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DOI: 10.1128/JCM.02320-08 · Source: PubMed

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Improvement in Mycobacterial Yield and Reduced Time to Detection in Pediatric Samples by Use of a Nutrient Broth Growth Supplement[∇]

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Received 3 December 2008/Returned for modification 5 February 2009/Accepted 4 March 2009

There is an urgent need to improve the methods used for the bacteriological diagnosis of childhood mycobacterial disease. This study compared the mycobacterial yields and the times to detection (in days) of mycobacteria in pediatric clinical specimens by using Mycobacterial Growth Indicator Tubes (MGITs) and solid Löwenstein-Jensen (LJ) slants with and without a nutrient broth supplement. A total of 801 specimens from 493 patients were processed: 82.8% were gastric aspirate specimens, 15.6% were sputum specimens, and 1.6% were fine-needle-aspiration biopsy specimens. The mycobacterial yield obtained with MGITs (with and without nutrient broth) was 11.0%, and that obtained with LJ slants was 1.6% ($P < 0.001$). Of the 88 positive cultures, 62 were detected in MGITs and 73 were detected in MGITs supplemented with nutrient broth ($P = 0.11$). The mean time to detection in MGITs (without nutrient broth) was 18.5 days, whereas it was 12.4 days in MGITs with nutrient broth ($P < 0.001$). Supplementation of standard MGITs improved the mycobacterial yield and significantly reduced the time to detection of mycobacteria in pediatric samples.

The accurate diagnosis of childhood tuberculosis remains a major challenge (6, 24), since the paucibacillary nature of the disease in children and the difficulty of specimen collection hamper bacteriological confirmation of the diagnosis. Clinical diagnosis is complicated by the nonspecific symptoms and signs associated with pediatric tuberculosis, particularly in children with human immunodeficiency virus (HIV) infection (3, 9, 14). The traditional growth media used to cultivate mycobacteria include solid egg-based Löwenstein-Jensen (LJ) slants and Middlebrook (7H9) liquid broth-based media, such as that used in the Bactec Mycobacterial Growth Indicator Tube (MGIT) (21, 23).

Growth supplements are widely used to improve the culture yield of fastidious organisms (10, 11). For the improved recovery of *Mycobacterium bovis* BCG, 7H9 broth (glycerol, bovine albumin fraction, glucose, sodium chloride) has been supplemented with Tween 80 (10% polyoxyethylene sorbitan monooleate solution), but the benefits appeared to be limited (7). The Bactec MGIT uses 7H9 broth supplemented with oleic acid, albumin, dextrose, and catalase (OADC) to enhance the growth of mycobacteria (21). The supplementation of 7H9 broth with a nutrient broth (modified Dubos liquid medium) containing beef extract and peptone has been recommended for the enhanced recovery of mycobacteria from inocula containing few organisms (5, 18), but data on its performance in a routine diagnostic laboratory and especially with pediatric specimens are limited. The development of tests that allow the

rapid and reliable confirmation of mycobacterial infection in children has been identified as a high research priority (15, 17).

This study evaluated whether the addition of a meat extract-based growth supplement (Bacto TB nutrient broth; Difco Laboratories Inc., Detroit, MI) to standard culture medium improves the mycobacterial yield and the time to detection of mycobacteria achieved during routine processing of specimens from children with suspected mycobacterial disease. The composition of the Bacto TB broth is available on request.

(This study was performed in partial fulfillment of the master's degree of W. Brittle.)

MATERIALS AND METHODS

Study setting and population. This prospective, hospital-based study was conducted in a biosafety level 2 mycobacteriology laboratory (Department of Medical Microbiology) of the National Health Laboratory Service at Tygerberg Children's Hospital, Tygerberg, South Africa. Tygerberg Children's Hospital serves as one of two major referral hospitals for the Western Cape Province, where the incidence of tuberculosis was 994.2 per 100,000 population in 2007. The incidence of tuberculosis among children aged 0 to 14 years was 620 per 100,000 population, accounting for 17.3% of the total tuberculosis caseload in the province (Western Cape Department of Health, unpublished data). From 2003 to 2005, 323 children (median age, 2.5 years) were diagnosed with culture-confirmed tuberculosis at Tygerberg Children's Hospital (19).

Specimen collection and processing. All mycobacterial specimens routinely collected from children ≤ 15 years of age from 1 June 2007 through 28 February 2008 were included. Fine-needle-aspiration biopsy (FNAB) specimens were collected directly into an MGIT, whereas respiratory specimens (gastric aspirates whose pH was adjusted with equal volumes of 10% sodium carbonate and sputa) were digested and decontaminated with *N*-acetyl-L-cysteine and 2% NaOH before centrifugation at 3,000 rpm for 20 min. The supernatant was discarded, and 1 drop of the sediment was used for smear preparation by the use of standard protocols (11, 21). The remaining sediment was reconstituted with sterile phosphate-buffered saline (pH 6.8) to a final volume of 1.5 ml. This volume was then split into four aliquots; and the four aliquots were inoculated as follows: (i) 0.5 ml was inoculated into a standard MGIT (Becton Dickinson, Cockeysville, MD), (ii) 0.5 ml was inoculated into an MGIT supplemented with nutrient broth (0.5 ml Bacto TB nutrient broth supplement was added prior to sample inoculation),

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[∇] Published ahead of print on 11 March 2009.

(iii) 0.25 ml was inoculated onto a solid LJ slant (National Health Laboratory Service, South Africa), and (iv) 0.25 ml was inoculated onto an LJ slant supplemented with nutrient broth (0.5 ml Bacto TB nutrient broth supplement was added prior to sample inoculation).

The Bacto TB nutrient broth supplement (Difco Laboratories) contains a unique beef extract not found in standard growth media. The growth supplement was prepared by dissolving 1.6 g Bacto TB nutrient broth in 200 ml distilled water and adding 0.2 ml Tween 80, before the mixture was dispensed in 5-ml aliquots into screw-cap tubes, which were autoclaved at 121°C for 15 min and stored at 4 to 6°C (5). Standard antimicrobials (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) were reconstituted in OADC, according to the manufacturer's instructions (Becton Dickinson), and the mixture was added to each MGIT prior to inoculation to avoid bacterial or fungal contamination (21). The MGITs were incubated at 37°C by using the fully automated fluorimetric Bactec MGIT 960 system (Becton Dickinson) and were monitored hourly for 42 days or until a positive fluorescence signal was recorded. FNAB specimens were aseptically collected from enlarged cervical lymph nodes by a pathologist and were inoculated into an MGIT at the bedside. On arrival in the laboratory, a 1-ml aliquot was aspirated, 0.5 ml was inoculated into an MGIT supplemented with nutrient broth, and the remaining 0.5 ml was divided between two LJ slants (with and without nutrient broth).

Positive MGIT cultures were examined microscopically to confirm the presence of mycobacteria by using smears stained with Ziehl-Neelsen stain and subcultured on a tryptose blood agar plate to exclude the possibility of bacterial contamination (21). The species from these positive MGIT cultures were then determined by standard PCR (4). Negative controls were included with each batch of clinical specimens processed to monitor for laboratory cross-contamination, as recommended elsewhere (1, 21). Solid LJ slants were incubated at 37°C and were examined weekly for 6 weeks or until colonies became visible. The mycobacterial yields, the times to detection of mycobacteria, and the contamination rates were recorded; and the values obtained by the four culturing methods were compared.

Anonymous coded data were entered into an Excel spreadsheet. Descriptive and comparative data analysis was performed with Statistica software (version 8; Statsoft). Mycobacterial yields were compared by using McNemar's chi-square test, and the paired *t* test was used to compare the times to detection. A two-sided alpha level of 0.05 was regarded as statistically significant. Ethics approval for the study was provided by the Institutional Review Board of Stellenbosch University (study no. 2003/005).

RESULTS

A total of 801 specimens from 493 children were evaluated; mycobacteria were isolated from 88/801 clinical specimens (70/493 children with culture-confirmed mycobacterial disease). Of the children with culture-confirmed disease, 50/70 were less than 5 years of age, with a median age of 18 months (age range, <1 month to 180 months). The gender distribution was nearly equal, with 36 males and 34 females. Of the 54/70 children tested for HIV infection, 14 were positive.

The majority of specimens collected ($n = 788$) were from a respiratory source: 125 were sputum specimens and 663 were gastric aspirate specimens. The 13 specimens collected from a nonrespiratory source were all FNAB specimens. Mycobacteria were isolated from 66/663 gastric aspirate specimens, 17/125 sputum specimens, and 5/13 FNAB specimens. The use of liquid medium allowed the detection of mycobacteria in all 88 cultures found to be positive: 73/88 in nutrient broth-supplemented MGITs and 62/88 in MGITs without nutrient broth ($P = 0.11$). The use of solid LJ medium allowed the detection of mycobacteria in only 15/88 of cultures found to be positive. The rate of bacterial contamination with MGITs (25/801; 3.1%) was significantly ($P < 0.001$) lower than that with LJ slants (118/801; 14.7%).

The mean time to detection was significantly shorter in nutrient broth-supplemented MGITs than in MGITs without nutrient broth (means, 12.4 days and 18.5 days, respectively; $P <$

TABLE 1. Mycobacterial species recovered from different specimen types

Specimen type	No. (%) of isolates recovered			Total
	<i>M. tuberculosis</i>	<i>M. bovis</i> BCG	NTM ^a	
Gastric aspirate	60	2	4	66 (75.0)
Sputum	16	0	1	17 (19.3)
FNAB	2	3	0	5 (5.7)
Total	78 (88.65)	5 (5.7)	5 (5.7)	88

^a NTM, nontuberculous mycobacteria.

0.001). The mean times to detection were similar on LJ slants with and without nutrient broth: 26.6 and 26.8 days respectively ($P = 0.99$). Of the 88 isolates detected, 78 were *Mycobacterium tuberculosis*, 5 were *M. bovis* BCG, and 5 were nontuberculous mycobacteria. Table 1 summarizes the mycobacterial species recovered from different sources.

DISCUSSION

This study demonstrates that the addition of an inexpensive growth supplement (Bacto TB nutrient broth) to a standard MGIT improves the mycobacterial yield and significantly reduces the time to detection of mycobacteria in routine pediatric clinical specimens. With the addition of the Bacto TB nutrient broth to MGITs, 12.5% more mycobacteria were recovered: 73 MGITs with the supplement and 62 MGITs without the supplement. To our knowledge, these are the first published data comparing mycobacterial recovery rates and times to detection for pediatric specimens. Apart from children for whom a more rapid and accurate diagnosis will improve clinical care, other patients with paucibacillary disease, such as HIV-infected patients with sputum smear-negative tuberculosis, may also benefit from culture methods with improved mycobacterial yields and reduced turnaround times.

As the present study was not sufficiently powered to demonstrate statistically significant differences in the mycobacterial yields, studies with larger cohorts of patients suspected of having paucibacillary tuberculosis are required to confirm our observations. It would also be of great interest to evaluate the performance of Bacto TB nutrient broth-supplemented liquid growth medium in combination with early detection techniques, such as the microscopic observation drug susceptibility assay (16), especially for the detection of paucibacillary disease.

The mean time to detection of 12.4 days obtained with MGITs supplemented with nutrient broth in the present study is comparable to the times to detection obtained with standard MGITs reported for adults (11.6 days by Lee et al. [12], 14.3 days by Tortoli et al. [23], 14.4 days by Hanna et al. [8], and 10.7 days by Chien et al. [2]). These studies enrolled adult tuberculosis patients with high bacillary loads; children, however, usually have paucibacillary disease. This study compared the mycobacterial yields and times to detection under identical and rigorous conditions (with identical specimen types, inoculation volumes, growth conditions, and detection systems) with supplemented and unsupplemented media. However, the mycobacterial yields may have been reduced by the limited inoculation volume, since we utilized only the sediment that re-

mained after smears were prepared for routine microscopy. In addition, a smaller inoculation volume (0.25 ml versus 0.5 ml) was applied to the LJ slants, according to standard methods (8, 22, 23), which probably biased the results in favor of the liquid media used in the MGITs. However, the difference in mycobacterial yield seems far in excess of what can be attributed to differences in inoculation volume. The superior mycobacterial yield achieved with MGITs compared to that achieved with LJ slants has been well established in previous comparative studies (12, 13, 20).

The addition of a meat extract growth supplement has been recommended for the cultivation of *M. tuberculosis* specifically from inocula with low bacillary loads (5, 18), but this has not been tested with pediatric specimens; the reason for the enhanced growth remains poorly elucidated. Tween 80 (10% polyoxyethylene sorbitan mono-oleate solution) seems to facilitate growth by allowing compounds essential for growth to enter the mycobacterial cell more efficiently (5, 18). The standard OADC supplement added to MGITs contains 10% oleic acid, albumin, dextrose, and catalase. Oleic acid plays an important role in the metabolism of mycobacteria, and albumin acts as a protective agent that binds to free fatty acids often found to be toxic to mycobacteria, while dextrose is included as an energy source and catalase destroys any toxic peroxides that may be present in the medium (5, 18, 21). However, the cost and laboratory infrastructure required for the use of liquid automated culture systems remain major limitations in resource-poor settings. The use by laboratories of alternative, less costly methods and equipment that have the added benefit of improved sensitivity, such as the Bactec manual MGIT reader, should be explored (21).

In conclusion, we showed that more pediatric cases of mycobacterial infection could be detected and that the time to detection could be significantly reduced with the addition of additional growth supplements to standard MGITs. Improved and more rapid mycobacterial detection may improve children's access to the appropriate clinical care. It should also reduce the turnaround times for drug susceptibility testing. Future studies should explore the use of additionally supplemented growth media with rapid detection systems such as the microscopic observation drug susceptibility assay.

ACKNOWLEDGMENTS

Financial support was received from the National Research Foundation and the National Health Laboratory Service (NHLS).

Marlein Bosman (NHLS) provided valuable technical advice.

No industry funding was received to perform this study, and none of the authors have any commercial interests to declare.

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