NOTES

Rapid Detection of *Brucella* spp. in Blood Cultures by Fluorescence In Situ Hybridization

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Brucellosis is a severe systemic disease in humans. We describe a new 16S rRNA-based fluorescence in situ hybridization assay that facilitates rapid and specific detection of all human pathogenic species of *Brucella* and that can be applied directly to positive blood cultures.

Brucellae are gram-negative bacteria which cause serious infections in animals and humans. Four species, namely, *Brucella abortus*, *Brucella canis*, *Brucella suis*, and especially *Brucella melitensis*, are able to infect humans and cause a systemic disease called brucellosis that is endemic in Mediterranean countries, the Middle East, Africa, and Latin America. DNA hybridization and multilocus enzyme electrophoresis studies show a high level of homology among the *Brucella* spp., indicating that the genus *Brucella* comprises only one species with several biovars (6, 15, 17). A reliable and early diagnosis of brucellosis is of major importance in order to initiate an adequate therapy. Culture of blood and other specimens, such as bone marrow aspirates and biopsy specimens, is the gold standard for diagnosis of human brucellosis (16, 17). However, culture and phenotypic identification are hampered by the slow and fastidious growth of the organisms and by possible misleading results of commercial biochemical identification systems (4). In addition, brucellae are associated with a high risk of laboratory-acquired infections since they are easily transmitted by aerosols and the infectious dosage is very low. Intensive handling of the organism in the laboratory often precedes identification as *Brucella* spp., and thus, protective measures are taken too late (8, 12). Serological methods for the detection of *Brucella*-specific antibodies are available, but their usefulness is limited by a high prevalence of *Brucella*-specific antibodies in areas where brucellosis is endemic, low sensitivity, especially in acute infections, and cross-reactions with other gram-negative bacteria (6, 17).

The development of new diagnostic techniques that facilitate rapid detection and identification of brucellae and minimize the risk of laboratory infection is of great practical importance. Fluorescence in situ hybridization (FISH) using fluorescently labeled oligonucleotide probes complementary to unique target sites on the rRNA has already been successfully

implemented in the field of clinical microbiology, including the detection and identification of pathogens in blood cultures and cerebrospinal fluid (9, 13). FISH is a rapid and easy-to-perform method that directly visualizes bacteria without prior amplification or cultivation steps.

Here, we describe a new FISH assay that facilitates rapid and specific detection of all human-pathogenic species of *Brucella* spp. that can be applied directly to positive blood cultures. The FISH assay was evaluated by means of 17 reference strains of brucellae, 19 other relevant bacterial species (43 isolates), and 33 positive blood cultures, including 2 from patients with brucellosis.

The FISH probe Bru-996 (5'-CCA CTA ACC GCG ACC GGG ATG), targeting the bacterial 16S rRNA gene, was designed using the ARB program package, available at <http://www.arb-home.de>. The probe was synthesized and directly 5' end labeled with Cy3 by Thermo Hybaid, Germany. Because the *Brucella* probe shows only one mismatch to *Ochrobactrum* spp., an unlabeled competitor was added with the purpose to prevent cross-binding (KBru-996, 5'-CCA CTA ACC GCG ATC GGG ATG [the competitor differed from the probe at one base, which is underlined]). A fluorescein isothiocyanate-labeled eubacterial probe (EUB 338 [3]) was added in all experiments as a control.

The sensitivity and specificity of the *Brucella* FISH test were evaluated in a blinded manner on a panel of *Brucella* reference strains, including all species that are pathogenic for humans and other pathogenic bacteria (Table 1). Approximately 10 μ l of a suspension of bacteria in 0.9% saline was applied to a glass slide, air dried, and fixed for 5 min in methanol. FISH was performed at 46°C using hybridization buffers with 30% formamide and corresponding washing buffers as described elsewhere (2, 10). FISH correctly detected all 17 isolates of *Brucella* spp. and showed no cross-reactions with other species, including the closely related species *Ochrobactrum anthropi*, thus resulting in a sensitivity and specificity of 100%. Two isolates of *Ochrobactrum anthropi* that were misidentified as *Brucella* spp. phenotypically (API 20 NE; BioMérieux, Germany) and by 16S rRNA gene sequencing did not react with

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TABLE 1. Strains used for evaluation of the FISH test

Strain (biotype)	Source	FISH result ^b
<i>Brucella abortus</i> (1)	ATCC 23448	+
<i>Brucella abortus</i> (2)	ATCC 23449	+
<i>Brucella abortus</i> (3)	ATCC 23450	+
<i>Brucella abortus</i> (4)	ATCC 23451	+
<i>Brucella abortus</i> (5)	ATCC 23452	+
<i>Brucella abortus</i> (6)	ATCC 23453	+
<i>Brucella abortus</i> (7)	ATCC 23454	+
<i>Brucella abortus</i> (9)	ATCC 23455	+
<i>Brucella melitensis</i> (1)	ATCC 23456	+
<i>Brucella melitensis</i> (2)	ATCC 23457	+
<i>Brucella melitensis</i> (3)	ATCC 23458	+
<i>Brucella suis</i> (1)	ATCC 23444	+
<i>Brucella suis</i> (2)	ATCC 23445	+
<i>Brucella suis</i> (3)	ATCC 23446	+
<i>Brucella ovis</i> (1)	ATCC 25840	+
<i>Brucella canis</i> (1)	ATCC 23365	+
<i>Brucella neotomae</i> (1)	ATCC 23459	+
<i>Acinetobacter calcoaceticus</i>	ATCC 23055	–
<i>Acinetobacter junii</i>	ATCC 17908	–
<i>Acinetobacter lwoffii</i>	ATCC 15309	–
<i>Agrobacterium tumefaciens</i>	CI (2) ^a	–
<i>Alcaligenes faecalis</i>	ATCC 35655	–
<i>Enterococcus faecalis</i>	CI (1)	–
<i>Escherichia coli</i>	CI (11)	–
<i>Haemophilus influenzae</i>	ATCC 10211	–
<i>Haemophilus parainfluenzae</i>	ATCC 33392	–
<i>Klebsiella pneumoniae</i>	CI (4)	–
<i>Neisseria cinerea</i>	ATCC 14685	–
<i>Neisseria lactamica</i>	ATCC 23970	–
<i>Ochrobactrum anthropi</i>	CI (2)	–
<i>Proteus mirabilis</i>	CI (2)	–
<i>Pseudomonas aeruginosa</i>	CI (6)	–
<i>Pseudomonas stutzeri</i>	ATCC 17558	–
<i>Salmonella enteritidis</i>	CI (1)	–
<i>Staphylococcus aureus</i>	CI (4)	–
<i>Stenotrophomonas maltophilia</i>	ATCC 13637	–

^a Clinical isolate (number of isolates tested).^b +, positive for brucellae; –, negative for brucellae.

the *Brucella*-specific FISH probe. Both isolates were also negative in the *Brucella*-specific BCSP-31 PCR (11) and the AMOS PCR (5).

In a second step, the applicability of the FISH test to detect brucellae directly in positive blood cultures was evaluated on 33 positive blood cultures (BACTEC 9240 system; BD), which showed gram-negative rods by Gram staining. For the FISH investigation, 10 µl of the positive blood culture medium (BACTEC plus aerobic/F medium) was transferred to glass slides and fixed with methanol on the same day that the BACTEC system reported the bottles as positive. FISH slides were examined as described above. Two blood culture bottles from two Turkish patients with brucellosis caused by *B. melitensis* biovars 1 and 2 were included. In both patients, gram-negative coccoid bacilli were detected in aerobic BACTEC 9240 blood culture bottles by Gram staining after 5 to 6 days of incubation in the automated BACTEC system. The brucellae showed bright fluorescence after hybridization with probe Bru-996. The 31 remaining blood cultures were negative by probe Bru-996 but positive with the eubacterial control EUB-338. In these bottles, *Escherichia coli* ($n = 16$ isolates), *Klebsiella pneumoniae* ($n = 5$), *Klebsiella oxytoca* ($n = 1$), *En-*

terobacter cloacae ($n = 4$), *Enterobacter* spp. ($n = 1$), *Pseudomonas aeruginosa* ($n = 2$), *Haemophilus parainfluenzae* ($n = 1$), and *Morganella morganii* ($n = 1$) were isolated by culture.

In order to evaluate whether the new FISH assay allows more rapid detection and identification of brucellae in blood cultures than the automated BACTEC system, blood cultures of healthy volunteers were spiked with serial dilutions of the two clinical isolates of *Brucella melitensis* (5×10^2 to 5×10^6 brucellae per blood culture bottle), and the FISH assay and Gram staining were performed daily until positive signaling of the bottles in the automated BACTEC system. By FISH, the brucellae were detected 1 to 2 days (16 to 38 h) before positive signaling in the automated BACTEC system. Remarkably, in the blood culture bottles that were spiked with the lowest concentration of brucellae, FISH detected the brucellae even 1 day earlier (day 3) than the Gram staining (day 4; BACTEC, day 5). This can be explained by the fact that low concentrations of bacteria are more easily detected by fluorescence than by Gram staining, due to the small size and faint gram-negative appearance of brucellae.

The new FISH assay described here is a valuable tool for both the identification of cultured isolates suggestive of *Brucella* spp. and the direct detection of brucellae in positive blood cultures. When applied directly to positive blood cultures, it allows rapid detection of brucellae without the need for further subcultures and phenotypical tests and thus accelerates diagnosis and reduces the risk of laboratory-acquired infections. In patients with clinical suspicion of brucellosis, FISH can detect the brucellae in the blood culture bottles even more rapidly than the automated BACTEC system. In contrast to 16S rRNA gene sequencing and real-time PCR strategies that also allow reliable molecular identification of brucellae (1, 5, 7, 11, 14), the FISH technique is a rapid and cheap method that is also applicable in resource-limited laboratories. Further studies are needed to determine the usefulness of FISH for the direct identification of brucellae in clinical specimens, such as bone marrow aspirates and tissue biopsy specimens.

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