Comparison of three alternatives cost-effective culture media for *Mycobacterium tuberculosis* detection and drug susceptibility determination using the MODS culture technique

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ABSTRACT

Tuberculosis is an endemic infectious disease in low- and mid-income countries (LMIC). Besides, drug-resistant tuberculosis is a significant public health concern. Rapid, low-cost, and reliable detection tests are crucial for an appropriate diagnosis and early treatment. Phenotypically detection assays are still used in LMIC. The MODS assay is a culture test to detect *Mycobacterium tuberculosis* (MTB) and determine the bacterial resistance profile. MODS implementation is still limited due to the low accessibility to culture supplies for the enriched media preparation. In this study, we evaluated three alternative dry culture media: an unsterilized powder-based mixed (PM), irradiated powder-based mixed (IM), and sterile-lyophilized media (LM). Mycobacterial growth and drug susceptibility to rifampin, isoniazid, and pyrazinamide were evaluated. A total of 282 sputum samples with positive acid-fast smear were assessed. MTB growth was reported since day four for all culture media, and no significant difference in mycobacterial growth was observed. A total of 47 samples were multidrug resistant. Evaluation of pyrazinamide susceptibility showed a good agreement between the alternative culture media and a reference consensus standard. In conclusion, these results showed that all alternative culture media are good candidates to facilitate MODS implementation in low resource setting countries.

Keywords: Tuberculosis, drug susceptibility test, MODS, diagnosis, culture media,

INTRODUCTION

Drug resistance in tuberculosis (TB) is a primary global health concern. In 2020, the WHO reported 9.9 million TB cases and 1.5 million deaths. Additionally, 206,000 multidrug-resistant TB (MDR-TB) new cases were reported (1). MDR-TB is caused by *Mycobacterium tuberculosis* (MTB) strains that are resistant to rifampicin (RIF) and isoniazid (INH), both drugs used in the first-line TB therapy. In the Americas, Peru represents 13.4% of all reported MDR-TB cases (2). Pyrazinamide (PZA) is another relevant antituberculosis drug used in the first-line TB therapy, along with new and repurposed drugs for drug-resistant TB treatment (3). However, PZA resistance has been reported at least in 50% of MDR-TB cases (4). Therefore, a rapid, consistent, affordable drug susceptibility test with high sensibility and specificity is necessary for early and personalized TB treatment. Mismanagement of anti-tuberculosis drugs increases MDR-TB incidence in TB endemic countries (5).

Drug susceptibility testing (DST) has been reported in phenotypic and molecular approaches. Molecular testing is based on the detection by DNA sequencing and amplification of gene regions that harbor resistance-related mutations (6, 7). There are several molecular tests such as the line-probe assays (LPA), the high-resolution melting assay (HRM), the Gene-Xpert test, and the DNA sequencing of promotor and target genes. Regardless of the high sensitivity and specificity of molecular DST and being recommended by the WHO, these tests are not widely used, especially in low- and mid-income countries (LMIC) where phenotypic DST is still used routinely (8, 9,10). Phenotypic testing includes classic assays such as the proportion method, the absolute concentration method, the resistant ratio method (7, 11), and the automatized mycobacterial growth indicator tube (MGIT). All phenotypic DST relies on isolating the patient's MTB strain, drastically increasing the test's overall turnaround time, and using a critical drug concentration that directly affects the DST performance (10, 12). Currently, the MGIT assay is recommended as the standard test for first- and second-line anti-TB drugs (13,14). Nevertheless, many LMIC does not routinely use that assay due to high costs (11).

The MODS (Microscopic Observation Drug Susceptibility) assay is an alternative low-cost, reliable, and validated phenotypic DST for susceptibility profile determination of both first- and second-line anti-TB drugs (15, 16). MODS test is based on the visualization of MTB growth in a liquid culture medium where an MTB growth pattern in the form of a cord is observed using an inverted optical microscope (15). A phenotypic adaptation of the MODS test based on the classic Wayne assay (MODS-Wayne) (17) indirectly determines PZA resistance by evaluating the pyrazinamidase enzymatic activity through direct detection of the metabolite pyrazinoic

acid. Despite the advantages of the MODS test compared to other available phenotypic DST, this assay is not routinely used. Two technical factors limit MODS assay's large-scale use, the required supplies and trained personnel for preparing the enriched culture media (18). The culture medium is supplemented with glycerol, casitone, and OADC (oleic acid, albumin, dextrose, and catalase) to favor MTB growth in culture (18, 19). The PANTA antibiotic-mixture solution (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) is used to minimize contamination by the remanent oral flora from decontaminated sputum samples (20). The OADC and PANTA are commercially available.

A commercial kit (Hardy Diagnostic, USA) containing liquid media was available a few years ago to facilitate the routine use of the MODS assay. The culture performance of the kit was compared to the conventional MODS culture medium, showing that no significant differences were observed between the kit and the conventional MODS assay (21). However, the kit required a cold chain (4 °C) to transport all the components, which increased the cost and limited the demand and use in LMIC. Alternatively, home-based reagents such as the OADC supplement can be used for the enriched culture medium to reduce the assay implementation cost (18). Nonetheless, the turnaround time of the test could increase by at least 72 hours, which represents a limitation in diagnostic laboratories with high-demand TB cases. Preliminary results of alternative cost-effective culture media for the MODS assay (22) showed that culture variants such as a powder-component mixed media (PM) or a sterile lyophilized liquid media (LM) have a similar performance to the standard MODS culture medium (i.e., commercial 7H9 medium enriched with OADC and PANTA) when evaluating MTB isolates and positive acid-fast smear sputum samples. The results showed that PM culture media enriched only with PANTA showed a time-to-positivity and sensitivity like the standard MODS medium, which is the simplest to prepare. It does not require any other sterilization process.

In this study, three powder-component mixed culture media based on the standard 7H9 medium were evaluated to determine drug susceptibility to rifampin (RIF), isoniazid (INH), and pyrazinamide (PZA) from clinical sputum samples. Alternative culture media performance was compared to the standard MODS culture medium. Drug susceptibility profile was compared for RIF and INH with standard MODS assay and for PZA with a consensus reference test that includes *pnc*A sequencing, MGIT-PZA, and classic Wayne assay.

MATERIAL AND METHODS

Clinical samples

Two hundred eighty-two remnants from clinical sputum samples were obtained from recently diagnosed and treated patients. Samples were collected from the Hospital Nacional Cayetano Heredia, Lima, Peru; Hospital Hipólito Unanue, Lima, Peru; and the Regional Tuberculosis Reference Laboratory, Callao, Lima, Peru. Ethical approval was obtained from the Universidad Peruana Cayetano Heredia (345-15-19).

Samples were decontaminated according to the standardized protocol (23). Briefly, the sample was mixed with DECO solution (NaOH, sodium citrate, and NaLc) in a ratio of 1:1 for a final volume of 4 mL and vortexed roughly. Then the sample was incubated at room temperature for 15 minutes and centrifuged at 3000 rpm for 15 min at 17°C. Finally, the pellet was resuspended with 2.5 ml of phosphate buffer saline.

Evaluated culture media

Standard MODS culture medium (MM): Middlebrook 7H9 broth was prepared according to the standard MODS protocol (24). Each 500 mL of culture broth base was enriched with 625 mg Bacto™ Casitone (BD, USA) and 1.55 mL glycerol (J.T. Baker, USA). The culture medium was sterilized and stored at 4°C. The OADC and PANTA antibiotic solutions were added before use.

Powder-component mixed media (PM): Mixed culture medium was prepared according to the standard MODS protocol (24) with the following modifications: Aliquots were prepared with 0.148 g of Middlebrook 7H9 broth base (BD, USA), 0.031 g Bacto™ Casitone (BD, USA), 0.125 g Bovine Serum Albumin (Sigma-Aldrich, USA), 0.021g NaCl (Merck, USA), and 0.05 g D-Glucose (Sigma-Aldrich, USA). The PM culture medium was not sterilized. Resuspension was done with water for a final volume of 25 mL, and glycerol and PANTA antibiotic solution were added before use.

Lyophilized liquid media (LM): Lyophilized culture medium was prepared according to the standard MODS protocol (22). After sterilization by autoclaving, the LM medium was enriched with OADC and aliquoted in 25 mL. Aliquots were frozen for 24 hours at -72°C. Lyophilization was performed at -46°C with a pressure between 50 x 10-3 to 70 x 10-3 MBAR in the lyophilizer LYPHLOCK 12 (Labconco, USA). Before use, aliquots were resuspended in 25 mL of distilled water, and PANTA antibiotic solution was added.

Irradiated powder-component mixed media (IM): Irradiated culture medium was prepared like the PM media. Then IM aliquots were irradiated with gamma radiation delivered at five kGy using Co-60 gamma radiation at 19°C and 968 hPa, with the Gamma cell 220 irradiator type 1 at the Instituto Peruano de Energía Nuclear. Before use, aliquots were resuspended with 25 mL of distilled water, then glycerol and PANTA antibiotic solution were added.

Mycobacterial culture assay

For culture assay, 500 µL of decontaminated samples were added to 7 mL of each culture medium (MM, PM, LM, and IM). The remaining volume of decontaminated sample was stored at 4°C. A culture assay was performed on a 24-well plate following the plate design shown in Figure 1. Briefly, 900 µL of the diluted decontaminated sample were transferred to each well that previously contained 100 µL of culture medium, four µg/mL isoniazid (INH), one µg/mL rifampicin (RIF), respectively. Pyrazinamide (PZA) was added after bacterial growth was reported in the control wells. Four samples were evaluated per culture plate. The plate was covered with a silicone cap and incubated at 37°C for up to twenty-one days. MTB strains such as the H37Rv and DM97 were used as drug-sensitive and drug-resistant controls at a final concentration of 2×10^5 CFU/mL.

Bacterial growth was recorded from day 5 of incubation using an inverted microscope (Nikon TS100) at 40X magnification. Bacterial growth was described according to the following scale (Figure 2): category 1, growth covering 25% of well area; category 2, growth covering 25 – 50% of well area; category 3: growth covering 50 – 75% of well area; and category 4, growth covering 100% of well area. Bacterial growth was reported on the first day of cordon-pattern growth observation and seven days after. Drug susceptibility to INH and RIF was reported when cordon-pattern growth was observed in the control wells (i.e., without antibiotics) (25). Drug susceptibility to PZA was evaluated according to the MODS-Wayne protocol (17). Briefly, after recording bacterial growth in the control wells, the plate was incubated for six days more. Then 100 µL of 8 mg/mL PZA was added, and the plate was incubated for three days more. Finally, 100 µL of 10% ferrous ammonium sulfate (FAS) was added. A PZA-sensitive result was indicated by a reddish color observed after FAS addition; color intensity was classified as low (+), mid (++), high (+++), and extreme (++++) (Figure 3). Instead, a PZA-resistant result was indicated by the absence of color.

Consensus reference test for PZA susceptibility determination (CRT)

The discrepancy between the activity of PZA *in vitro* and *in vivo* conditions has been reported (26). Therefore, there is no reference DST to determine PZA susceptibility (27). The WHO

recommends the DNA sequencing of the promotor and *pnc*A gene and the MGIT-BACTEC-960 as molecular and phenotypic DST, respectively (17,28). However, technical limitations regarding both tests have been reported. For the DNA sequencing, not all mutations reported in the *pnc*A gene are related to phenotypic PZA resistance, and the prevalence of mutations is highly variable depending on geographical distribution (29). On the other hand, high technical expertise is required to prepare MTB isolates for the MGIT-960. The bacterial load of the inoculum and the used PZA concentration are critical factors in the performance of the MGIT-960 to determine PZA susceptibility (30). A consensus reference test (CRT) has been indicated to be an excellent alternative to evaluate PZA susceptibility (27). For this study, only MDR and mono-resistant isolates were assessed by the CRT test to determine PZA susceptibility. Nonresistant isolates were not assessed due to the low PZA resistance prevalence. Three assays were considered for the CRT: the MGIT-BACTER-960, DNA sequencing of the promotor and *pnc*A gene, and the classic Wayne assay. Therefore, a PZA-sensitive result was defined for samples that showed sensitivity in at least two of the used assays for the CRT. On the contrary, a PZA resistant result was determined when a sample showed resistance in at least two of the used assays for the CRT (28).

To perform the assays for the CRT, a 100 µL aliquot from the control wells was inoculated in agar 7H10 enriched with OADC and incubated at 37°C for three weeks. MTB isolates then were pretreated as follows for each assay:

DNA sequencing: DNA extraction was performed using a modified proteinase K-chloroform protocol as described in (31). The *pncA* gene was amplified using the primer P1 (5'GTCGGTCATGTTCGCATCG-3') and P6 (5'-GCTTTGCGGCGAGCGCTCCCA-3'). Amplification was performed as follows: 10 µL master mix Phusion Taq polymerase, 1 µL of each primer, 2 µL of genomic DNA, for a final volume of 20 µL. The PCR cycling parameters were initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 1 second, annealing at 60°C for 5 seconds, elongation at 72°C for 12 seconds, and final elongation at 72°C for 60 seconds. Amplification products were visualized in 1% agarose gel (70 V for 45 min). The 720-bp amplification products were sequenced using the same primers used for PCR (Psomagen, USA). To determine the presence of mutations in the promotor and *pncA* gene, a pairwise sequence alignment was performed with the nucleotide sequence of the MTB H37Rv reference strain (NCBI RefSeq accession no. NC_000962.3).

BACTEC MGIT 960: Bacterial suspension was prepared from one full loop of 14-days MTB culture suspended in physiological saline solution. The suspension was vortexed and left to

rest for 20 minutes, then 3 mL of suspension were adjusted to a turbidity equivalent to 0.5 McFarland. Two dilutions were prepared in a ratio of 1:5 and 1:10 from the MTB suspension, then 0.5 mL was transferred to the test tube and control tube, respectively. Pyrazinamide was added to the test tube at a final 100 µg/mL concentration. The tubes were placed in the BACTEC equipment, where they were incubated between 8 to 15 days to compare the fluorescent signal of the control and test tubes.

Classic Wayne assay: Agar Dubos was prepared according to (32) with 0.1 g of PZA (i.e., the final concentration at 100 µg/mL) and 2 g of sodium pyruvate. Two heavy loops of 21-days MTB culture were transferred to agar Dubos. Then the tubes were incubated at 37°C for seven days. A positive (H37Rv strain) and negative (DM97 strain) controls were included. For test reading, 1 mL of fresh 1% FAS solution was added and incubated at 4°C in darkness. A reddish color indicated a PZA-sensitive result in the tube. The absence of color meant a PZA-resistant result.

Statistical analysis

Mycobacterium tuberculosis growth, test positivity-day, and contamination results among all culture media tested were compared using Wilcoxon's signed-rank tests for paired samples. On the other hand, the frequency of susceptibility to drugs (INH and RIF) was compared among all the culture media using the proportions test. For the hypothesis contrast, a Friedman test was performed for completely randomized block designs, considering as a factor: the week in which bacterial growth was evaluated (initial, week one, and week two) and the type of culture. Each culture media was compared to the traditional MM culture medium. Finally, the agreement between the MODS-Wayne dichotomous results and the CRT was evaluated using the Kappa index.

RESULTS

Culture performance of the alternative cost-effective culture media compared to the standard MODS medium.

Two hundred eighty-two sputum samples were evaluated in two alternative culture media, LM and PM, and the standard MM medium. Additionally, only 253 samples were evaluated in the IM medium. The contamination rate for all culture media was between 8.2% to 13.5%. The highest contamination rate was reported for the PM, which was not sterilized. No significant difference was observed in the contamination rate between the IM and LM compared to the

standard MM medium (Table 1). On the other hand, test positivity rates of the PM (86.5%), LM (89.5%), and IM (83.6%) did not show a significant difference compared to the MM medium (Table 1). No significant difference was observed for the median time of positivity between the LM (8.5 days; P = 0.131) and PM (9.5 days; P = 0.621) compared to the MM medium (Table 1). Regarding the bacterial growth, no significant difference was observed between the LM (P = 0.404) and the standard MM medium. The IM (P = 0.059) and PM (0.041) showed a similar growth level to the MM culture medium. More than 90% of samples showed bacterial growth ranging from low to high (i.e., growth covering up to 75% of the well area) (Figure 4, Table 2). Bacterial growth level was evaluated in two additional reading plate times after 7 and 14 additional days of incubation to determine the effect of longer incubation periods on bacterial growth. No significant differences were observed for the PM (P = 0.621) and LM (P = 0.131) media compared to the MM medium. The evaluated culture media showed a similar bacterial growth level along the three reading plate times (Figure 4).

Drug susceptibility determination for INH and RIF

According to the MODS standard medium, 85 (37.7%) samples showed a drug-resistant profile. Of these, three (3.53%) samples were mono-resistant to RIF (RIFr), 33 (38.82%) samples were mono-resistant to INH (INHr), and 49 (57.64%) showed resistance to both drugs (i.e., MDR). Sensitivity ranged 83.6 – 91.8% and specificity 78.9 – 95.5% for detection of MDR-TB. No significant differences were observed in the sensitivity and specificity for the PM, LM, and IM culture media compared to the standard MODS medium (Table 3).

Drug susceptibility determination for pyrazinamide

A reference consensus test (CRT) for 83 samples that showed a drug-resistant profile (RIF and INH resistance) was used to determine pyrazinamide resistance. Twenty-four samples (28.92%) were reported as PZA-resistant isolates according to the CRT assay. On the other hand, 38 samples (45.78%) were resistant to PZA by MGIT, 19 samples (22.89%) were resistant to PZA by the classic Wayne test, and 25 samples (30.12%) showed at least one mutation in the promotor or *pncA* gene sequence (Table 4). Of 47 MDR samples, 44% (21/47 samples) were reported as PZA resistant according to the CRT test. Of 33 samples with mono-resistance to INH, 9.09% (3/33 samples) were registered as PZA resistant. No PZA resistance was observed in the samples with mono-resistance to RIF isolates. Finally, eight samples from the RIF/INH sensitive isolates were PZA resistant.

According to the MODS-Wayne, the number of PZA-resistant isolates ranged between 13 – 16% in the alternative culture media and the traditional MM medium (Table 5). MODS-Wayne

performance was evaluated based on the 83 samples that reported resistance to RIF and INH. Sensitivity and specificity were calculated by comparison with the PZA resistance profile determined by the CRT. No significant difference was observed between the alternative culture media and the traditional MM medium. An 85% agreement value was observed for each of the evaluated culture media (Table 6). It is worth noting that the results in the MODS-Wayne in all the culture media were similar; the minimal differences in sensitivity and specificity are due to distinct samples evaluated in each media due to contamination.

DISCUSSION

In the present study, we demonstrated that three alternative presentations of dehydrated culture media allow detection of *M. tuberculosis* (MTB) growth in the MODS test, with a similar percentage of positivity and without significant differences in the test turnaround time when compared to the standard MODS culture medium (MM). The mycobacterial growth showed the same pattern in all evaluated culture media (Figure 4). Around eight days of incubation (initial detection), more than 90% of the samples showed low to moderate growth. After an additional 14 days of incubation, more than 90% of the samples showed abundant growth in both the alternative culture media (PM, LM, and IM) and the MM medium. No differences were observed in the pattern of cord formation, which is characteristic of MTB growth in liquid culture (20). In an evaluation study of a telediagnosis system for the MODS test, it was reported that, from 14 days of incubation, MTB growth was in the form of conglomerates covering the entire culture well (36), like the bacterial growth observed in this study. The difference observed in the level of growth between the PM medium, and the standard MM medium could be due to the absence of catalase. It has been reported that the supplementation of culture media with catalase favors the growth of MTB (24) since it allows for reducing the concentration of reactive forms of oxygen (ROS), which can be produced during aerobic metabolism (37). A previous study (22) showed that culture media lacking catalase showed a lower MTB growth level in liquid culture than in supplemented culture media.

On the other hand, sterility is a critical factor in using culture media for diagnostic purposes. Different methods for sterilizing culture media (i.e., moist-heat and dry-heat based, pasteurization, sterile filtration, and radiation) have been reported (38,39). The present study evaluated the use of antibiotics and gamma radiation to control contamination. The commercial antibiotic mixture PANTA has been reported as a supplement to reduce the viability of the contaminating flora of clinical samples when evaluated in culture for the diagnosis of tuberculosis (20). The use of PANTA in liquid culture media has been reported to reduce

contamination (2.2%) compared to solid media such as Lowenstein-Jensen (6%) (40). Gamma radiation is used commercially to sterilize agricultural products, packaging, and certain foods, but principally for various medical devices (41). Its use to sterilize culture media has also been reported (19), with bacterial growth found to be similar in irradiated and autoclaved culture media. We observed that the LM and IM culture media showed no significant differences in the percentage of contamination recorded compared to the standard MM medium, suggesting that both methods can be used without compromising the culture performance. According to the CDC guidelines (42), a contamination rate of up to 5 ± 2% is acceptable in clinical sample cultures. A slightly higher contamination rate is accepted for liquid culture media, between 7 - 9%. Our study reported a contamination percentage between 8.2 - 8.9%, except for the PM medium. This culture medium was not subjected to radiation or any other sterilization method during its preparation. Although PANTA was added to the medium once hydrated, a contamination percentage of 13.5% was observed. In thin layer agar (TLA), 26% of the sputum culture samples were contaminated, and still, it is a recommended method because it is rapid and does not need of inverted microscope (42). Suggestions such as improving the decontamination process and technical capacitation are also valid for this study (43). In contrast, a very low contamination rate (less than 3%) is suggested to indicate an overly stringent decontamination process, which would also affect the growth of mycobacteria and could reduce the positivity rate or cause an increase in the detection time of positive mycobacterial culture (42).

Despite the technical advantages of the MODS test (i.e., short turnaround time, simultaneous evaluation of drug susceptibility profile, low cost per test, high sensitivity - specificity, and being patent-free) (15,16), this diagnostic method is not yet widely used. The MODS test was specially designed to be applied in low- and mid-income countries (LMIC) (44), which are countries that usually have a considerable prevalence of tuberculosis. In Peru, the MODS test is still only used in some health reference laboratories (Callao, Lima Sur, Arequipa, and Ica), despite being the country where the test was developed and validated (45). One factor limiting the large-scale application of the MODS test is the elaboration of the culture medium. An enriched culture medium is used, so trained personnel and specific reagents are required to ensure adequate preparation. The culture medium used consists of the 7H9 Middlebrook broth, enriched with casitone, glycerol, OADC (oleic acid, albumin, dextrose, and catalase), and a cocktail of antibiotics (PANTA) (20,46,47). The components are acquired or prepared individually to reduce the costs for the routine preparation of the culture medium. The hydrated base medium is supplemented with casitone and glycerol and then sterilized in an autoclave. While the OADC supplement is sterilized by filtration, and the antibiotic cocktail is commercially acquired in

dehydrated form. These last two components are added before using the culture medium (18). The relatively extensive preparation of the culture medium is a limitation in diagnostic laboratories with high demand for tuberculosis cases, generating a more significant workload and delay in diagnosis.

Implementing a more straightforward methodology in terms of handling, preparing, and sterility of the culture medium for the MODS test could facilitate the implementation of this assay on a larger scale. Previously, a commercial kit for the MODS test was developed and marketed in 2014 (48). However, its production was discontinued due to the technical limitations related to the transport chain since it needed to have a cold chain (4 – 8°C). According to our results, the three dehydrated media evaluated showed to be viable options to be used in the MODS test. For the evaluated alternative culture media, a cold chain is not required, which would reduce the costs of a potential kit. We believe that the PM medium represents a better option for laboratories that are limited in resources and infrastructure. In contrast, LM and IM media could be suitable alternatives for when it is necessary to transport and distribute the culture media.

Drug-resistant tuberculosis is a major global health problem. The prevalence of multidrug-resistant tuberculosis (MDR-TB) has been reported to be between 3-4 % worldwide (1) and 13.4% in Peru in the region of the Americas (1,2). Currently, semi- or automated tests such as BACTEC MGIT and molecular tests allow determining resistance to isoniazid (INH) and rifampicin (RIF). However, there are limitations to its implementation in LMIC, where microbiological drug susceptibility tests (DSTs) based on solid culture media are still used (11, 49). The MODS test also allows the detection of susceptibility to first- and second-line drugs (50,51). Sensitivity and specificity values of 97.8% and 99.6%, respectively, have been reported to determine susceptibility to INH and RIF using the MODS test (20,40). In our study, the alternative culture media evaluated showed no significant difference in diagnostic performance values. A sensitivity between 87.8 – 93.9% and a specificity between 78.6 – 94.7% were found to determine resistance to INH. While determining resistance to RIF, a sensitivity of 100% and a specificity between 79.5 – 86% were found. Though, only three samples with monoresistance to RIF were obtained. In detecting MDR-TB isolates, sensitivity values between 83.6 – 91.8% and specificity between 78.9 – 95.5% were reported. These values are comparable with the reported sensitivity (96.95%) and specificity (96.8%) data of other microbiological DSTs such as genotype MTBDRrplus assay (51,52).

No DST is currently considered the gold standard for pyrazinamide (PZA) resistance determination (53). Tests such as the BACTET MGIT or the DNA sequencing of promoter and *pnc*A gene are recommended by the WHO to determine susceptibility to PZA (30,54). However, both tests have technical limitations, including false negative results due to the amount of used inoculum, PZA concentration, and the pH of the culture medium for the MGIT assay (55). On the other side, the low frequency and great diversity of mutations, different geographical prevalence of mutations, and not all mutations associated with phenotypic resistance restrict the use of molecular assays (33,34,56,57). Our research group reported a variant of the MODS test to assess susceptibility to PZA, the MODS-Wayne assay (17). This test is based on the detection of pyrazinoic acid (POA) produced by MTB in the presence of PZA. This molecule is made from the deamination of PZA by the action of pyrazinamidase (PZasa). Mycobacterial strains sensitive to PZA have a functional PZasa that transforms PZA to POA, which is released into the extracellular environment where it can be detected by adding ferrous ammonium sulfate (32). Unlike the BACTEC MGIT assay, the MODS-Wayne test does not require an acidic pH. It can be performed directly from cultures of clinical samples, eliminating the need for prior isolation (17).

Our study compared the diagnostic performance to determine the PZA susceptibility of the standard culture medium (MM) and the alternative media with a consensus reference test (CRT). The results of the CRT were obtained from the analysis of susceptibility results of three DST assays, as previously reported (28). Our results showed good agreement (percentage of agreement of more than 85%) for the MM medium and the three alternative culture media compared with the CRT results. Sensitivity values between 79 – 82% and specificity values between 87 – 88% were reported for the evaluated dehydrated culture media. No significant differences in the sensitivity and specificity were observed regarding the standard MM medium. Concerning the mutations detected, it was observed that 100% of the mutations correspond to SNPs. No mutations were found at the level of the promoter region. We found 13 isolates without mutations at the *pnc*A gene and with a positive result in Wayne's classic assay. However, they showed a resistant phenotype in the BACTEC MGIT. It has been suggested that the resistance mechanism to PZA does not depend exclusively on the activity of PZasa (56,57). But other mechanisms can participate in resistance to PZA, among which is considered the activity of efflux pumps (58,59) and other molecular targets such as mutations in the *panD* gene, mutation in *RpsA* encoding ribosomal protein S1, a mutation in *clpC1* encoding an ATP-dependent ATPase involved in protein degradation is associated with pyrazinamide resistance or other unidentified genes involved in PZA resistance (60-62). On the other hand, some isolates

(X%) showed mutations in the *pnc*A gene, which has been reported as associated with phenotypic resistance to PZA (i.e., H51R and Q10T), showed a positive result in the detection of POA in Wayne's classic assay. These cases may likely represent a picture of polyclonal or mixed infection, which seems to be more frequent than expected, where a patient may live with several strains of the same bacteria (63). However, this hypothesis was not confirmed.

CONCLUSIONS

Our results showed that the evaluated dehydrated culture media (PM, LM, and IM) as alternatives to the standard culture medium for the MODS test showed similar diagnostic performance for detecting MTB in clinical sputum samples. Likewise, the three alternative culture media allowed the determination of susceptibility to INH, RIF, and PZA with high sensitivity and specificity values. The different forms of preparation and manipulation of the alternative culture media did not affect the diagnostic performance of susceptibility to three first-line drugs (INH, RIF, and PZA). However, a larger sample size is required for a new assessment. The culture LM medium showed the highest positivity rate (89.5%), as well as the sensitivity and specificity values for MDR-TB (91.8% and 95.5%, respectively) and PZA (81% and 87%, respectively). Based on its preparation, we consider that this alternative culture medium presentation is an excellent candidate to be used as a base medium for the design of a new MODS test diagnostic kit, which will allow decentralization of the MODS test to more regional laboratories in LMIC.

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