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Short communication

A DNA sequence capture extraction method for detection of *Mycobacterium avium* subspecies *paratuberculosis* in feces and tissue samples

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Abstract

Culturing of *Mycobacterium avium* subspecies *paratuberculosis* (Map) remains difficult and is time consuming. An alternative for the rapid detection of Map in samples is PCR. We have developed a sensitive DNA-extraction method based on sequence capture for the rapid detection of *M. avium* subspecies *paratuberculosis* by PCR in fecal and tissue samples. The method detected 10^2 Map/g feces using spiked samples, and reached a diagnostic sensitivity of 33,7% compared to 22% for culture. Analysis of tissue samples gave 65 polymerase chain reaction (PCR)-positive (42.2%) and 49 culture-positive samples (31.8%). Therefore, the detection limit of the DNA-extraction is the same as previously reported for culture, the PCR assay could detect more positive samples than the culture method.

Keywords: Mycobacterium; Paratuberculosis; Feces; DNA-extraction

1. Introduction

Cultivation of *Mycobacterium avium* subspecies *paratuberculosis* (Map) has a low sensitivity (Fang et al., 2002), and is laborious and time consuming due to a long incubation time (12 to 16 weeks).

The development of a rapid, sensitive and specific diagnostic method for the detection of Map is essential in the control of Johne's disease in economically important animals. Detection of the Map specific insertion sequence IS900 by the polymerase chain reaction (PCR) has improved the diagnosis of paratuberculosis (Collins et al., 1989). However, recovering DNA from paucibacillary samples or from a complex matrix as feces is difficult (Widjojoatmodjo

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et al., 1992; Christopher-Hennings et al., 2003). Excessive non-specific DNA derived from the host or other microbes can also inhibit amplification (Marsh et al., 2000). The sequence capture technique eliminates essentially all non-specific DNA and inhibitory substances present in crude samples (Marsh et al., 2000) resulting in a higher sensitivity in combination with a nested PCR (Englund et al., 2001). Mangiapan et al. (1996) used this method successfully for the detection of *M. tuberculosis* in paucibacillary clinical sputum samples from patients. This sequence capture approach has also been used to detect Map from tissue (Roring et al., 2000) and feces or tissue (Millar et al., 1995; Marsh et al., 2000; Halldorsdottir et al., 2002) but specificity and methodology could be improved.

In this study, we have developed, tested and evaluated a new more specific and easy to perform DNA-extraction method based on sequence capture to detect Map in feces and tissue samples.

2. Materials and methods

2.1. Spiked fecal samples

Map-culture negative fecal samples from Belgian cattle were spiked with a known amount of Map isolate ATCC19698 in a range of 10–10^6 Map/g feces. In the serial dilution range, the numbers of Map were confirmed using solid phase cytometry (Chemscan, Chemunex, Ivry-sur-Seine, France). Spiked samples were tested 10 times by PCR and used as a positive extraction control in each DNA-extraction.

2.2. Feces of experimentally infected cattle

Five young calves were experimentally infected by the oral route and sampled every 2 weeks by Dr. Godfroid and Dr. Walravens at CERVA, Brussels. A total of 249 fecal samples of these animals were tested.

2.3. Fecal samples of naturally infected dairy herds

Two Belgium dairy herds with a known Map history were sampled ($n = 154$) once (collection 4) or repeatedly (collections 1–3).

2.4. Fecal and tissue samples from wild red deer (Cervus elaphus)

During necropsy of hunter-killed free-living cervids (from southern Belgium), fecal and tissue samples were collected by Dr. Linden and colleagues (University of Liège, Belgium). A total of 81 mesenteric lymphenodes, 69 spleens, 2 small intestines, 2 large intestines and 25 feces were sampled from Map-suspected animals based on macroscopic inspection and serology (HerdChek, IDEXX).

2.5. Decontamination and culture

All fecal samples (except the spiked samples) were cultured on Löwenstein–Jensen medium supplemented with mycobactin J (1 mg/l), PANTA plus (40 ml/l) and sodium pyruvate (4 g/l) for MAP, and on Stonebrink and Löwenstein–Jensen for other isolation of other mycobacteria. Two different decontamination methods were used. The double incubation method of Whitlock and Rosenberger (1990) was used for decontamination of the fecal samples from the Belgian dairy herds. The sediment of 2–5 g feces was used to seed the culture tubes. Fecal samples from deer and the experimental infected cattle were decontaminated with the oxalic acid method (Beerwerth and Schurmann, 1969). A suspension from approximately 0.5 g feces was used to inoculate the culture tubes. The tissue samples were decontaminated with the reverse Petroff method (adapted from Petroff, 1915). A suspension of approximately 1 g of minced tissue was used to inoculate the LJ media and incubated at 37 °C for up to 8 months and examined regularly for signs of bacterial growth.

2.6. Sample preparation before sequence capture

In case of tissue samples, 250 μl of decontaminated suspension was transferred to a 1.5 ml tube and mixed with 250 μl of buffer A (200 mM Tris–HCl pH 7.4, 3000 mM NaCl, 100 mM EDTA). One gram of non-decontaminated feces was suspended in 10 ml of a 2% Tween-solution and glass beads were added. The samples were mixed vigorously on a vortex mixer for 1 min, left standing for 30 min. One milliliter supernatant was transferred to a new microcentrifuge tube and centrifuged at 6000 × *g* for 1 min. Supernatant

Table 1
Primers and probes used in the study

Primer	DNA sequence	Product size
IS900S1	5′ ggg ttg atc tgg aca atg acggtt a 3′	572 bp
IS900R3 (2)	5′ agc gcg gca cgg ctc ttg tt 3′	
IS900S2	5′ gga ggt ggt tgt ggc aca acc tgt 3′	452 bp
IS900R1	5′ cga tca gcc acc aga tcg gaa 3′	
Probe	DNA sequence	
IS900SB	5′ biotine-gtt cgg ggc cgt cgc tta ggc t 3′	
IS900RB	5′ biotine-gag gat cga tcg ccc acg tga 3′	

was transferred to a new microcentrifuge tube, centrifuged at 14,000 × *g* for 10 min and the pellet suspended in 500 μl of a 100 mM Tris–HCl solution containing 150 mM NaCl and 50 mM EDTA.

2.7. Sequence capture

Five hundred microliters of 0.1 mm-diameter glass microspheres and 50 μl of a 20 mg/ml proteinase K solution (Boehringer Mannheim, Germany) were added to the samples. The samples were agitated for 50 s, allowed to digest overnight and agitated again for 50 s. The supernatant was recovered by centrifugation and transferred into a new microcentrifuge tube, heated at 100 °C for 10 min and cooled on ice to 0 °C. Two hundred microliters of a 3.75 M NaCl solution containing 2.5 pmol of biotinylated capture oligonucleotides IS900SB and IS900RB (Table 1) was added. Tubes were incubated at 60 °C for 3 h under agitation to allow hybridization. Afterwards 10 μl of streptavidin dynabeads (washed according to the manufacturer's instructions, Dynal, Oslo, Norway) was added and the incubation was continued for 2 h at room temperature. The magnetic beads were captured, washed twice with a 10 mM Tris–HCl solution containing 0,1 mM EDTA (pH 8) and resuspended in 20 μl water. Ten microliter was used for amplification.

2.8. Nested PCR

The primary PCR of 40 cycles was performed in 50 μl containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 200 μm of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase and 10 μl of sample. Primers were IS900S1 and IS900R3 (Table 1)

Table 2
PCR and culture results

	Total nr of samples tested	PCR+/Cult+				PCR+/Cult−				PCR−/Cult+				PCR−/Cult−			
		Total	ZN+	ZN−	ZN NT	Total	ZN+	ZN−	ZN NT	Total	ZN+	ZN−	ZN NT	Total	ZN+	ZN−	ZN NT
Experimental inf animals	249	44	10	9	0	40	5	34	1	11	2	9	0	156	17	137	2
Belgian dairy herds	154																
Sample collection 1	20																
Sample collection 2 + 3	34																
Sample collection 4	100																
Cervids	179																
Lymph node	81	35	14	11	10	6	2	4	0	7	1	4	2	34	2	13	19
Spleen	69	14	5	4	5	6	5	1	0	7	0	3	4	42	2	22	18
Feces	25	16	0	0	16	2	0	0	2	0				7	0	0	7
Small intestine	2	1	1	0	0	1	1	0	0	0				0			
Large intestine	2	2	2	0	0	0				0				0			

ZN+: positive on microscopy, using Zielh–Neelsen. ZN−: negative on microscopy, using Zielh–Neelsen. ZN NT: not tested on microscopy.

Table 3
Results on tissue samples and fecal samples

Samples	Number	PCR positives	Culture positives
Tissue	154	65 (42.2%)	49 (31.8%)
Feces	409	135 (33.0%)	70 (17.1%)
Total	563	200 (35.5%)	119 (21.1%)

with annealing temperature at 68 °C. The secondary PCR of 25 cycles was performed in 24 μl standard PCR mix and 1 μl of the first PCR solution. Primers were IS900S2 and IS900R1 with annealing temperature at 68 °C.

3. Results

The fecal sequence capture method had a detection limit of 100 Map/g feces for spiked samples, and a diagnostic sensitivity of 33.7% (84/249) for fecal samples from experimental infected animals compared to 22% (55/249) by culture. Eleven culture positive samples remained negative by PCR. Systematic evaluation of feces specimens from Belgian dairy herds with a known Map history, was not possible in a reliable way due to fungal overgrowth for part of the cultures. In sample collection 1 ($n = 20$), where no overgrowth was observed, PCR detected 4 samples of which 3 were culture positive. In sample collections 2 and 3 ($n = 34$), with fungal overgrowth, 11 PCR positive fecal samples were obtained. In sample collection 4 ($n = 100$), 4 samples were both PCR and culture positive. All extractions were repeated and PCR results were reproducible. The results of PCR and culture on tissue and fecal samples of the wild red deer are summarized in Tables 2 and 3. Al smear-positive specimens were paucibacillary, i.e. per 300 fields we detected 1–3 separate acid fast bacilli or a single small cluster of about 10 bacilli.

4. Discussion

In this study, we report a DNA-extraction method based on the nucleic acid sequence capture technique giving highly purified templates for the detection of Map in fecal and tissue samples (Marsh et al., 2000). Although magnetic-particle technology has been reported as a mechanism for isolating specific DNA targets from complex mixtures, it results rarely in a complete absence of PCR inhibition. This phenomenon could explain that 18 culture positives (11 fecal and 7 tissues) were negative on PCR.

We achieved a fecal detection limit of 100 Map/g feces for spiked samples, which is comparable to fecal culture (Merkal, 1973) and other fecal extraction methods like the xylene (Challans et al., 1994) and freeze-boiling method (van der Giessen et al., 1992). Millar et al. (1995) also described a similar sequence capture technique for detecting Map in feces samples, based on the use of a 513 bp probe giving a detection limit of 2500 Map/g of feces. However, our probe and primer set proved more specific, not amplifying IS902 or IS900-like sequences causing false positive results in current IS900 amplification assays (Vansnick et al., 2004; Vansnick, 2004).

Halldorsdottir et al. (2002) described a complex detection method using buoyant density centrifugation followed by a single sequence capture PCR with 60 bp primers and a dot blot hybridization to increase the sensitivity to 1000 CFU/g of feces. We used a nested PCR approach with shorter primers (20 bp) to increase the sensitivity and overcome the influence of inhibitory substances to some extent. In nested-PCR systems false positive results caused by carry-over or cross contamination could occur more frequently and precautions have to be taken to circumvent this problem (Belak and Ballagi-Pordany, 1993). Real-time PCR could be an alternative to reduce the chance of DNA contamination, but a major disadvantage of real-time PCR is the higher cost of the instrumentation.

We detected more positive fecal samples by sequence capture (33.7%) than by culture (22%). The possibility of PCR contamination in these cases is unlikely given the results of negative controls included in each PCR reaction and strict application of a ‘‘three room system’’. A possible explanation is the non-uniform distribution of fecal samples due to clumping, a phenomenon that has been previously described (Sockett et al., 1992) and seems to be more pronounced in light shedders, as was the case in this set of samples. Another possibility may relate to the decontamination step that reduces the number of viable cells that cannot be cultured but can still be detected by PCR. For 34/428 fecal samples we did not

obtain culture results due to contamination, a major problem in culturing Map that can easily mask the presence of Map colonies or even prevent Map growth. The use of a PCR overcomes this contamination problem associated with culture.

The sequence capture technique can also be used on a variety of other clinical samples by using an appropriate sample preparation protocol. We tested the performance of sequence capture PCR on tissue samples by comparing culture and PCR results of 154 tissue samples from cervids. Again, more tissue samples were found positive with PCR (42.2%) than with culture (31.8%).

5. Conclusion

We have reported here a sensitive 2-days procedure to detect Map in fecal and tissue samples without the use of hazardous reagents (phenol–chloroform). A detection limit of 100 Map/g of feces was achieved, which is comparable with the sensitivity obtained by fecal culture. The diagnostic sensitivity was higher for PCR compared to culture. It can be concluded that the sequence capture PCR as described in this article is a valid alternative for the time-consuming Map culture.

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