

# Direct Urease Test and Acridine Orange Staining on Bactec Blood Culture for Rapid Presumptive Diagnosis of Brucellosis

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## Abstract

Brucellosis is one of the most common zoonotic diseases in Iran and human brucellosis is endemic in all parts of the country. Growth of *Brucella* is slow and blood culture of these bacteria by use of classical methods is time-consuming. Furthermore, in endemic area culture is required for definitive diagnosis. In the present study, direct urease test and acridine orange staining were tried on the BACTEC blood culture broths for early presumptive identification of *Brucella* growth. Blood cultures were attempted in 102 seropositive patients. In the forty one blood cultures positive for *Brucella*, coccobacilli were seen in broth smears stained with acridine orange stain, and also were urease test positive, thus providing presumptive identification of *Brucella* growth. Urease test was negative and bacteria were not seen in the broth smears of the remaining 61 broths negative for *Brucella* growth. Because of simplicity, reliability and reproducibility, these tests can be routinely incorporated in the laboratory for diagnosis of brucellosis.

**Keywords:** *Brucella* spp, Brucellosis, Urease test, Acridine orange, BACTEC blood culture, Iran

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## Introduction

Brucellosis is a worldwide zoonosis caused by members of the genus *Brucella*. The disease is accidentally transmitted to humans during occupational contact with infected animals or by consumption of contaminated animal products (1). The incidence of the disease has decreased markedly in industrialized countries. However, it remains a major public health problem in many developing countries (2). In Iran, human brucellosis is endemic in all parts of the country. Patients recorded in 1988 were 71,051 (132.4 per 100,000) (3). The laboratory confirmation of human brucellosis is based either on isolation of *Brucella* from blood cultures or detection of specific antibodies. Blood cultures provide the best yield for microbiological diagnosis with sensitivity ranging from 53 to 90%, depending on the disease stage, *Brucella* spe-

cies, culture medium, quantity of circulating bacteria, and culture technique employed (4). Despite recent developments, like nucleic acid probes, PCR, and other molecular techniques for microbiological diagnosis, blood cultures still remain the most practical and reliable method in the diagnosis of blood stream infections (5). In endemic areas culture is required for a definitive diagnosis because the symptoms and signs of brucellosis are non-specific, and the interpretation of agglutinating antibody titer can be confounded by persistently elevated titer in persons without active disease who have been repeatedly exposed to infected animals (6). Blood culture by the classical Castaneda method can take up to 35 days of incubation before growth is detected (7). Over the last few years, experience on the isolation of *Brucella* spp by use of automated blood culture

systems has been accumulating at a slow pace (8). Rapid detection of microorganisms in blood cultures is one of the most important tasks of the clinical microbiology laboratory. In 1980, McCarthy and Senne evaluated the use of acridine orange (AO) for the detection of microorganisms in blood cultures (9). They found acridine orange a rapid, inexpensive alternative to blind subcultures and more sensitive than Gram stain for detecting microorganisms in smears (9). In 1994, Larson et al. described the use of AO staining of broth blood cultures for detection of *Bartonella quintana* (10). In 2003, Adler et al. found that AO staining can replace subcultures of false-positive blood cultures (11). Our study is the first that suggests the use of AO staining as an early indicator for the presence of *Brucella* spp.

## Materials and Methods

During 2002 to 2004, blood culture samples were taken from 102 suspected patients of brucellosis who came to the Dept. of Infectious Diseases, School of Medicine, Tehran University of Medical Sciences, Iran. Suspicious of brucellosis was serologically confirmed by rose Bengal antigen test. All the patients were adults, and had high titer anti-brucella antibodies that were performed at the time of diagnosis. High titer was defined as a titer of  $\geq 1:160$  by the standard tube agglutination (STA) method. Only one blood culture was processed per patient. Blood for culture was drawn by the staff physicians, and between 5 to 7 ml of blood from patients was inoculated in each of standard aerobic/F BACTEC bottle at the patient's bedside. Bottles were incubated for up to 7 d in the incubator-rocker compartment of the BACTEC 9120 instrument and examined for the presence of growth. Each bottle contained a chemical sensor which detected increases in CO<sub>2</sub> produced by the growth of microorganisms. The sensor was monitored by the instrument every ten min for an increase in fluorescence, proportional to the amount of CO<sub>2</sub> pre-

sent. A positive reading indicated the presumptive presence of viable microorganisms in the bottle. The bottle with positive and negative growth index was Gram and acridine orange stained and subcultured to chocolate blood agar at 37° C in CO<sub>2</sub>. In addition, subculture of (0.9-1 ml) BACTEC broths onto urea slants was done. Tubes were incubated at 37° C in carbon dioxide and examined for the red color that was characteristic of urease activity every two h for six h and again after overnight incubation. At the end of the first week, bottles with negative growth index was kept for an additional three weeks and blind subcultures of samples from the blood culture bottles were performed on days 7, 14, 21, and 28. Cultures were considered negative for *Brucella* only after four weeks of incubation. Slides for AO staining were air dried and then fixed in absolute methanol for two min. Without being rinsed, the slides were air dried and then stained for one min with AO stain. After being rinsed, the slides were air dried and viewed under the high dry objective of a microscope equipped with a vertical fluorescence illuminator. Known positive and negative specimens were stained each day for quality assurance. Positive controls consisted of a known positive culture, and negative controls consisted of filter-sterilized broth.

## Results

A total of 102 blood culture bottles were processed during the evaluation period: 41 of them (40.2%) gave a growth index positive (GIP) by the BACTEC system and in 61(59.8%) cases, no growth was detected by BACTEC system and they were growth index negative (GIN). Regardless of the bottles growth index, all blood culture bottles were subcultured onto urea slants. A Gram stain and AO stain of the blood culture bottles also were performed, and the fluid was subcultured on sheep blood agar, chocolate agar and MacConkey plates. Seeded media were incubated at 35° C in a 5% CO<sub>2</sub>-enriched atmosphere and examined daily for

four d. All 41 GIP gave a positive urease reaction, 38 gave a positive reaction within four h and three were positive after overnight incubation but all 61 GIN were urease test negative after overnight incubation. Subculture of 41 GIP BACTEC broths on sheep blood and chocolate blood agar eventually yields *Brucella* organisms within two to three d. Thirty nine BACTEC broths gave a visible colony within two d, but two isolates gave a visible colony after three d (Table1). All cultures positive with the BACTEC instrument were detected within a week and blind subcultures of 61 GIN BACTEC broths on days 7, 14, 21 and 28 were negative. No positive blood culture bottles were missed by the BACTEC 9120 instrument and detected by blind subcultures. AO staining detected organisms in all of these GIP culture broths. Gram staining detected organisms in 26 of 41 GIP BACTEC broths. Acridine orange and Gram stain of 61 GIN BACTEC broths for *Brucella* were negative.

**Table 1:** Comparison of blood culture with urease test and acridine orange staining during time

Type of test	Positive (in/wk) (% agreement w/culture)	Negative (4 wk) (% agreement w/culture)
Blood culture	41	61
Urease	41(100)	61(100)
Acridine orange	41(100)	61(100)

## Discussion

Isolation of the *Brucella* organisms is the definitive means of diagnosis, but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In the last decade, automated blood culture system, based on continuous monitoring and colorimetric detection of CO<sub>2</sub> production by growing organisms has been introduced into clinical practice. Recent studies have demonstrated that this technology enable detection of

genus *Brucella* within the 7-day routine incubation period used by most clinical microbiology laboratories (8). The major drawback of culture for *Brucella* is that it is time consuming. We have tried to overcome this drawback by using simple and inexpensive methods, i.e., use of direct urease test and AO staining for detecting the growth of *Brucella* in blood culture broths. There is only one study on use of direct urease test on BACTEC blood culture for presumptive diagnosis of brucellosis. (12).

Although AO staining has been effectively utilized for laboratory diagnosis of bacteria such as *Bartonella quintana* and *Streptococcus pneumoniae*, our report is the first study that uses AO for detection of *Brucella* spp. No data on the performance of BACTEC blood culture system and use of direct urease test for the detection of *Brucella* spp have been published from Iran and this is the first and the largest reported study. Our data for use of urease test agree with those of Rich et al (12) who have reported that 100 % of 4-h urease test on signal-positive blood culture broths (BACTEC 9240) were indicative of *Brucella* bacteremia. These methods provide a presumptive indication of *Brucella* growth at least two to three d earlier than the conventional identification methods. Positive urease test and AO staining results obtained from all BACTEC bottles with positive growth index, indicate that these tests have a high sensitivity in detection of *Brucella* in blood culture. Therefore, we contend that positive urease and AO tests are good predictors of *Brucella* growth in subsequent culture from original BACTEC bottle. In other words; blood cultures from patients suspected of brucellosis, which present with negative urease and AO test, are not likely to isolate any *Brucella* organisms. The agreement between direct urease test and AO stain results and culture confirmation was 100 percent (Table1).

Therefore, we strongly suggest that the urease test and AO staining can be utilized for rapid detection of *Brucella* spp even in conventional blood cultures. Furthermore, we suggest that

clinical microbiology laboratories in areas that brucellosis is endemic can integrate the simple, inexpensive and rapid urease and AO test into their routine diagnostic procedures.

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