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# Bacteremia is as unpredictable as clinical manifestations in human brucellosis

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Lymph node aspirate culture

**Summary**
*Objectives:* Because of the suboptimal recovery rate of brucellae from blood, it has been proposed that cultures of bone marrow, liver tissue, and lymph nodes may improve the recovery rate of the organism. Data in support of these recommendations are limited and not clearly convincing, especially that of bone marrow culture. The main purpose of this work was to evaluate the roles of blood, bone marrow, liver, and lymph node cultures in the diagnosis of human brucellosis.
*Methods:* Blood and bone marrow cultures were evaluated in parallel in 103 cases of human brucellosis using Castaneda's biphasic technique. Simultaneous cultures of blood, bone marrow, liver, and lymph node aspirates were also carried out for 13 of these 103 cases.
*Results:* Blood culture identified 47 (45.6%) cases and bone marrow culture identified 85 (82.5%) cases. Faster recovery of *Brucella spp* was accomplished with the bone marrow culture ($2.8 \pm 0.7$ days, $p < 0.05$). When the results of cultures of blood and bone marrow were compared with each other in the 13 cases, it was found that bone marrow specimens could be sterile (six cases (46%)) when bacteremia was present, but *Brucella melitensis* was detected in liver aspirate in all these six bacteremic cases.
*Conclusions:* Our data indicate that it is worthwhile practicing bone marrow culture by conventional biphasic technique for the definitive and rapid diagnosis of brucellosis; this is particularly the case in developing countries where diagnostic facilities by advanced technologies such as automated culture systems with PCR are not available. Bone marrow culturing would be a better gold standard in areas where antibiotic pretreatment is common. Also, adopting the

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practice of culturing liver/lymph node fluids may enhance bacterial isolation and aid in the establishment of a diagnosis of brucellosis in cases for whom blood and bone marrow cultures are negative.


## Introduction

Brucellosis is a major zoonotic disease, the incidence of which is uncertain, but which is certainly underestimated.[1] It is now regarded as one of the emerging infections.[2] The wide spectrum of symptoms of human brucellosis contributes greatly to its underdiagnosis.[3,4] Therefore laboratory testing is indispensable for diagnosis. Laboratory tools include Brucella-specific antibody demonstration and molecular diagnosis by PCR, as well as isolation of the brucellae.

The presence of antibodies does not always mean an active case of brucellosis, and therefore serological results must be interpreted in the light of clinical and epidemiological data. Although in the last few years, PCR-based laboratory tests have been proposed,[5–7] they cannot be considered a routine diagnostic tool yet especially in developing countries where brucellosis is endemic. The unequivocal proof of an active Brucella infection is the culture, and blood broth culture is the simplest and most often used procedure.[8] However, conventional Castaneda blood cultures for *Brucella spp* present several problems. Failure to detect the pathogen is a frequent occurrence. In addition, since the majority of conventional Castaneda blood cultures for *Brucella spp* are positive between days 7 and 21 and 2% are positive after day 27,[9] long incubation periods are essential before a blood culture can be declared as negative for *Brucella spp*.

In addition to evasion of the polymorphonuclear leukocytes, brucellae can survive and multiply within mononuclear phagocytes of the reticuloendothelial system. Because of the suboptimal recovery rate of brucellae from blood, it has been proposed that cultures of bone marrow,[10] liver tissue,[11] and lymph nodes[12] may improve the recovery rate of the organism. Data in support of these recommendations are limited and not clearly convincing, especially those for bone marrow culture. The main purpose of this work was to evaluate the roles of blood, bone marrow, liver, and lymph node cultures in the diagnosis of human brucellosis.

## Materials and methods

In this prospective study, a total of 103 patients suffering from brucellosis presenting to BLDEA's Shri BM Patil Medical College Hospital, Bijapur, Karnataka, India ($N = 78$) and Dr Bidari's Ashwini Institute of Child Health and Research Centre, Bijapur, Karnataka, India ($N = 25$) from September 2004 to March 2006, were studied.

A case of brucellosis was identified if the standard tube agglutination titers were $\geq$1:160[13] in the presence of clinical features suggestive of brucellosis along with epidemiological indication. Clinical features taken into consideration for the diagnosis of brucellosis were prolonged fever, joint pain, sweats, anorexia, fatigue, and enlargement of the spleen, liver, and lymph nodes. Epidemiological indications such as belonging to high-risk groups (farm laborers, shepherds, farmers, butchers, veterinarians), animal contact, and ingestion of high-risk foods in various combinations were also taken into consideration.

The patients were divided into three groups according to the evolution of the disease: acute cases had had symptoms for less than 2 months (64 cases), subacute cases had had symptoms for 2 months to 1 year (11 cases), and chronic cases had had symptoms for more than 1 year (28 cases). A detailed clinical history including epidemiological data, prior antibiotic therapy, and examination findings were recorded in proforma and analyzed. An informed consent was taken from all patients enrolled in the study.

*Brucella abortus* plain antigen for the test was obtained from the Indian Veterinary Research Institute (IVRI), Izatnagar, India. Blood and bone marrow cultures were performed on all 103 patients. All samples from a given patient were processed simultaneously. Five milliliters of venous blood was inoculated aseptically into the broth phase of Castaneda's biphasic medium consisting of brain heart infusion agar and broth or trypticase soy agar and broth (High Media, Mumbai, India). Bone marrow aspirate (1–2 ml) was collected from the sternum/iliac crest in adult cases, and from the iliac crest in child cases, taking full aseptic precautions. The aspirate of bone marrow obtained was inoculated as mentioned for blood cultures. The biphasic media were incubated at 37 °C and examined for bacterial growth once a day for 30 days, performing subculture by washing the broth-blood/bone marrow mixtures over the agar slant every day. The date of the appearance of the first colony was recorded for comparison of growth rates. In addition, 13 other specimens (liver aspirates and lymph node aspirates) were available for comparative evaluation and were cultured simultaneously using Castaneda's biphasic technique described above. Identification of Brucella strains was performed using standard classification tests, including growth characteristics, Gram-staining, a modified Ziehl–Neelsen stain, oxidase activity, urease activity, $H_2S$ production (4 days), dye sensitivity such as basic fuchsin (1:50 000 and 1:100 000) and thionin (1:25 000, 1:50 000, and 1:100 000), and seroagglutination. *Brucella abortus* and *Brucella melitensis* monospecific antisera (Murex Biotech Ltd, Dartford, UK) were used for the seroagglutination test. Isolates were sent to IVRI for confirmatory identification.

### Statistical analysis

Differences in sensitivity and time to culture positivity of blood and bone marrow cultures were compared using the Chi-square test with Yates' correction. A value of $p < 0.05$ was considered significant.

## Results

A total of 103 patients were reported with a diagnosis of brucellosis during the study period. Their ages ranged from

**Table 1** Diagnostic yield of simultaneous cultures of blood and bone marrow from 103 cases of brucellosis

| Stage of illness | Patients (*n*) | Positive by blood culture (*n* (%)) | Positive by bone marrow culture (*n* (%)) |
|---|---|---|---|
| Acute (<2 months) | 64 | 35 (54.7) | 59 (92.2) |
| Subacute (2–12 months) | 11 | 4 (36.4) | 8 (72.7) |
| Chronic (>1 year) | 28 | 8 (28.6) | 18 (64.3) |
| Total | 103 | 47 (45.6) | 85 (82.5) |

Chi-square values: (a) acute – 6.13, $p < 0.02$; (b) chronic – 3.84, $p < 0.05$.

2 to 81 years with mean ± SD age of 24.5 ± 17.4 years. Seventy-four patients were under 35 years of age and 29 were over 35 years of age. Thirty-six patients were of pediatric age (14 years and less) and 67 were adults. There were 77 males and 26 females, giving a male to female ratio of 3:1. A seasonal variation in the distribution of cases was not observed.

Of the 103 patients, information regarding exposure to risk factors for transmission of brucellosis was recorded in 89. The majority were farmers or farm labourers (50.4%) and shepherds (35.3%). The source of infection was unknown in 11/103 (10.7%) cases since the patients could not recall any exposure events. More than 60% of the patients had a history of both consumption of fresh unpasteurized milk and close animal contact.

The illness was acute in 64 (62.1%) cases, subacute in 11 (10.7%), and chronic in 28 (27.2%). A substantial number of patients (75.7%) presented with fever, this being the only complaint in 52.4% of the cases. Joint pain alone was found in 11 cases. Hepatosplenomegaly was noticed in 34 patients, splenomegaly alone in ten, and hepatomegaly alone in five. Lymphadenopathy was recorded in 15 cases.

There was no correlation between culture positive rates in blood and bone marrow specimens with age and sex of the patients. The diagnostic yields of cultures of blood and bone marrow are shown in Table 1.

Bone marrow culture was found to be more sensitive than blood culture in detecting brucellae in acute ($p < 0.02$), as well as in chronic cases ($p < 0.05$). Of the 64 acute cases, bone marrow culture grew brucellae in 59 (92.2%) cases whereas blood culture picked up only 35 (54.7%) cases. Of the 28 chronic brucellosis cases, bone marrow culture identified brucellae in 18 (64.3%) cases whereas blood culture identified brucellae in only eight (28.6%) cases. In subacute cases, though statistically insignificant ($p > 0.05$), bone marrow culture detected more cases (eight (72.7%)) than blood culture (four (36.4%)). When the results were taken together, bone marrow culture recovered brucellae from 82.5% of cases whereas blood culture was positive in only 45.6% of cases, however this was statistically insignificant ($p > 0.05$).

Bone marrow culture shortened the mean time to detection of circulating brucellae to 2.8 ± 0.7 (SD) days compared to 7.2 ± 2.4 (SD) days by blood culture ($p < 0.05$).

Information concerning the history of prior antibiotic therapy was available for 19 patients (Table 2). In untreated cases, bone marrow culture was superior (83.3%; $p < 0.01$) to blood culture (46.4%), however, in treated cases, though bone marrow showed a higher positivity (78.9%) as compared to blood culture (42.1%), this difference was statistically insignificant ($p > 0.05$).

Table 3 summarizes the results of the culture of blood, bone marrow, liver, and lymph node done simultaneously for 13 of the 103 cases. No single specimen identified 100% cases. When the results of cultures of blood and bone marrow were compared with each other in these 13 cases, it was found that bone marrow specimens could be sterile in cases when bacteremia was present (six cases, 46%); however Brucella was detected in the liver aspirate in all these six bacteremic cases, and Brucella was recovered from lymph node fluid in three cases. Liver

**Table 2** Influence of previous antibiotic therapy on the recovery of Brucella from blood and bone marrow cultures

| History of previous antibiotic therapy | Patients (*n*) | Positive by blood culture (*n* (%)) | Positive by bone marrow culture (*n* (%)) |
|---|---|---|---|
| Present | 19 | 8 (42.1) | 15 (78.9) |
| Absent | 84 | 39 (46.4) | 70 (83.3) |
| Total | 103 | 47 (45.6) | 85 (82.5) |

Chi-square values: (a) for patients on previous antibiotic therapy – 2.14, $p > 0.05$; (b) for patients without previous antibiotic therapy – 8.82, $p < 0.01$.

**Table 3** Results of parallel cultures of blood, bone marrow, liver, and lymph node from 13 of 103 cases of brucellosis

| Blood culture | Bone marrow culture | Liver aspirate culture | Lymph node fluid culture | No. (%) of cases |
|---|---|---|---|---|
| Positive | Positive | Positive | Positive | 4 (30.8) |
| Positive | Negative | Positive | Negative | 3 (23.1) |
| Negative | Negative | Positive | Negative | 2 (15.4) |
| Positive | Negative | Positive | Positive | 3 (23.1) |
| Negative | Positive | Negative | Positive | 1 (7.7) |

aspirate alone was efficient in picking up two additional cases of brucellosis in the present study making a total of 87 bacteriologically proven cases of brucellosis. All isolates were identified as *B. melitensis* biotype 1.

## Discussion

Brucella infections are difficult to diagnose because of the wide spectrum of clinical manifestations associated with them. Human brucellosis is underdiagnosed and underreported, with estimates that at least 25 cases go unrecognized for every case that is diagnosed.[14] The diagnosis of brucellosis is made with certainty when Brucella organisms are recovered from the blood, bone marrow, or other tissues. Blood culture is the method of choice but is frequently hampered by the low sensitivity and delay in growth on account of the low concentration of bacteria usually found in patients with Brucella bacteremia.[15]

In this study, *B. melitensis* was isolated from 45.6% of patients by blood culture, a finding that is comparable with the results of positive cultures of blood reported by others,[16–18] however bone marrow culture showed a high recovery rate of Brucella. In the present series, a statistically significant difference was seen in the performance of bone marrow culture over blood culture in the recovery of Brucella both in acute ($p < 0.02$) and chronic ($p < 0.05$) cases of brucellosis. In one study of 50 patients who were eventually diagnosed with brucellosis, cultures of blood and bone marrow were positive in 70% and 92% of the patients, respectively.[10] Ozkurt et al.[19] compared the BacT/Alert system with traditional Brucella broth culture using 50 blood and 50 bone marrow paired cultures. Bone marrow cultures were positive in 70% and blood cultures in 48%. Bone marrow cultures were examined in five cases by Tsolia et al.[20] and were positive in four including one with negative blood cultures.

The greater ability of the bone marrow to yield isolates could be on account of the relatively high concentration of Brucella in the bone marrow. This shows that the sequestration of brucellae may be responsible for the higher rates of bone marrow positivity; this needs further elucidation. But the interesting finding of the present series was that isolation rates from bone marrow by the conventional Castaneda biphasic technique are comparable with the results of the BACTEC system reported in other studies,[19,21] noteworthy because our culture technique is inexpensive; however harvesting bone marrow for culture remains an invasive, painful technique.

Despite the small volume of bone marrow cultured (1–2 ml) in the present study compared to the much larger volume of 2–4 ml used by some workers,[19] we were able to pick up a significant number of cases – a finding that has relevance to the laboratory technique. The reasons for our findings could be due to the stringent clinical as well as epidemiological criteria along with the serological evidence in the selection of brucellosis cases. Although automated culture systems are also reliable in isolating brucellae, to maximize detection of the organism by the BacT/Alert, a prolonged incubation time and periodic performance of subcultures for at least four weeks are required. This could be an infectious hazard as well as costly in terms of resources for developing countries where brucellosis is endemic.

Magill et al.[22] reported that blood cultures were more reliable than bone marrow cultures, and Shehabi et al.[23] found that, in their experience, blood cultures had a sensitivity of 44.4% compared to 27.7% for bone marrow cultures. Iseri et al.[24] compared the diagnostic value of blood and bone marrow cultures in 102 patients using the BACTEC 9050 system. The rate of positive blood cultures was found to be 48% while the rate was 34% for bone marrow cultures. Ozturk et al.[21] compared 23 blood cultures with 18 bone marrow obtained simultaneously, using the BACTEC 9240 system; Brucella was isolated in 82.6% from blood culture and in 81.2% from bone marrow.

In many previous studies,[21–23] ill-defined clinical and epidemiological criteria might have influenced the positivity rate of blood and bone marrow cultures. When the results of cultures of blood and bone marrow specimens were compared with each other in the present study in 13 patients, it was observed that bone marrow samples could be negative when bacteremia was seen and vice versa suggesting that the bone marrow culture results are not universally reproducible. It is worthwhile to note that *B. melitensis* was isolated in liver aspirate in all six bacteremic cases, along with lymph node fluid picking up *B. melitensis* in three cases in which bone marrow cultures were sterile. Our data clearly illustrate that brucellae may not be uniformly distributed in the bone marrow and that bacteremia might also be maintained from other sources of the reticuloendothelial system. Perhaps this could be the reason for the discrepancy in the results of blood and bone marrow cultures reported in the literature. Two cases were additionally recognized by liver aspirate alone. This is a remarkable finding since detection of Brucella in clinical specimens elicits prompt consideration of therapeutic measures especially in endemic areas of the world where the interpretation of the results of serological tests is difficult. This also gives us reason to propose multiple sampling (blood, bone marrow, liver, and lymph node) for the bacteriological confirmation of human brucellosis, an area that needs further clinical studies.

The results of the effect of antibiotic pretreatment on the isolation rate in blood and bone marrow were consistent with the finding of Gotuzzo et al.[10] Prior antibiotic therapy has given varying results in the bacteriological confirmation of brucellosis. The findings that blood culture-negative patients were positive by bone marrow culture may be useful in settings where antibiotic use is high.

The major finding of our study was the significant difference between the blood and bone marrow cultures with respect to growth time of the Brucella compared to previous studies.[10,19,21] Our detection time for bone marrow culture seems to be faster even with conventional methods compared to a similar study.[10] Our bone marrow cultures were found to be superior to the BacT/Alert system when time to detection of Brucella is considered with some other studies;[19,21] however with regards to blood samples, the cultural confirmation seems to be inferior to the BacT/Alert system. Our findings could be attributed to the application of a biphasic medium and also to the fact that broth-blood/bone marrow mixtures were tilted over the solid phase every day at the daily examination.

Rapid detection of infecting Brucella is of paramount importance for the administration of effective anti-brucellar therapy in order to decrease the morbidity and mortality associated with the brucellosis. However, liver/lymph node aspirates did not result in any significant difference in the

rapid detection of brucellae compared to bone marrow. In this work, bone marrow culture has been shown to substantially increase the rate of isolation over blood culture. Bone marrow culturing would be a better gold standard in areas where antibiotic pretreatment is common. Bone marrow culture has the advantage of having colonies immediately available for further characterization and effective case management.

To conclude, it is worthwhile practicing bone marrow culture by conventional biphasic technique for the definitive and rapid diagnosis of brucellosis in developing countries, where diagnostic facilities by advanced technologies like automated culture systems with PCR are unavailable, since an additional 36.9% of brucellosis cases were diagnosed by this technique as shown in our data. Also, adopting the practice of culturing liver/lymph node fluids may enhance bacterial isolation and aid in the establishment of the diagnosis of brucellosis in cases in whom blood and bone marrow cultures are negative, since harvesting of these specimens is less invasive compared to harvesting of bone marrow.

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*Conflict of interest:* No conflict of interest to declare.

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