^3 or more MAP organisms [Grant *et al*. 2005]. In previous studies, DNA was prepared either by heating the bead suspension at 100°C for 15 min [Grant *et al*. 2005] or by bead beating only [Grant *et al*. 2006]. Inhibition in the PCR product signal intensity on agarose gel in studies by Grant *et al*. [2005] might have been caused by the presence of various inhibitory substances that were not effectively removed by IMS alone. The addition of digestion and extraction steps also improved the PCR signal intensity when larger volumes of milk were spiked. In the present study, an integrated procedure for isolation and lysis of *MAP* to maximize the yield of high-quality DNA was developed. In contrast to previous studies, we demonstrated that 100 or fewer *MAP* organisms were consistently detected in milk samples by using Adiapure and Roche kits in combination with multiplex real-time PCR beside internal control. By quantification of the extracted DNA, even less than 5 fg of DNA could be detected by assay developed in this study.

In summary, a multiplex real-time PCR assay targeting IS900 and ISMAV2 in combination with two commercial DNA extraction kits could be an ideal sensitive and specific protocol for routine large-scale analysis of milk samples and other clinical specimens from man and animals. Also, it overcomes the limitations of detection and significantly reduces the time and costs relative to those for standard bacteriological culture.

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