Eur. J. Clin. Microbiol. Infect. Dis., June 1993, p. 425–429
0934-9723/93/06 0429-05 $ 3.00/0

Multicentre Evaluation of a Biphasic Culture System for Recovery of Mycobacteria from Clinical Specimens

E. Tortoli[1]*, F. Mandler[2], M. Bartolucci[3], G. Bertoli[4], D. Costa[5], A. Goglio[6], B. Innocenti[7], C. Lacchini[8], F. Marchesani[9], C. Passerini Tosi[6], V. Penati[8], M.T. Simonetti[1]

A new biphasic system (MB Check, Roche) for isolation of mycobacteria from clinical specimens was evaluated by eight different microbiological laboratories in comparison with methods routinely used in the respective laboratories. Altogether 1125 clinical specimens were processed; pretreatment, if performed, was by a variety of methods. Mycobacteria were recovered from 167 specimens with the biphasic system and 165 specimens with the other methods. The average time required for isolation of *Mycobacterium tuberculosis* was 22.6 days with the biphasic system and 24.7 days with egg-based media; for other mycobacterial species it was 23.5 versus 20.8 days. The inclusion of a chocolate agar section in the biphasic system facilitated the early detection of contaminants, while the NAP-containing section appeared unable to differentiate *Mycobacterium tuberculosis* from other mycobacterial species. The biphasic system, which enables recovery of mycobacteria in small laboratories without specialized equipment, is more practical than conventional methods and at least as sensitive.

The resurgence of mycobacterial infections has strengthened the need for improved methods for their detection which are suitable for use in clinical laboratories. The clinically most important species of mycobacteria grow very slowly, and thus quite a long time is required until the final laboratory results become available. The only really rapid method still relies on examination of acid-fast smears, which however lacks sensitivity and does not provide any information about the species of the acid-fast rods detected. The radiometric method (Bactec, Becton-Dickinson, USA) has earned wide recognition because of its sensitivity and the considerably shorter time required for detection of mycobacterial growth, but still requires subculture on solid media before identification and susceptibility testing can be carried out.

Conventional methods for isolation of mycobacteria on the other hand are still poorly standardized, and require specially equipped laboratories and skilled personnel. A self-contained biphasic system (MB Check, Roche, Switzerland) has therefore been developed for recovery of mycobacteria based on the assumptions that liquid medium might optimize initial growth of the inoculum (as in the Bactec system), and solid media might afford easy isolation, and furnish additional information on contaminating organisms and on the species of the mycobacterial isolates. In an independent collaborative study the biphasic system compared well with established methods under strictly controlled laboratory conditions in tests on both clinical material and on reconstituted specimens (1).

Depending on the nature of the specimen to be processed, prior decontamination and liquefaction treatments may be required; recommended methods allow for wide variations in the actual handling of clinical material (2). The method suggested by the manufacturer of the biphasic system for pretreatment of contaminated, viscous samples employs sodium lauryl-sulfate and N-acetyl-L-cysteine.

[1] Bacteriological and Virological Laboratory, Careggi Hospital, Viale Pieraccini 24, 50139 Florence, Italy.
[2] Fondazione Monzino, Milan, Italy.
[3] Clinical Laboratory, S. Salvatore Hospital, Pesaro, Italy.
[4] Microbiological Laboratory, University Hospital, Modena, Italy.
[5] Hygiene Institute, University Hospital, Bari, Italy.
[6] Microbiological Laboratory, United Hospitals, Bergamo, Italy.
[7] Bacteriological Laboratory, S. Chiara Hospital, Pisa, Italy.
[8] Institute for Chest Diseases, Tuberculosis Laboratory, Milan, Italy.
[9] Locatelli Hospital, Piario, Italy.

As clinical laboratories may receive a variety of specimens and use different methods for pretreatment of specimens and isolation of organisms, we performed a prospective multicentre study comparing the performance of the biphasic system in the recovery of mycobacteria with that of methods currently used in a number of laboratories selected for their expertise in mycobacterial microbiology.

Materials and Methods

To qualify for inclusion in the study, the eight participating centres had to fulfill the following requirements: a) inoculation of the biphasic system and other media with equal amounts of the same material after decontamination and/or homogenization if required; b) use of the same incubation period and reading of results three times a week for the methods used for comparison; c) observation of the manufacturer's instructions for use of the biphasic system; d) completion of a questionnaire for each positive specimen; e) recovery of at least ten positive specimens. Laboratories were requested to use their own established methods for the pretreatment of samples, and their preferred inoculum volumes and media for isolation. They were also free to decide which specimens were to be included in the comparison, with the provision that any kind of specimen from any site was acceptable. Some laboratories decided to select random specimens, whereas others used only microscopically positive specimens.

As anticipated, normally sterile materials (blood, cerebrospinal fluid, cavity fluids etc) for a total of 277 specimens were not pre-treated in any laboratory. Decontamination of other specimens was done with 2 % NaOH in three laboratories (48 specimens); with 3 % NaOH in one laboratory (374 specimens); with 4 % NaOH in two laboratories (82 specimens); with Nekal BX (Biotest AG, Germany) in one laboratory (112 specimens); and with a quaternary ammonium compound (Bactofen, Ciba Geigy, Switzerland) in one laboratory (287 specimens). Specimen treatment differed among laboratories, but in all cases the centrifuged sediments were resuspended in distilled water before inoculation. Sediments from the Bactofen treatment were washed twice in order to remove traces of the cationic detergent which is known to interfere with isolation on non-egg-based media. Several laboratories liquefied and homogenized thick sputa: three laboratories used 0.5 % N-acetyl-L-cysteine (42 specimens) and two used 0.1 % dithiothreitol (51 specimens).

The routine media used for comparison with the biphasic system were Lowenstein-Jensen in four laboratories, International Union against Tuberculosis Medium (IUTM) in one laboratory, both Lowenstein-Jensen and IUTM in two laboratories, and Bactec 12B (Becton-Dickinson) together with IUTM in one laboratory.

Inoculum volumes ranged in the various laboratories from 0.5 ml to 2 ml.

The biphasic system consists of a bottle containing Middlebrook 7H9 broth and air enriched with CO_2. Before inoculation, to each bottle must be added 1 ml of a supplement containing glucose, glycerol, oleic acid, pyridoxal hydrochloride, catalase, albumin, polyoxyethylene-40 stearate, azlocillin, nalidixic acid, trimethoprim, polymyxin B and amphotericin B. Immediately after inoculation, which may be done with a Pasteur pipette, the cap of the bottle is replaced by a transparent plastic tube containing a slide covered with three media: modified Middlebrook 7H11, the same medium containing approximately 8 µg/ml nitroacetylaminohydroxypropiophenone (NAP), and chocolate agar. The system is incubated upright at 37 °C and has to be observed for growth of colonies on the solid media at regular intervals. The bottle must be inverted daily during the first seven days and then weekly to obtain inoculation of the slide, on which isolated colonies can eventually grow. The NAP-containing medium allows rapid differentiation of *Mycobacterium tuberculosis*, the growth of which is inhibited by this substance. The chocolate agar allows early detection of contaminants.

Results

A total of 1125 clinical specimens were processed, including 477 sputum samples, 122 blood samples, 117 pleural fluid samples, 107 bronchial aspirate samples, 106 urine samples and 196 other kinds of specimens, roughly representative of the range of specimens routinely received for recovery of mycobacteria in clinical laboratories. Mycobacteria were isolated from 181 specimens.

Recovery rates could not be compared between laboratories on account of the study design, which allowed laboratories to use their own criteria for specimen selection and their own methods for processing. Therefore, results were averaged among the laboratories, according to the technique of decontamination (Table 1) and liquefaction (Table 2). Specimens yielding mycobacteria on any medium were counted, and the recovery rates were also computed as the percentage of samples positive in the biphasic system or on one of the conventional media. The recovery rates were very similar in the biphasic system and on conventional media. Altogether, 14 isolates were recovered only in the biphasic system, while 12 were recovered only on the conventional media. The small differences between the pretreatment groups were not significant.

Analysis of positive results according to the species of mycobacteria isolated as finally identified by established criteria, showed that the recovery rates were identical (145/158 = 91.8 %) for the biphasic system and conventional media for *Mycobacterium tuberculosis*, which was by far the most frequently isolated species. One of 13

Table 1: Comparison of mycobacterial recovery rates related to the decontamination treatment used.

Decontamination treatment	No. of specimens	No. of positive specimens			P value*
		Any method	Biphasic system	Other methods	
None	277	19	16 (84.2 %)	15 (78.9 %)	0.500
NaOH 2%	48	43	42 (97.7 %)	42 (97.7 %)	–
NaOH 3%	374	14	14 (100.0 %)	14 (100.0 %)	–
NaOH 4%	82	37	35 (94.6 %)	31 (83.8 %)	0.130
Nekal BX	232	44	40 (90.9 %)	42 (95.4 %)	0.338
Bactofen	112	24	20 (83.3 %)	21 (87.5 %)	0.500

*Two-tailed p values using the chi-square Fisher exact test.

Table 2: Comparison of mycobacterial recovery rates related to the liquefaction treatment used.

Liquefaction treatment	No. of specimens	No. of positive specimens			P value*
		Any method	Biphasic system	Other methods	
None	694	88	84 (95.5 %)	76 (84.4 %)	0.359
N-acetyl-L-cysteine	45	42	39 (92.8 %)	41 (97.6 %)	0.305
Dithiothreitol	386	51	44 (86.3 %)	48 (94.1 %)	0.183

*Two-tailed p values using the chi-square Fisher exact test.

Table 3: Comparison of times for isolation of mycobacteria related to the organism isolated.

Organism	No. of isolates	Average number of days required for isolation		P value*
		Biphasic system	Egg-based media	
M. tuberculosis	158	22.59	24.70	>0.05
MOTT	23	23.52	20.85	>0.05

*Two-tailed probability, Student's t test for paired samples.

isolates of *Mycobacterium avium* complex, the one isolate of *Mycobacterium flavescens* and the one isolate of *Mycobacterium xenopi* were recovered only in the biphasic system, whereas 1 of 4 isolates of *Mycobacterium chelonae* was recovered only on the egg-based media; 3 isolates of *Mycobacterium kansasii* and 1 of *Mycobacterium bovis* were isolated both on conventional media and in the biphasic system. The rate of recovery of 9 isolates of *Mycobacterium tuberculosis* and 1 of *Mycobacterium chelonae* was identical in the Bactec radiometric system and biphasic system.

The average time required for recovery of *Mycobacterium tuberculosis* was 22.6 days in the biphasic system and 24.7 days on conventional media; the difference was not significant (Table 3). With mycobacteria other than *Mycobacterium tuberculosis* (MOTT) the average time was 23.5 days versus 20.9 days; again the difference was not significant. These times do not refer to the time needed to detect mycobacterial growth in liquid media, which was substantially shorter in the few cases where the Bactec radiometric system was used along with a conventional medium.

The presence of contaminants was more frequently detected in the biphasic system than on the conventional media (36 versus 22 specimens, $p < 0.001$). To our surprise, in several cases colonies of *Mycobacterium tuberculosis* or

MOTT grew on the chocolate agar section of the biphasic system culture slide: perhaps the repeated floodings with rich broth provided the required nutrients.

The NAP-containing section of the culture slide often allowed growth of colonies which later were identified as *Mycobacterium tuberculosis*; this happened with 62 of the 145 isolates of that species.

Discussion

In our study the overall sensitivity of the biphasic system and the time necessary to obtain visible colonies were at least as good as those of conventional procedures.

Previous studies performed with reconstituted specimens also gave favourable results, however those results are not directly comparable with ours due to the nature of the specimens (1, 3). In the study of Giger and Burkardt (3) about a thousand routine clinical specimens were processed in a single laboratory according to a fixed protocol, and inoculated into the biphasic system and onto two different egg-based media; 17 isolates of *Mycobacterium tuberculosis* were obtained with the former, 14 with the latter. Assuming maximum possible concordance, the comparative recovery rates would be 17/17 (100 %) for the biphasic system versus 14/17(82.4 %) for the egg-based media considered together. Results for MOTT were puzzling, with 12 isolations in the biphasic system and none on either of the egg-based media. The low number of isolates obtained by Giger and Burkardt (3) attest to the low prevalence of viable mycobacteria in their clinical material (29/995 = 2.9 %), as the detection sensitivity of the entire procedure (particularly after pretreatment with sodium dodecyl-sulphate) is supported by their results in artificially inoculated sputum specimens.

In the large collaborative study of Isenberg et al. (1) the overall rate of positive specimens was also much lower than in our study (312/3773 = 8.27 % versus 181/1125 = 16.08%) but the rates varied considerably between centres (from 3.56 % to 87.41 %), with corresponding wide variations in the recovery rates on different media (from 55 to 84 % on Lowenstein-Jensen, and from 85 to 98 % in the biphasic system. Moreover, their way of reporting the study results makes it impossible to analyze pairwise the concordance of isolations on different media, and the species distribution of their isolates (about 50 % MOTT) was quite different from ours, where *Mycobacterium tuberculosis* accounts for 87 % of the positive specimens.

With respect to the time needed to detect growth, we felt that only the appearance of colonies on the solid media was sufficiently objective, as is already apparent from discrepancies between published reports (1, 3). We consider that the biphasic system compares well with conventional media in this respect.

The greater ability of the biphasic system to detect contaminants early might be considered an advantage, as repeat cultures can be set up sooner, possibly using alternative decontamination procedures. However, further studies should be conducted to ensure that this result is not due to lack of selectivity of the liquid phase.

The differentiating ability of the NAP-containing section of the slide appears to be insufficient to be useful in a clinical laboratory, as already noted in previous studies (1, 3). This is at variance with the satisfactory differentiation afforded by NAP in the Bactec radiometric system, which however requires timely subculture and is rather cumbersome (2). Changes in the NAP medium might improve results.

In conclusion, our results are in general agreement with those of previous studies. The design of our multicentre study allows us to conclude that the biphasic system for the isolation of mycobacteria could be adopted for use in a variety of clinical laboratories, where it could be expected to perform reliably, with no sacrifice in sensitivity and speed in comparison to conventional methods. The latter methods are generally slow and not very sensitive; moreover, they require specialized equipment and personnel. Like other isolation methods, the biphasic system does not have 100 % sensitivity, nor can it compete with radiometric methods for speed of growth detection. However, the biphasic system is compact, self-contained, safe and simple to use; it does not require gas supplies or radioactive tracers and it minimizes laboratory risks. All these advantages make it suitable for use in small clinical laboratories.

Acknowledgement

We thank Pasquale Urbano, Institute of Microbiology, University of Florence, for his critical and careful review of this manuscript.

References

1. **Isenberg HD, D'Amato RF, Heifets L, Murray PR, Scardamaglia M, Jacobs MC, Alperstein P, Niles A:** Collaborative feasibility study of a biphasic system (Roche Septi-Check AFB) for rapid detection and isolation of mycobacteria. Journal of Clinical Microbiology 1991, 29: 1719–1722.
2. **Roberts GD, Koneman EW, Yoo KK:** *Mycobacterium.* In: Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shadomy HJ (ed): Manual of clinical microbiology. American Society for Microbiology, Washington, DC, 1991, p. 304–339.
3. **Giger T, Burkardt HJ:** Evaluation of a new biphasic culture system for the recovery of mycobacteria. European Journal of Clinical Microbiology & Infectious Diseases 1990, 9: 428–431.