Chapter 8

Blood Cultures for the Diagnosis of Human Brucellosis

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Additional information is available at the end of the chapter

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Abstract

Brucellosis represents a serious health threat to human populations living in areas endemic for the disease. The clinical manifestations of brucellosis are protean and non-specific, and laboratory confirmation of the diagnosis is crucial for an adequate management of the patient and implementation of infection control measures aimed to control the disease in affected herds. Although brucellosis can be confirmed by serologic tests and nucleic acid amplification assays, culture detection of circulating *Brucella* organisms remains a diagnostic cornerstone. Traditionally, prolonged incubation of media and performance of blind subcultures of negative blood culture vials have been recommended to maximize isolation of the organism. In recent years, modern automated blood culture systems have revolutionized the diagnosis of human brucellosis by improving sensitivity and enabling detection of brucellae within the routine one-week incubation protocol followed in most Clinical Microbiology laboratories. Development of molecular techniques and mass-spectrometry technology have also shortened the time needed to identify members of the genus, whereas use of biological safety cabinets considerably reduce the risks of contagion to laboratory personnel.

Keywords: Human brucellosis, blood cultures, diagnosis, identification, safety

1. Introduction

Because of the non-specific clinical manifestations of human brucellosis and the need for prolonged combination therapy with antibiotics that are not routinely prescribed for other infectious diseases, laboratory confirmation of the diagnosis is of paramount importance for

the adequate patient management. In addition, evidence of brucellosis has serious public health implications because it discloses exposure to a contaminated source (infected animals or their products, unsafe laboratory practices, or a potential biological warfare attack).

The current laboratory diagnosis of human brucellosis is based on culture, serology, and nucleic acid amplification assays. Although the culture strategy is hampered by the slow growing features of *Brucella* species, safety problems, and the reduced sensitivity of the method for detecting chronic cases, isolation of the organism remains a diagnostic cornerstone. Recovery of the brucellae is an irrefutable evidence of the disease; it permits speciation and typing of the recovered strain for epidemiological studies [1] and enables determination of antibiotic susceptibility, when indicated. Blood cultures may also allow diagnosis of brucellosis in the acute period of the disease, when serological test results may still be negative or exhibit borderline antibody titers [2]. An additional advantage of the culture approach is the fact that it enables the diagnosis in cases in which brucellosis is not suspected. This is an important consideration because the clinical presentation of human brucellosis is frequently not specific, and patients may present with symptoms and signs suggestive of other diagnoses, including a variety of infections, rheumatic, hematologic, or neurologic conditions, hepatitis, etc. If the possibility of brucellosis is not considered, specific serologic tests or nucleic acid amplification assays will not be ordered and, under these circumstances, the diagnosis of the disease can be missed altogether, unless a positive blood culture was obtained. Isolation of *Brucella* organisms can be, then, the first and only proof of the disease. For instance, in a study conducted in a highly endemic area for *B. melitensis* in southern Israel, 27 blood cultures obtained from 21 patients with suspected brucellosis grew the organism, as did 42 cultures drawn from 27 patients in whom possibility of the disease was not entertained [3].

The current prevalence of brucellosis in most Western countries is low and, therefore, microbiology laboratories are frequently unfamiliar with the tools available for isolating the organism. The purpose of this review is to summarize published information on the performance of the different blood culture techniques for the detection of brucellae. Because anaerobic conditions do not support growth of the strictly aerobic members of the genus, only data on the performance of aerobic media will be included in the chapter.

1.1. Role of blood cultures in the diagnosis of human brucellosis

Brucellosis is a systemic infection in which the bacterium initially localizes in the regional lymph nodes and then disseminates by the hematogenous route to macrophages-rich tissues where it adopts an intracellular lifestyle [4]. In the early stages of the disease, patients experience continuous brucellemia, facilitating the culture diagnosis of the disease. As the infection progresses, bacteremia tends to wane, making the recovery of the organism increasingly difficult [5]. However, *Brucella* organisms may reappear in the bloodstream intermittently [5], and their isolation is associated with an increased risk of relapse, probably because a demonstrable bacteremia implies a high bacterial burden [6, 7]. Even in localized infections, the pathogenesis of brucellosis in the human host always implies a bacteremic phase and, therefore, blood cultures may represent an adequate tool for establishing the diagnosis, although their sensitivity varies widely (between 10% and 90%) in different series [5].

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2. Blood culture methods

The sensitivity of blood cultures for detecting circulating brucellae may be negatively influenced by a variety of factors such as patient's age [8], prolonged or chronic clinical course [9–12], or previous exposure to antibiotics [12, 13], as well as technical aspects including blood sample volume, incubation time, frequency of growth monitoring, or the performance of blood culture media and detection systems. Despite these drawbacks, blood culture techniques have also been adopted for the isolation of *Brucella* spp. from normally sterile specimens other than blood [14], such as bone marrow [12, 15, 16], synovial fluid aspirates [17], pancreatic exudate [18], or cerebrospinal fluid [19], and have been shown to be comparable or more sensitive than conventional culture methods on solid media.

2.1. Manual monophasic methods

Although *Brucella* organisms may be recovered by routine bacteriological culture methods, detection of the organism in clinical specimens is frequently hindered by its slow growth. Because seemingly negative blood culture vials are routinely discarded after a one-week incubation period, unless physicians and laboratory personnel are aware of the possibility of brucellosis, the diagnosis may be missed altogether. To maximize the detection of fastidious members of the genus, incubation of blood cultures for 30 days and performance of blind subcultures have been advised [20, 21]. This approach has obvious drawbacks: it is labor intensive, prolonged incubation of blood culture vials requires large laboratory space and costly equipment, and diagnosis of the disease is substantially delayed.

2.2. Biphasic methods

To circumvent the necessity of making repeat subcultures, an ingenious biphasic flask, containing solid agar and a liquid phase, was developed by Ruiz-Castañeda in the late 1940s [11, 14, 21, 22]. After inoculation, the flask is supplemented with 10% CO_2 and tilted so that the liquid covers the solid medium and incubated in the upright position. Flasks are examined every 3 days for the presence of colonies [14, 21, 22]. If no growth is observed, flasks are tilted again and re-incubated, and the cycle is repeated for at least 35 days [14, 21, 22].

Gotuzzo *et al*. reported their experience with the Castañeda method in Peru and observed that brucellae colonies developed within one week, with a mean time-to-detection of 4.3 days when seeded with bone marrow specimens, and 6.7 days when inoculated with peripheral blood, and all positive results were obtained within 15 days of incubation [10]. In a Spanish study, however, the time-to-detection was more prolonged, and the majority of flasks required between one and three weeks of incubation [23]. Differences in the patients' population, the biological characteristics of the *Brucella* strains, or the composition and quality of homemade media may explain the observed discrepancies in the performance of the method.

The capability of a commercial biphasic blood culture flask (Hémoline biphasic medium, bioMérieux, Marcy l'Etoile, France) to recover *Brucella melitensis* was prospectively assessed by Ruiz et al. [24]. Flasks were inoculated with 10 ml of blood obtained from patients with

suspected brucellosis, incubated for three weeks, and subjected to blind subcultures on day 21. Although the median time-to-positivity was 5 days only, four out of 19 (21.1%) positive cultures were detected after 7 to 9 incubation days [24].

2.3. Lysis centrifugation: In-house and commercial methods

Braun and Kelsh developed a membrane filter technique for isolating *Brucella* spp. and evaluated its performance in a rabbit animal model [25]. A heparinized blood specimen obtained from animals experimentally inoculated with *Brucella* organisms was subjected to osmotic lysis and filtered through a sterile Millipore filter under negative pressure. Filters were placed on the surface of solid media and incubated, and organisms trapped in the membrane developed as colonies on the agar. The technique was abandoned because it was too cumbersome, time and labor intensive, and filters became easily plugged with cellular components of the blood.

A new and original method was subsequently developed in which blood cells were osmotically lysed, and this step was followed by centrifugation and spread of the lysate on the surface of solid culture media [26, 27]. In 1984, Etemadi et al. evaluated this lysis centrifugation procedure —also known as lysis concentration—and compared its performance with that of the Castañeda flask for the detection of *B. melitensis* from blood and other normally sterile body fluids [26]. All cultures, including 14 peripheral blood samples, two bone marrow, and two cerebrospinal fluid specimens, were positive by the lysis centrifugation method within 48 hours, whereas all 18 Castañeda flasks remained negative after 21 days of incubation [26].

A similar lysis centrifugation method was used by Mantur and Mangalgi who compared it with the biphasic Castañeda vial in patients with acute and chronic brucellosis confirmed by a standard agglutination test (SAT) titer ≥160 [28]. Of 121 patients with acute brucellosis, the Castañeda method identified 87 (71.8%), whereas the lysis centrifugation was positive in 110 (90.9%) patients (*P=0.001*), and the time-to-detection was 6.7±2.2 and 2.4±0.9 days, respectively (*P<0.001*). Of the 27 patients with chronic disease, the detection rates were 3.3% (n=9) for the Castañeda flask and 74.1% (n=20) for the lysis centrifugation method (*P=0.087*), and the time-to-detection was 7.2±2.6 and 2.7±1.4 days, respectively (*P=0.001*). In a more recent study, the lysis centrifugation recovered *B. melitensis* in 73 (43.1%) of 169 serologically-confirmed human cases, compared to 42 (24.8%) detected by the blood clot culture and 59 (34.9%) by the Castañeda technique, and the detection time was significantly shorter [29].

Encouraging results were also obtained in Peru by Espinosa et al. who compared the performance of the traditional Castañeda method with that of Etemadi's lysis centrifugation technique in 88 patients in which the disease was suspected on the bases of compatible clinical symptoms and a SAT titer ≥1:25 [9]. The two methods were similar in terms of sensitivity: the lysis centrifugation procedure detected *Brucella* organisms in 38 (43.2%) patients while the Castañeda flask succeeded in 31 (35.2%) patients (*P>0.05*). However, the detection times differed significantly and were 3.8±0.8 days for the lysis centrifugation and 13.6±6.5 days for the Castañeda method (*P<0.001*). In a prospective study, Kolman et al. obtained blood cultures from Israeli patients with serologically proven brucellosis [27]. Blood sample aliquots were subjected to an in-house lysis centrifugation procedure and inoculated into an aerobic

radiometric BACTEC system (Becton Dickinson Diagnostic Instrument Systems, Towson, Md., USA) vial [27]. The lysis centrifugation detected *B. melitensis* in only 15 (27.8%) out of 54 patients, whereas the comparator succeeded in 19 (35.2%) patients. The lysis centrifugation method, however, detected brucellae after an average of 3.5 days (range 2–4 days) vs.14 days (range 7–30 days) by the automated system.

The traditional in-house lysis concentration methods have been replaced in recent decades by a commercial blood culture system (the Isolator Microbial Tube, Wampole Laboratories, Cranbury, NJ, USA). Collected blood samples are inoculated into a vial containing a mixture of the anticoagulant sodium polyethol sulfonate (SPS) and detergent. The detergent lyses the blood cells releasing already phagocytized but still viable organisms, and the lysate is then seeded onto appropriate solid media and incubated. The system has two versions: a small tube for use in pediatric patients that accommodate up to 1.5 ml of blood and, therefore, is plated directly; and a larger 10 ml-containing tube for use in adult patients that require a preliminary centrifugation step to concentrate the lysate before plating.

In a prospective study, Navas et al. inoculated 10 ml of blood obtained from patients with presumptive brucellosis into an Isolator Microbial Tube, and two 5 ml-aliquots were inoculated into one aerobic (NR6A) and one anaerobic (NR7A) BACTEC NR660 vials [30]. The two methods were comparable in terms of sensitivity, the Isolator Microbial Tube detected 7 positive cultures system vs. 6 identified by the automated system. [30]. The lysis concentration technique reduced the time-to-detection to 2–5 days vs.17 to 29 days with a mean of 20.6 days for the BACTEC blood culture system. It should be noted, however, that because anaerobic bottles do not support the growth of strictly aerobic *Brucella* organisms, the effective blood volume inoculated into the BACTEC system was, in fact, only half of that seeded onto the Isolator Microbial Tube plates [30].

A study conducted in a region endemic for *B. melitensis* in Israel confirmed the capability of the Isolator Microbial Tube system to accelerate the detection of the organism as compared to traditional methods, and 15 out of 22 (68.2%) blood cultures were already positive after 72 hours [31]. When compared with the automated BACTEC 9240 system, however, the Isolator Microbial Tube was inferior in terms of both time-to-detection and sensitivity (see "Comparative studies involving fully automated blood culture systems" section).

2.4. Automated blood culture systems

In the past, detection of positive blood culture vials relied on periodic examination of inoculated vials for the presence of turbidity as an indication that microorganisms have multiplied in the broth and reached a high concentration. Over the last few decades, the diagnosis of bacteremic infections has been revolutionized by the development of automated blood culture systems. The novel technologies are based on detections of increasing concentrations of CO_2 released by the metabolic activity of a growing mass of organisms, or consumption of the available oxygen. Significant changes in the gas content of the blood culture vials can be detected before cloudiness becomes visible, resulting in the gain of precious time and allowing early diagnosis of bacteremia. The detecting technology evolved over the years; the pioneer semi-automated BACTEC 460 detected release of radioactive CO_2 generated by the metabolism

of ^{14}C-containing substrates by penetrating the vial top and aspirating the headspace above the fluid level. The subsequent generations consisted of fully-automated instruments that employed either detection of CO_2 levels by invasive infrared reading (BACTEC NR), non-invasive measurement of increasing fluorescence as the concentration of CO_2 increases or the O_2 content decreases (the BACTEC 9000 and FX series of instruments), colorimetric CO_2 measurement (BacT/ALERT, bioMérieux, Marcy l'Etoile, France), or quenching of fluorescence by CO_2 production and acidification or reduction of the culture broth (VITAL, bioMérieux, Marcy l'Etoile, France). Overall, published studies indicate that technical advances in the detection technologies and improvements in the composition of broth culture media have resulted in gradual increase in sensitivity, shortened time-to-detection of *Brucella* organisms, enabling labor saving by continuous hands-off monitoring of a large number of blood culture vials and decrease in culture contamination rates.

Experience with the isolation of *Brucella* spp. by automated blood culture systems has been accumulating at a slow pace. Although the disease is still prevalent in many developing countries, use of modern bacteriologic techniques in endemic areas is limited because of their high cost, whereas in the more affluent Western world, where use of modern automated systems is widespread, brucellosis has been successfully controlled or eradicated altogether.

2.5. Factors influencing detection of brucellae by automated systems

In general terms, detection of CO_2 production in blood culture broths depends on the initial number of bacteria inoculated (which reflects the concentration of circulating organisms and the volume of the blood sample drawn), duplication time of the species, its intrinsic metabolic activity, composition of the media, presence of growth promoters or inhibitory factors, frequency of readings, sensitivity of the sensor, and threshold levels.

Obviously, obtaining a large blood specimen should improve the sensitivity of the blood culture tool for detecting bacteremia. In practice, the volume of blood inoculated in the bottle varies little (usually between 1 to 3 ml per bottle in children and 3 to 5 ml in adults) because of the requirement to keep at least a 1:5 to 1:10 blood-to-broth ratio to reduce the concentration of detrimental factors such as complement, antibodies, or antibiotics contained in the clinical specimen.

The magnitude of *Brucella* bacteremia is frequently low with a median of 88 CFU/ml [31, 32] and a range of 1.3 CFU/ml to >1,000 CFU/ml in children [31]. As it should be expected, the time-to-positivity of automated blood culture systems correlates inversely with the concentration of circulating organisms, validating the results of experimental studies with simulated blood cultures [33, 34]. In addition, *Brucella* organisms have a relatively long (2.5 to 3.5 hours) doubling time compared to other pathogenic bacteria [32]. This feature, coupled with the low CO_2 production by members of the genus, results in delayed detection of brucellae by some automated blood culture systems. In a series of in vitro studies using the BacT/ALERT system, a slow release of CO_2 by *B. melitensis* compared with other human pathogens was observed, and the peak concentrations of the gas were inferior [33]. In a series of experiments with BACTEC NR730 vials inoculated with brucellae, Gamazo et al. reported that noticeable

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turbidity was noted in the vial on average 24 hours earlier than detection by the automated instrument [32].

With the purpose of improving CO_2 production by *Brucella* organisms, the effect of adding a variety of supplements (pyruvate, alanine, glutarate, urea, glucose, and erythritol), as well as changing the pH of the culture broth was investigated [32]. Only alanine and pyruvate resulted in a mild increase in the CO_2 production, while lowering the pH of the medium from 7.2 to 6.2 coupled with pyruvate supplementation, induced a more pronounced increment. Although these experimental results suggest that modifications in the formulation of blood culture media may reduce the time-to-detection of *Brucella* bacteremia, changes in the broth composition may not necessarily sustain growth of other bacterial species. In the same study, a harmful effect of the anticoagulant SPS contained in the blood culture vials was demonstrated. Unfortunately, blood culture systems cannot dispense with the use of SPS because there are no good alternatives to the antiphagocytic, anticomplementary, and aminoglycoside-neutralizing effects of this compound. In the vials of the 9000 series of BACTEC instruments, the concentration of SPS has been reduced to 0.025% compared with 0.035% in the NR660 and BacT/ALERT media and the total volume of broth has been increased from 30 ml in the NR660 system to 40 ml in the BACTEC 9000 instruments vials, improvements that may explain the better performance of the latter systems for detecting fastidious *Brucella* organisms [35].

2.6. Radiometric detection of brucellae

The BACTEC 460, developed in the early 1970s was the first in a series of modern blood culture systems. Published experience with the use of this method for the recovery of brucellae from blood is limited and obtained results were suboptimal [27, 36–38]. In 1984, Arnow et al. investigated a cluster of foodborne *B. melitensis* infections among travelers to endemic Spain [36]. Overall, 15 out of 19 (78.9%) blood cultures derived from 6 patients were detected by the automated instrument between 4 and 8 days of incubation. In another report, brucellae were only recovered from a blind subculture performed in a three-day-old vial that remained radiometrically negative despite having been incubated for 6 additional days [37].

In a comparative study, Serrano et al. obtained 83 blood culture sets from 42 patients with positive *Brucella* agglutinin titers [38]. Five ml of blood were inoculated into an aerobic BACTEC 460 vial and an identical volume was inoculated into a Castañeda flask, incubated for 10 days, and subjected to blind subcultures on days 5 and 10. By day 5, 14 cultures were positive. The Castañeda method detected 12 positive cultures (85.7%) and the BACTEC bottle 10 (71.4%), of which only 2 were detected radiometrically and the remaining by subculture only. On day 10, 49 cultures were already positive by the biphasic flask and 56 by the radiometric medium ($P>0.05$), of which only 27 reached the radiometric positivity threshold [38].

2.7. Infrared detection system

Data on the use of infrared detection technology (BACTEC NR instruments) for the detection of *Brucella* spp. are also scarce [3, 27, 30, 34, 39, 40]. Zimmerman et al. recovered *B. abortus* by subculture of two five-day-old blood cultures and from a seven-day-old bone marrow culture

inoculated into aerobic BACTEC NR vials [34]. Once the diagnosis was made, additional blood cultures were obtained and 15 vials, including aerobic, osmotically stabilized (aerobic hypertonic), and anaerobic media were inoculated and monitored by the automated instrument. All five aerobic and four osmotically stabilized vials became positive between 7 and 20 days, whereas, as expected, all five anaerobic bottles remained negative.

In a Spanish study, inoculated BACTEC NR vials and biphasic Hémoline flasks were monitored for three weeks, and negative media were blindly subcultured on day 21. The biphasic system detected 28 positive cultures, obtained from 18 patients, after an average of 7 days. The BACTEC NR system detected only 12 positive bottles, missed 10 patients, and the mean time-to-positivity was substantially longer (19.6 days) [39]. Furthermore, 11 of these 12 BACTEC NR positive vials gave negative infrared readings during the three-week incubation period, and the organism was detected by subculture only [39].

In the aforementioned study by Navas et al., the BACTEC NR instrument detected only 12 out of 16 (75.0%) blood culture sets obtained from 7 patients and missed the diagnosis in 1 patient, whereas the Isolator Microbial Tube detected all 7 patients, and the time-to-positivity was significantly shorter [30]. Employing the BACTEC NR system, Gedikoglu isolated brucellae in 22 patients with a median detection time of 72 hours [40]. Because vials were only kept for 7 days and no blind subcultures of negative bottles were performed, the study does not allow assessment of the sensitivity of the system for detecting brucellae within the routine one-week incubation protocol.

To assess the capability of the BACTEC NR blood culture system to detect *B. melitensis* within the conventional one-week incubation schedule, we conducted a prospective study in southern Israel [3]. Blood culture vials were monitored by the automated instrument and subcultured once a week for four weeks, and the proportion of positive cultures detected by the instrument within the first week was determined. During the two-year study period, 27 of 373 (7.2%) blood cultures, drawn from 21 patients, were positive for brucellae. Twenty-one (78.8%) of these cultures were detected by the BACTEC NR instrument within 7 days, and 6 positive cultures (22.2%) were detected by subculture after two or three weeks, corroborating that prolonged incubation and periodic performance of subcultures of negative bottles were still required to optimize the detection of *B. melitensis* by the non-radiometric BACTEC technology.

2.8. Continuous monitoring systems

BacT/ALERT system. The published experience with the use of the BacT/ALERT system for the recovery of circulating brucellae remains limited [18, 33, 41]. In 1992, Solomon and Jackson isolated *B. melitensis* in a traveler to the Middle East after only 2.8 days [33]. Two years later, Casas et al. drew blood cultures from 6 patients with serologically-confirmed infection [41]. Inoculated bottles were monitored by the BacT/ALERT instrument for 10 consecutive days and were then transferred to a regular incubator for 10 additional days, and blind subcultures on solid media were performed on days 10 and 20. Only 1 of 9 positive bottles was detected by the automated instrument after 2.9 days, while the remaining bottles were detected by subculture only: 7 on day 10, and 1 on day 20 [41]. A different experience was reported by Roiz et al. who found that all 9 blood cultures, obtained from 5 patients, yielded the organism within

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3.7 days, and a blood culture vial, inoculated with pancreatic fluid, was detected positive after 13.3 hours only [18].

BACTEC 9000 instruments. In 1996, Gedikoglu et al. summarized the experience accumulated in a Turkish hospital with the use of the BACTEC 9120 system with a one-week monitoring protocol [40]. Thirty blood cultures, drawn from 15 patients grew *B. melitensis* within 84 hours of incubation. Akcam et al. compared the BACTEC 9120 blood culture system and conventional cultures for culturing normally-sterile body fluids other than blood employing the aerobic pediatric vial and a 7-day incubation protocol, and reported that the five clinical specimens containing *B. melitensis* were only detected by the automated instrument [14].

Kurtoglu et al. summarized the experience accumulated with the BACTEC small 9050 instrument and the medium-size 9120 model for culturing blood in an endemic area for brucellosis in Turkey. The study employed a routine 5-day protocol but extended the incubation period to 14 days when brucellosis was suspected [42]. All brucellae were recovered within 10 days but no precise information on the time-to-positivity was reported, and the fraction of organisms detected within the routine protocol's timetable was not stated. Using the BACTEC 9240, a larger version of the system, and a similar incubation protocol, we detected 59 of 77 (76.6%) positive *Brucella* cultures within 4 days (unpublished data).

Despite these encouraging results, limiting incubation of blood culture vials to the traditional one-week period instituted in most clinical laboratories cannot be adopted in regions endemic for brucellosis, unless it is convincingly demonstrated that no significant number of positive cultures are missed by a short incubation schedule. Adequate assessment of the capability of any blood culture system to detect brucellae within the routine one-week incubation protocol requires keeping of inoculated vials for a longer period and performance of blind subculture of negative vials to assure that no positive cultures are overlooked.

The capability of the BACTEC blood culture system to detect brucellae within 7 days was prospectively investigated among febrile children in southern Israel [43]. Following the traditional recommendations by the World Health Organization [20] and the American Society for Microbiology [21], inoculated aerobic pediatric blood culture vials were monitored by the BACTEC 9240 instrument for four consecutive weeks, and blind subcultures of negative vials were performed once a week [43]. Of a total of 2,579 blood cultures drawn, 42 (1.6%) were positive for *B. melitensis*, of which 41 (97.6%) were detected by the automated instrument within 2 to 6 days, and the remaining positive vial was missed by the instrument and detected by blind subculture performed on day 7. Cumulative positivity rates by the automated detection were 0.0%, 23.6%, 78.9%, 86.8%, 92.1%, 97.6%, and 97.6% for days 1 through 7, respectively.

Similar results were obtained in a study conducted in Saudi Arabia in a mixed population of children and adult patients [35]. BACTEC 9240 aerobic/F (for adults) and Peds Plus vials (used for pediatric patients) were kept for up to 21 days, but no blind subcultures of negative vials were performed, precluding an adequate assessment of the sensitivity of the method. Overall, the BACTEC instrument detected 90 out of 97 (92.7%) positive cultures, of which 85 yielded *B. melitensis* and 12 *B. abortus* isolates within 5 days of incubation, and only 3 cultures (3.1%) became positive after the seventh day (2 on day 8 and 1 on day 9) [35].

Durmaz et al. reviewed their five-year experience with the BACTEC 9120 system in a Turkish hospital [44]. Vials were monitored by the automated instrument for 7 days and vials negative at the end of period were Gram-stained and subcultured. Overall, 20 vials yielded *B. melitensis* after a median 69.9 hours (mean: 30.0 hours, range: 31.2–117.5 hours), and no false negative automated readings were recorded.

However, in another Turkish study, 8 of 136 cultures obtained from 60 patients, remained undetected by the instrument and were recovered on blind subcultures performed after 30 days of incubation [45]. A similar experience was recorded in the investigation of an outbreak of *B. melitensis* infections involving 16 adult Spanish patients [46]. The researchers employed the BACTEC 9050 that differs from the other instruments of the BACTEC 9000 series in that agitation of the bottles is continuous, a factor that may accelerate bacterial growth, whereas in the other models is intermittent. Inoculated bottles were incubated for 21 days, and negative vials were subcultured blindly at the end of the study period [46]. Overall, 13 patients had demonstrable *Brucella* bacteremia. Growth of the organism was detected by the instrument within one week in only 9 (69.2%) bacteremic individuals, in 2 additional patients on the 8th and 11th day, and in the remaining 2 patients, brucellae were entirely missed by the instrument and detected by the final subculture.

Although the reasons for these discrepancies are not obvious, the superior performance of the automated BACTEC system in the aforementioned communication by Yagupsky et al. [43] could be explained by the fact that their study population consisted entirely of children presenting to the Pediatric Emergency Department with an acute febrile disease, probably characterized by continuous high-magnitude bacteremia, whereas other investigations enrolled mostly adult patients with a more prolonged disease and, therefore, a lower bacterial load.

The BACTEC MYCO/F LYTIC medium has been recently developed to improve the recovery of intracellular pathogens such as fungi and mycobacteria by lysing leucocytes with saponin [47]. Because brucellae are facultative intracellular bacteria, it was assumed that use of the automated blood culture system coupled with this novel medium would improve both sensitivity and time-to-detection of circulating organisms. However, in a prospective study in which the performance of the traditional pediatric (Peds Plus /F) and adult (PlusAerobic/F) aerobic vials were compared with that of the MYCO/F LYTIC vial, the sensitivity was comparable but the time-to-positivity was significantly longer in the latter (101.4±46.7 hours) vs. 65.5±18.9 hours for the traditional media combined (*P=0.004*), and after 72 hours of incubation, only 5 out of the 16 (31.2%) MYCO/F LYTIC vials were already positive, compared to 16 out of 19 (84.2%) aerobic adult and pediatric vials (*P=0.005*).

2.9. Comparative studies involving fully automated blood culture systems

In a prospective study in which blood aliquots drawn from children with suspected brucellosis were inoculated into a BACTEC 9240 aerobic vial and into an Isolator Microbial Tube, the sensitivity and time-to-positivity of the two methods were compared [31]. Overall, 122 pairs of blood cultures were obtained and 28 (22.8%) were positive by at least one method. The BACTEC system detected all 28 positive cultures and the Isolator Microbial Tube detected 22

positive cultures (sensitivity: 78.6%, *P<0.023*). Among those 22 cultures positive by both methods, 21 (95.5%) and 15 (68.2%) were found to be positive within 3 days by the BACTEC and by the lysis centrifugation systems, respectively. Eight cultures (36.4%) were detected at least 1 day earlier by the BACTEC instrument, and the remaining 14 were detected by the two systems on the same day (*P<0.05*). In summary, the automated BACTEC system was significantly superior than the compactor in terms of sensitivity and also reduced the time-to-positivity.

The performance of the two most popular automated blood culture systems for the detection of brucellae was compared in a single study [48]. BacT/ALERT and BACTEC 9,240 vials were inoculated with 10 ml of adult patients' blood and monitored for 7 days. Overall, the BACTEC system detected 9 out of 17 (52.9%) positive cultures whereas the BacT/ALERT detected 14 (82.3%) (*P=0.067*), and the time-to-detection of the positive vials were similar (2.8 vs. 2.5 days, respectively). Apparently, no blind subcultures of negative vials were performed, and it is unknown whether a more prolonged incubation would have improved the recovery rate.

The performance of three blood culture systems [the automated BACTEC 9120 and VITAL (bioMérieux) systems, and the Hémoline biphasic flask] was compared in a prospective study involving 19 positive blood cultures drawn from Spanish patients with brucellosis [24]. The Hémoline medium detected all 19 positive cultures, whereas the BACTEC and the VITAL systems missed one positive culture each (sensitivity: 94.7%). By using a 5-day incubation protocol, 47.4%, 78.9%, and 10.5% cultures were detected by the three blood culture systems, respectively. When the incubation was extended to 7 days, the results were 73.7%, 94.7%, and 47.4%, respectively, indicating that the BACTEC system was significantly faster than the comparators (*P<0.05*). The delayed detection of brucellae by the VITAL system was confirmed in two later studies in which the time-to-positivity for members of the genus was 119.7 and 211.7 hours [49, 50].

2.10. Bone marrow vs. blood cultures

Because of the suboptimal recovery rate of brucellae from blood, it has been suggested that cultures of bone marrow [14, 10, 11, 51, 52], liver tissue [53, 54], or lymph nodes [55] may improve the recovery of the organism. The rationale for these alternative approaches is that *Brucella* organisms survive the intracellular killing by phagocytes and polymorphonuclear leukocytes and localize in the reticuloendothelial system [10, 52].

Ganado and Bannister demonstrated that in one-fifth of patients in whom bone marrow cultures were positive for brucellae, the organism could not be isolated from the blood [39]. Gotuzzo et al. reported that among 50 patients with proven brucellosis detected by cultures of blood, bone marrow, or both, bone marrow cultures were positive in 46 (92.0%) patients whereas blood cultures were positive in only 35 (70.0%) [10]. Despite the small volume of bone marrow cultured (usually less than 1 ml) compared to the much larger blood volumes (between 5 and 10 ml), brucellae grew more rapidly from bone marrow samples, suggesting that higher bacterial concentrations may be present in this macrophages-rich specimen type. In a prospective study by Mantur et al., blood samples and bone marrow aspirates obtained from 103 Indian patients with serologically confirmed brucellosis were inoculated into Castañeda flasks

[15]. The sensitivity of bone marrow cultures was significantly superior and recovered *Brucella* organisms in 85 (82.5%) patients vs. 47 (45.6%) detected by blood cultures *(P<0.001),* and the time-to-recovery was significantly shorter (2.8±0.7 and 7.2±2.4 days, respectively, *P=0.001)* [15]. It is noticeable that the superior performance of the bone marrow culture was observed in acute, as well as in chronic cases.

Özkurt et al. obtained blood and bone marrow samples from 50 Turkish patients with suspected brucellosis, of which 48 exhibited SAT titers ≥1:160 [12]. Specimens were inoculated into BacT/ALERT vials and into a homemade *Brucella* broth medium. Seeded BacT/ALERT vials were incubated for 7 days. Negative vials at day 7 were incubated for two additional weeks and subcultured on solid media every 2 days. The non-commercial *Brucella* broth media were incubated for four weeks and subcultured blindly every 2 days. The bone marrow specimens proved to be more sensitive for the detection of *B. melitensis* and, overall, 35 of 50 (70.0%) bone marrow cultures, but only 24 of 50 (48.0%) blood cultures grew the organism (*P<0.05*).

On the other hand, Magill and Killough found that in their experience, blood cultures were more reliable (sensitivity: 90%) than bone marrow cultures (sensitivity: 40%) [56]. Similarly, Shehabi et al. reported a sensitivity of 44.4% for blood cultures compared to 27.7% for bone marrow cultures [57]; and Iseri et al., employing the BACTEC 9050 instrument, also found peripheral blood cultures to be more sensitive than bone marrow aspirates [detection rates 39 out of 102 (48.0%) and 35 out of 102 (34.3%), respectively (*P<0.05*)] [16].

Although current evidence regarding the relative merits of bone marrow vs. peripheral blood cultures remains controversial, most experts considered the former as the gold standard specimen for diagnosis [4]. However, it should be pointed out that blood cultures have the clear advantage of being easy to obtain and repeat, and the fact that they can serendipitously identify cases in which the diagnosis was not entertained; whereas aspiration of bone marrow samples for detecting brucellae requires, *a priori,* a high index of suspicion.

2.11. Blood clot cultures

Because the serum of patients with brucellosis may have antibacterial activity, culture of the blood clot, where organisms phagocytized by leukocytes may be trapped, appears as a rational strategy. The method consists of collecting a blood sample in a sterile tube and allowing it to cloth. The tube is then centrifuged and the serum is separated aseptically and used for serological assays, whereas the clot is disrupted by shaking the tube and seeded into appropriate media [29]. Available data on the advantages of this technique, however, are limited and contentious. Escamilla et al. employed two types of clot cultures, one with added taurocholate-streptokinase and the other with bile, and compared their yield with that of conventional cultures of whole blood in an area endemic for brucellosis in Peru and found the clot cultures were far less sensitive and more labor-intensive than the comparator method [58]. Whereas the conventional cultures detected 28 of 30 (93.3%) positive cultures, the taurocholate-streptokinase was positive in 21 (70.0%) and the bile-clot recovered the organism in a single culture (3.3%). It is unclear whether culturing of the clot without the additives could have provided better results.

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In a comparative study of 169 serologically confirmed patients, Mangalgi and Sajjan reported a detection rate of 34.9% for the clot culture, 24.8% for the Castañeda flask, and 43.1% for the lysis concentration method; the mean±SD recovery times were 5.8±1.4, 9.6±1.7, and 4.1±0.9 days, respectively [29]. In a second study by the same research group, blood clot cultures were clearly superior to conventional broth cultures of whole blood for isolating brucellae, increasing the yield by >20% and shortening the time-to-positivity from an average of 8.2 days to 3.1 days [59]. If these favorable results are confirmed by additional research, this simple and inexpensive method could represent a real contribution to the diagnosis of brucellosis in developing countries where more advanced and expensive laboratory technologies are not available.

3. From detection to identification

3.1. Conventional identification of blood culture isolates

Once bacterial growth is detected in a blood culture vial, prompt and precise identification of the isolate is of paramount importance for adequate patient management and avoidance of exposure of laboratory technicians to infective *Brucella* organisms. Traditionally, a Gram stain of the positive broth is performed and, unless the biphasic Castañeda method is employed, it is subcultured onto solid media. Identification of members of the genus *Brucella* is based of the presence of typical small Gram-negative coccobacilli (see Figure 1); positive oxidase, catalase, and urease tests; no fermentation of sugars; CO_2 requirement; lack of motility; and confirmed by a positive agglutination reaction with specific antiserum [14] or, alternatively, the isolate'sbiochemical profile is determined by a commercial system. The main drawbacks of this traditional approach is the slow turnaround time (2 to 3 days) and the possible misidentification of brucellae as *Ochrobactrum anthropi* [60], *Ochrobactrum intermedium* [61], *Bergeyella zoohelcum* [62], or *Moraxella phenylpyruvica* by commercial kits; a serious mistake that has already lead to an outbreak of laboratory-acquired infection [63].

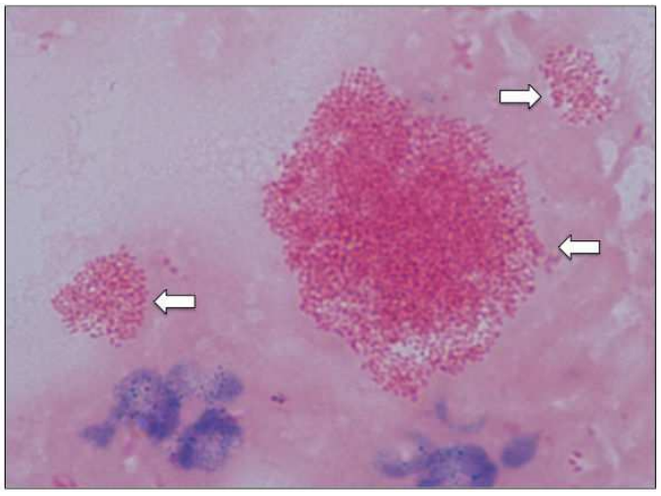

Figure 1. Gram stain of a positive aerobic BACTEC blood culture vial showing *Brucella melitensis* microcolonies (white arrows).

3.2. Rapid phenotypic identification methods

A simple and rapid method was proposed by Rich and co-investigators in Saudi Arabia for the presumptive identification of brucellae from signal-positive BACTEC 9240 blood culture vials [64]. Thirty-three positive BACTEC broths containing Gram-negative coccobacilli and 32 with no visible organisms were subcultured on urea slants and incubated in a CO_2-enriched atmosphere. Of the 44 *Brucella* isolates eventually recovered, 37 gave a positive urease reaction within 4 hours and the remaining were positive after overnight incubation. The urease test showed good specificity and only 2 isolates other than brucellae (both *Haemophilus influenzae*) gave a delayed positive urease reaction. Favorable results were also reported by Maleknejad et al. in an endemic area of Iran using a slight modification of the procedure [65]. The investigators combined the routine Gram staining procedure of positive vials with the high sensitivity of the acridine orange staining, and inoculated positive media onto urea slants. The procedure correctly identified the 41 blood cultures positive for brucellae within 4 hours and was negative in 61 slants seeded with blood culture broths that grew other bacterial species.

In recent years, introduction of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) technology in the Clinical Microbiology laboratory has revolutionized the field of bacterial speciation, enabling precise, reproducible, and cost-effective identification of isolates within minutes. The method obviates the need for biochemical testing and, thus, is suitable for high-throughput by less skilled laboratory personnel [66, 67]. This novel approach can be employed on bacterial colonies growing on agar plates, as well as from positive culture broths and, therefore, it enables direct identification of organisms from blood culture vials [68]. Available data with type strains and simulated blood cultures indicate that the MALDI-TOF procedure reliably identifies isolates as members of the genus *Brucella*. It should be pointed out that because of the high transmissibility of *Brucella* organisms, an initial bacterial inactivation step with absolute ethanol was added as a measure of caution, to be followed by extraction with formic acid and acetonitrile [69, 70]. Although in some studies, the method also enabled discrimination at the species level and even at the biovar level for *B. suis* [66, 67], other investigators reported unreliable discrimination between the different *Brucella* species [68].

In summary, major advancements in spectrometry technology over the last decade have opened the possibility of accurate and rapid identification of brucellae directly from blood culture vials. Data on the use of MALDI-TOF method for this purpose, however, are still limited because, although the cost for specimen processing is low, MALDI-TOF instruments are expensive and, thus, unavailable in most resources-poor rural areas endemic for brucellosis. Although experimental results are promising, this encouraging experience awaits confirmation with real cultures derived from actual patients.

3.3. Identification of brucellae by DNA technology

A variety of molecular approaches have also been proposed to shorten the identification process and enable correct identification of *Brucella* isolates. A fluorescence *in-situ* hybridization (FISH) assay targeting a part of the *16S* rRNA gene and containing an unlabeled competitor differing from the probe at one base with the purpose of preventing cross-binding, has been

developed and evaluated with actual blood cultures [71]. The test was employed directly in positive blood culture broths and enabled rapid and correct identification of *B. melitensis* at a low cost, and was negative in cultures that grew a variety of other bacterial species.

Sequencing of the *16S* rRNA gene, which is in widespread use for bacterial identification, can be misleading and *Brucella* organisms cannot be accurately distinguished from the closely related α-protobacterial *Ochrobacterium* species [72]. A novel *recA* gene-based, multi-primer, single-target PCR assay has been recently developed and succeeded in differentiating between brucellae and *Ochrobacterium anthropi* and *O. intermedium* [73], although the test has a more prolonged turnaround time and is more expensive than the FISH test.

4. Blood cultures for brucellae and laboratory safety

Brucellosis remains among the most commonly recognized causes of laboratory-transmitted infections, and 2% of all brucellosis cases are laboratory-acquired [74]. Several biological characteristics make brucellae easily transmissible within the close confinement of the Clinical Microbiology laboratory: the infecting dose for humans is very low (10 to 100 bacteria); the organism may enter the body in many ways relevant to laboratory practices, including through the respiratory mucosa, conjunctivae, gastrointestinal tract, or abraded skin [74]; and the long-term persistence of viable microorganisms on inanimate surfaces [13, 75].

Because of the protean manifestations of brucellosis in humans, a wide array of clinical samples submitted to the Clinical Microbiology laboratory for culture, including normally sterile body fluids, exudates, and tissues, may contain viable bacteria, although blood cultures represent the largest number of specimens. The concentration of circulating brucellae in the patients' blood is frequently low [31], and unless a serious breach of safety practices has occurred, blood specimens do not pose a tangible threat of contagion to laboratory personnel. In addition, current automated blood culture instruments monitor CO_2 production without penetrating the blood culture vial and, thus, avoid creation of risky aerosols. However, the danger of significant exposure increases exponentially after incubation, and routine bacteriologic procedures such as preparing, centrifuging, and vortexing of bacterial suspensions, performing subcultures and biochemical testing, particularly the catalase test, entail a substantial potential for nebulization of bacteria, accidental spillage, and contamination of the laboratory environment [76].

In regions endemic for brucellosis, the number of positive cultures for the organism and, consequently, the risk for transmission to laboratory personnel can be extremely high. In a Clinical Microbiology laboratory in Ankara, Turkey, an annual average of 400 cultures were positive for *Brucella* spp. and the disease was diagnosed in 10 (18%) of 55 laboratory workers, representing a calculated hazard of 8% per employee-year [77]. In a study conducted in 1997 at the Soroka University Medical Center (SUMC) that serves an endemic area for the disease in southern Israel, 127 of 3,974 (3.2%) aerobic blood culture vials detected as positive by the automated BACTEC instrument, as well as 11 of 126 (8.7%) Isolator Microbial Tube cultures, grew *B. melitensis* [78]. From 2002–2009, the organism was isolated from 514 of 20,620 (2.5%)

positive blood culture vials and, as expected, the detection rate showed a significant seasonal pattern and was higher between April and September (3.3%) compared with the October-March period (0.9%, *P<0.001*) [79].

To increase laboratory safety, the Centers for Disease Control (CDC) has strongly recommended that all manipulations with live *Brucella* cultures should be confined to a Class II biologic safety cabinet [80]. However, by the time bacterial isolates are identified as brucellae, extensive manipulation of culture media has usually been performed and inadvertent exposure of laboratory personnel may have already occurred. Following a large outbreak of laboratory-acquired brucellosis at the SUMC in 1997 [78], all positive blood cultures are initially processed in safety cabinets until the presence of the organism is ruled-out, and performance of unnecessary antibiotic susceptibility testing of *Brucella* isolates and aerosol-generating procedures has been discontinued, and no further cases of the disease have been detected ever since. It seems, then, prudent to recommend that all positive blood culture vials in endemic areas should be processed in a safety cabinet, when available, pending final identification of the isolate.

5. Conclusions

Although the diagnosis of human brucellosis can be established by serologic and nucleic acid amplification assays, culture confirmation of the disease has not lost its traditional clinical and epidemiological importance. In the past, isolation of brucellae was hindered by the slow growth of the organism and the lack of a suitable commercial blood culture system. To improve recovery of this fastidious bacterium, use of biphasic media, prolonged incubation of vials, and periodic performance of blind subcultures have been traditionally recommended. Development of automated blood culture systems in recent decades has resulted in the gradual increase in sensitivity and shortening of detection time of *Brucella* species. Nowadays, use of modern blood culture systems makes possible the diagnosis of more than 95% of positive cultures within the routine 7-day incubation protocol, and performance of subcultures of negative media is no longer necessary. Additional advances, especially the development of MALDI-TOF technology and nucleic acid amplification and hybridization assays, in recent years, enable a rapid and precise identification of the genus.

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References

[1] Lee S, Hwang KJ, Park MY, Hwang SD, Chai HY, Chu H, Park SH. Evaluation and selection of multilocus variable-number tandem-repeat analysis primers for genotyping *Brucella abortus* biovar 1 isolated from human patients. Osong Public Health Res Perspect 2013; 4:265-270.

[2] Shemesh AA, Yagupsky P. Limitations of the standard agglutination test for detecting patients with *Brucella melitensis* bacteremia. Vector Borne Zoonotic Dis 2011; 11:1599-1601.

[3] Yagupsky P. Detection of *Brucella melitensis* by BACTEC NR660 blood culture system. J Clin Microbiol 1994; 32:1889-1901.

[4] Pappas G, Akritidis, Bosilkovski M, Tsianos E. Brucellosis. N Eng J Med 2005; 352:2325-2336.

[5] Pappas G, Papadimitriou P. Challenges in *Brucella* bacteremia. Int J Antimicrob Agents 2007; 30; Supp. l1:S29-S31.

[6] Ariza J, Corredoira J, Pallares R, Viladrich PF, Rufi G, Pujol M, Gudiol F. Characteristics of and risk factors for relapse of brucellosis in humans. Clin Infect Dis 1995; 20: 1241-1249.

[7] Solera J, Martinez-Alfaro E, Espinosa A, Castillejos ML, Geijo P, Rodriguez-Zapata M. Multivariate model for predicting relapse in human brucellosis. J Infect 1998; 36:85-88.

[8] Apa H, Devrim I, Memur S, Günay I, Gülfidan G, Çelegen M, Bayram N, Karaarslan U, Bag O, Isgüder R, Özturk A, Inan S, Ünal N. Factors affecting *Brucella* spp. blood culture positivity in children. Vector Borne Zoonotic Dis 2013; 13:176-180.

[9] Espinosa BJ, Chacaltana J, Mulder M, Franco MP, Blazes DL, Gilman RH, Smits HL, Hall ER. Comparison of culture techniques at different stages of brucellosis. Am J Trop Med Hyg 2009; 80:625-627.

[10] Gotuzzo E, Carrillo C, Guerra J, Llosa L. An evaluation of diagnostic methods for brucellosis-the value of bone marrow culture. J Infect Dis 1986; 153:122-125.

[11] Ruiz Castañeda, M. Laboratory diagnosis of brucellosis in man. Bull World Health Org 1961; 24:73-84.

[12] Özkurt Z, Erol S, Tasyaran MA, Kaya A. Detection of *Brucella melitensis* by the BacT/ALERT automated system and *Brucella* broth culture. Clin Microbiol Infect 2002; 8:749-752.

[13] Doganay M, Aygen B. Human brucellosis: An overview. Int J Infect Dis 2003; 7:173-182.

[14] Akcam FZ, Yayli G, Uskun E, Kaya O, Demir C. Evaluation of the Bactec microbial detection system for culturing miscellaneous sterile body fluids. Res Microbiol 2006; 157:433-436.

[15] Mantur BG, Mulimani MS, Bidari LH, Akki AS, Tikare NV. Bacteremia is as unpredictable as clinical manifestations in human brucellosis. Int J Infect Dis 2008; 12:303-307.

[16] Iseri S, Bulut C, Kinikli S, Demiröz AP, Tülek N. Comparison of the diagnostic value of blood and bone marrow cultures in brucellosis. Microbiyol Bul 2006; 40:201-206.

[17] Yagupsky P, Peled N. Use of the Isolator 1.5 Microbial Tube for detection of *Brucella melitensis* in synovial fluid. J Clin Microbiol 2002; 40:3878.

[18] Roiz MP, Peralta FG, Valle R, Arjona R. Microbiological diagnosis of brucellosis. J Clin Microbiol 1998; 36:1819.

[19] Raj A, Gautam V, Gupta PK, Sethi S, Rana S, Ray P. Rapid detection of *Brucella* by an automated blood culture system at a tertiary care hospital of north India. Indian J Med Res 2014; 139:776-778.

[20] Alton GG, Jones LM. Bacteriological methods, 1967; pp. 13-17. *In* Laboratory techniques in brucellosis, World Health Organization, Geneva.

[21] Moyer NP, Holcomb LA. *Brucella*, 1995; pp. 549-555. *In* Murray PR, E J Baron, Pfaller MA, Tenover FC, Yolken RH (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.

[22] Ruiz Castañeda M. A practical method for routine blood cultures in brucellosis. Proc Soc Exp Biol Med 1947; 64:114-115.

[23] Rodríguez-Torres A, Fermoso J, Landinez R. Brucelosis. Medicine (Spanish Ed.) 1983; 48:3126-3136.

[24] Ruiz J, Lorente I, Perez J, Simarro E, Martínez-Campos L. Diagnosis of brucellosis by using blood cultures. J Clin Microbiol 1997; 35:2417-2418.

[25] Braun W, Kelsh J. Improved method for cultivation of *Brucella* from the blood. Proc Soc Exp Biol Med 1954; 85:154-155.

[26] Etemadi H, Raissadat A, Pickett MJ, Zafari Y, Vahedifar P. Isolation of *Brucella* spp. from clinical specimens. J Clin Microbiol 1984; 64:115.

[27] Kolman S, Maayan MC, Gotesman G, Rozenszajn LA, Wolach B, Lang R. Comparison of the BACTEC and lysis concentration methods for recovery of *Brucella* species from clinical specimens. Eur J Clin. Microbiol Infect Dis 1991; 10:647-648.

[28] Mantur BG, Mangalgi SS. Evaluation of conventional Castañeda and lysis centrifugation blood culture techniques for diagnosis of human brucellosis. J Clin Microbiol 2004; 42:4327-4328.

[29] Mangalgi S, Sajjan A. Comparison of three blood culture techniques in the diagnosis of human brucellosis. J Lab Physicians 2014; 6:14-17.

[30] Navas E, Guerrero A, Cobo J, Loza E. Faster isolation of *Brucella* spp. from blood by Isolator compared with BACTEC NR. Diagn Microbiol Infect Dis 1993; 16:79-81.

[31] Yagupsky P, Peled N, Press J, Abramson O, Abu-Rashid M. Comparison of BACTEC 9240 Peds Plus medium and Isolator 1.5 Microbial Tube for detection of *Brucella melitensis* from blood cultures. J Clin Microbiol 1997; 35:1382-1384.

[32] Gamazo C, Vitas AI, Lopez-Goñi I, Diaz R, Moriyon I. Factors affecting detection of *Brucella melitensis* by BACTEC NR730, a nonradiometric system for hemocultures. J Clin Microbiol 1993; 31:3200-3203.

[33] Solomon HM, Jackson D. Rapid diagnosis of *Brucella melitensis* in blood: some operational characteristics of the BACT/ALERT. J Clin Microbiol 1992; 30:222-224.

[34] Zimmerman SJ, Gillikin S, Sofat N, Bartholomew WR, Amsterdam D. Case report and seeded blood culture study of *Brucella* bacteremia. J Clin Microbiol 1990; 28:2139-2141.

[35] Bannatyne RM, Jackson MC, Memish Z. Rapid diagnosis of *Brucella bacteremia* by using the BACTEC 9240 system. J Clin Microbiol 1997; 35:2673-2674.

[36] Arnow PM, Smaron M, Ormiste V. Brucellosis in a group of travelers to Spain. J Am Med Assoc 1984; 251:505-507.

[37] Menard RR, Choudhury SP, Naini MG, Wilk JT. A case of brucellosis: The importance of physician/laboratory communication. Clin Microbiol Newsl 1989; 11:157-158.

[38] Serrano ML, Llosa J, Castells C, Mendoza J, Navarro JM, de la Rosa M. Detección radiométrica de bacteriemias por *Brucella*. Enferm Infec Microbiol Clin (Spain) 1987; 5:139-142.

[39] García Rodríguez JA, García Sánchez JE, Muñoz Bellido JL, Canut-Blasco A. 1988. Comparison of a biphasic system and non-radiometric system for blood culture. Eur J Clin Microbiol Infect Dis 1988; 7:666-668.

[40] Gedikoglu S, Helvaci S, Ozakin C, Gokirmak F, Kilicturgay K. Detection of *Brucella melitensis* by BACTEC NR 730 and BACTEC 9120 systems. Eur J Epidemiol 1996; 12:649-650.

[41] Casas J, Partal Y, Llosa J, Leiva J, Navarro JM, de la Rosa M. Detección de *Brucella* por un sistema automático de hemocultivos: Bact/ALERT. Enferm Infec Microbiol Clin 1994; 12:497-500.

[42] Kurtoglu MG, Bozkurt H, Tuncer O, Kesli R, Berktas M. Distribution, optimum detection time and antibiotic susceptibility rates of the microorganisms isolated from blood cultures over a 4-year time period in a Turkish university hospital and a review of the international literature. J Intern Med Res 2008; 36:3-13.

[43] Yagupsky P, Peled N, Press J, Abu-Rashid M, Abramson O. Rapid detection of *Brucella melitensis* from blood cultures by a commercial system. Eur J Clin Microbiol Infect Dis 1997; 16:605-607.

[44] Durmaz G, Us T, Aydinli A, Kiremitci A, Kiraz N, Akgun Y. Optimum detection times for bacteria and yeast species with the BACTEC 9120 aerobic blood culture system: evaluation for a 5-year period in a Turkish university hospital. J Clin Microbiol 2003; 41:819-821.

[45] Ayaslioglu E, Kiliç D, Kaygusuz S, Kücük S, Ceken S, Erol O, Alpay Y. The detection of *Brucella* spp. by BACTEC 9050 blood culture system. Microbiyol Bul 2004; 38:415-419.

[46] Lepe JA, Guerrero FJ, Garrido A, Perea R. Deteccion de *Brucella melitensis* por el sistema BACTEC 9050. Enferm Infecc Microbiol Clin 2001; 19:267-269.

[47] Yagupsky P. Use of the MYCO/F LYTIC medium for detection of *Brucella melitensis* bacteremia. J Clin MIcrobiol 2004; 42:2207-2208.

[48] Baysallar M, Aydogan H, Kilic A, Kucukkaraaslan A, Doganci L. Evaluation of the BacT/ALERT and BACTEC 9240 automated blood culture systems for growth time of *Brucella* species in a Turkish tertiary hospital. Med Sci Monitor 2006; 12:235-238.

[49] Giménez M, Prat C, Vallés X, Matas L, Arnal J, Ausina V. Evaluation of the VITAL (bioMérieux) automated blood culture system using blind subculture. Clin Microbiol Infect 2002; 8:222-228.

[50] Melo-Cristino J, Salgado MJ. Detection of *Brucella* bacteremia by using the VITAL automated system and trypticase broth culture. Clin Microbiol Infect 1999; 5:706-709.

[51] Ganado W, Bannister W. Bacteremia in human brucellosis. Br Med J 1960; 1:601-603.

[52] Young EJ. An overview of human brucellosis. Clin Infect Dis 1995; 21:283-290.

[53] Cohen FB, Robins B, Lipstein W. Isolation of *Brucella abortus* by percutaneous liver biopsy. New Eng J Med 1957; 257:228-230.

[54] Fogel R, Lewis S. Diagnosis of *Brucella melitensis* by percutaneous needle biopsy of the liver. Ann Intern Med 1960; 53:204-206.

[55] Poston MA, Parsons PB. Isolation of *Brucella* from lymph nodes. J Infect Dis 1940; 66:86-90.

[56] Magill GB, Killough JH. Cortisone and combined antibiotic therapy of acute brucellosis melitensis. Am J Med 1954; 16:810-817.

[57] Shehabi A, Shakir K, El-Khateeb M, Qubain H, Fararjeh N, Abu Shamat AR. 1990. Diagnosis and treatment of 106 cases of human brucellosis. J Infect 1990; 20:5-10.

[58] Escamilla J, Florez-Ugarte H, Kilpatrick ME. Evaluation of blood clot cultures for isolation of *Salmonella typhi*, *Salmonella paratyphi*-A, and *Brucella melitensis*. J Clin Microbiol 1986; 24:388-390.

[59] Mantur BG, Bidari LH, Akki AS, Mulimani MS, Tikare NV. Diagnostic yield of blood clot culture in the accurate diagnosis of enteric fever and human brucellosis. Clin Lab 2007; 53:57-61.

[60] Elsaghir AA, James EA. Misidentification of *Brucella melitensis* as *Ochrobactrum anthropi* by API 20NE. J Med Microbiol 2003; 52:441-442.

[61] Hubálek Z, Sholz HC, Sedláceck I, Melzer F, Sanogo Y, Nesbadbová JJ. Brucellosis of the common vole (*Microtus arvalis*). Vector Borne Zoonotic Dis 2007; 7:679-687.

[62] Dash N, Al-Zarouni M, Rattan A, Panigrahi D. Misidentification of *Brucella melitensis* as *Bergeylla zoohelcum* by Microscan WalkAway: A case report. Med Princ Pract 2012; 21:495-497.

[63] Batchelor BI, Brindlr RJ, Gilks GF, Stelton JB. Biochemical mis-identification of *Brucella melitensis* and subsequent laboratory acquired infections. J Hosp Infect 1992; 22:159-162.

[64] Rich M, Bannatyne RA, Memish ZA. Direct urease test on BACTEC blood cultures. Early presumptive diagnosis of brucellosis in an area of endemicity. J Clin Microbiol 2000; 38:1706.

[65] Maleknejad P, Hashemi FB, Fatollahzadeh B, Jafari S, Peeri Dogaheh H. Direct urease test and acridine orange staining on BACTEC blood culture for rapid presumptive diagnosis of brucellosis. Iranian J Public Health 2005; 34:52-55.

[66] Lista F, Reubsaet FAG, De Santis R, Parchen RR, de Jong AL, Kieboom J, van der Laaken AL, Voskamp-Visser IA, Fillo S, Jansen HJ, Van der Plas J, Paauw A. Reliable identification at the species level of *Brucella* isolated with MALDI-TOF. BMC Microbiol 2011; 11:267. doi: 10.1186/1471-2180-11-267.

[67] Karger A, Melzer F, Timke M, Bettin B, Kostrzewa M, Nöcler K, Hohman A, Tomasoo H, Neubauer H, Al Dahouk S. Interlaboratory comparison of intact-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry results for identification and differentiation of *Brucella* spp. J Clin Microbiol 2013; 51:3123-3126.

[68] Ferreira L, Vega Castaño S, Sánchez-Juanes F, González-Cabrero S, Menegotto F, Orduña-Domingo A, González-Buitrago JM, Muñoz-Bellido JL. Identification of *Brucella* by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. PloS ONE 2010; 5:e14235. doi:m10.1371/journal.pone.0014235.

[69] Drevinek M, Dresler J, Klimentova J, Pisa L, Hubalek M. Evaluation of sample preparation methods for MALSI-TOF MS identification of highly dangerous bacteria. Lett Appl Microbiol 2012; 55:40-46.

[70] Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T, Wenzel T, Kostrzewa M, Bierbaum G, Hoerauf A, Sahl HG. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol 2009; 47:2912-2917.

[71] Wellinghousen N, Nöckler K, Sigge A, Bartel M, Essig A, Poppert S. Rapid detection of *Brucella* spp. in blood cultures by fluorescence in situ hybridization. J Clin Microbiol 2006; 44:1828-1830.

[72] Horvat RT, El Atrouni W, Hammoud K, Hawkinson D, Cowden S. Ribosomal RNA sequence analysis of *Brucella* infection misidentified as *Ochrobactrum anthropi.* J Clin Microbiol 2011; 49:1165-1168.

[73] Scholz HC, Pfeffer M, Witte A, Neubauer H, Al Dahouk S, Wernery U, Tomaso H. Specific detection and differentiation of *Ochrobactrum anthropi* and *Ochrobactrum intermedium* and *Brucella* spp. by a multi-primer PCR that targets the *recA* gene. J Med Microbiol 2008; 57:64-71.

[74] Yagupsky P, Baron EJ. Laboratory-exposures to brucellae and implications for bioterrorism. Emerg Infect Dis 2005; 11:1180-1185.

[75] Calfee MW, Wendling M. The effects of environmental conditions on persistence and inactivation of *Brucella suis* on building material surfaces. Lett Appl Microbiol 2012; 54:504-510.

[76] Noviello S, Gallo R, Kelly M, Linberger RJ, DeAngelis K, Cain L, Wallace B, Duma N. Laboratory-acquired bucellosis. Emerg Infect Dis 2004; 10:1848-1850.

[77] Ergonul O, Celikbas A, Tezeren D, Guvener E, Dokuzoguz B. Analysis of risk factors for laboratory-acquired *Brucella* infections. J Hosp Infect 2004; 56:223-227.

[78] Yagupsky P, Peled N, Riesenberg K, Banai M. Exposure of hospital personnel to *Brucella melitensis* and occurrence of laboratory-acquired disease in an endemic area. Scand J Infect Dis 2000; 32:31-35.

[79] Shemesh AA, Yagupsky P. Isolation rates of *Brucella melitensis* in an endemic area and implications for laboratory safety. Eur J Clin Microbiol Infect Dis 2012; 31:441-443.

[80] Centers for Disease Control and National Institutes of Health Biosafety in Microbiological and Biomedical Laboratories, Publication no. 17-40-508-3. 1988. U.S. Government Printing Office, Washington, DC.