Diagnosis of disseminated mycobacterial infection: testing a simple and inexpensive method for use in developing countries

F.C.O. Fandinho,[1] B. Grinsztejn,[2] V.G. Veloso,[2] M.C.S. Lourenço,[3] E. Werneck-Barroso,[4] E. João,[5] S.A. Nogueira,[6] & L. de S. Fonseca[7]

With the development of the acquired immunodeficiency syndrome (AIDS) epidemic, the isolation of mycobacteria from blood has become a common problem for clinical laboratories. In this study two methods were used for the recovery of mycobacteria from blood specimens obtained from AIDS patients: (1) direct inoculation of a biphasic medium, and (2) a non-commercial lysis–centrifugation method. A total of 3 consecutive blood samples were taken at 15-minute intervals from each of 50 AIDS patients with clinical suspicion of disseminated mycobacterial disease. Mycobacterium growth was noted in 70/138 blood specimens from 30 (60%) patients. These cultures yielded Mycobacterium tuberculosis *in 19 (63%) and* Mycobacterium avium *complex organisms in 11 (37%) patients. Cultures using the lysis–centrifugation method were positive in 54% of the patients while cultures using biphasic medium were positive in 44% (*$P > 0.05$*). The positivity for* M. avium *complex was higher with lysis–centrifugation (91%) than with biphasic medium (45.4%) (*$P < 0.05$*). However, the positivities for* M. tuberculosis *with the lysis–centrifugation method (89.5%) and direct inoculation in biphasic medium (100%) were similar (*$P > 0.05$*). The use of a non-commercial lysis–centrifugation technique is inexpensive, reliable, and can be an alternative method for the diagnosis of mycobacteraemia in developing countries.*

Introduction

Following the development of the acquired immunodeficiency syndrome (AIDS) epidemic, an increasing number of secondary disseminated mycobacterial infections has prompted the search for a sensitive method of recovering mycobacteria from blood. The highest diagnostic yield for *Mycobacterium avium* complex organisms is achieved by lysis–centrifugation culture methods (*1*, *2*). Such systems are commercially available and are commonly used in the USA and in Western Europe (*3*, *4*). Diagnosis with the radiometric BACTEC culture medium is more rapid and has a similar sensitivity but requires a radioactive substrate and does not yield isolated colonies (*5*, *6*). Both methods are too expensive for routine use in developing countries. This prospective study compares the efficiency of two different methods for detecting mycobacterial growth from the peripheral blood of AIDS patients: (1) direct inoculation onto a biphasic medium and (2) an inexpensive non-commercial lysis–centrifugation method.

Methods

Patients

During the period December 1993 to August 1994, blood specimens were obtained from 50 AIDS patients with suspected disseminated mycobacterial

[1] Graduate Student, Microbiology Institute, Federal University of Rio de Janeiro and Department of Technological Development, Immunobiological Products Institute, Fiocruz, Rio de Janeiro, Brazil.
[2] Physician, Evandro Chagas Hospital, Fiocruz, Rio de Janeiro, Brazil.
[3] Chief, Bacteriology Laboratory, Evandro Chagas Hospital, Fiocruz, Rio de Janeiro, Brazil.
[4] Associate Researcher, Evandro Chagas Hospital, Fiocruz, Rio de Janeiro, and Pulmonary physician, Pneumology and Physiology Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
[5] Physician, Servidores do Estado do Rio de Janeiro Hospital, Rio de Janeiro, Brazil.
[6] Associate Professor, Department of Infectious Diseases, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
[7] Associate Professor, Department of Medical Microbiology, Microbiology Institute, Federal University of Rio de Janeiro, Centre for Health Sciences, Bloco 1, Ilha do Fundão, 21.941-590, Rio de Janeiro, Brazil. Requests for reprints should be sent to this author.
Reprint No. **5789**

disease. The main characteristic used to select patients was fever of unknown origin plus one of the following: hepatosplenomegaly, lung infiltrate, anaemia, or elevated alkaline phosphatase. Furthermore, when possible, a CD4 lymphocyte T cell count (cells per μl) was performed.

Blood specimens

A total of 3 blood samples were taken from each patient at 15-minute intervals by a trained phlebotomist. Each sample was collected in 2 Vacutainer tubes (Becton Dickinson, MD, USA) containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The temperature of the patients was measured at each sample. A total of 138 blood samples were obtained from the patients studied. Other specimens (e.g. sputum) were obtained for routine mycobacterial culture according to clinical indications.

Procedure

Upon arrival in the laboratory the contents of one Vacutainer tube were mixed with 30 ml of 0.3% (w/v) sodium deoxicholate solution and incubated for 15 min at room temperature. After centrifugation for 25 min at 3000 *g* (4 °C), the pellet was suspended in 1 ml of 0.2% bovine albumin saline (Fraction V, Sigma, St. Louis, MO, USA), and 0.2 ml of the suspension was added to one tube with Middlebrook 7H9 broth and to two Löwenstein–Jensen slants.

The contents of the second Vacutainer were directly added to biphasic medium containing 5 ml of Middlebrook 7H9 broth and 15 ml of Middlebrook 7H10 agar. All mycobacterial isolates were identified using standard biochemical methods.

Statistical analysis

The χ^2 test (with Yates' correction for 2×2 tables) and Fisher's exact test were used to compare statistics between the patient groups. For continuous variables Student's *t*-test was used. In each case, a two-sided test with a 0.05 significance level was used.

Results

Mycobacterial growth was obtained from 70/138 specimens (from 30/50 (60%) patients). Positive cultures yielded *M. tuberculosis* in 19 (63%) and *M. avium* complex organisms in 11 (37%) patients. Only 4 patients with *M. avium* complex cultured from blood had mycobacteria isolated from cultures obtained from other clinical specimens. Of these, one patient had *M. avium* complex isolated from a liver sample (patient No. 3). Two patients with *M. avium* complex isolated from blood showed *M. tuberculosis* in cultures from either sputum (patient No. 46) or a lymph node biopsy (patient No. 1). One patient had *Mycobacterium gordonae* isolated from faeces (patient No. 13). The majority of patients with positive blood cultures for *M. tuberculosis* had the same mycobacteria species isolated from other clinical samples. However, 5 patients could be diagnosed with tuberculosis only after the results of blood culture (patients No. 6, 7, 18, 19, and 32). These results, including CD4 lymphocyte T cell counts, are depicted in Table 1.

The mean of CD4 lymphocyte T cell counts for patients with positive blood cultures for *M. avium* complex was 15.9 cells per μl, while for patients with positive *M. tuberculosis* cultures the mean was 71.1 cells per μl (range, 0–449 cells per μl).

A total of 19 (63%) patients with positive mycobacterial cultures were positive by both methods used. With the lysis–centrifugation method blood cultures were positive for 54% (27/50) of patients, while with biphasic medium blood cultures were positive for 44% (22/50). A comparative analysis of the two methods showed no statistically significant difference ($P > 0.05$). The mean detection time for lysis–centrifugation and biphasic medium was 42.8 and 58.5 days, respectively. This probably means that the lysis–centrifugation method recovered more viable mycobacteria from blood than did the biphasic medium, despite the lack of a statistically significant difference between them. A comparison of the results obtained with the Löwenstein–Jensen slants and the Middlebrook 7H9 broth used for the isolation of mycobacteria in the lysis–centrifugation method showed that for *M. tuberculosis* (34% and 42%, resp.) as well as for *M. avium* complex (45% and 36%, resp.) no statistically significant difference was observed ($P > 0.05$, results not shown). However, a striking difference was observed when we compared the two blood-culture methods according to the species of mycobacteria recovered (Table 2). The positivity for *M. avium* complex was higher with lysis–centrifugation (10/11 = 91%) than with biphasic medium (5/11 = 45.4%) ($P < 0.05$), although the positivity for *M. tuberculosis* by the lysis–centrifugation method (89.5%) and direct inoculation of biphasic medium (100%) was similar ($P > 0.05$) (Fig. 1). Samples from the same patient showed concordant results in 39/50 (78%) patients.

Table 1: **Mycobacterium culture results for the 50 AIDS patients studied**

Patient No.	CD4 lymphocyte T cell counts	Blood culture: Biphasic medium	Blood culture: Lysis–centrifugation method	Blood culture: *Mycobacterium* species identified	Other specimen culture: Collection sites	Other specimen culture: *Mycobacterium* species identified
1	0	+	+	*M. avium* complex	lymph node	*M. tuberculosis*
2	52	+	+	*M. avium* complex	—	—
3	12	+	+	*M. avium* complex	liver	*M. avium* complex
4	40	+	+	*M. tuberculosis*	BAL[a]	*M. tuberculosis*
5	100	–	–	—	—	—
6	46	NP[b]	+	*M. tuberculosis*	—	—
7	86	NP	+	*M. tuberculosis*	—	—
8	50	–	+	*M. avium* complex	—	—
9	0	+	+	*M. tuberculosis*	sputum	*M. tuberculosis*
10	0	–	+	*M. avium* complex	—	—
11	20	–	+	*M. avium* complex	—	—
12	0	–	–	—	—	—
13	19	–	+	*M. avium* complex	faeces	*M. gordonae*
14	31	–	–	—	—	—
15	165	–	–	—	sputum	*M. tuberculosis*
16	89	–	–	—	—	—
17	49	–	–	—	—	—
18	0	+	+	*M. tuberculosis*	—	—
19	50	+	+	*M. tuberculosis*	—	—
20	32	–	–	—	sputum	*M. tuberculosis*
21	38	–	–	—	—	—
22	34	–	–	—	—	—
23	80	–	–	—	—	—
24	265	–	–	—	—	—
25	48	+	+	*M. tuberculosis*	bone marrow	*M. tuberculosis*
25				—	sputum	*M. tuberculosis*
25					faeces	*M. tuberculosis*
26	151	+	+	*M. tuberculosis*	bone marrow	*M. tuberculosis*
27	15	–	–	—	—	—
28	59	+	+	*M. tuberculosis*	lymph node	*M. tuberculosis*
29	33	+	+	*M. tuberculosis*	bone marrow	*M. tuberculosis*
30	135	+	+	*M. tuberculosis*	sputum	*M. tuberculosis*
30					bone marrow	*M. tuberculosis*
30					lymph node	*M. tuberculosis*
31	53	–	–	—	—	—
32	20	+	+	*M. tuberculosis*	—	—
33	25	+	+	*M. tuberculosis*	liver	*M. tuberculosis*
33					sputum	*M. tuberculosis*
34	75	+	+	*M. tuberculosis*	lymph node	*M. tuberculosis*
35	0	+	–	*M. tuberculosis*	sputum	*M. tuberculosis*
36	449	+	+	*M. tuberculosis*	sputum	*M. tuberculosis*
37	5	+	+	*M. tuberculosis*	sputum	*M. tuberculosis*
38	13	+	+	*M. tuberculosis*	sputum	*M. tuberculosis*
39	116	+	–	*M. tuberculosis*	bone marrow	*M. tuberculosis*
40	0	–	–	—	sputum	*M. tuberculosis*
41	0	–	–	—	—	—
42	15	+	–	*M. avium* complex	—	—
43	100	–	–	—	sputum	*M. tuberculosis*
44	2	–	–	—	sputum	*M. tuberculosis*
45	0	–	–	—	sputum	*M. tuberculosis*
46	5	–	+	*M. avium* complex	sputum	*M. tuberculosis*
47	2	+	+	*M. avium* complex	—	—
48	0	–	+	*M. avium* complex	—	—
49	2	–	–	—	BAL	*M. tuberculosis*
50	5	–	–	—	lymph node	*M. tuberculosis*

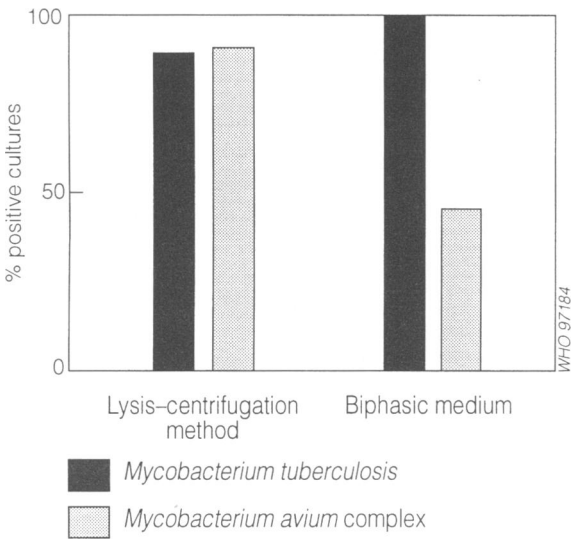
[a] BAL = bronchoalveolar lavage.
[b] NP = not performed.

Table 2: **Comparison of different culture media for the recovery of mycobacteria from the blood of AIDS patients studied**

	M. tuberculosis:			*M. avium* complex:		
	LC-7H9[a]	LC-LJ[b]	Biphasic medium	LC-7H9[a]	LC-LJ[b]	Biphasic medium
Positive	16	13	17	8	10	5
Negative	3	6	0	3	1	6
Not performed	0	0	2	0	0	0

[a] Blood processed by lysis–centrifugation and added to Middlebrook 7H9 broth.
[b] Blood processed by lysis–centrifugation and added to Löwenstein–Jensen slants.

Fig. 1. **Comparison of the positivity of the two culture methods studied, by species isolated.** Biphasic medium, $P < 0.05$.



Discussion

Despite culture techniques making the detection of mycobacteria in blood easier (*2*, *7*), few studies have focused on *M. tuberculosis* bacteraemia in AIDS patients (*6*, *8*, *9*). This study suggests that *M. tuberculosis* bacteraemia is common among AIDS patients in Brazil, and some workers have suggested that such bacteraemia has become more frequent (*5*). This study also suggests that blood culture is a valuable diagnostic tool when tuberculosis is suspected, even when the culture of other specimens fails to yield the organism. Thus, our observations concur with those of Bouza et al. (*10*), but contrast with those of Shafer et al. (*6*), who did not find blood culture useful for the diagnosis of tuberculosis.

M. avium complex is the most common bacterial infection in AIDS patients in the USA and Europe (*3*, *8*), but its frequency in developing countries is almost unknown (*11*). Our results and those of Barreto et al (*12*) suggest that *M. avium* complex infection might be more common in AIDS patients in Brazil than is realized.

The higher yield for isolation of *M. avium* complex with the lysis–centrifugation method arises because *M. avium* complex organisms are not usually found free in plasma (*1*). The lysis of peripheral blood leukocytes releases intracellular mycobacteria, which are concentrated by centrifugation, increasing the sensitivity of blood culture (*4*). As *M. tuberculosis* is a true pathogenic bacterium with greater capacity for causing disseminated disease, both methods were sensitive for detecting *M. tuberculosis* bacteraemia. Although Middlebrook 7H9 broth has generally been found to be more sensitive than tests on solid media, such as the Löwenstein-Jensen slants, the lack of significant difference found here may be due to the relatively small sample size.

The low CD4 lymphocyte T cell counts in patients with positive *M. avium* cultures from blood (15.9 cells per μl) are concordant with the findings of some workers who estimate the risk for the occurrence of *M. avium* bacteraemia to be much greater when the CD4 lymphocyte T cell count is less than 50 cells per μl (*13–15*). However, the mean CD4 lymphocyte T cell count of 71.1 cells per μl in patients with *M. tuberculosis* cultured from blood was lower than most of those reported elsewhere (range, 23–742 cells per μl) at the moment of tuberculosis diagnosis. Human immunodeficiency virus (HIV) positive patients with extrapulmonary tuberculosis have lower CD4 lymphocyte T cell counts (mean, 153 cells per μl) than patients with pulmonary tuberculosis only (*16*, *17*). The characteristics used to select patients for this study probably favoured the selection of more complicated cases of tuberculosis. This is supported by the high percentage of patients

with disseminated tuberculosis (78%) in this study and by the fact that all patients had negative tuberculin skin tests.

All three consecutive blood samples from an individual showed concordant results in 39 patients (78%), suggesting that one blood sample is sufficient for the diagnosis of mycobacteraemia. Similar results were found in a retrospective study evaluating the usefulness of paired blood cultures, which demonstrated concordant results in 96% of pairs (*18*). None of our patients had fever at the moment of blood collection, which contrasts with the criteria for taking blood samples for the diagnosis of bacteraemia caused by non-acid-fast organisms.

In conclusion, this study shows that blood culture can be performed using a non-commercial, sensitive, inexpensive method for the diagnosis of disseminated mycobacterial disease, and we recommend it for routine use in mycobacterial laboratories in developing countries; moreover, this study demonstrated a high frequency of disseminated *M. avium* complex infection in HIV-positive patients in Brazil.

Acknowledgements

This work was supported by CNPq and FINEP, Brazil. We thank M.G. Silva and O.M. Santos for their technical support, and Dr A. Silva for her help with the *Résumé*.

Résumé

Diagnostic de la mycobactériose généralisée: essai d'une méthode simple et bon marché utilisable dans les pays en développement

Avec le développement de l'épidémie de syndrome d'immunodéficience acquise (SIDA), l'isolement des mycobactéries dans le sang est devenu un problème fréquent pour les laboratoires d'analyses médicales. Deux méthodes ont été utilisées dans la présente étude pour cultiver les mycobactéries à partir des prélèvements de sang pratiqués chez les patients atteints de SIDA: 1) inoculation directe d'un milieu biphasique, et 2) méthode non commercialisée par lyse et centrifugation. Au total, trois échantillons de sang consécutifs ont été prélevés à 15 minutes d'intervalle chez chacun des 50 patients présumés cliniquement atteints de mycobactériose généralisée. Les mycobactéries ont cultivé pour 70 des 138 prélèvements de sang réalisés chez 30 patients (soit 60%). Ces cultures ont permis d'obtenir *Mycobacterium tuberculosis* chez 19 patients (soit 63%), et des germes appartenant au complexe *Mycobacterium avium* chez 11 patients (soit 37%). Les cultures obtenues par lyse-centrifugation étaient positives chez 54% des patients, tandis que les cultures sur milieu biphasique étaient positives chez 44% des patients ($p > 0,05$). Le nombre de cultures positives pour le complexe *M. avium* était plus grand avec la lyse-centrifugation (91%) qu'avec le milieu biphasique (45,4%) ($p < 0,05$). Pour *M. tuberculosis*, la sensibilité de la lyse–centrifugation (89,5%) et celle de l'inoculation directe en milieu biphasique (100%) étaient toutefois comparables ($p > 0,05$). La lyse–centrifugation non commercialisée est une technique bon marché, fiable, et qui peut être utilisée comme méthode de remplacement pour le diagnostic de la mycobactériémie dans les pays en développement.

References

1. **Meylan PR, Richman DD, Kornbluth RS.** Characterization and growth in human macrophages of *Mycobacterium avium* complex strains isolated from the blood of patients with acquired immunodeficiency syndrome. *Infection and immunity*, 1990, **58**: 2564–2568.
2. **Salfinger M et al.** Comparison of three methods for recovery of *Mycobacterium avium* complex from blood specimens. *Journal of clinical microbiology*, 1988, **26**: 1225–1226.
3. **Inderlied CB, Kemper CA, Bermudez LE.** The *Mycobacterium avium* complex. *Clinical microbiology review*, 1993, **6**: 266–310.
4. **Pitchenik AE, Fertel D.** Tuberculosis and non-tuberculous mycobacterial diseases. *Medical clinics of North America*, 1992, **76**: 121–171.
5. **Baber TW, Craven DE, McCabe WR.** Bacteremia due to *Mycobacterium tuberculosis* in patients with human immunodeficiency virus infection. *Medicine*, 1990, **69**: 375–383.
6. **Shafer RW et al.** Frequency of *Mycobacterium tuberculosis* bacteremia in patients with tuberculosis in an endemic area for AIDS. *American review of respiratory diseases*, 1989, **140**: 1611–1613.
7. **Kiehn TE et al.** *Mycobacterium tuberculosis* bacteremia detected by the isolator lysis–centrifugation blood culture system. *Journal of clinical microbiology*, 1985, **21**: 647–648.
8. **Saltzman BA et al.** *Mycobacterium tuberculosis* bacteremia in the acquired immunodeficiency syndrome. *Journal of the American Medical Association*, 1986, **256**: 390–391.
9. **Truffot–Pernot C et al.** Results of blood cultures for detection of mycobacteria in AIDS patients. *Tubercle*, 1989, **70**: 187.
10. **Bouza E et al.** High prevalence of tuberculosis in AIDS patients in Spain. *European journal of clinical microbiology and infectious disease*, 1988, **7**: 785–788.
11. **Gilks CF et al.** Disseminated *Mycobacterium avium* infection among HIV-infected patients in Kenya. *Journal of acquired immune deficiency syndrome and human retrovirology*, 1995, **8**: 195–198.

12. **Barreto JA et al.** Isolation of *Mycobacterium avium* complex from bone marrow aspirates of AIDS patients in Brazil. *Journal of infectious diseases*, 1993, **168**: 777–779.
13. **Crowe SM et al.** Predictive value of CD4 lymphocyte numbers for the development of opportunistic infection and malignancies in HIV infected persons. *Journal of acquired immune deficiency syndrome*, 1991, **4**: 770–776.
14. **Nightingale SD et al.** Incidence of *Mycobacterium avium* complex bacteremia in human immunodeficiency virus-positive patients. *Journal of infectious diseases*, 1992, **165**: 1082–1085.
15. **Horsburg Jr CR.** Current concepts: *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. *New England journal of medicine*, 1991, **324**: 1332–1338.
16. **Theuer CP et al.** Human immunodeficiency virus infection in tuberculosis patients. *Journal of infectious diseases*, 1990, **162**: 08–12.
17. **Jones BE, Young SMM, Barnes PF.** Relationship of the manifestations of tuberculosis to the CD4 counts in patients with human immunodeficiency virus infection. *American review of respiratory diseases*, 1993, **148**: 1292–1297.
18. **Stone BL et al.** Utility of paired blood culture and smears in diagnosis of disseminated *Mycobacterium avium* complex infection in AIDS patients. *Journal of clinical microbiology*, 1994, **32**: 814–842.