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Rapid Direct Testing of Susceptibility of *Mycobacterium tuberculosis* to Isoniazid and Rifampin on Nutrient and Blood Agar in Resource-Starved Settings

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In this study, we evaluated the performance of blood agar (by macroscopic growth) and nutrient agar (by a microcolony detection method) for drug susceptibility testing of *Mycobacterium tuberculosis* against rifampin (RIF) and isoniazid (INH), using 67 smear-positive sputum specimens. The direct proportion method on Lowenstein-Jensen (LJ) medium was used as the "gold standard." Compared with LJ medium, results for both media were in 100% agreement for RIF, while for INH the agreement levels for blood agar and nutrient agar were 98% and 95%, respectively. Within 2 weeks, 100% of specimens yielded results on blood agar, while 96.8% of specimens yielded results on nutrient agar. Our study showed that blood agar and nutrient agar can be used as alternative media for direct susceptibility testing of RIF and INH, especially in resource-poor settings.

Despite recent advancements in the field of mycobacteriology, multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) still remain public health nuisances (9, 19). Conventional culture methods are slow, requiring about 6 to 8 weeks for diagnosis and subsequent drug susceptibility testing (DST). In the last decade, the Bactec MGIT 960 automated system was introduced for early diagnosis and DST of *Mycobacterium tuberculosis* from various clinical specimens. However, this system is costly, its consumables are not easily available, and it requires technical expertise. Parallel to the progress in automated systems, much work has also been published on the development of rapid but economical methods which are equally effective and comparable to automated systems, especially for resource-limited countries (1, 11, 12, 15, 18).

Drancourt et al. were the first to report the incidental growth of *M. tuberculosis* colonies on blood agar and termed this finding the "end of dogma" (7). Their report led to further research on blood agar for cultivation of *M. tuberculosis* (2, 13). In addition, it was demonstrated that blood agar can be used instead of Middlebrook 7H10/11 agar or Lowenstein-Jensen (LJ) medium for susceptibility testing of *M. tuberculosis* (3–6). In this study, we evaluated for the first time the performance of blood agar and nutrient agar for DST of *M. tuberculosis* against rifampin (RIF) and isoniazid (INH) directly on smear-positive sputum specimens.

MATERIALS AND METHODS

Setting. This comparative study was carried out at the Department of Pathology, Combined Military Hospital, Dera Ismail Khan, Pakistan. A total of 70 fresh smear-positive sputum specimens were obtained from the Tuberculosis Control Program health setting.

Specimen processing. All of the sputum specimens were from patients diagnosed clinically and radiologically along with having a positive acid-fast bacillus (AFB) smear. Informed consent was obtained from all patients, as approved by the hospital's ethical committee. In the protocol followed, all of the clinical specimens (one per patient) were homogenized and decontaminated by a standard sodium hydroxide–*N*-acetyl-L-cysteine method using 2% NaOH (final concentration) (14). A direct smear was made from the sediment, stained by Ziehl-Neelsen (ZN) staining, and scored according to the bacillary load as described by Kent and Kubica (8). Remaining sediment was diluted in 3 ml of sterile distilled water and used subsequently for inoculation.

Preparation of nutrient agar and blood agar with drugs. Autoclaved nutrient agar (Oxoid, United Kingdom) was divided into two equal parts, and 5% sheep blood was added to one part. Both were further subdivided into four equal parts; *para*-nitrobenzoic acid (PNB) (500 μg/ml), INH (0.2 μg/ml), and RIF (1.0 μg/ml) were added to the first three parts, while the fourth part was used without antibiotic (control). About 5 ml of respective medium was poured into the quadrants of sterile labeled petri dishes.

Inoculation of sputum specimens on media. In order to ensure adequate but not excessive growth, concentrated sputum specimens were diluted as follows: 10^{-1} for 1 to 9 AFB/10 fields (2+), 10^{-2} for 1 to 9 AFB/field (3+), and 10^{-3} for >9 AFB/field (4+). Samples of 0.1 ml of concentrated specimen were inoculated on both nutrient agar and blood agar, while 0.5 ml was added to the LJ slants. All plates were sealed with CO_2-permeable tape, placed in plastic bags, and incubated at 37°C in 5 to 10% CO_2. *M. tuberculosis* H37Ra strain ATCC 25177 was used as a control.

Direct proportion method on blood agar and LJ medium. DST on LJ and blood agar media was carried out by the proportion method according to the standard laboratory protocol (8). In brief, the LJ medium was prepared per the manufacturer's recommendations, with and without antibiotics. The critical concentrations of INH and RIF were the same as those used in nutrient agar and blood agar. Each specimen was diluted 1:100, and 0.2 ml of the undiluted suspension was inoculated onto antibiotic-containing LJ slopes, while 0.2 ml of diluted specimen was added to drug-free LJ slopes. All of the agar plates were incubated at 37°C in 5 to 10% CO_2.

Reading of plates. Blood agar plates were examined after 2 days for any contamination and then on days 7, 14, and 21 for appearance of any visible colonies. Nutrient agar plates were examined under a simple mi-

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TABLE 1 Comparison of results of LJ proportion method with results of sensitivity on blood agar[a]

Drug	LJ result	No. of samples with blood agar result		% Sensitivity	% Specificity	PPV (%)	NPV (%)	Agreement (%)
		R	S					
RIF	R	11	0	100	100	100	100	100
	S	0	52					
INH	R	14	1	100	98	94	100	98
	S	0	48					

[a] R, resistant; S, susceptible; PPV, positive predictive value; NPV, negative predictive value.

croscope (10× objective) for microcolonies (cording) twice weekly for up to 4 weeks, as described by Martin et al. for 7H11 agar (10). Growth on plain blood agar medium with no growth on PNB medium confirmed the isolate as *M. tuberculosis*. In the case of LJ medium, susceptibility results were reported after 6 weeks. If growth on drug-containing medium was more than 1% of that on the drug-free medium, the isolate was considered resistant to that drug.

Statistical analysis. In our study, sensitivity reflects the ability to detect true resistance in an isolate, whereas specificity is the ability to detect a true sensitive isolate. We calculated these parameters, the positive predictive value (PPV), and the negative predictive value (NPV) by using SPSS (Statistical Package for the Social Sciences), version 19.

RESULTS AND DISCUSSION

Among a total of 70 smear-positive sputum specimens, 3 specimens on LJ medium were contaminated, with 2 contaminated specimens on blood agar and 2 contaminated specimens on nutrient agar, all yielding growth of other bacterial colonies. These 7 clinical specimens were excluded from the final results. The results for the remaining 63 clinical specimens are summarized in Tables 1 and 2. Overall agreement levels for testing of *M. tuberculosis* strains against RIF and INH were 100% and 98%, respectively, between LJ medium and blood agar, while the levels were 100% and 95%, respectively, between LJ medium and nutrient agar. After observing growth on day 14, blood agar and nutrient agar plates were further incubated until day 21. In our study, two specimens revealed growth on the control quadrant on blood agar plates on day 14, with no growth on drug-containing quadrants, but on day 21, growth was seen on drug-containing quadrants in both cases. In cases of visible growth, colonies were stained with ZN stain for confirmation of AFB. None of the specimens yielded growth of nontuberculous mycobacteria. As far as time to detection of growth is concerned, the microcolony detection method gave results as early as day 5, while growth on blood agar was seen as early as day 7. The mean times to detection of growth for the three methods are shown in Fig. 1, with results by the microcolony method being obtained earliest. Results were available in 2 weeks for 63 (100%) specimens in the case of blood agar, while 61 (96.8%) specimens yielded results in 2 weeks on nutrient agar (microcolony method). Colonies of *M. tuberculosis* on blood agar were light yellow, glistening, and smooth (Fig. 2), in contrast to the cream-colored, rough colonies on LJ medium.

Blood agar and nutrient agar are the most commonly used media in clinical laboratories. They are easily available, cost-effective, and simple to prepare. The reasons for using the microcolony detection method on nutrient agar are that this medium is clear and can be prepared simultaneously while preparing blood agar and colonies can easily be visualized through the medium by simple microscopy. The approximate cost of microcolony detection in agar is 2.5 euros per isolate, compared to 6 euros per isolate for the MGIT 960 system (10). In recent years, various studies have reported the use of blood agar for diagnosis and susceptibility testing of *M. tuberculosis*. In one recent study, Satana et al. evaluated blood agar for susceptibility testing of 35 MDR-TB clinical isolates against second-line drugs and compared the results with those of the Bactec 460 TB system and the agar proportion method using Middlebrook 7H10 medium (17). They found 100% agreement for kanamycin (KM), rifabutin (RBU), *p*-aminosalicylic acid (PAS), capreomycin (CAP), and clofazimine (CFM) compared with the Bactec 460 TB system. However, in our study, we evaluated only susceptibilities to INH and RIF on nutrient and blood agar, using smear-positive specimens, and compared the results with the direct proportion method on LJ medium.

In addition to rapid culture of *M. tuberculosis*, direct susceptibility testing of smear-positive clinical specimens on blood agar and nutrient agar has the added advantage that both culture and susceptibility results are available at the same time. This is crucial for breaking the chain of TB transmission, especially in resource-

TABLE 2 Comparison of results of LJ proportion method with results of sensitivity on nutrient agar (by microcolony detection method)[a]

Drug	LJ result	No. of samples with nutrient agar result		% Sensitivity	% Specificity	PPV (%)	NPV (%)	Agreement (%)
		R	S					
RIF	R	11	0	100	100	100	100	100
	S	0	52					
INH	R	12	3	100	95	80	100	95
	S	0	48					

[a] R, resistant; S, susceptible; PPV, positive predictive value; NPV, negative predictive value.

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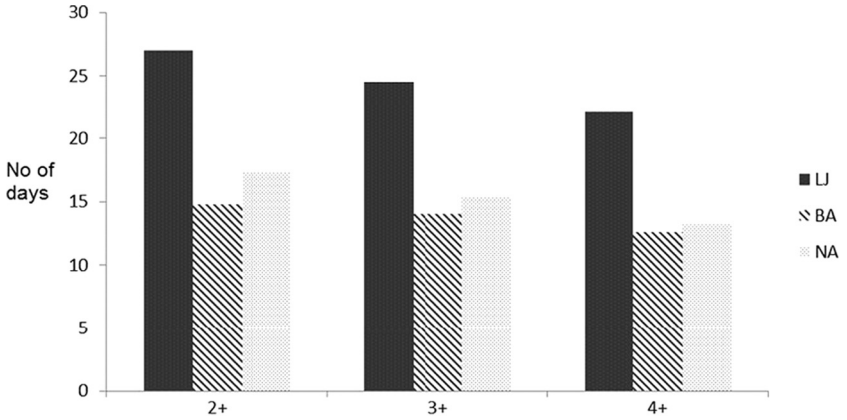

FIG 1 Average time (in days) for detection of growth by three culture methods, grouped by bacillary smear load. BA, blood agar; NA, nutrient agar.

poor countries with areas of high TB burden. In our study, susceptibility results were available within 2 weeks for both blood agar and nutrient agar in most cases (more than 96%), which is in accordance with the Centers for Disease Control and Prevention guidelines (16).

In our study, there was 100% agreement for both new culture methods for susceptibility testing of RIF, while agreement levels for INH on blood agar and nutrient agar were 98% and 95%, respectively, which is reasonably good. This is very essential for TB patients, as these two drugs are the most important anti-TB agents, and also, in most cases, RIF resistance is associated with INH resistance (20). The results of our study are comparable to those of a study done by Coban et al. with 100% and 94.1% agreement levels for RIF and INH, respectively (6). In another study, Coban et al. used blood agar for susceptibility testing of *M. tuberculosis* isolates against first-line drugs and compared the results with the radiometric proportion method, with 100% agreement for both INH and RIF (4).

In our study, we used the proportion method on LJ medium as the "gold standard" instead of the recommended Middlebrook 7H10 medium or automated systems. This is because of the fact that in our region, automated systems such as MGIT 960 systems are available only in limited reference laboratories, and thus most peripheral laboratories use conventional LJ medium for diagnosis and susceptibility testing for purposes of economy. We used only smear-positive specimens in our study, thus minimizing the chances of growth failure.

To the best of our knowledge, this is the first report on the evaluation of DST on nutrient agar (by the microcolony method) and blood agar, using smear-positive sputum specimens instead of known strains or growth. Our results show that blood agar and nutrient agar can be used for the detection and susceptibility testing of *M. tuberculosis*. Both of these media are easily available and economical, with no problems of procurement of consumables, give timely results compared to conventional LJ medium, and give results that are comparable to those of more costly automated systems. However, further studies on these two methods are required for their application to second-line drugs, especially in resource-limited laboratories.

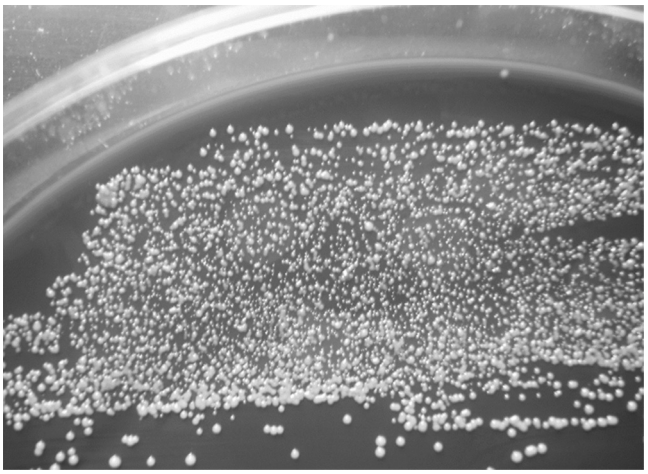

FIG 2 Colonies of *M. tuberculosis* on blood agar plate after 14 days of incubation.

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