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Inhibitory Effects of Polyoxyethylene Stearate, PANTA, and Neutral pH on Growth of *Mycobacterium genavense* in BACTEC Primary Cultures

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We report on the influences of polyoxyethylene stearate (POES), PANTA, and pH on primary cultures of *Mycobacterium genavense* in BACTEC vials. As a model for primary cultures from tissue, seven different strains first isolated from AIDS patients (five from Switzerland and two from the United States) were inoculated into nude mice in order to obtain large amounts of bacilli to test different conditions simultaneously. Our results demonstrate that the size of the inoculum (10^6 acid-fast bacilli/vial), an acid pH (pH 6.0), and the absence of additives (POES and PANTA) significantly ($P < 0.001$) increased the probability of a successful culture in 1 month, considering growth index (GI) of ≥100 or a GI of ≥999 as criterion of success. In logistic regression analysis, all factors maintained a significant ($P < 0.001$) independent effect, and no interactions were observed between them. The best conditions for the primary cultures of *M. genavense* were the use of Middlebrook 7H12 medium at pH 6.0 without any additives.

Mycobacterium genavense causes disseminated infections in patients with late-stage AIDS and has now been found in Europe (2, 20), North America (1, 7, 16), and Australia (11). In primary cultures of samples from approximately half of the patients confirmed at autopsy to be infected with *M. genavense*, this mycobacterium showed little growth in broth medium (radiometric Middlebrook 7H12 medium) (4), and the organism failed to grow on the solid media routinely used in the mycobacteriology laboratory. Because of its fastidious growth, it is possible that the prevalence of disseminated infections caused by *M. genavense* has been underestimated. *M. genavense* was reported to cause disseminated infection in 12.8% of AIDS patients in Switzerland from 1990 to 1992 (14). Until now, definite identification of *M. genavense* has been based on the determination of the 16S rRNA gene sequence (3). BACTEC 7H12 medium has been reported to yield positive cultures from clinical specimens more than other media, to improve the ability to detect *Mycobacterium avium* complex, and to significantly reduce the time of detection for positive growth of *Mycobacterium tuberculosis* as well as nontuberculous mycobacteria (13). For those reasons the radiometric culture method is the most commonly used liquid medium for the recovery of mycobacteria. We report here on the influence of polyoxyethylene stearate (POES), PANTA, and pH on primary isolation in radiometric liquid medium of seven strains of *M. genavense* obtained from infected nude mice.

MATERIALS AND METHODS

Inoculation in mice. Since *M. genavense* multiplies in nude mice (10), 8-week-old adult female congenitally athymic (nude) BALB/c mice (IFFA Credo, Lyon, France) were inoculated intraperitoneally with 0.25-ml suspensions of *M. genavense* containing at least 10^5 acid-fast bacilli (AFB) per ml. Seven different *M. genavense* strains obtained from seven different patients with AIDS were used for the present study. The origin and the strain reference numbers are as follows. Institute of Tropical Medicine (ITM) strain ITM 95-786 was first isolated from a patient living in Lausanne, Switzerland; strains ITM 95-975, 95-976, 95-1184, and 95-1328 were isolated from four different patients living in Geneva, Switzerland; strain ITM 95-1329 was isolated from one patient from Seattle, Wash.; and strain ITM 96-387 was isolated from a patient from Iowa City, Iowa.

The mice were killed between 8 and 9 months after inoculation, and the spleens were kept at −70°C (for up to 4 months).

Inoculum preparation. The spleens were thawed, weighed, minced with sterile scissors, and homogenized with a pestle and mortar in 20% (wt/vol) phosphate-buffered saline (PBS). The numbers of AFB were counted by the method of Shepard and McRae (17). Briefly, disposable micropipettes were used to deliver 5 μl of the suspension to be counted onto circular areas marked on microscope slides. Fixation was carried out by using formaldehyde vapor for 3 min, heat (on the lid of a boiling bath) for 2 min, application of gelatin-phenol with a pipette for 4 min, heat for 2 min, formaldehyde vapor for 3 min, and heat for 2 min. The slides were stained by Ziehl-Neelsen staining. The numbers of AFB on three circular areas were counted, and quantification was as described by Shepard and McRae (17).

Dilutions of the tissue homogenate were made in PBS. No decontamination was performed.

Culture media and incubation conditions. The following radiometric Middlebrook 7H12 broths were inoculated: BACTEC 12B (pH 6.8 ± 0.2) and BACTEC pyrazinamide control medium (pH 6.0 ± 0.2), both as such or supplemented with either 100 μl of PANTA (polymyxin B, 50 U/ml; amphotericin B, 5 μg/ml; nalidixic acid, 20 μg/ml; trimethoprim, 5 μg/ml; azlocillin, 10 μg/ml; Becton Dickinson) reconstituted as recommended by the manufacturer in PANTA Reconstituting Fluid (Becton Dickinson) containing POES (final concentration, 100 μg of POES/ml), 100 μl of PANTA reconstituted in sterile distilled water, or 100 μl of PANTA Reconstituting Fluid (POES) at the same concentration described above. The vials were inoculated with 100 μl of the *M. genavense* suspension to obtain 10^6, 10^5, and 10^4 AFB/vial and were incubated at 37°C. All specimens were processed in triplicate. Readings of the release of $^{14}CO_2$ in the BACTEC 460 TB instrument were made twice a week for 1 month or until the growth index (GI) reached 999. The gas mixture used was air supplemented with 7% CO_2. When the GI reached 999 or at the end of the experiment, purity control was performed for all vials by inoculation of the contents onto thioglycolate, blood agar, and Middlebrook 7H11 medium.

Statistical analysis. A primary liquid culture of undecontaminated *M. genavense* was defined as being successful within 1 month by analyzing two criteria of growth detection in the BACTEC system: (i) when the GI reached 100, the GI recommended for performing an AFB smear, and (ii) when the GI reached 999, the maximum metabolic activity that the BACTEC system can detect. The influence of the parameters tested (strains, bacillus concentrations [10^6 to 10^4 AFB/vial], pH of the medium [pH 6.8 or 6.0], and the different additives [POES and PANTA by themselves and PANTA reconstituted in POES]) was first explored by univariate and stratified analyses. Subsequently, a logistic regression model was built to control for strain influences and confounding among other growth determinants to explore possible interactions and to test

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TABLE 1. Influence of pH and additives on the growth of seven strains of *M. genavense* in BACTEC system

pH	Additive	No. of strains positive after 1 mo of incubation under the following conditions:					
		GI, ≥999			GI, ≥100		
		Inoculum (no. of AFB/vial)			Inoculum (no. of AFB/vial)		
		10^4	10^5	10^6	10^4	10^5	10^6
6.8	None	0	3	4	2	4	6
	POES alone	0	1	3	1	3	5
	PANTA alone	0	1	3	1	3	6
	POES + PANTA	0	0	3	0	1	4
6.0	None	4	4	7	4	6	7
	POES alone	2	4	6	4	5	7
	PANTA alone	2	4	6	4	5	7
	POES + PANTA	0	3	4	2	4	5

the statistical significance of the observed effect (while allowing for the two definitions of success). The strength of the effects was summarized by an odds ratio (OR), which can be interpreted as the relative chance of detecting growth under the different conditions tested (e.g., absence versus presence of POES).

RESULTS

Table 1 presents the total numbers of strains reaching a GI of ≥999 and a GI of ≥100 within 1 month as a function of the inoculum size (10^6 to 10^4 bacilli/vial), the pH (6.8 and 6.0), and the presence of different additives (POES alone, PANTA alone, and PANTA reconstituted in POES). The maximum metabolic activity (GI of 999) could be detected in the BACTEC system within 1 month for all seven strains tested when a high concentration of bacilli (10^6) was inoculated into vial under acidic conditions without any additives. POES and PANTA clearly have inhibitory effects at pH 6.8 and pH 6.0 for all concentrations of bacilli tested, as indicated by the lower number of positive strains. The GI never reached 999 when an inoculum of 10^4 *M. genavense* was inoculated in a vial at neutral pH with or without additives. At a GI of 100, the best results were obtained with 10^6 AFB/vial in acidic medium without any additives or supplemented with either POES or PANTA. Considering the number of strains reaching GIs of ≥100 at the different concentrations tested, neutral pH and additives had an inhibitory effect. When 10^4 *M. genavense* organisms/vial were inoculated into neutral medium supplemented with POES and PANTA (the conditions recommended for *M. tuberculosis*), the GI did not reach 100 within 1 month for any of the seven strains tested. At 10^5 and 10^6 bacilli/vial, one and four strains, respectively, reached GIs of 100 under the same cultures conditions. Considering the three inocula used in this study and the two criteria for positive cultures (GI of ≥100 and GI of ≥999), the best culture conditions were acidic medium with no additives.

Figure 1 presents the growth of the seven different strains of *M. genavense* under all the conditions tested. The most representative concentration of bacilli for each strain is shown. In acidic medium and in neutral medium, PANTA and POES had a negative influence on *M. genavense* growth, as indicated by the curves. When PANTA reconstituted in POES was added to the BACTEC vials, there was a drastic effect on the growth of *M. genavense*. This effect was evident at both pHs tested (6.0 and 6.8). Differences between strains could be observed: Strain 96-387 was much more inhibited by neutral pH than strain 95-1328 or strain 95-786. The inhibitory effect of POES alone seemed to be similar to that of PANTA alone for all strains, as indicated by both curves at pH 6.0 or 6.8. Only strain 95-976 seemed to be more inhibited by POES than by PANTA, which is more clear at pH 6.0. In univariate statistical analysis, all the factors (strain, bacillus concentration, pH, and absence of additives) had a significant ($P < 0.001$) influence on the growth. For both criteria of growth success (GI of ≥100 and GI of ≥999), the size of the inoculum (10^6 AFB/vial), an acid pH (pH 6.0), and the absence of additives (POES and PANTA) significantly increased the probability of a successful culture in 1 month. In logistic regression analysis, all factors maintained a significant ($P < 0.001$) independent effect, and no interactions were observed between them. The ORs for concentrations of 10^5 and 10^6 bacilli/vial versus a concentration of 10^4 bacilli/vial were 3.8 and 19.0, respectively, for pH 6.0 versus pH 6.8, the OR was 6.2; the OR for the absence of POES versus the presence of POES was 2.4; and the OR for the absence of PANTA versus the presence of PANTA was also 2.4. The magnitude of the effects of the different parameters was not influenced by the definition of successful cultures.

The purity of cultures was checked by inoculation of the vials on thioglycolate, blood agar, and Middlebrook 7H11 medium. None of the seven strains tested grew on thioglycolate and blood agar under any of the conditions tested. Very small and transparent colonies were obtained by subculturing positive vials with acidic medium (with or without additives) onto Middlebrook 7H11 medium: after 3 months of incubation for strains ITM 95-976 and ITM 95-1184 and after 2.5 months of incubation for strains ITM 95-1329 and ITM 96-387. These colonies were identified as *M. genavense* by characterization of specific sequences in the *rrn* operon (data not shown). For the other three strains and all the other conditions tested, no growth was obtained on Middlebrook 7H11 medium even after 3 months of incubation.

DISCUSSION

Our results demonstrate that the pH of the medium and the additives (POES and PANTA) influence the growth of primary cultures of *M. genavense* in the BACTEC 460 TB system. *M. genavense* grew better in primary cultures in acidic medium. This confirms the results of others (19, 20) and is not surprising: the majority of mycobacterial species grow better at a slightly acid pH (pH 6) than at a neutral pH (15).

The inhibition of *M. genavense* growth in primary culture, as detected in the BACTEC system, by the addition of POES to the medium was quite unexpected, if we refer to studies of other mycobacteria (8, 18). POES enhances the growth of many mycobacteria, especially *M. tuberculosis* and *M. bovis*, in radiometric Middlebrook 7H12 medium (18). To our knowledge, an inhibitory effect of POES has been described for *M. leprae* (9) and for one strain of *M. genavense* when it was subcultured into the BACTEC system (20). One explanation could be that *M. genavense* metabolizes POES, which is not labelled like [^{14}C]palmitic acid is. Therefore, *M. genavense* growth could occur in the BACTEC system but would not be detected. Camargo et al. (5) have reported the poor oxidation of stearic acid by *M. tuberculosis* and *M. bovis*, but nothing is known concerning the oxidation of stearic acid by *M. genavense*. Recently, Miller et al. (12) reported growth inhibition of *M. tuberculosis* by POES in the BACTEC pyrazinamide susceptibility test. They suggested that this inhibitory effect could be pH dependent. In the present study, POES had an inhibitory effect on the growth of *M. genavense* in Middlebrook 7H12 medium at both pH 6.8 and 6.0.

PANTA reconstituted in water also had an inhibitory effect

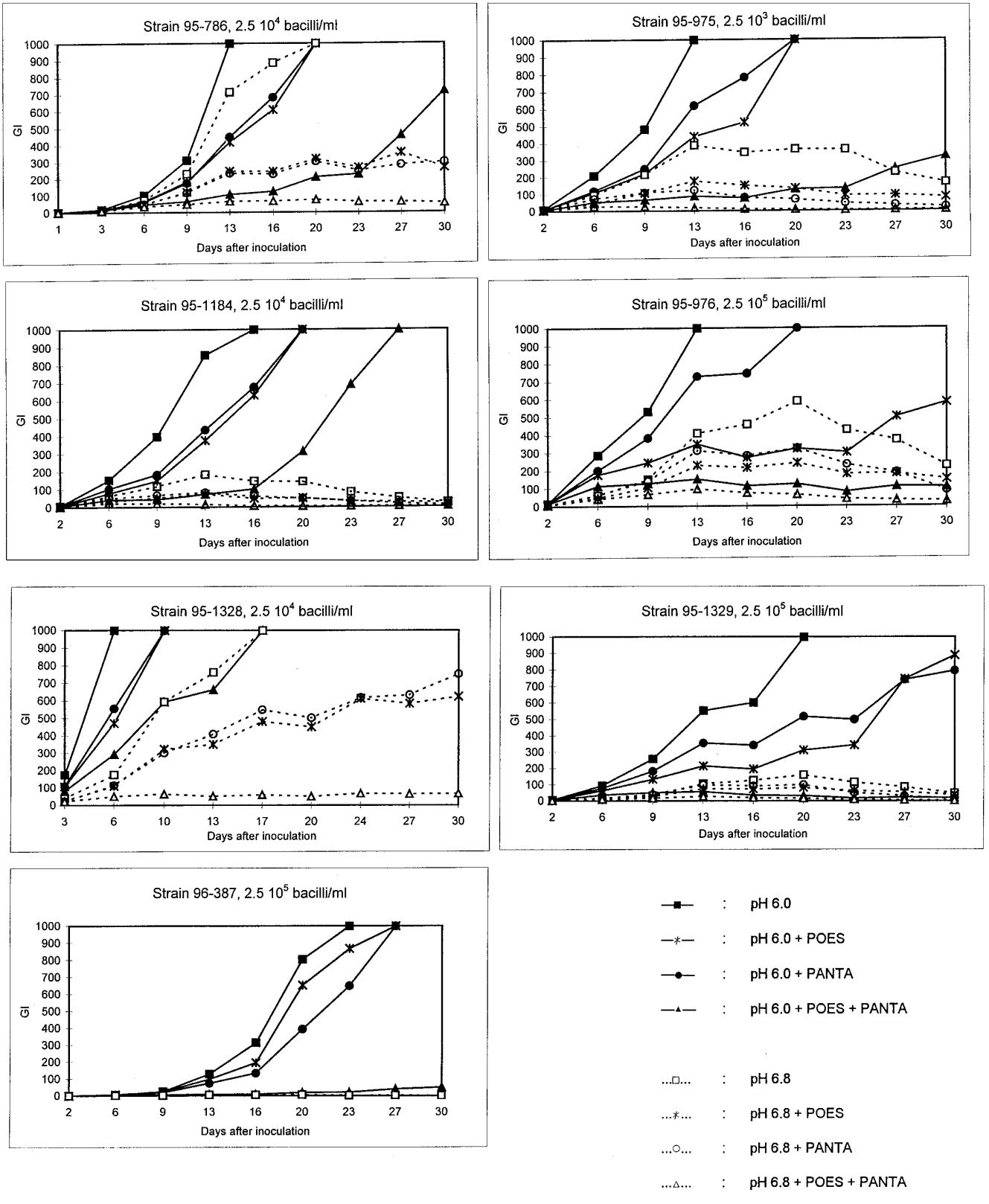

FIG. 1. Effect of pH and additives on growth of seven strains of *M. genavense* in the BACTEC system.

on the growth of *M. genavense*, as shown by the delay in growth obtained at both pHs. There is a report on the possible presence in PANTA of a factor inhibiting the growth of *M. kansasii* in BACTEC 12B medium (6). Those investigators found that nalidixic acid alone inhibited the growth of *M. kansasii* isolates. Concerning *M. genavense*, preliminary experiments showed that polymyxin B and azlocillin by themselves exerted some growth inhibition.

The combined effect of adding PANTA and POES to BACTEC vials was drastic for primary cultures of *M. genavense.*

Another factor important to the success of growth, as we defined it in the BACTEC system, is the strain itself. Statistical analysis showed a significant difference ($P < 0.001$) in growth between the seven different strains of *M. genavense* tested. In vials with acidic medium without any additives, 10^4 AFB/vial were sufficient to obtain a GI of 999 within 1 month for strains ITM 95-1328, ITM 95-786, ITM 95-1184, and ITM 95-975, all from Switzerland, whereas for the other three strains (strains ITM 95-1329 and ITM 96-387 from the United States and strain ITM 95-976 from Switzerland), inocula of 10^6 AFB/vial were necessary. A correlation between the observed growth of the strains and the time that the tissues were kept at −70°C (data not shown) was not observed. Only one freezing-thawing cycle of the organs from infected nude mice was performed before inoculation. The differences between the strains that we observed could be related to the viability of each strain.

In the present study, as a model for cultures from tissues, we used organs from nude mice in order to obtain enough bacilli to test different conditions simultaneously. We have found that the best conditions for the primary culture of *M. genavense* in the BACTEC system are the use of Middlebrook 7H12 medium at pH 6.0 without any additives and propose that other researchers evaluate these conditions with clinical specimens.

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REFERENCES

1. **Bessesen, M. T., J. Shlay, B. Stone-Venohr, D. L. Cohn, and R. R. Reves.** 1993. Disseminated *Mycobacterium genavense* infection: clinical and microbiological features and response to therapy. AIDS **7:**1357–1361.
2. **Böttger, E. C., A. Teske, P. Kirschner, S. Bost, H. R. Chang, V. Beer, and B. Hirschel.** 1992. Disseminated "*Mycobacterium genavense*" infection in patients with AIDS. Lancet **340:**76–80.
3. **Böttger, E. C., B. Hirschel, and M. B. Coyle.** 1993. *Mycobacterium genavense* sp. nov. Int. J. Syst. Bacteriol. **43:**841–843.
4. **Böttger, E. C.** 1994. *Mycobacterium genavense*: an emerging pathogen. Eur. J. Clin. Microbiol. Infect. Dis. **13:**932–936.
5. **Camargo, E. E., J. A. Kertcher, S. M. Larson, B. S. Tepper, and H. N. Wagner, Jr.** 1982. Radiometric measurement of differential metabolism of fatty acid by mycobacteria. Intern. J. Lepr. **50:**200–204.
6. **Conville, P. S., J. W. B. Andrews, and F. G. Witebsky.** 1995. Effect of PANTA on growth of *Mycobacterium kansasii* in BACTEC 12B medium. J. Clin. Microbiol. **33:**2012–2015.
7. **Coyle, M. B., L. C. Carlson, C. K. Wallis, R. B. Leonard, V. A. Raisys, J. O. Kilburn, M. Samadpour, and E. C. Böttger.** 1992. Laboratory aspects of "*Mycobacterium genavense*," a proposed species isolated from AIDS patients. J. Clin. Microbiol. **30:**3206–3212.
8. **Cutler, R. R., P. Wilson, and F. V. Clarke.** 1987. The effect of polyoxyethylene stearate (POES) on the growth of mycobacteria in radiometric 7H12 Middlebrook TB medium. Tubercle **68:**209–220.
9. **Franzblau, S. G.** 1989. Drug susceptibility testing of *Mycobacterium leprae* in the BACTEC 460 system. Antimicrob. Agents Chemother. **33:**2115–2117.
10. **Hirschel, B., H. R. Chang, N. Mach, P. F. Piguet, J. Cox, J. D. Piguet, M. T. Silva, L. Larsson, P. R. Klatser, J. E. R. Thole, L. Rigouts, and F. Portaels.** 1990. Fatal infection with a novel, unidentified *Mycobacterium* in a man with the acquired immunodeficiency syndrome. N. Engl. J. Med. **323:**109–113.
11. **Jackson, K., A. Sievers, B. C. Ross, and B. Dwyer.** 1992. Isolation of a fastidious *Mycobacterium* species from two AIDS patients. J. Clin. Microbiol. **30:**2934–2937.
12. **Miller, M. A., L. Thibert, F. Desjardins, S. H. Siddiqi, and A. Dascal.** 1996. Growth inhibition of *Mycobacterium tuberculosis* by polyoxyethylene stearate present in the BACTEC pyrazinamide susceptibility test. J. Clin. Microbiol. **34:**84–86.
13. **Nolte, F. S., and B. Metchock.** 1995. Mycobacterium, p. 400–437. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
14. **Pechère, M., M. Opravil, A. Wald, J. P. Chave, M. Bessesen, A. Sievers, R. Hein, J. Vonoverberch, R. A. Clark, E. Tortoli, S. Emler, P. Kirschner, V. Gabriel, E. C. Böttger, and B. Hirschel.** 1995. Clinical and epidemiological features of infection with *Mycobacterium genavense*. Arch. Intern. Med. **155:** 400–404.
15. **Portaels, F., and S. R. Pattyn.** 1982. Growth of mycobacteria in relation to the pH of the medium. Ann. Microbiol. (Institut Pasteur). **133B:**213–221.
16. **Shafran, S. D., G. D. Taylor, and J. A. Talbot.** 1995. Disseminated *Mycobacterium genavense* infection in Canadian AIDS patients. Tubercle Lung Dis. **76:**168–170.
17. **Shepard, C. C., and D. H. McRae.** 1968. A method for counting acid-fast bacteria. Int. J. Lepr. **36:**78–82.
18. **Siddiqi, S. H., J. P. Libonati, M. E. Carter, N. M. Hooper, J. F. Baker, C. C. Hwangbo, and L. E. Warfel.** 1988. Enhancement of mycobacterial growth in Middlebrook 7H12 medium by polyoxyethylene stearate. Curr. Microbiol. **17:**105–110.
19. **Siddiqi, S. H., A. Laszlo, W. R. Butler, and J. O. Kilburn.** 1993. Bacteriologic investigations of unusual mycobacteria isolated from immunocompromised patients. Diagn. Microbiol. Infect. Dis. **16:**321–323.
20. **Tortoli, E., M. T. Simonetti, D. Dionisio, and M. Meli.** 1994. Cultural studies on two isolates of *Mycobacterium genavense* from patients with the acquired immunodeficiency syndrome. Diagn. Microbiol. Infect. Dis. **18:**7–12.