Eur. J. Clin. Microbiol. Infect. Dis., 1995, 14: 6–10

Rapid Detection of Tuberculous and Non-Tuberculous Mycobacteria by Microscopic Observation of Growth on Middlebrook 7H11 Agar

P. Idigoras, E. Pérez-Trallero*, M. Alcorta, C. Gutiérrez, I. Muñoz-Baroja

The rate of recovery and time to the detection of mycobacteria in clinical specimens were measured in traditional egg-based media cultures and on Middlebrook 7H11 agar plate cultures using microcolony detection. In the 5438 specimens processed, a total of 293 (5.4 %) clinically relevant mycobacterial isolates were detected (*Mycobacterium tuberculosis*, n = 231; *Mycobacterium avium* complex, n = 60; *Mycobacterium kansasii*, n = 2). Of these, 227 (77 %) and 237 (81 %) isolates were detected on Lowenstein-Jensen medium and Coletsos medium, respectively, and 265 (90 %) isolates were detected on Middlebrook 7H11 plates examined microscopically. The detection time was shorter with the microcolony detection method. While the Lowenstein-Jensen and Coletsos media required an average of 23 and 25 days, respectively, for first detection of mycobacteria, microcolony detection on Middlebrook 7H11 required an average of only 12 days. For acid-fast, stain-positive specimens that were culture positive for *Mycobacterium tuberculosis*, the average interval to positivity was nine days for the microcolony method compared with 20 and 21 days for the Lowenstein-Jensen and Coletsos media, respectively. Microscopic detection on Middlebrook 7H11 agar plates is a rapid, accurate and inexpensive method of detecting *Mycobacterium tuberculosis* and other clinically important mycobacteria.

Tuberculosis, has become more prevalent in recent years in both developed and developing countries (1, 2). This increase in prevalence is mainly associated with the spread of HIV infection; an added problem of HIV-infected patients is that they often carry *Mycobacterium tuberculosis* strains with multiple drug resistance (2–4).

One of the main problems with tuberculosis is the difficulty in achieving early microbiological diagnosis. Many advances are being made in this field, notably, the polymerase chain reaction (5, 6). However, until new technologies become available at a reasonable cost, in the majority of countries the diagnosis of tuberculosis will still be based on traditional culture methods.

The use of the Middlebrook 7H11 plate (7H11) to visualize microcolonies with the aid of a low power microscope was proposed in the 1960s and 1970s by Runyon (7) and Vestal and Kubica (8). The method was described as being rapid, effective and technically simple. It has not been used much routinely in laboratory diagnostics and, because of the use of liquid media and radiometric detection methods, has not really been evaluated properly.

Following recommendations by Welch et al. (9), we undertook this study in our region to evaluate microscopic observation of mycobacterial growth on 7H11 plates.

Materials and Methods

Nuestra Señora de Aránzazu Hospital in San Sebastián, Spain, is a 900-bed teaching hospital that cares for pediatric and adult patients and has an AIDS unit. Over a 16-month period, all specimens sent to the laboratory for mycobacterial culture were cultured using both conventional methods in Coletsos and Lowenstein-Jensen tubes and the microcolony method on 7H11 plated medium. Coletsos is an egg-based medium similar to Lowenstein-Jensen medium supplemented with sodium pyru-

Servicio de Microbiología, Hospital Nuestra Señora de Aránzazu, Apartado 477, 20080 San Sebastián, Spain.

vate, sodium glutamate and oseine. For blood culture, the specimens were pre-treated using a lysis-centrifugation method (Isolator, Wampole, USA). Specimens expected to contain other microbial agents were subjected to a routine protocol of digestion and decontamination with sodium lauryl sulfate and sodium hydroxide (10). An inoculum of 0.02 ml was seeded on each of the following media: Lowenstein-Jensen (Biomedics, Spain) and Coletsos (bioMérieux, France), in slanted tubes, and 7H11 agar on a thinly poured (18 ml) plate (10 by 90 mm). The 7H11 agar medium was prepared in-house from dehydrated powder (Difco, USA) and Middlebrook OADC enrichment (Difco, or BBL, USA). During the study period, 5438 specimens were seeded on the three culture media: 3153 sputa, 457 bronchial aspirates, 137 bronchoalveolar lavage fluid, 207 cerebrospinal fluid, 1077 urine, 109 feces, 134 blood and 164 of various other origins.

After inoculation, all media were incubated at 35°C. Plated and tubed media were incubated, in an atmosphere enriched with 5 % carbon dioxide for two and one weeks, respectively, and then the incubation continued in an atmosphere without carbon dioxide for a total duration of 40 and 90 days, respectively.

The 7H11 plates were studied microscopically by focusing through the bottom of the plate at x 100 magnification using a conventional microscope. The streak line in the agar surface makes the correct plane of focus easier. The plated media were screened microscopically every two days for the first 15 days and were then examined by sight every week. Tubed media were evaluated by sight every week or every two days if the 7H11 medium showed growth. Plates were sealed with an adhesive tape (surgical tape Micropore, 3M, USA) until growth was detected. Colonies were picked and stained for acid fastness; acid-fast colonies were studied using DNA probes (Gen-Probe, USA) for *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex and *Mycobacterium kansasii* species.

A specimen was considered positive if it grew in at least one of the three media.

Data analysis was conducted with conventional tests. Isolation rates were evaluated using the McNemar modification of the chi-square test. Repeated measures analysis of variance (ANOVA, Instat V2.04a, GraphPad Software, USA) was used to compare time to the detection of mycobacteria. The level of significance chosen was $\alpha = 0.05$.

Results

Of the 5438 specimens cultured, 293 (5.4 %) from 110 patients were positive. Tuberculosis was diagnosed in 98 patients (231 specimens), mycobacteriosis by *Mycobacterium avium* complex in 11 patients (60 specimens) and disseminated *Mycobacterium kansasii* infection in one patient (2 specimens). Isolates of other non-pathogenic mycobacteria were omitted from this study.

Initial screening with auramine stain was positive in 121 specimens, which corresponded to 41 % of the specimens that were culture positive (Table 1). The 7H11 agar had the highest recovery rate of all the media, effectively isolating 265 (90 %) of the strains compared with 237 (81 %) strains isolated with Coletsos medium and 227 (77 %) with Lowenstein-Jensen medium. Recovery rates calculated by comparing the number of isolates obtained with 7H11 to the number of isolates obtained with the egg-based conventional media were: $\chi^2 = 11.20$, $p < 0.001$ for 7H11 versus Coletsos and $\chi^2 = 19.51$, $p < 0.001$ for 7H11 vs. Lowenstein-Jensen. There was no statistically significant difference between growth on the Coletsos and Lowenstein-Jensen media ($\chi^2 = 2.08$, $p = 0.15$). Table 2 shows the results of culture by group or species of *Mycobacterium*. The mean time to the detection of positive cultures on 7H11 was shorter than for other media: 12 days (11.69, SD 6.61), 23 days (23.08, SD 9.28), and 25 days (25.33, SD 10.35) with 7H11, Coletsos, and Lowenstein-Jensen, respectively (Table 3). To make statistical comparison (ANOVA) possible, we excluded the

Table 1: Results of direct acid-fast bacilli (AFB) smear detection and culture by type of specimen.

Specimen source	No. of mycobacteria isolated	No. (%)			
		AFB smear positive	Isolation on 7H11	Isolation in Coletsos	Isolation in LJ
Sputum	189	83 (44)	173 (92)	161 (85)	156 (83)
Bronchial aspirates	24	8 (33)	20 (83)	20 (83)	17 (71)
BAL	10	1 (10)	9 (90)	6 (60)	7 (70)
Urine	38	19 (50)	34 (89)	28 (74)	28 (74)
CSF	7	1 (14)	6 (86)	5 (71)	6 (86)
Blood	11	0 (0)	9 (82)	9 (82)	6 (55)
Feces	7	5 (71)	7 (100)	3 (43)	3 (43)
Other	7	4 (57)	7 (100)	5 (71)	4 (57)
Total	293	121 (41)	265 (90)	237 (81)	227 (77)

BAL: bronchoalveolar lavage; CSF: cerebrospinal fluid; 7H11: Middlebrook 7H11 agar medium; LJ: Lowenstein-Jensen.

Table 2: Results of culture by group or species of *Mycobacterium* in each medium used.

Organism (n)	No. (%)			
	AFB smear positive	7H11	Coletsos	LJ
M. tuberculosis complex (231)	114 (49)	210 (91)	191 (83)	187 (81)
M. avium complex (60)	5 (8)	53 (88)	45 (75)	39 (65)
M. kansasii (2)	2 (100)	2 (100)	1 (50)	1 (50)
Total	121 (41)	265 (90)	237 (81)	227 (77)

AFB: Acid-fast bacilli; 7H11: Middlebrook 7H11 agar medium; LJ: Lowenstein-Jensen.

Table 3: Average number of days to the time of first detection of positive culture by the microcolony method and conventional method.

	Mean no. of days (range)		
	7H11	Coletsos	LJ
M. tuberculosis			
Smear-positive	9 (3–32)	20 (10–55)	21 (10–60)
Smear-negative	16 (6–40)	27 (14–65)	29 (14–90)
M. avium complex		11 (9–13)	
Smear-positive	5 (4–6)	24 (7–42)	24 (9–40)
Smear-negative	8 (3–21)		31 (7–58)
M. kansasii	6 (3–10)	10	10
Total	12 (3–40)	23 (7–65)	25 (7–90)

7H11: Middlebrook 7H11 agar medium; LJ: Lowenstein-Jensen.

specimens that were grown in only one or two media, leaving 197 specimens that were grown in all three media. The mean time, in days, to the detection of these 197 positive cultures was: 11 (11.06, SD 6.14); 21 (21.46, SD 7.76), and 24 (23.92, SD 8.59) with 7H11, Coletsos, and Lowenstein-Jensen media, respectively (ANOVA $f = 416$, $p < 0.001$).

Specimens containing acid-fast staining bacilli grew faster than those containing a negative initial smear (Table 3). The mean time to the detection of *Mycobacterium tuberculosis* positive cultures was 9, 20 and 21 days for positive acid-fast smears versus 16, 27 and 29 days for negative acid-fast smears ($t = 8.15$, $p < 0.001$; $t = 5.50$, $p < 0.001$; $t = 5.98$, $p < 0.001$) with 7H11, Coletsos, and Lowenstein-Jensen media, respectively.

Of the 28 negative cultures on 7H11 which were positive on the other media, four were plates discarded at the beginning of incubation because of contamination. Of the remaining 24, 23 cultures were positive on either the Coletsos or Lowenstein-Jensen media after 28 days of incubation; the last one grew on the Coletsos and Lowenstein-Jensen media in 21 and 41 days, respectively. Thirty-seven cultures (12 *Mycobacterium avium* complex, 1 *Mycobacterium kansasii* and 24 *Mycobacterium tuberculosis*) were grown only on 7H11; ten cultures (3 *Mycobacterium avium* complex and 7 *Mycobacterium tuberculosis*) only on Coletsos; and seven cultures (7 *Mycobacterium tuberculosis*) only on Lowenstein-Jensen.

Discussion

Tuberculosis is a chronic disease. Active cases are usually contagious and spread easily to close con-

tacts. The number of AIDS patients is increasing and tuberculosis in these patients is a much more fulminant disease. Therefore, early diagnosis and adequate treatment are critical for the control of the disease. Using 7H11-medium plates and microscopic detection, the time required to detect colonies was appreciably shorter than for traditional culture media and was similar to that reported for radiometric detection media (11–13). Moreover, the mycobacteria recovery rates for the 7H11 agar medium were superior to those of conventional media. Other comparisons of mycobacterial recovery rates between 7H11 and Lowenstein-Jensen media have shown diverse results (14, 15). Welch et al. (15), in a similar study, found a lower sensitivity using commercially prepared 7H11 plates than with Lowenstein-Jensen medium although the time of first detection was found to be even shorter than that reported by this study. The higher recovery rate we found for 7H11 might be due to the fact that the medium was prepared in-house and was incubated in an atmosphere enriched with 5 % carbon dioxide for two weeks. By comparison, the egg-based media were obtained from a commercial source and incubated for only one week in a carbon dioxide-enriched atmosphere.

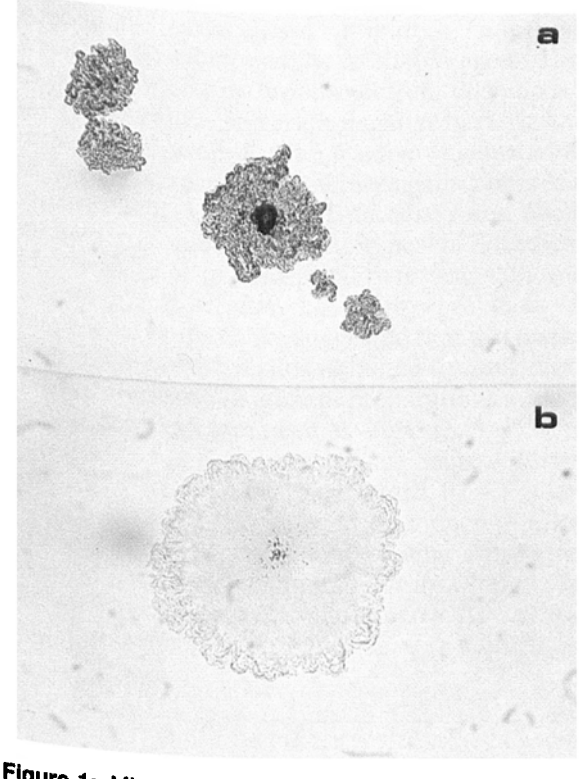

Figure 1: Microcolonies of *Mycobacterium tuberculosis* (a) and *Mycobacterium avium* complex (b) as they appear on Middlebrook 7H11 agar (100 x).

Microscopic detection of colony growth, in spite of being somewhat more laborious than optical readings of traditional media, does not require an investment in sophisticated technology nor does it present special safety problems. We estimate that an average of five minutes more per specimen is required for the entire process of microscopic follow-up of plate cultures. Supplementary use of DNA probes enables same-day species identification of microcolonies. In our experience, microcolonies detected on 7H11 plates can be presumptively identified by their appearance much more easily than those grown on traditional media; therefore, it is easier to detect different species of mycobacteria in the same culture (mixed cultures) (Figure 1). Making a presumptive identification by microcolony appearance can be helpful both in guiding early therapy and in determining which DNA probe to use. One limitation of using the microscopic detection method on plates is that the culture medium dries out more quickly. Furthermore, the smaller the number of organisms in the inoculum, the more time required for a visible colony to develop. Positive specimens requiring more than four weeks for growth cannot be easily identified by this procedure; therefore, the specimen should also always be seeded in the traditional tube for prolonged surveillance. The use of humid chambers and plates with a thicker layer of 7H11 agar prolongs the incubation time on plates somewhat. In our study, most of the strains that grew in tubes with egg-based media and not on 7H11 plates were among the minority that needed more than four weeks for growth. We agree with Welch et al. (15) that the use of this method precludes having to check the Lowenstein-Jensen tubes before three weeks.

Training is needed to follow-up the 7H11 plates, but the technique is quickly learned because a minimum of experience with microscopic visualization of colonies of the genus *Mycobacterium* suffices to achieve a high degree of skill. The main advantage of this technique is that it does not require special microscopes or uncommon technology, hence, it can be used in any laboratory. Our only recommendation is that a biological safety cabinet must be used. We recommend the method of detecting microcolonies on 7H11 agar, which notably reduces the time to diagnosis of tuberculosis and mycobacteriosis, for laboratories in which the liquid media with radiometric detection methods are not used.

References

1. Raviglione MC, Sudre P, Rieder HL, Spinaci S, Kochi A: Secular trends of tuberculosis in Western Europe. Bulletin of the World Health Organization 1993, 71: 297–306.
2. Narain JP, Raviglione MC, Kochi A: HIV-associated tuberculosis in developing countries: epidemiology and strategies for prevention. Tubercle and Lung Disease 1992, 73: 311–321.
3. Perronne C: Multiple-drug-resistant tuberculosis: current aspects in industrialized countries, and future strategies. Research in Microbiology 1993, 144: 129–133.
4. Horsburgh CR, Pozniak A: Epidemiology of tuberculosis in the era of HIV. AIDS 1993, 7, Supplement: 109–114.
5. Fauville-Dufaux M, Vanfleteren B, De Wit L, Vincke JP, Van Vooren JP, Yates MD, Serruys E, Content J: Rapid detection of tuberculous and non-tuberculous mycobacteria by polymerase chain reaction amplification of a 162 bp DNA fragment from antigen 85. European Journal of Clinical Microbiology & Infectious Diseases 1992, 11: 797–803.
6. Shawar RM, El-Zaatari FA, Nataraj A, Clarridge JE: Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase chain reaction and nonisotopic hybridization methods. Journal of Clinical Microbiology 1993, 31: 61–65.
7. Runyon EH: Identification of mycobacterial pathogens utilizing colony characteristics. American Journal of Clinical Pathology 1970, 54: 578–586.
8. Vestal AL, Kubica GP: Differential colonial characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar-base medium. American Review of Respiratory Disease 1965, 94: 247–252.
9. Welch DF, Guruswamy AP, Shaw CH, Gilchrist MJR: Implications of multi-drug-resistant tuberculosis for clinical laboratories. ASM News 1992, 58: 136–137.
10. Tacquet A, Tison F: Nouvelle technique d'isolement des mycobactéries par le lauryl-sulfate de sodium. Annales de l'Institut Pasteur 1961, 100: 676–680.
11. Abe C, Hosojima S, Fukasawa Y, Kazumi Y, Takahashi M, Hirano K, Mori T: Comparison of MB-Check, BACTEC, and egg-based media for recovery of mycobacteria. Journal of Clinical Microbiology 1992, 30: 878–881.
12. Kirihara JM, Hillier SL, Coyle MB: Improved detection times for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* with the BACTEC radiometric system. Journal of Clinical Microbiology 1985, 22: 841–845.
13. Shah DH, Devdhar MN, Ganatra RD, Kale PN, Virdi SS, Deshmukh MD: Modified rapid radiometric method for detection of *Mycobacterium tuberculosis* from sputum samples. International Journal of Nuclear Medicine and Biology 1985, 12: 333–335.
14. Stager CE, Libonati JP, Siddiqi SH, Davis JR, Hooper NM, Baker JF, Carter ME: Role of solid media when used in conjunction with the BACTEC system for mycobacterial isolation and identification. Antimicrobial Agents and Chemotherapy 1991, 29: 154–157.
15. Welch DF, Guruswamy AP, Sides SJ, Shaw CH, Gilchrist MJ: Timely culture for mycobacteria which utilizes a microcolony method. Journal of Clinical Microbiology 1993, 31: 2178–2184.