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DIRECT DETECTION OF *Mycobacterium avium* SUBSP. *paratuberculosis* IN BOVINE MILK BY MULTIPLEX REAL-TIME PCR

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Abstract: This study aimed to 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 *Mycobacteriacea* 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.

Key words: milk, paratuberculosis, multiplex real-time PCR, internal control

Introduction

Paratuberculosis or Johne´s disease is a chronic and incurable granulomatous enteric disease affecting cattle, sheep, goat and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Aside great individual variability of subclinical and clinical symptoms 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 cachexia and finally to death (*Cocito et al., 1994; Harris and Barletta, 2001*). Diseases caused by MAP disease have become a worldwide problem. Contaminated milk may be a source of exposure to the organism in human and need to be further investigated. 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 is a high priority task not only for animal breeders but also for the food production industry and for public health concern.

In control programs 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 this 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 be regularly 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. intracellulare*-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 at least three copies in MAP-cells (*Stratmann et al., 2006*). Our objective was comparison between 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).

Materials and methods

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

Bacterial strains. For the determination of the sensitivity of the multiplex real-time PCR protocols six *MAP* isolates and 22 non *MAP*-isolates representing 14

further subspecies of *Mycobacteria* were included for evaluation of specificity (Table 1).

Table 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 hominisuis						
(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	Environment	–	–	–	–
M. fortuitum (M134/1)	TS	Human	–	–	–	–
M. intracellulare (M136/1)	TS	*a*	–	–	–	–
M. nonchromogenicum (M433/1)	FI-J	Environment	–	–	–	–
M. abuense (03A0262/3)	TS	Human	–	–	–	–
M. palustre (04A0610/1)	TS	Environment	–	–	–	–
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

The origin and detailed characterization of these *Mycobacteria* were described earlier (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 fecal 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 approximately 10^8 organisms/ml.

Target sequences for MAP-detection using real-time PCR. For detection of *MAP* real-time PCR targeting two different genomic regions were evaluated (Table 2). The identification of the most frequently used IS900 was based on the procedure of 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		
	ISMAV 2	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 the 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 of 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 volume. 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 program: 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 by spectrophotometry at 260 and 280 nm. Afterwards 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 for with following calculation of mean values and standard deviation (SD).

Analytical specificity. For determination of the analytical specificity of real-time PCR for IS900, ISMAV2 sequences 22 *Mycobacteria* were analyzed 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, we tested 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 ranged between 10^8 to 10 per ml two commercial kits were evaluated.

High pure template preparation kit (Roche). One hundred microliters of Triton X 100 (Calbioche, Germany) was 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 discarding, 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 was added, then incubate at 65°C until milk pellets were dissolved. Then 300µl binding buffer of high pure PCR template preparation kit was 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 incubate immediately at 70 °C for 10 min. Then the mixtures were short centrifuge, 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 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 tabulated in Table 3, the slandered 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.

Table 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

Analytical Specificity. All six MAP-strains analysed for the determination of the analytical sensitivity could be correctly detected by multiplex-PCR-protocol (Table 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 determined 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 mil 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 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.

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

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 und 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 examined matrix. Results from this study showed 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 considerable 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 analyzing unique dilution series of MAP-DNA, *Möbius et al. (2008)* found detection limits 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-*Mycobacteria* 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 to be a difficult specimen for the detection of organisms by PCR, due to the presence of large amounts of fat and calcium ion (*Lantz et al., 1994*). Apart from these considerations, the cell walls of *Mycobacteria* are highly specialized with a wide diversity of lipids, which shield them from various stresses and make them 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 affecting 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., 2000*). In previous studies, DNA was prepared either by heating the bead suspension at 100°C for 15 min (*Grant et al., 2000*) or by bead beating only (*Grant et al., 1998*). Inhibition in the PCR product signal intensity on agarose gel in studies by Grant et al. (2000) 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.

Conclusion

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 overcoming the limits of detection and significantly reducing the time and costs relative to those for standard bacteriological culture.

Direktna detekcija *Mycobacterium avium* subsp. *paratuberculosis* u goveđem mleku metodom Multipleks Real-Time PCR

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Rezime

Ovo istraživanje ima za cilj direktnu detekciju *Micobacterium avium subsp. paratuberculosis* (MAP) u mleku ocenjivanjem multipleks real-time PCR analize ciljanjem IS900 i ISMAV2 sekvence uključujući amplifikacije PUC19-plazmida kao unutrašnje kontrole. Osetljivost testova je ocenjivana testiranjem *MAP* izolata u širokom spektru linearnom DNK (50 ng – 5 fg/µl). Za validaciju specifičnosti, 6 *MAP* izolata i 22 izolata roda *Micobacteriacea* su testirani. Rezultati su otkrili da je izvodljiva granica za real-time PCR ciljanje IS900 i ISMAV2 bila 5 fg/µl i 50 fg/µl. Ciljajući ISMAV2 sekvencu, 100% specifičnosti je otkriveno. Međutim, unakrsna reakcija sa 5 ng/µl genoma od 3 *M. avium* podvrste ptičjih sojeva je detektovana ciljanjem IS900 i negativno u donjem genomu u količini (5pg/µl). Da biste povećali osetljivost detekcije, efikasna strategija za MAP-DNK ekstrtakciju iz mleka sa povišenin nivoom je ocenjena. Ciljanje na IS900 je bilo osetljivo i ciljanje ISMAV2 je veoma specifično. Dakle, multipleks real-time PCR test analiza za ciljanje IS900 i ISMAV2 u kombinaciji sa dva komercijalna kompleta za ekstrakciju DNK mogla bi da bude idealni protokol osetljivosti i specifičnosti za rutinske analize velikih uzoraka mleka i drugih kliničkih uzoraka ljudi i životinja.

References

COCITO C., GILOT P., COENE M., DE KESEL M., POUPART P., VANNUFFEL P. (1994): Paratuberculosis. Journal of Clinical Microbiology Review, 7, 823-345.

COUSINS D.V., WHITTINGTON R., MARSH I., MASTERS A., EVANS R.J., KLUVer P. (1999): Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: Implications for diagnosis. Molecular Cellular Probes, 13, 431-442.

GIESE S.B., AHRENS P. (2000): Detection of Mycobacterium avium subsp. paratuberculosis in milk from clinically affected cows by PCR and culture. Veterinary Microbiology, 77, 291–297.

GRANT I.R., BALL H.J. ROWE M.J. (1998): Isolation of Mycobacterium paratuberculosis from milk by immunomagnetic separation. Applied Environmental Microbiology, 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. Veterinary Microbiology, 77, 369–378.

HARRIS N.B., BARLETTA R.G. (2001): Mycobactreium avium subsp. paratuberculosis in veterinary medicine. Clinical Microbiology Review, 14, 489-512.

HUGHES V.M., STEVENSON K., SHARP J.M. (2001): Improved preparation of high molecular weight DNA for pulsed-field gel electrophoresis from mycobacteria. Journal of Microbiological Methods, 44, 209-215.

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 PCR. Journal of Clinical Microbiology, 42, 1075-1081.

KIM S.G., SHIN S.J., JACOBSON R.H., MILLER L.J., HARPENDING P.R., STEHMAN S.M., ROSSITER C.A., LEIN D.A. (2002): Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *Paratuberculosis*. Journal of Veterinary Diagnostic Investigation, 14, 126-131.

KÖHLER H., BURKERT B., PAVILK I., DILLER R., GEUE L., CONRATHS F.J., MARTIN G. (2008): Evaluation of five ELISA test kits for the measurment of antibodies against *M.avium* subsp. *paratuberculosis* in bovine serum. Berliner Müncher Tierärztlich Wochenschrichlicer, 121, 203-210.

LANTZ P.G., HAHNHAGERDAL B., RADSTROM P. (1994): Sample preparation methods in PCR-based detection of food pathogens. Trends Food Science Technology, 5:384–389.

MÄDE D., REITING R., STRAUCH E., KETTERITZSCH K., WICKE A. (2008): A real-time PCR for Detection of Pathogenic *Yersinia enterocolitica* in food combined with an Universal Internal Amplification Control System. Journal für Verbraucherschutz und Lebensmittelsicherheit, 3, 141-151.

MÖBIUS P., HOTZEL H., RAßBACH A., KÖHLER H. (2008): Comparison of 13 single-rounds and nested PCR assays targeting IS900, ISMAV2, F57 and locus 255 for detection of *Mycobacterium avium* subsp. *Paratuberculosis*. Veterinary Microbiology, 126, 324-333.

ODUMERU J., GAO A., CHEN S., RAYMOND M., MUTHARIA L. (2001): Use of the bead beater for preparation of Mycobacterium paratuberculosis template DNA in milk. Canadian Journal of Veterinary Research, 65, 201–205.

RAVVA S.V., STANKER L.H. (2005): Real-time quantitative PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* and differentiation from other mycobacterium using SYBR Green and TaqMan assays. Journal of Microbiological Methods, 63, 305-317.

RODRIGUEZ-LAZARO D., D´AGOSTINO M., HERREWEGH A., PLA M., COOK N., IKONOMOPULOS J. (2005): Real-time PCR-based methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. International Journal of Food Micorbiology, 101, 93-104.

SCHÖNENBRÜCHER H., ABDULMAWJOOD A., FAILING K., BÜLTE M. (2008): New Triplex Real-time PCR Assay for detection of *Mycobacterium avium* subsp. *Paratuberculosis* in Bovine faces. Applied and Environmental Microbiology, 47, 2751-2758.
SELIM A. (2011): Evaluation of detection of Mycobacterium avium subsp. paratuberculosis in bovine feces by real-time PCR. (Dissertation med. Vet). Leipzig University. Leipzig.
STABEL J.R. (1998): Johne´s disease: a hidden threat. Journal of Dairy Science, 81, 283-288.
STABEL J.R., BANNANTINE J.P. (2005): Development of nested PCR method targeting a unique multicopy element, ISMAV02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faecal samples. Journal of Clinical Microbiology, 43, 4744-4750.
STRATMANN J., STEVENSON K., GERLACH G.F. (2006): Peptide aMptD-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bulk milk samples. Applied Environmental Microbiology, 72, 5150-5158.
TASARA T., HOELZLE L.E., STEPHAN R. (2005): Development and evaluation of a *Mycobacterium avium* subsp. *paratuberculosis* specific multiplex PCR assay. International Journal of Food Microbiology, 104, 279-287.
VAN SOOLINGEN D., HERMANS P.W.M., DE HAAS P.E., SOLL D.R., VAN EMBDEN J.D. (1991): Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. Journal of Clinical Microbiology, 29, 2578-2586.
VANSNICK E., DERIJIK, P., VERCAMMEN F., GEYSEN D., RIGOUTS L., PORTAELS F. (2004): Newly developed primers for detection of *Mycobacterium avium* subsp. *Paratuberculosis*. Veterinary Microbiology, 100, 197-204.

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