JOURNAL OF CLINICAL MICROBIOLOGY, July 1993, p. 1794–1798
0095-1137/93/071794-05$02.00/0

Vol. 31, No. 7

# Reproducibility of Lysis-Centrifugation Cultures for Quantification of *Mycobacterium avium* Complex Bacteremia

DIANE HAVLIR,[1]* CAROL A. KEMPER,[2,3,4] AND STANLEY C. DERESINSKI[2,3,4]

*Department of Medicine, Division of Infectious Diseases, 8208, UCSD Medical Center, 200 West Arbor Drive, San Diego, California 92103[1]; Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center, San Jose, California[2]; Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California[3]; and AIDS Community Research Consortium, Redwood City, California[4]*

Received 1 February 1993/Accepted 12 April 1993

**While quantitative mycobacterial blood cultures have been accepted as the standard for evaluating response to various *Mycobacterium avium* complex (MAC) treatment regimens, variability in this methodology has not been evaluated in a rigorous fashion. We thus studied the reproducibility of quantitative MAC cultures by a lysis-centrifugation culture system within and among five institutions. To measure the intralaboratory variation in mycobacterial colony counts, colony counts from duplicate blood specimens collected from 52 AIDS patients with MAC bacteremia were determined. Colony counts ranged from 0 to 50,000 CFU/ml. Nonparametric analyses revealed there was no significant difference in colony counts between the 52 duplicate specimens. The agreement between the intralaboratory paired specimens, as measured by the intraclass correlation coefficient, was 0.997. To measure the interlaboratory variation, multiple 10-ml aliquots from 12 patients were distributed to five institutions and processed within 24 to 32 h by lysis-centrifugation. For the 12 specimens distributed to the five laboratories, two-way analysis of variance for repeated measures revealed no significant difference in an individual patient's colony counts between laboratories ($P > 0.2$). We conclude that quantitation of mycobacterial colony counts by the lysis-centrifugation system is reproducible within and between institutions. Clinical trials evaluating response to therapeutic interventions for MAC can use multiple laboratories for quantitation of mycobacteremia. Furthermore, a 24- to 32-h delay in processing appeared to have no impact on reproducibility.**

The optimal therapy of disseminated *Mycobacterium avium* complex (MAC) in AIDS patients has been the focus of intense clinical investigation (2, 4, 5). Early in the AIDS epidemic, the observation was made that patients with MAC often had a heavy organism burden with large numbers ($>10^4$) of CFU per milliliter of blood (3, 6, 8, 10). Because of the accessibility of peripheral blood and the ability to measure changes in CFU, this technique has been utilized to measure quantitative changes in mycobacteria. The change in CFU while on therapy has been adapted as the standard microbiologic measurement of drug efficacy (2, 5). The reproducibility of this technique, the variability at low CFU, and the effect of antibiotics and transport on patient specimens have not been critically evaluated to date.

The purpose of the current investigation was to determine the feasibility of the utilization of multiple laboratories and of shipment of specimens in the conduct of clinical therapeutic trials which rely upon accurate quantitation of MAC bacteremia.

(This work was presented in part at the 1992 General Meeting of the American Society for Microbiology, 28 May 1992.)

## MATERIALS AND METHODS

**Patients.** All patients were adults ≥18 years of age who were infected with the human immunodeficiency virus, had MAC bacteremia, and were cared for at one of five participating institutions (University of California, San Diego; Santa Clara Valley Medical Center, San Jose; University of California, Irvine; University of Southern California, Los Angeles; and University of South Florida, Gainesville). All of the patients who participated in the intralaboratory analysis and 9 of the 12 patients in the interlaboratory analysis were studied before antimycobacterial therapy was initiated. Two patients in the interlaboratory analysis were receiving clofazimine, and one patient was receiving a combination of clofazimine and rifampin. All patients in the interlaboratory analysis participated under informed consent.

**Microbiologic methods.** For the evaluation of intralaboratory reproducibility, two pretreatment 10-ml blood samples from each subject were separately inoculated into an Isolator-10 tube (Wampole Laboratories, Cranbury, N.J.). Within 1 h of collection, 0.6 ml was plated directly onto 7H10 agar plates (Remel, Lenexa, Kans.), 0.3 ml was diluted 1:10 in 7H9 broth (BBL Cockeysville, Md.), and 0.1 ml of this dilution was plated in duplicate on two 7H10 plates. The rest of the 1:10 dilution was held at 2 to 8°C for later use in case the organism count was too high and further dilutions were necessary. The volume of the remainder of the blood was recorded and centrifuged per the manufacturer's instructions. The concentrate was plated on 7H10 agar plates and incubated at 37°C in an atmosphere of 5% $CO_2$; the plates were inoculated surface up. Plates were examined weekly for 8 weeks, and the numbers of CFU per milliliter of blood were determined. Colony counts per milliliter of whole blood were enumerated from the dilution containing 30 to 300 colonies or the dilution containing <30 colonies if this was

* Corresponding author.

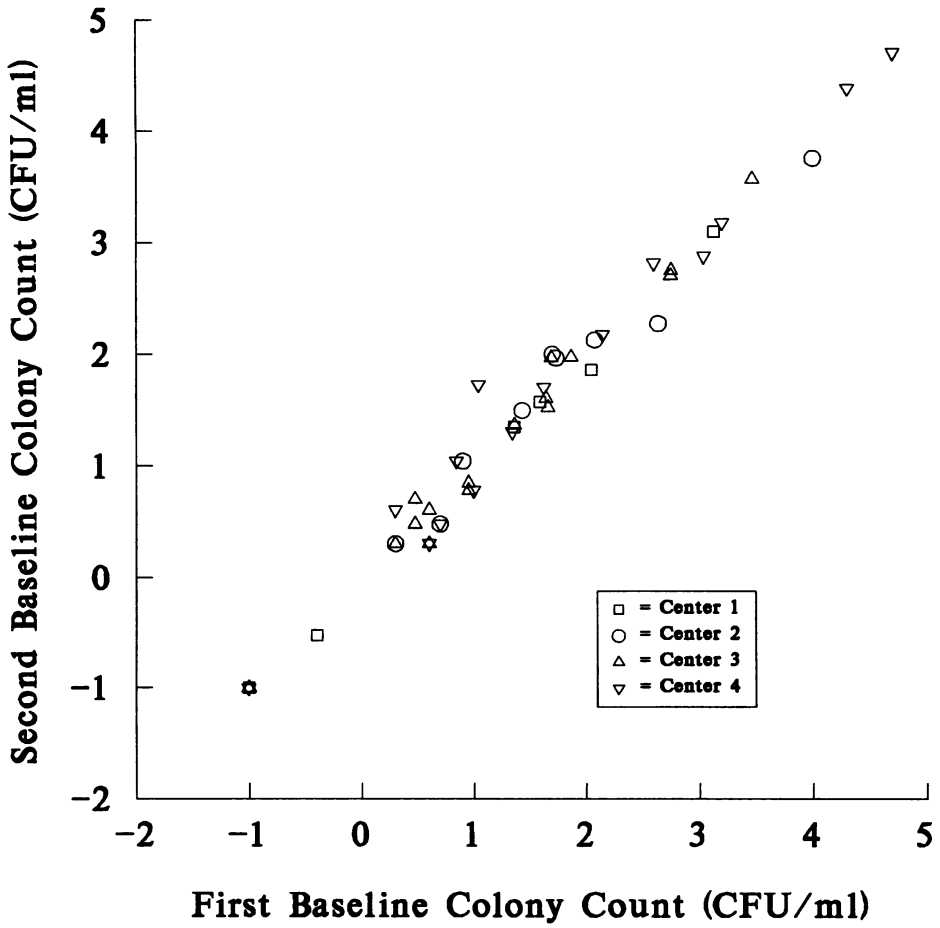


FIG. 1. Duplicate baseline culture results in CFU/ml of whole blood at the four participating centers (shown by center). The intraclass correlation coefficient is 0.997, indicating very close agreement between paired specimens within the individual laboratories.

the only dilution with colony growth. For plates with >300 colonies, the 1:10 dilution was further diluted to 1:100 and 1:1,000 with 7H9 broth and plated. The potential range of detection using this method was −1.0 to 6.0 $\log_{10}$ CFU/ml.

For the assessment of interlaboratory reproducibility, samples from 12 patients were evaluated. One Isolator-10 tube was obtained and, in addition, up to five 10-ml samples were drawn into acid-citrate-dextrose tubes (100 by 16 mm; Becton-Dickinson, Sparks, Md.). The Isolator tube was processed immediately as described above at the initiating institution. Each of the other samples was shipped by overnight express to the other participating centers. Upon receipt, the blood was transferred to an Isolator tube and processed in the manner described above within 24 to 32 h of the initial draw. To control for the 24-h time delay in processing, a fifth acid-citrate-dextrose tube was processed at the initiating institution 24 h after the initial draw in five individual cases.

**Statistical methods.** A nonparametric (Kruskal-Wallis) one-way analysis of variance was used to compare the colony counts between the two paired specimens and between the laboratories. Intraclass correlation was used to calculate the agreement of colony counts between the paired specimens. The Wilcoxon matched-pairs test was used to compare colony counts between the paired specimens.

To evaluate differences in reproducibility which might occur at either very low or very high colony counts, paired specimens were stratified by colony count. Standard deviations and confidence intervals of the colony counts were calculated from the mean estimated variances of the paired colony counts. The difference between individual paired specimens was compared to the mean difference between the pairs, and the standard error of this difference was determined. Calculations of logarithmic colony counts were performed, with negative cultures defined as those with 0.099 CFU/ml, which is below the lower limit of Isolator culture sensitivity.

On the specimens distributed to the five laboratories, nonparametric two-way analysis of variance for repeated measures was used to compare colony counts between laboratories. The intraclass correlation coefficient was used to calculate the agreement within patient values between laboratories (despite possible differences in methodology between centers).

## RESULTS

Of the 104 paired quantitative cultures, colony counts ranged from 0 to 50,000 CFU/ml (Fig. 1). Paired cultures from six patients were negative. One additional patient had one negative culture and one culture with 0.1 CFU/ml. Nonparametric analyses revealed no significant difference in

TABLE 1. Mean colony counts and confidence intervals stratified by log range as determined from the analysis of paired specimens obtained from 46 patients with MAC bacteremia before the administration of antimycobacterial agents[a]

| Range of colony counts (CFU/ml) | No. of pairs of specimens | Mean $\log_{10}$ colony count (95% CI)[b] ($\log_{10}$ CFU/ml) | $\log_{10}$ SD[c] | Geometric mean colony count (95% CI) (CFU/ml) | Mean difference in paired determinations ± SE[d] ($\log_{10}$ CFU/ml) |
|---|---|---|---|---|---|
| $10^{-1}$–$10^{1}$ | 19 | 0.31 (0.25, 0.37) | 0.12 | 2.04 (1.80, 2.32) | 0.04 ± 0.04 |
| $>10^{1}$–$10^{2}$ | 14 | 1.62 (1.53, 1.71) | 0.17 | 42.07 (34.43, 51.40) | −0.09 ± 0.06 |
| $>10^{2}$–$10^{3}$ | 7 | 2.55 (2.46, 2.64) | 0.12 | 356.45 (288.60, 439.64) | 0.04 ± 0.07 |
| $>10^{3}$ | 6 | 3.79 (3.72, 3.85) | 0.08 | 6,151.79 (5,296.63, 7,144.96) | 0.02 ± 0.05 |
| $>10^{-1}$ | 46 | 1.51 (1.47, 1.54) | 0.13 | 31.99 (29.24, 34.99) | 0.001 ± 0.03 |

[a] Excluding 6 pairs of negative cultures from the original 52 pairs of specimens.
[b] CI, confidence interval.
[c] The standard deviation was determined from the mean variance of the paired data for each stratification.
[d] The standard error of the mean difference between the paired determinations (i.e., the standard error of the effect of replication).

colony counts between the two paired specimens or between the laboratories ($P > 0.2$). The intraclass correlation coefficient was 0.997. The arithmetic mean difference (± standard deviation) between the two pairs of colony counts, excluding the six pairs of negative cultures, was 3.24 ± 896.04 CFU/ml.

Mean $\log_{10}$ colony counts, geometric mean colony counts, and the mean difference between the paired colony counts were determined for colony counts stratified by log range (Table 1). For each stratification, the mean difference between the paired colony counts (i.e., the effect of the replication) was small relative to the standard error, indicating that the variance of the colony counts is primarily due to their distribution within a stratum, not forgiving the difference in the paired laboratory determination.

Colony counts of simultaneously obtained patient specimens, as determined by each of the five laboratories, are shown in Fig. 2. The mean colony counts ranged from 0.02 to 15,530 CFU/ml. There was no significant difference in an individual patient's colony counts between laboratories ($P > 0.2$). The intraclass correlation comparing the results within patient's values was 0.887, indicating close agreement among all five laboratories.

Furthermore, there was a high degree of correlation between the mean counts of the 12 specimens processed immediately compared with the mean of those processed after a 24- to 32-h delay ($P < 0.001$). The mean colony count of the specimens processed immediately compared with that of the delayed specimens was 1.94 log CFU/ml (compared with 1.87 log CFU/ml) ($P > 0.2$). Compared with counts of the respective baseline specimens, the colony counts of 22 delayed specimens were higher, 21 specimens had lower colony counts, and counts of 5 specimens were the same. In addition, in five cases, there was no apparent difference between the immediate and delayed colony counts as determined by an individual laboratory.

The effect of antimycobacterial agents on delayed quantitation could not be specifically determined because of the small number of patients ($n = 3$), but there was no apparent decrease in colony counts in the two patients who were receiving clofazimine. Colony counts in the third patient, who was receiving a combination of clofazimine and rifampin, however, did appear to be lower after a delay in processing. Colony counts were 1,475 CFU/ml at the originating institution and 460, 390, and 297 CFU/ml at three other sites.

## DISCUSSION

This study demonstrates that experienced laboratories can achieve highly reproducible quantitative culture results for a wide range of mycobacterial colony counts. These data have several clinical and research implications. First, the correlation between paired specimens suggest that results are highly reproducible on an individual specimen tested in a single laboratory. Because of the importance of the baseline isolate in a research investigation, two quantitative cultures are often performed to ensure that there is at least one baseline value obtained in case a culture is lost because of a technical error or contamination problem. Our data suggest that results from a single baseline culture would be reliable in this situation. Second, in clinical practice where laboratories utilize lysis-centrifugation, one culture is adequate for a diagnostic evaluation for MAC: there appears to be no increased yield with a simultaneous culture. Reves found a similarly high degree of concordance in paired specimens in terms of a positivity in cultures processed by hypotonic lysis, centrifugation, and inoculation of sediment into Bactec 12B or solid media (8).

While evaluating the reproducibility of quantitative cultures in five laboratories, we obtained data addressing the issue of the impact of delayed processing on quantitation of MAC. von Reyn et al. previously reported on delayed processing when they examined blood samples from healthy volunteers seeded with known quantities of MAC (9). Organisms remained viable for up to 28 days in all five of the samples which were inoculated, and colony counts actually appeared to increase when specimens were held for 7 days.

The current investigation differs from that of von Reyn et al. in that all samples were obtained from AIDS patients with active MAC disease. Human immunodeficiency virus-infected patients are uniquely predisposed to MAC and may be infected with multiple strains of MAC. Our study addressed the impact of delayed processing by using clinical samples relevant to therapeutic trials. No significant impact on a 24-h delay of processing of MAC cultures was found despite the fact that specimens were transported via air express to geographically distant sites and temperature and agitation were not controlled. Metchock et al. also evaluated the effect of the delay in processing on 25 pairs of Isolator tubes with MAC specimens from AIDS patients. This investigation reported that MAC isolates survived in Isolator tubes with delayed processing overnight as well as they did in Isolator tubes processed in ≤8 h (7). The impact of a greater than 24-h processing time is less clear. Limited data suggest that

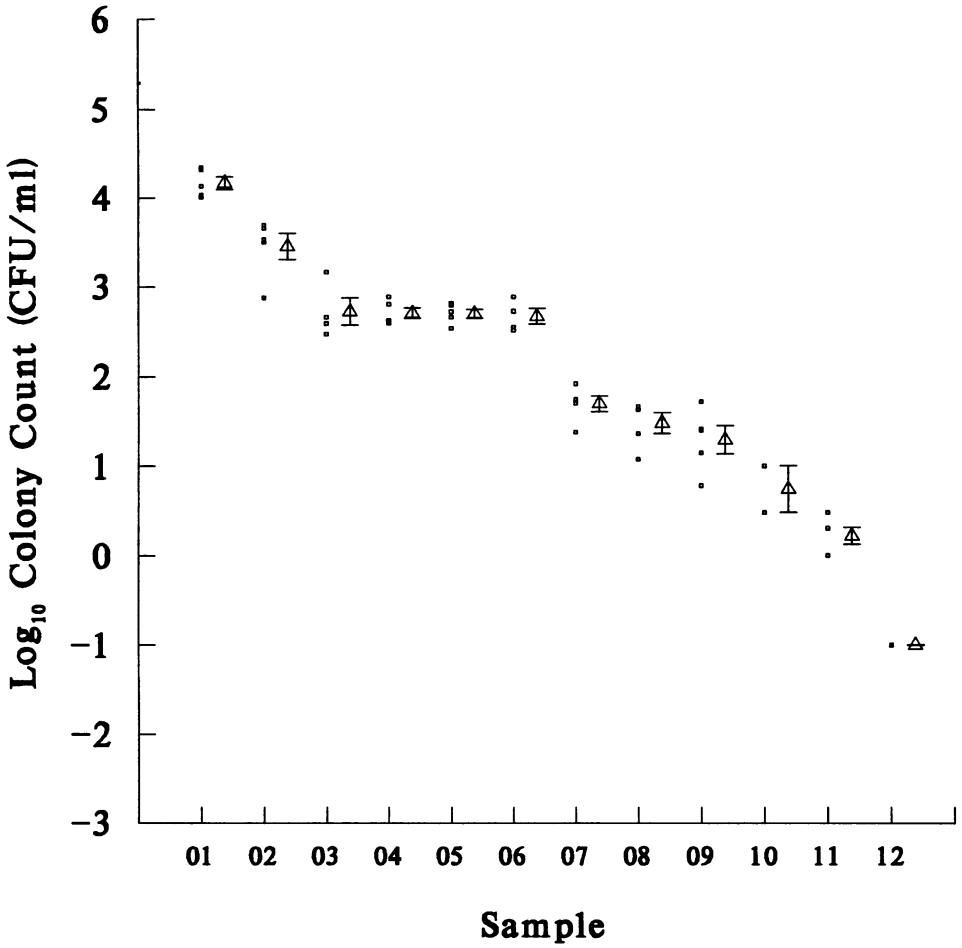


FIG. 2. A scattergram of the colony counts ($log_{10}$ CFU/ml) from each of the 12 patients as determined by the five laboratories (shown in descending order). Triangles with error bars represent the median colony counts and the standard deviations. Two-way analysis of variance revealed no significant differences within patients' colony counts between laboratories ($P > 0.2$).

a delay of greater than 24 h in processing clinical specimens may decrease sensitivity, particularly at low levels of mycobacteremia (1).

Another important question for which our study provides only limited information is the impact of antimycobacterial antibiotics on colony counts during delays in processing. In the von Reyn study, quantitative bacteremia was determined in one patient with AIDS and MAC who was receiving a combination of rifampin and clofazimine; colony counts were similar at baseline and at 7 days (11 and 14 CFU/ml, respectively). In the study by Metchock and associates, therapy did not appear to influence outcome. Only one of three of our patients on antimycobacterial therapy appeared to have significantly lower counts (460, 390, and 297 CFU/ml), compared with 1,475 CFU/ml at the originating institution. It appears that lysis and dilution of the Isolator sample minimize any negative impact of antibiotic in the culture sample and therefore, even for patients on antibiotics, a 24-h delay is not detrimental. This, however, requires further investigation with other agents.

In conclusion, while a central laboratory consolidates resources and laboratory personnel needed for processing of quantitative cultures, our data support the alternative approach of using multiple laboratories to generate colony count data. Multicenter clinical trials of MAC bacteremia can be done with independent laboratories after appropriate quality control measures have been instituted. The impact of antimycobacterial therapy on delayed quantitation (>24 h) of mycobacterial colony counts remains to be determined.

## ACKNOWLEDGMENTS

This work was supported by the California Universitywide AIDS Research Program (grant R91-CC86SD), through the California Collaborative Treatment Group. (D.H., C.A.K., and S.C.D. are members of this group).

We thank the following individuals for their participation in this study: Liz Keays, University of California, San Diego; John R. Hamilton, Carol A. Kane, and Karen Cisek, Santa Clara Valley Medical Center; William Lewis, Los Angeles County Hospital; Dennis Haghighat and Rita Lu, University of California, Irvine, Medical Center; and Kalliope P. Halkias and Bienvenido Yangco, University of South Florida. We also thank Daniel A. Bloch, professor of biostatistics, Stanford University School of Medicine, Stanford, Calif., for his expert advice in the statistical analysis; Angie Bartok for assistance in statistical analysis; and Abbe Cavender for secretarial assistance.

## REFERENCES

1. **Agy, M. B., C. K. Wallis, J. J. Plorde, L. C. Carlson, and M. B. Coyle.** 1989. Evaluation of four mycobacterial blood culture media: BACTEC 13A, Isolator/BACTEC 12B, Isolator/Middle-

brook Agar, and biphasic media. Diagn. Microbiol. Infect. Dis. **12:**303–308.

2. **Chiu, J., J. Nussbaum, S. Bozzette, J. G. Tilles, L. S. Young, J. Leedom, P. N. R. Heseltine, J. A. McCutchan, and the California Collaborative Treatment Group.** 1990. Treatment of disseminated *Mycobacterium avium* complex infection in AIDS with amikacin, ethambutol, rifampin, and ciprofloxacin. Ann. Intern. Med. **113:**358–361.
3. **Greene, J. B., G. S. Sidhu, S. Lewin, J. F. Levine, H. Masur, M. S. Simberkoff, P. Nicholas, R. C. Good, S. B. Zolla-Pazner, A. A. Pollock, M. L. Tapper, and R. S. Holzman.** 1982. *Mycobacterium avium-intracellulare*: a cause of disseminated life-threatening infection in homosexuals and drug abusers. Ann. Intern. Med. **97:**539–546.
4. **Horsburgh, C. R.** 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:**1332–1338.
5. **Kemper, C. A., T. C. Meng, J. Nussbaum, J. Chiu, D. F. Feigel, A. E. Bartok, J. M. Leedom, J. G. Tilles, S. C. Deresinski, J. A. McCutchan, and the California Collaborative Treatment Group.** 1992. Treatment of *Mycobacterium avium* complex bacteremia in AIDS with a four-drug oral regimen: rifampin, ethambutol, clofazamine and ciprofloxacin. Ann. Intern. Med. **116:**466–472.
6. **Macher, A. M., J. A. Kovacs, V. Gill, G. D. Roberts, J. Ames, C. H. Park, S. Straus, H. C. Lane, J. E. Parrillo, A. S. Fauci, and H. Masur.** 1983. Bacteremia due to *Mycobacterium avium-intracellulare* in the acquired immunodeficiency syndrome. Ann. Intern. Med. **99:**782–785.
7. **Metchock, B., L. Diem, J. Havlik, S. Gordon, C. R. Horsburgh, and J. E. McGowan, Jr.** 1992. Effect of delayed processing of Isolator tubes (Iso) on recovery of *Mycobacterium avium* complex (MAC) from blood, abstr. C-87, p. 435. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
8. **Reves, R.** Utility of paired blood cultures in the diagnosis of disseminated *Mycobacterium avium* complex, abstr. 45, p. 29. Abstr. Frontiers in Mycobacteriology: *M. avium*, The Modern Epidemic. 1992.
9. **von Reyn, S. F., S. Hennigan, S. Niemczyk, and N. J. Jacobs.** 1991. Effect of delays in processing on the survival of *Mycobacterium avium-M. intracellulare* in the Isolator blood culture system. J. Clin. Microbiol. **29:**1211–1214.
10. **Wong, B., F. F. Edwards, T. E. Kiehn, E. Whimbey, H. Donnely, E. M. Bernard, J. W. M. Gold, and D. Armstrong.** 1985. Continuous high-grade *Mycobacterium avium-intracellulare* bacteremia in patients with the acquired immune deficiency syndrome. Am. J. Med. **78:**35–40.