Specific detection and differentiation of *Ochrobactrum anthropi*, *Ochrobactrum intermedium* and *Brucella* spp. by a multi-primer PCR that targets the *recA* gene

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Ochrobactrum anthropi, *Ochrobactrum intermedium* and *Brucella* spp. are phenotypically and genetically closely related pathogens that may cause disease with similar clinical presentation. Consequently, difficulties in their identification and differentiation have been reported. In this study, a sensitive *recA* gene-based multi-primer single-target PCR (MP-ST-PCR) was developed that allowed the specific detection and differentiation of these clinically relevant pathogens. The specificity of the assay was evaluated using a representative panel of 50 *O. anthropi* and 16 *O. intermedium* strains and the type strains of all *Brucella* spp. Detection limits for purified DNA from *O. anthropi*, *O. intermedium* and *Brucella melitensis* were 100, 10 and 100 fg, respectively. *Brucella* DNA was also successfully detected in various clinical specimens from a human patient with culture-proven brucellosis and from a *Brucella*-infected sheep and its aborted fetuses. The sensitivity of the MP-ST-PCR was comparable to that of an evaluated in-house *Brucella* real-time PCR assay. The developed assay closes a diagnostic gap and provides a simple but robust tool for the sensitive detection and correct identification of *O. anthropi*, *O. intermedium* and *Brucella* spp.

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INTRODUCTION

Ochrobactrum and *Brucella* are genetically closely related genera of the family *Brucellaceae* within the class *Alpha-proteobacteria* (Velasco *et al.*, 1998), with *Ochrobactrum intermedium*, the closest relative to *Brucella*, sharing 98.8 % rRNA gene similarity with *Brucella* spp.

Whereas brucellae are well recognized as important pathogens that cause brucellosis in man and many animal species, *Ochrobactrum anthropi* and *O. intermedium* have only recently emerged as human pathogens (Alnor *et al.*, 1994; Möller *et al.*, 1999; Mahmood *et al.*, 2000;

Apisarnthanarak *et al.*, 2005; Vaidya *et al.*, 2006; Kämpfer *et al.*, 2007). In the past, *O. anthropi* and *O. intermedium* have been regarded as opportunistic human pathogens of low virulence, infecting only immunocompromised patients with underlying diseases. Recent reports on *Ochrobactrum* infections, however, have demonstrated the occurrence of severe *O. anthropi* infections in immunocompetent hosts without underlying diseases with a clinical presentation similar to brucellosis (Kettaneh *et al.*, 2003; Romero Gomez *et al.*, 2004; Perez-Blanco *et al.*, 2005; Ozdemir *et al.*, 2006). A report on a pelvic abscess in an immunocompetent host and a comprehensive review of the literature on *O. anthropi* infections in patients with normal immune status was recently published by Vaidya *et al.* (2006).

Abbreviations: C_t, cycle threshold; MP-ST-PCR, multi-primer single-target PCR.

Because of the clinical relevance of *Brucella* infections and the potential use of *Brucella* as a biological agent (Pappas *et al.*, 2006), a battery of PCR assays for the specific detection of *Brucella* spp. has been developed in recent years (Al Dahouk *et al.*, 2004, 2007; Garcia-Yoldi *et al.*, 2006). However, in many clinical laboratories, the initial diagnosis is made by conventional identification methods, mainly culture and biochemical analysis using commercially available test systems. These test systems often do not allow correct identification of the agent, and consequently misidentifications of *Brucella* spp. as *O. anthropi* (Elsaghir & James, 2003) or *O. intermedium* (Hubalek *et al.*, 2007) have been reported. Furthermore, as *O. anthropi* is the only species of *Ochrobactrum* in the database of commercially available test systems such as API 20NE, differentiation of *O. anthropi* and *O. intermedium* is impossible (Teyssier *et al.*, 2005; Kämpfer *et al.* 2007). Likewise, *O. intermedium* was recently misidentified as *O. anthropi* in a severely diseased patient with a pelvic abscess using the API 20NE test and partial 16S rRNA gene sequencing (Vaidya *et al.*, 2006; Kämpfer *et al.* 2007). In this case, partial 16S rRNA gene sequencing was misleading because of the high sequence identity of >98% between *O. anthropi* and *O. intermedium*.

Reports on the misidentification of *O. anthropi*, *O. intermedium* and *Brucella* spp. emphasize the urgent need for a diagnostic tool that allows the correct identification and differentiation of these pathogens. Accordingly, we have developed a sensitive multi-primer single-target PCR (MP-ST-PCR) based on sequence variations in the *recA* gene that allows the accurate identification and differentiation of *O. anthropi*, *O. intermedium* and *Brucella* spp. in a single reaction.

METHODS

Bacterial strains. A total of 71 *Ochrobactrum* strains (50 *O. anthropi*, 16 *O. intermedium* and one each of *Ochrobactrum tritici*, *Ochrobactrum oryzae*, *Ochrobactrum grignonense*, *Ochrobactrum gallinifaecis* and *Ochrobactrum lupini*) and the type strains of the eight *Brucella* species (*Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, *Brucella neotomae*, *Brucella ceti* and *Brucella pinnipedialis*) were examined (Table 1). An additional 23 strains of closely related or clinically relevant bacteria were used as a negative-control panel.

Strains were obtained from the American Type Culture Collection (ATCC), the Laboratorium voor Microbiologie (BCCM/LMG), the Culture Collection University of Goeteborg (CCUG), the National Collection of Type Cultures (NCTC), the German Collection of Micro-organisms and Cell Cultures (DSMZ) and the Czech Collection of Micro-organisms (CCM). Strains TA13, E276, RR, TD30 and TM73 were kindly provided by A. Sessitsch (ARC Seibersdorf Research GmbH, Seibersdorf, Austria). Strains ALM4, OaC13a, CLM6, CLM7, OpsIndien, OiC8-5, OiC8-6 and OgA9a were kindly provided by M. Lebuhn (GSF-Forschungszentrum für Umwelt und Gesundheit, Munich, Germany). Strain LUP21^T was provided by M. E. Trujillo (Departamento de Microbiología, Salamanca, Spain). Strain RMA 16994 was kindly provided by E. J. Goldstein and S. A. Vaidya (Medical Scientist Training Program, University of California,

Los Angeles, USA). The type strains of *O. anthropi* (LMG 3331^T, CCUG 24695^T) and *O. intermedium* (LMG 3301^T, CCUG 24694^T) were each obtained from two different strain collections, CCUG and LMG, respectively.

DNA preparation from pure cultures. One distinct colony of each strain was transferred from an agar plate to 200 µl lysis buffer [5 × buffer D (PCR Optimization kit; Invitrogen) diluted 1:5 in double-distilled H₂O, 0.5% Tween 20 (ICI American), 12 U proteinase K (Roche Diagnostics)]. After incubation at 56 °C for 1 h followed by inactivation for 10 min at 95 °C, 2 µl of the cleared lysate was used as template in the PCR assays.

For determination of the PCR detection limits, the type strains *B. melitensis* 16M^T, *O. anthropi* LMG 3331^T and *O. intermedium* LMG 3301^T were grown at 37 °C in liquid medium to OD₆₀₀ 0.7 (mid-exponential growth phase). Genomic DNA was prepared with a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. PCR was carried out as described below.

DNA preparation from clinical samples. Total DNA was prepared from various formalin-inactivated (10%, v/v) clinical samples and body fluids of an aborting sheep with brucellosis and its two fetuses. Specimens examined were: placental fluid, serum, urine, stomach content, placenta, lung, liver, spleen, uterus, udder and kidney (see Fig. 2(a), left panel).

Additionally, DNA was prepared from liver biopsy material of a human patient with culture-proven brucellosis. After removing the formalin by washing twice in 10 ml deionized water and further incubation in sterile saline overnight at 4 °C, 50 mg of each sample was homogenized in 500 µl PBS (pH 7.5) using a Bio101 Fast Prep System (Qbiogene). Total DNA was prepared from 100 µl homogenized tissue using a DNeasy Tissue kit (Qiagen). The DNA was eluted twice with 60 µl double-distilled H₂O, of which 5 µl was used in PCRs.

Primer design and MP-ST-PCR. Primers were designed on the basis of a multi-sequence alignment of known *recA* gene sequences from *Ochrobactrum* spp. and *Brucella* spp. (Scholz *et al.*, 2006; H. C. Scholz, unpublished data). The GenBank accession numbers of the various *recA* genes are given in Table 1. Details of the primers and amplification sizes of the partial *recA* gene sequences from *O. anthropi*, *O. intermedium* and *Brucella* spp. are given in Table 2.

PCR conditions were optimized for each primer pair using purified template DNA from *O. anthropi* LMG 3331^T, *O. intermedium* LMG 3301^T and *B. melitensis* 16M^T prior to use in MP-ST-PCR. Optimized MP-ST-PCR was performed in 50 µl Ready-to-go MasterMix (Eppendorf) using 15 pmol each primer. Amplification was carried out in a Perkin-Elmer GeneAmp 2400 thermal cycler with 30 cycles of 30 s denaturation at 94 °C, 40 s annealing at 62 °C and 1 min elongation at 72 °C, with a final elongation step of 7 min at 72 °C. The expected sizes of the amplification products were 544, 402 and 167 bp for *O. anthropi*, *O. intermedium* and *Brucella* spp., respectively. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in 1 × Tris/borate/EDTA running buffer at a constant voltage of 120 V for 1 h. Fragments were visualized with UV light after staining in ethidium bromide.

Detection limit of the MP-ST-PCR. Purified DNA preparations of *O. anthropi* LMG 3331^T, *O. intermedium* LMG 3301^T and *B. melitensis* 16M^T were quantified in triplicate using an ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies). The detection limits were determined three times by analysing a dilution series of the prepared DNA in PCR-grade water ranging from 1 ng to 1 fg. In the first step, the detection limit for each primer pair was determined separately using 1 µl of the corresponding template DNA of each

Table 1. List of strains analysed in this study

NA, Not available.

Strain	Source	Species	recA GenBank accession number	MP-ST-PCR result		
				<i>Brucella</i> spp.	<i>O. anthropi</i>	<i>O. intermedium</i>
<i>Brucella</i>						
NCTC 10093 ^T	Cattle	<i>B. abortus</i>	AM113730	+	—	—
ATCC 23365 ^T	Dog	<i>B. canis</i>	AM113731	+	—	—
ATCC 23456 ^T	Goat	<i>B. melitensis</i>	AM087912	+	—	—
ATCC 23459 ^T	Rat	<i>B. neotomae</i>	AM113732	+	—	—
ATCC 25840 ^T	Sheep	<i>B. ovis</i>	AM113733	+	—	—
NCTC 10316 ^T	Wild boar	<i>B. suis</i>	AM113734	+	—	—
NCTC 12890 ^T	Sea lion	<i>B. pinnipedialis</i>	AM113735	+	—	—
NCTC 12891 ^T	Whale	<i>B. ceti</i>	AM113736	+	—	—
<i>Ochrobactrum</i>						
LMG 33	Hospital environment	<i>O. anthropi</i>	AM422738	—	+	—
LMG 35	Dextran	<i>O. anthropi</i>	AM422745	—	+	—
LMG 371	NA	<i>O. anthropi</i>	AM422746	—	+	—
LMG 394	Human cerebrospinal fluid	<i>O. anthropi</i>	AM422747	—	+	—
LMG 395	Human incision	<i>O. anthropi</i>	AM422748	—	+	—
LMG 2134	Unused contact lens	<i>O. anthropi</i>	AM422880	—	+	—
LMG 2136	Industrial dust	<i>O. anthropi</i>	AM422736	—	+	—
LMG 3298	Environment	<i>O. anthropi</i>	AM422737	—	+	—
LMG 3305	Hospital environment	<i>O. anthropi</i>	AM422739	—	+	—
LMG 3307	Paint	<i>O. anthropi</i>	AM422740	—	+	—
LMG 3309	Human tracheal secretion	<i>O. anthropi</i>	AM422741	—	+	—
LMG 3312	Wheat rhizoplane	<i>O. anthropi</i>	AM422742	—	+	—
LMG 3313	Soil	<i>O. anthropi</i>	AM422743	—	+	—
LMG 3329	Human faeces	<i>O. anthropi</i>	AM422744	—	+	—
LMG 3331 ^T	Sewage plant in Bora	<i>O. anthropi</i>	AM087899	—	+	—
LMG 3333	Blood culture	<i>O. anthropi</i>	AM087908	—	+	—
LMG 5140	Estuary sediment	<i>O. anthropi</i>	AM158983	—	+	—
LMG 5438	Human vagina	<i>O. anthropi</i>	AM422749	—	+	—
LMG 5440	Human peritoneal fluid	<i>O. anthropi</i>	AM422750	—	+	—
LMG 5441	Human blood	<i>O. anthropi</i>	AM422751	—	+	—
LMG 5442	Human blood	<i>O. anthropi</i>	AM422752	—	+	—
LMG 5444	Human	<i>O. anthropi</i>	AM422753	—	+	—
LMG 7991	Human eye	<i>O. anthropi</i>	AM087909	—	+	—
LMG 18952	Human blood	<i>O. anthropi</i>	AM422734	—	+	—
LMG 18953	Arsenite cattle-dip tray	<i>O. anthropi</i>	AM422735	—	+	—
CCUG 772	Human blood	<i>O. anthropi</i>	AM422728	—	+	—
CCUG 1047	Human abscess	<i>O. anthropi</i>	AM422707	—	+	—
CCUG 1076	Hospital environment	<i>O. anthropi</i>	AM422708	—	+	—
CCUG 1838	NA	<i>O. anthropi</i>	AM422714	—	+	—
CCUG 7349	NA	<i>O. anthropi</i>	AM422727	—	+	—
CCUG 12303	NA	<i>O. anthropi</i>	AM422709	—	+	—
CCUG 12860	Human blood	<i>O. anthropi</i>	AM422710	—	+	—
CCUG 13083	Human peritoneal fluid	<i>O. anthropi</i>	AM422711	—	+	—
CCUG 16508	NA	<i>O. anthropi</i>	AM422712	—	+	—
CCUG 17894	Contaminant	<i>O. anthropi</i>	AM422713	—	+	—
CCUG 18525	Unused contact lens	<i>O. anthropi</i>	AM422715	—	+	—
CCUG 18681	Industrial dust	<i>O. anthropi</i>	AM422716	—	+	—
CCUG 20020	Human blood	<i>O. anthropi</i>	AM422717	—	+	—
CCUG 20050	Human blood	<i>O. anthropi</i>	AM422718	—	+	—
CCUG 24695 ^T	Human	<i>O. anthropi</i>	AM422719	—	+	—
CCUG 25934A	Human cerebrospinal fluid	<i>O. anthropi</i>	AM422720	—	+	—
CCUG 28303	Human blood	<i>O. anthropi</i>	AM422721	—	+	—
CCUG 30656B	Human eye	<i>O. anthropi</i>	AM422722	—	+	—

Table 1. cont.

Strain	Source	Species	recA GenBank accession number	MP-ST-PCR result		
				<i>Brucella</i> spp.	<i>O. anthropi</i>	<i>O. intermedium</i>
ALM4	Soil	<i>O. anthropi</i>	AM422706	—	+	—
OaC13a	Soil	<i>O. anthropi</i>	AM422755	—	+	—
CLM6	Soil	<i>O. anthropi</i>	AM422730	—	+	—
CLM7	Soil	<i>O. anthropi</i>	AM422731	—	+	—
WS 4312	Dairy waste	<i>O. anthropi</i>	AM422759	—	+	—
WS 4290	Dairy waste	<i>O. anthropi</i>	AM422757	—	+	—
WS 4292	Dairy waste	<i>O. anthropi</i>	AM422758	—	+	—
LMG 379	Human ear	<i>O. intermedium</i>	AM422946	—	—	+
LMG 3301 ^T	Human blood	<i>O. intermedium</i>	AM422944	—	—	+
LMG 3306	Soil	<i>O. intermedium</i>	AM422945	—	—	+
LMG 5425	Human urine	<i>O. intermedium</i>	AM422947	—	—	+
LMG 5426	Human urine	<i>O. intermedium</i>	AM422948	—	—	+
CCUG 24694 ^T	Human blood	<i>O. intermedium</i>	AM087913	—	—	+
RMA 16994	Human pelvic abscess	<i>O. intermedium</i>	AM422878	—	—	+
OspIndien	Deep-water rice	<i>O. intermedium</i>	AM422943	—	—	+
OiC8-5	Soil	<i>O. intermedium</i>	AM422949	—	—	+
OiC8-6	Soil	<i>O. intermedium</i>	AM422950	—	—	+
TA13	Industrial environment	<i>O. intermedium</i>	AM422952	—	—	+
E276	Industrial environment	<i>O. intermedium</i>	AM422942	—	—	+
RR	Industrial environment	<i>O. intermedium</i>	AM422951	—	—	+
TM73	Industrial environment	<i>O. intermedium</i>	AM422954	—	—	+
TD30	Industrial environment	<i>O. intermedium</i>	AM422953	—	—	+
CCM 7036	Sand fly	<i>O. intermedium</i>	AM422941	—	—	+
Other <i>Ochrobactrum</i> species						
LMG 18957 ^T	Wheat rhizoplane	<i>O. tritici</i>	AM087914	—	—	—
DSM 17471 ^T	Rice	<i>O. oryzae</i>	AM263421	—	—	—
DSM 15295 ^T	Chicken	<i>O. gallinifaecis</i>	AM422962	—	—	—
OgA9a ^T	Soil	<i>O. grignonense</i>	AM422960	—	—	—
LUP21 ^T	Plant	<i>O. lupini</i>	AM113737	—	+	—
Possible sample contaminants						
LMG 1267 ^T	Fresh water	<i>Chromobacterium violaceum</i>	NA	—	—	—
ATCC 10145 ^T	NA	<i>Pseudomonas aeruginosa</i>	NA	—	—	—
IMB P739	Plant	<i>Ralstonia solanacearum</i>	NA	—	—	—
IMB P26	Human	<i>Xanthomonas maltophilia</i>	NA	—	—	—
LMG 559	<i>Brassica napus</i>	<i>Xanthomonas campestris</i>	NA	—	—	—
Differential diagnosis: clinical presentation						
ATCC 27294 ^T	Human	<i>Mycobacterium tuberculosis</i>	NA	—	—	—
ATCC 19430 ^T	Human	<i>Salmonella typhi</i>	NA	—	—	—
ATCC 12600 ^T	Human	<i>Staphylococcus aureus</i>	NA	—	—	—
Differential diagnosis: potential biological (B) agents						
BH	Environment	<i>Bacillus anthracis</i>	NA	—	—	—
3520	Cattle	<i>Ba. anthracis</i>	NA	—	—	—
4675	Cattle	<i>Ba. anthracis</i>	NA	—	—	—
ATCC 23344 ^T	Human	<i>Burkholderia mallei</i>	NA	—	—	—
ATCC 23343 ^T	NA	<i>Burkholderia pseudomallei</i>	NA	—	—	—
FSC 041	Tick	<i>Francisella tularensis</i> subsp. <i>tularensis</i>	NA	—	—	—
FSC 043	Human	<i>F. tularensis</i> subsp. <i>tularensis</i>	NA	—	—	—
EV76	Human	<i>Yersinia pestis</i> var. <i>orientalis</i>	NA	—	—	—

Table 1. cont.

Strain	Source	Species	recA GenBank accession number	MP-ST-PCR result		
				<i>Brucella</i> spp.	<i>O. anthropi</i>	<i>O. intermedium</i>
Exu 21	Human	<i>Y. pestis</i> var. <i>orientalis</i>	NA	—	—	—
PKH4	Human	<i>Y. pestis</i> var. <i>medievalis</i>	NA	—	—	—

dilution. In the MP-ST-PCR, the six primers were mixed in equal amounts and template DNA from *O. anthropi* LMG 3331^T, *O. intermedium* LMG 3301^T or *B. melitensis* 16M^T was added. Amplification products were analysed by electrophoresis through a 1.5% agarose gel, followed by ethidium bromide staining and visualization with UV light.

Brucella-specific real-time PCR. An in-house LightCycler real-time PCR was used for the detection of *Brucella* DNA in clinical samples. This PCR was evaluated recently (Al Dahouk *et al.*, 2007) and uses the specific primers B4/B5, originally developed by Bailly *et al.* (1992), which targets the *omp31* gene of *Brucella* spp., in combination with *Brucella*-specific hybridization probes. Briefly, a single-tube duplex LightCycler PCR was performed using a FastStart DNA Hybridization Probes kit (Roche Diagnostics). The 20 µl reaction mixture contained 2.5 mM MgCl₂, 2 µl 10 × reaction mix, 0.5 µl each primer (20 pmol µl⁻¹), 0.5 µl each hybridization probe (8 pmol µl⁻¹) and 2 µl template DNA. Cycling was started with a 10 min denaturation step at 95 °C, followed by 45 cycles of 10 s denaturation at 95 °C, 10 s annealing at 55 °C and 12 s extension at 72 °C. As an internal amplification control, we included a bacteriophage λ PCR system as described previously (Tomaso *et al.*, 2003). No-template controls containing 2 µl molecular-grade water instead of DNA and positive controls containing *Brucella* DNA of the reference strains *B. melitensis* 16M^T (biovar 1), *B. abortus* 1119-3 (biovar 1) and/or *B. suis* 1330 (biovar 1) were included in each run to detect any amplicon contamination or amplification failure. Temperature transition rates were set at 20 °C s⁻¹. Fluorescence values were measured during each cycle at 640 and 705 nm. Melting curves were generated by continuous measurement of fluorescence while raising the temperature from 45 to 95 °C with a transition rate of 0.2 °C s⁻¹. Data analysis was performed using LightCycler software version 3.32. The second-derivative maximum algorithm was used to

calculate the cycle threshold (C_t) value. Primer sequences and hybridization probes are listed in Table 2.

RESULTS AND DISCUSSION

Brucella and *Ochrobactrum* are phenotypically and genetically closely related micro-organisms that may cause diseases with similar clinical presentation (Elsaghir & James, 2003; Vaidya *et al.*, 2006). Although *Brucella* and *Ochrobactrum* can be differentiated phenotypically by means of their growth behaviour, motility, lysis by *Brucella*-specific bacteriophages and agglutination with *Brucella*-specific antisera, misidentification of these organisms using biochemical test systems such as API 20NE has been reported (e.g. Elsaghir & James, 2003; Hubalek *et al.*, 2007). Currently, no PCR assay is available for the discrimination of *O. anthropi*, *O. intermedium* and *Brucella* spp. Thus, the aim of this study was to establish and evaluate a discriminatory PCR assay for the sensitive and specific detection of each of these pathogens. For this purpose, the *recA* gene was selected as the target gene, as it is highly conserved between *Ochrobactrum* and *Brucella* but exhibits enough stable nucleotide substitutions to establish a discriminatory PCR assay between *Ochrobactrum* and *Brucella* and also between *O. anthropi*, *O. intermedium* and other non-pathogenic *Ochrobactrum* spp. (Scholz *et al.*, 2006). In a recent study, the *recA* genes of 106 *Ochrobactrum* strains and the *Brucella* type strains were

Table 2. PCR primers used in this study

Abbreviations: FL, fluorescein; LC Red640, LightCycler Red640.

Organism	Primer/probe	Sequence (5′→3′)	Nucleotide position	Amplicon (bp)
MP-ST-PCR				
<i>O. anthropi</i>	Anth-f	GCAAGCTGGGTGTCGATCTGG	347–367	544
	Anth-r	TTCTCGACGACACCGGCCTTTA	890–869	
<i>O. intermedium</i>	Inter-f	CGGCGTTGGTGGCTTGCCTAA	186–206	402
	Inter-r	GGAACGAGAGATAGACGCGGTA	588–567	
<i>Brucella</i> spp.	Bruc-f	AACCACGCTTGCCTTGACACACC	249–270	167
	Bruc-r	TTTCAAGCGCCTGTTACCCCG	395–415	
Real-time PCR				
Primers	B4	TGGCTCGGTTGCCAATATCAA		
	B5	CGCGCTTGCCTTTCAGGTCTG		
Probes	BruFL	AGGCAACGTCTGACTGCGTAAAGCC-FL		
	BruLC	LC Red640-ACTCCAGAGCGCCCGACTTGATCG		

sequenced and analysed in detail (H. C. Scholz *et al.*, unpublished data). In the present study, we used this information to construct *O. anthropi*-, *O. intermedium*- and *Brucella*-specific primers to set up and evaluate a differentiating PCR for these pathogens.

To assess the specificity of the developed MP-ST-PCR, the type strains of each *Brucella* species and a collection of 50 *O. anthropi* and 16 *O. intermedium* strains from both clinical and environmental sources, including one isolate (RMA 16994) from a recently reported severe *O. anthropi* infection in an immunocompetent host (Vaidya *et al.*, 2006), were used (Table 1). The negative panel comprised 23 strains of closely related organisms, including the type strains of a number of *Ochrobactrum* spp., and other diagnostically relevant bacteria.

In the first step, the primer pairs were tested in separate ST-PCR for optimization of the cycle conditions and determination of the detection limit for each PCR. As expected, specific fragments of 544, 402 and 167 bp were amplified using template DNA from all tested *O. anthropi*, *O. intermedium* and *Brucella* strains, respectively, using the appropriate primer pairs (data not shown). Each primer pair was specific for its template and did not amplify *recA* gene sequences from other bacteria, with the exception of *O. lupini*, which yielded a 544 bp fragment with the *O. anthropi*-specific primers (Table 1). However, *O. lupini* is a highly adapted plant bacterium infecting only the roots of *Lupinus albus*, and thus does not interfere with the diagnosis of brucellosis or *Ochrobactrum* infections. Strain RMA 16994, initially identified by Vaidya *et al.* (2006) as *O. anthropi*, was positive in the *O. intermedium* PCR but not in the *O. anthropi*-specific PCR (Table 1). This is in agreement with a recent detailed molecular analysis, which demonstrated that this strain is indeed a member of the species *O. intermedium* (Kämpfer *et al.*, 2007).

The detection limits in single PCR were 10, 10 and 100 fg for *O. anthropi*, *O. intermedium* and *Brucella* spp.,

corresponding to 2, 2 and 20 genome equivalents, respectively. MP-ST-PCR analysis using a mixture of equal amounts of each primer also resulted in the amplification of the expected products. Additional bands as a result of interfering primers were not observed. The detection limit of *O. anthropi* in the MP-ST-PCR was reduced by a factor of 10 (100 fg), whereas the other detection limits were unaffected. The detection limits for *O. anthropi*, *O. intermedium* and *B. melitensis* of the MP-ST-PCR are shown in Fig. 1. No amplification was detected with template DNA of the other bacteria investigated.

Specific amplification products were also generated when mixed-template DNA from *Brucella* spp. with either *O. anthropi* or *O. intermedium* was used (data not shown). The detection limit was not reduced using this mixed-template DNA. A mixture of *Brucella* spp. together with DNA from *O. anthropi* and *O. intermedium* resulted in strong primer interference, giving uninterpretable results. However, this was only a theoretical scenario; in practice, it is unlikely that two of these agents, and even less three, would be co-isolated from one patient. However, the multiplex PCR may help to identify laboratory-derived cross-contamination of these organisms.

Brucella DNA from clinical material was detected using an in-house real-time LightCycler PCR that has been evaluated recently (Al Dahouk *et al.* 2007) and compared with the novel MP-ST-PCR described here. Template DNA was prepared from various clinical specimens from a sheep and its fetuses with culture-proven brucellosis (Fig. 2). Real-time PCR was carried out with template DNA from 31 different clinical samples using 45 cycles of amplification (Fig. 2a). Template DNAs that were positive in this PCR (nos 2, 4, 6, 8, 9, 10, 11, 17 and 23) were subjected to the MP-ST-PCR using 37 amplification cycles. In this PCR, samples with a C_t value of <37 reacted positively (Fig. 2b, nos 2, 8, 9, 10 and 11). A faint band was visible with template 6 (C_t value of 37) in the MP-ST-PCR (Fig. 2b).

Fig. 1. Detection limits of the *recA* gene-based MP-ST-PCR. Agarose gels of the partial *recA* PCR products amplified with 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 0.1 fg DNA (lanes 2–9, respectively) of *O. anthropi* LMG 3331^T (a), *O. intermedium* LMG 3301^T (b) and *B. melitensis* 16M^T (c). Lane 10, no-template control; lanes 1 and 11, molecular mass standards.

Fig. 2. Detection of *Brucella* DNA in clinical samples by real-time PCR (a) and the *recA*-based MP-ST-PCR (b). Clinical samples that were used are shown on the left in (a). The numbers of the samples that were positive in the real-time PCR are given on the corresponding amplification curves. Samples with a C_t value of <37 were positive in both PCR assays.

Templates with a C_t value of >37 (nos 4, 17 and 23) were negative. Thus, both PCR assays exhibited almost equal detection limits. *Brucella* DNA was also amplified from liver abscess material from a patient with culture-proven brucellosis (data not shown). The applicability of the assay for the detection of *O. anthropi* and *O. intermedium* in clinical specimens could not be investigated as no clinical material was available, but it can be assumed that no loss in analytical sensitivity would occur.

Our results demonstrate that the developed PCR assay allows accurate differentiation of *O. anthropi*, *O. intermedium* and *Brucella* spp. The developed assay is intended mainly for use with pure cultures where biochemical tests have given uninterpretable results. Although we demonstrated that the developed PCR can also be used for the specific and sensitive detection of *Brucella* DNA in different tissues, we recommend well-evaluated real-time PCRs for the detection of *Brucella* in clinical samples, as investigated by Al Dahouk *et al.* (2007).

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