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Evaluation of the Usefulness of the MODS Assay for the Detection of TB and MDR TB among PTB Suspects in the Philippines

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Introduction

In a developing country with high prevalence of pulmonary tuberculosis (PTB) like the Philippines, a reliable low cost diagnostic tool for the detection of *Mycobacterium* tuberculosis (*M. tuberculosis*) in clinical specimens is needed [1]. Case detection is mainly based on sputum microscopy, a diagnostic tool that has low sensitivity [2,3]. Mycobacterial cultures are more sensitive; however the standard egg based culture needs 6 weeks-8 weeks of incubation before a result is released. The more rapid liquid based automated culture systems give results earlier than the standard methods but these are found only in big tertiary hospitals in our country and are generally considered to be expensive [4-6].

Moreover multi-drug resistant *M. tuberculosis* (MDR-TB) defined as resistance to Isoniazid (H) and Rifampicin (R) and is a growing concern in the country. The 1997 National Tuberculosis Prevalence Survey reported an MDR rate of 4.3% [7]. Methods available for drug susceptibility testing (DST) with solid media also takes time. On the average, DST results are released 3 weeks after a positive specimen is detected. *M. tuberculosis* culture and DST results therefore are known to the clinicians in about 3 months after a positive specimen is submitted. This is a long delay in the proper management of a patient with MDR-TB.

According to the World Health Organization global tuberculosis control update in 2013, of the 8.6 million incident cases of tuberculosis (TB), 450,000 (5%) have MDR-TB, and there was an estimated 170,000 deaths from this disease [8]. Globally, only 48% of MDR-TB patients in the 2010 cohort of detected cases were successfully treated, reflecting high mortality rates and loss to follow-up. In addition, TB detection in general is still a problem. About 75% of the estimated 2.9 million missed TB cases were in 12 countries, including the Philippines [8].

The microscopic observation drug susceptibility (MODS) assay, a low cost rapid technique to detect *M. tuberculosis* from clinical specimens has been developed. This assay utilizes liquid culture media in plastic microwells where specimens are inoculated and microbial growth is observed through an inverted light microscope for the formation of the characteristic cording pattern of growth of *M. tuberculosis* [9]. This method easily permits *M. tuberculosis* detection and determination of drug susceptibility [10,11]. Positive results are usually observed within a week with an average detection time of 7 days-9 days [11]. The advantage of MODS for early detection of *M. tuberculosis* from clinical specimens has been evaluated in earlier

studies in Peru and other countries where the burden of TB is high [12,13]. MODS have also been evaluated for early detection of drug resistant *M. tuberculosis*. With MODS, drug susceptibility tests with Isoniazid and Rifampicin were shown to be comparable with the standard LJ and agar based proportion method. Concordance rates reported were 99%-100% [13,14].

Recent publications of MODS studies show excellent results with *M. tuberculosis* detection with sensitivity and specificity of 73%-89.1% and 91%-99.1% respectively [15-17]. Comparing the sensitivity and specificity with recognized standards, MODS may turn out to be more accurate in isolating *M. tuberculosis* and predicting MDR-TB. Prompt detection of *M. tuberculosis* will result in a significant improvement in patient care management as well as implementation of infection control procedures.

In the Philippines, the development of a rapid, low cost and accurate diagnostic method in detecting tuberculosis in clinical specimens is urgently needed. With the limitations of various TB/MDR-TB diagnostic tests available in the country, there is a need to validate the usefulness of MODS, given its promising accuracy rates as reported from the literatures. This study is thus conducted to validate early detection of *M. tuberculosis* and MDR-TB using the MODS Assay in an actual bench setting in a TB diagnostic laboratory.

This study aims to compare the performance of the MODS assay in the detection of *M. tuberculosis* and MDR-TB from sputum specimens with the standard egg-based solid culture medium (Lowenstein Jensen) and the liquid MB BacT automated culture system. The specific objectives are to:

- determine time to detection of *M. tuberculosis* and MDR-TB from sputum specimen of patients with pulmonary tuberculosis using the MODS assay
- compare the accuracy rates of rifampicin (R) and isoniazid (H) drug susceptibility test results of the MODS assay with the drug susceptibility tests results done with the agar-based disk proportion method
- determine the effect of on-going treatment on the isolation rate of *M. tuberculosis* by the MODS assay, standard egg based LJ medium and liquid based MB BacT automated culture system
- compare the cost of the advantage of the MODS assay with the standard egg-based medium (LJ), the liquid based automated MB BacT (MB) and the disk agar proportion method for detection of HR resistance.

Method

Ethics

The study protocol was approved by the Ethics Review Board of the University of the Philippines-Philippine General Hospital (UP-PGH) and the Research Institute of Tropical Medicine by the Department of Health (DOH).

Study population and setting

Sputum specimens were gathered from patients suspected to have pulmonary tuberculosis (PTB) were screened from two study populations: patients at the outpatient clinics of the departments of internal medicine, family medicine and TB DOTS clinic of the University of the Philippines-Philippine General Hospital (UP-PGH) and from patients referred by the Department of Health's Programmatic Management of Drug-Resistant tuberculosis (PMDT). PTB suspects were patients with signs and symptoms of chronic cough >2 weeks plus weight loss, with or without night sweats, unexplained fever, anorexia, chest pain, easy fatigability and body malaise. Patients referred by PMDT were mostly patients suspected to have multidrug resistant tuberculosis (MDRTB). MDR-TB suspects were patients who were for re-treatment and/or patients with past history of at least 1 month of treatment for PTB. Patients with on-going treatment were not excluded. These patients submitted 1-2 sputum specimens for *M. tuberculosis* culture and drug susceptibility tests.

All laboratory procedures were done at the UP-PGH TB Research Laboratory, a level II mycobacteriology laboratory. This laboratory was also identified as the culture center for PMDT by the DOH.

Laboratory method

We compared the result of the MODS Assay with the standard TB culture isolation procedures. This was conducted in parallel with the standard lowenstein jensen (LJ) culture method; MB BacT automated broth culture and the agar disk 1% proportion method for drug susceptibility testing. Only one medical technologist was specially trained to perform the MODS assay and he was blinded from the results of the culture performed using the LJ media, MB/BacT system and the results of the drug susceptibility proportion method. Another medical technologist interpreted the drug susceptibility testing using the agar disk 1% proportion method as described in CLSI and by Kent & Kubica [18,19].

Processing of sputum specimens

Direct acid fast bacilli (AFB) microscopy smears were done using Ziehl-Neelsen staining method to all specimens. The sputum specimens were digested and decontaminated using the Petroff's Method [19]. The 4% sodium hydroxide is the reagent routinely used at the TB Research Laboratory in UP-PGH. An equal volume of sputum was added to 4% sodium hydroxide. The mixture is homogenized by vortex and then left to stand for 15 minutes, after which the tube was filled with phosphate buffer solution (pH 6.8). This was then centrifuged at 3000 Xg for 15 minutes. The supernatant was poured off and the sediment was resuspended with PBS. An aliquot was used for parallel cultures with LJ, MB BacT system and MODS assay.

Culture technique for *M. tuberculosis* detection

After decontamination, 100 µl of the resuspended sediment was inoculated onto each two tubes of LJ medium. Each tube was properly labeled with the sample number and date of inoculation. These tubes were incubated at 37°C and examined after 48 hours and then weekly up to 8 weeks. For positive LJ cultures, each isolate was examined for cultural morphology, growth rate and pigmentation. In addition, niacin production and nitrite reduction (described by Kent and Kubica) [19,20] were used for identification of *M. tuberculosis*.

The MB BacT culture bottle was inoculated with 500 µl of resuspended sample and cultures were monitored by the automated culture detection machine for growth up to 42 days (6 weeks). A bottle of MB BacT contains Middlebrook 7H9 broth with nutrients and antibiotic supplements. All positive cultures from LJ and MB were processed for indirect drug susceptibility using the 1% proportion method to check for drug resistance.

Drug susceptibility test for MDR-TB

Indirect agar-based disk submerged 1% proportion method was done for all positive LJ cultures and MB Culture System [21]. This method used appropriate anti-tuberculosis drug disks submerged in Middlebrook 7H10 Agar (Difco) supplemented with OADC (Becton Dickinson) and glycerol. Critical drug concentrations used were as follows: 0.2 µg/ml for Isoniazid and 1 µg/ml for Rifampicin. Procedures and Interpretations were based on Clinical Laboratory Standards (CLSI) for Antimycobacterial Drug Susceptibility and as described by Kent and Kubica [18,19].

The MODS assay

The MODS assay was performed according to the methods described by Caviedes and Moore [9,11]. Briefly, MODS medium was prepared using Middlebrook 7H9 broth base, Bacto Casitone, OADC Growth Supplement (oleic acid, albumin, dextrose and catalase) and PANTA Antibiotic Supplement (polymixin, amphotericin B, nalidixic acid, trimethoprim, azlocillin). The assay was prepared in a 48-well tissue culture plastic plates. Each run included a positive control of the standard *M. tuberculosis* H37Rv, a negative control of MODS medium and the test sample from the processed specimens. The positive control well contained 100 µl of standard *M. tuberculosis* H37Rv and 900ul of MODS medium. The negative control well contained 1000 µl of MODS medium. The test sample well contained 250 µl of resuspended test sample and 750 µl of MODS medium only. Plates were sealed with optical film (BioRad), covered with low evaporation lid and incubated at 37°C for up to 40 days. The culture plates were examined under an inverted light microscope at 40X magnification every day from day 3 to 15 and twice weekly afterwards up to 40 days of incubation. Positive cultures were identified by its characteristic cord formation. Bacterial and fungal contaminants were noted during and up to the end of the observation period. Contamination was compared with the parallel cultures in MB and LJ medium.

In the same 48-well tissue culture plastic plates described above, direct drug susceptibility testing was performed at the same time. Two wells contained Isoniazid and Rifampicin each. The drug concentrations used were as follows: isoniazid (H) 0.2 µg/ml and rifampicin (R) 1 µg/ml, these are similar to the critical drug concentrations of the indirect agar disk proportion method. The standard H37Rv positive control well served as the drug sensitive

control in each run. Rifampicin and isoniazid resistance were detected as growth or presence of cord formation in the drug containing wells.

Data analysis

M. tuberculosis detection rates for MODS, MB and LJ were summarized. We compared the time to a positive culture for LJ, MB and MODS, respectively, in samples positive in at least one method using the Wilcoxon test and presented using the Kaplan-Meier survival curves.

The accuracy of MODS for diagnosis of MDRTB was then assessed using the indirect disk agar proportion method as the gold standard reference test. Confidence intervals for accuracy measures (sensitivities, specificities, positive and negative predictive values) were calculated. All statistical analyses and graph were performed with Stata version 12.

Results

We included a total of 795 sputum specimens collected from January 2012 to November 2013. There were 405 sputum samples from UP-PGH patients and 390 from PMDT patients. Out of the 795 sputum specimens, 770 were analyzed when the contaminated samples and samples with NTM were removed (Figure 1). Of the 770 specimens 269 were smear-negative and 501 were smear-positive for AFB. Table I shows the detection rates with the three culture techniques based on the smear results. Higher yield for *M. tuberculosis* was observed from smear positive specimens. Contamination rates were 2.6% for LJ and 1.1% for both MB and MODS.
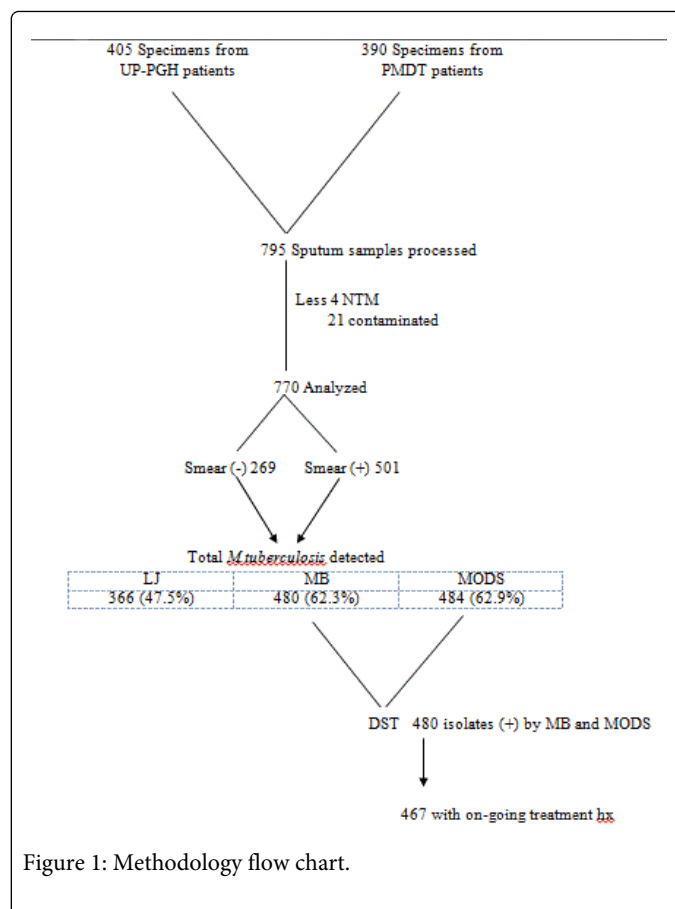

Figure 1: Methodology flow chart.

Culture Technique	LJ	MB	MODS
Smear-negative (N=269)	33 (12.3%)	73 (27.1%)	77 (28.6%)
Smear-positive (N=501)	333 (66.5%)	407 (81.2%)	407 (81.2%)
Total number of *M. tuberculosis* detected	366 (47.5%)	480 (62.3%)	484 (62.9%)
NTM detected	2	4	4
Contamination rate	21 (2.6%)	9 (1.1%)	9 (1.1%)

Table 1: Total number of *M. tuberculosis* detected in 770 specimens.

The rates of *M. tuberculosis* detection of the three techniques are shown in Figure 2. The median time for culture detection is 11 days for MODS, 17 days for MB and 37 days for LJ. The early detection of *M. tuberculosis* as shown in Figure 2 was statistically significant [Wilcoxon (Breslow) test], p value<0.0001. Good agreement was obtained between LJ and MODS while excellent agreement was observed between MB and MODS (Kappa Statistic 0.73 and 0.99 for LJ vs MODS and MB vs MODS respectively p<0.0001).

The average incubation period before culture detection was shorter in specimens with more bacillary load based on AFB smear grade. Compared with the negative smears, specimens with 1+ and more had shorter incubation periods for positive MODS Assay (p=0.000) (Table 2).

The rifampicin and isoniazid drug susceptibility test results using MODS and the standard disk proportion method are shown in Table 3. Computation of rates was based on 480 isolates that grew in both MB and MODS since both culture techniques utilized similar liquid culture media. There is better agreement of the isoniazid resistance rate compared with the standard disk proportion method.

The accuracy rates and predictive values of MODS in MDRTB detection are as follows: sensitivity 98.21% (CI 95, 99), specificity 99.60% (CI 98, 99), PPV 99.56% (CI 98, 100), NPV 98.41% (CI 96, 100).

Out of the 480 culture positive specimens, 13 specimens had no source data on the history of treatment, 399 were from patients without history of treatment and 68 were from patients with on-going anti-TB treatment. *M. tuberculosis* was still isolated from sputum samples of patients with on-going anti-TB treatment and majority were multi-drug resistant (Table 4).
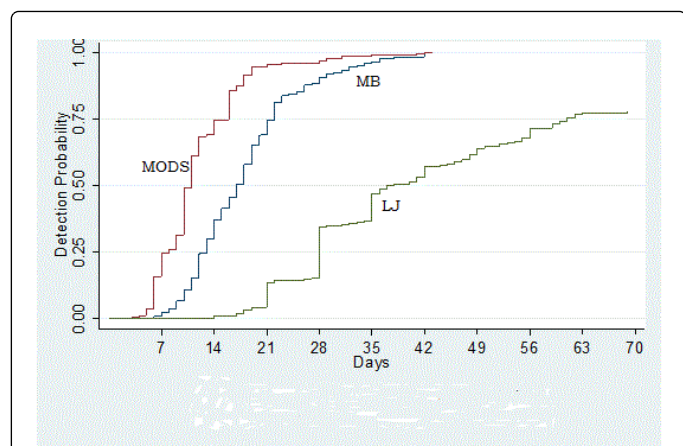
Figure 2: Time of detection of *M. tuberculosis*.

Sputum Smear Grade	Number of Specimens	Mean Growth Rate (Days)		
		LJ	MB	MODS
Negative	269	43	20	15
1-9/slide	125	41	20	13
1+	123	37	19	11
2+	104	35	18	11
3+	149	31	15	10

Table 2: Average incubation time based on smear grading (N=770).

The cost per sample tested using the three culture techniques were compared. Prices were based on the year 2009 cost of culture and drug susceptibility of *M. tuberculosis* at the UP-PGH laboratory. The automated culture system (MB) is still the most expensive compared to LJ and MODS (Table 5). The cost of the added benefit of MODS over the standard LJ was computed as total cost of one test with MODS minus total cost with LJ divided by the % difference in culture detection. The added cost benefit of MODS is only P5.70/test or approximately less than $1.00.

Resistance Pattern	Method of drug susceptibility test (DST)	
	Proportion Method No. (%)	MODS No. (%)
Susceptible to HR	206 (42.9)	204 (42.5)
Resistant to both Isoniazid and Rifampicin (HR)	231 (48.1)	228 (47.5)
Resistant to Rifampicin only (R)	18 (3.8)	22 (4.6)
Resistant to Isoniazid only (H)	25 (5.2)	26 (5.4)

Table 3: Result of drug resistance from 480 *M. tuberculosis* isolates.

Discussion

Based on our findings, as early as first week of incubation, 25% of the culture positive specimens were detected by MODS assay. Also, by the second week of incubation, almost 90% of the positive specimens were detected. Mean growth rate even for smear negative specimens was 15 days. This is one week earlier than the MB and three weeks earlier than LJ. Instead of continuing incubation for 42 weeks, incubating for 30 days is enough to detect positive specimens for the MODS technique.

Treatment Duration	Total Samples	Culture Technique					
		LJ		MB		MODS	
		No. (+)	No. MDR	No. (+)	No. MDR	No. (+)	No. MDR
No treatment history	317	244	90	317	109	317	110
1-2 months	108	80	64	108	86	108	82
3-6 months	15	9	5	15	10	15	10
>6 months	13	10	8	13	11	13	11
Unknown duration	14	13	8	14	8	14	8
No data	13	10	6	13	7	13	7
Total	480	366	181 (49.4%)	480	(48.1%)	480	228 (47.5%)

Table 4: Effect of on-going treatment on the isolation rate of *M. tuberculosis* (N=480).

	LJ	MB	MODS
Initial Outlay			
MB Machine	-	1,200,000	
Inverted Microscope			349,000

Medical Technologist training			12,443 (10 days training)
Average cost of culture media/reagents	880	2200	960 (including HR powders for DST
Average cost of drug susceptibility test	-	1,369.47	-
Total cost (TC) for culture and DST	880	3,569.47	960
Computation for added cost of benefit of MODS=P5.70 [TC (MODS)-TCLJ/%difference in yield]			

Table 5: Cost comparison market price of the three culture techniques in Philippine peso.

M. tuberculosis detection rate in our study was lower compared with the previous MODS evaluation studies because we did not exclude patients with on-going treatment. In a single-center study with high HIV prevalence by Shiferaw, their detection rate was 94% because they excluded patients on treatment [14]. Among non HIV patients, Minh Ha on the other hand excluded patients on treatment and their detection rate was only 50.5% [20]. Compared to a multi-center study in Brazil, we also included sputum samples of patients with on-going anti TB treatment [13]. Our overall detection rate was better (62.9% vs 44.4%).

Despite on-going treatment, *M. tuberculosis* was isolated in all culture techniques, but 56%-85% of the isolates were MDR. Both MB and MODS had similar detection rates for MDR. Even among those without treatment, the proportion of MDR were also high (43%-46%) and we attribute this to population bias since 49% of the sputum specimens came from MDRTB suspects. The early detection of MDR TB by MODS has important public health implications in terms of early case detection and management. During TB treatment, the usual intensive phase of HRZE for two months is reduced to HR for the four month maintenance period, without the knowledge of the DST [1]. Ideally the DST result should be known before drug regimen is reduced to HR especially for MDRTB suspects. Before shifting to maintenance treatment, MODS assay can provide this timely DST result to guide clinicians in the management of patients with MDRTB.

With the MODS assay, culture detection and HR resistance rate results are concomitantly available at the same time. In comparison to previous studies which showed sensitivity of 77.8%-92%our study provided better sensitivity results [17,20,21]. We attribute this to the concentration of isoniazid used for the MODS assay in this study. Isoniazid concentration was decreased to be similar to the critical concentration used for standard agar disk proportion method e.g. 0.2 μg/ml. In the prospective evaluation of MODS assay in Thailand, the isoniazid concentration used was 0.1 μg/ml when they compared MODS DST to MGIT 960. The MODS sensitivity and specificity for MDRTB in that study were 85.7% and 97.5% respectively [21].

We compared the cost of using the three techniques. All costs included media, reagent cost, medical technologist time, machine depreciation and 20% mark up for overhead cost which reflect the prices in 2009 when the protocol was made. LJ was the cheapest for MTB culture. With the MODS assay, for an additional cost of P5.70, the patient and clinician can receive the result 2 weeks earlier than LJ for the *M. tuberculosis* detection. And instead of waiting for an additional 3 to 4 weeks for the drug susceptibility test result, MODS can provide MDR test results at the same time as the culture result. Appropriate treatment can therefore be provided early to the patient especially for the MDRTB suspect.

Limitation

Our study is limited to clinical specimens. We did not take into account the condition of the patients especially those on treatment. The skill of the operator outside of the research laboratory could be a potential limitation of MODS due to the proficiency in discriminating the characteristic cording of *M. tuberculosis* from NTM. This however can be remedied with proper training. There are reports on MODS training of laboratory staff outside of research laboratories and results are encouraging [22,23]. Furthermore, other potential challenges include medical technologist time requirement and the need for detailed attention to laboratory procedures to prevent contamination. Standardization of the technique and quality control procedures should be addressed.

Conclusion and Recommendations

The MODS Assay is a rapid and cost-effective way of detecting *M. tuberculosis* comparable with MB automated culture system which also utilizes the liquid medium Middlebrook 7H9. We recommend the use of Isoniazid concentration of 0.2 μg/ml for the assay as this is similar to the critical drug concentration being used in the agar disk proportion method for DST.

With adequate training of medical technologists, the MODS Assay can be adopted in the regional TB laboratory centers in our country. This will actually lessen the waiting time for the results in a cheaper way. Since MODS can be applied to direct samples, this will give the clinician the edge of knowing the culture and sensitivity results earlier. MODS is cost beneficial to the patients.

Acknowledgement

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