Validation of a rapid method for detection of *M. tuberculosis* resistance to isoniazid and rifampin in Lima, Peru

L. A. Solis,* S. S. Shin,†‡ L. L. Han,§ F. Llanos,¶ M. Stowell,§ A. Sloutsky§

* Instituto Nacional de Salud, Lima, Peru; † Division of Social Medicine and Health Inequalities, Brigham and Women's Hospital, Boston, Massachusetts, USA; ‡ Socios en Salud, Lima, Peru; § TB Laboratory, Massachusetts State Laboratory Institute, Jamaica Plain, Massachusetts, USA; ¶ Departamento de Salud Publica, Universidad de Cayetano Heredia, Lima, Peru

_____ S U M M A R Y

SETTING: Treatment of multidrug-resistant tuberculosis (MDR-TB) is often based on drug susceptibility testing (DST) results; for this reason, rapid, simple DST methods are sought which could be applied in resource-poor countries. One such method is a nitrate reductase colorimetric assay known as the Griess method. In Peru, where the incidence rate of TB is among the highest in South America, the National Institute of Health recently undertook the validation and implementation of the direct Griess method.

OBJECTIVE: To describe the process of validation and implemention of the direct Griess method at the Peruvian National Institute of Health.

DESIGN: Prospective study comparing the sensitivity and specificity of the direct Griess method with the Löwenstein-Jensen proportion method in determining resistance to

isoniazid (INH) and rifampin (RMP) among clinical isolates.

RESULTS: Among 192 specimens, the sensitivity and specificity of the Griess method for detection of INH resistance was 99.1% and 100%, respectively. For identification of RMP resistance, the sensitivity and specificity was 93.5% and 100%, respectively.

CONCLUSIONS: In addition to its high sensitivity and specificity and rapid turn around time, the Griess method uses simple, inexpensive reagents and requires minimal laboratory space and technical expertise, thus providing an ideal screening tool for resource-poor settings with high rates of MDR-TB.

KEY WORDS: tuberculosis; diagnosis; drug resistance; MDR

MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB) is now recognized as one of the most significant emerging infectious diseases, with cases documented in more than 100 countries or territories throughout the world.[1,2] In the Americas, Peru has one of the highest rates of MDR-TB, with a national prevalence of 3% among patients never before treated for TB, and 12.3% among previously treated patients.[3] Although treatment with standardized directly observed therapy with short-course chemotherapy (DOT-SCC) has been successfully implemented in Peru since the early 1990s, MDR-TB continues to be a major public health problem, in part because of the difficulty and expense associated with its treatment.

Worldwide, programs for MDR-TB treatment have demonstrated promising cure rates;[4–11] the majority of these programs utilize a strategy of individualized treatment regimens, based on drug susceptibility testing (DST) results for each patient. Unfortunately, conventional DST methods used by most programs are

time-consuming, often resulting in delays of up to 3 or 4 months, sometimes more.[12] To optimize treatment outcomes for MDR-TB patients, timely initiation of proper therapy is crucial.[11] For these reasons, more rapid DST assays have been sought, and numerous novel methods have been described,[13,14] including the line probe assay (Innogenetics, Ghent, Belgium),[15] the Alamar Blue assay,[16] assays using Mycobacteria Growth Indicator Tubes (Becton Dickinson, Sparks, MD, USA)[17] and others.

While many of these methods have been developed specifically for use in resource-poor settings with high MDR-TB prevalence, none have been implemented to date for programmatic use in such settings. Many of these methods have proven impractical for use in resource-poor countries because of their prohibitive technical demand, laboratory requirements (space, equipment, disposal of radioactive waste, etc), and/or cost of equipment and supplies. An ideal method would be simple, inexpensive and reliable enough to

be implemented on a local level, to minimize additional delays associated with transport of specimens to specialized central laboratories.

One rapid DST method that could potentially satisfy these criteria is a nitrate reductase colorimetric assay known as the Griess method.[14,18] This assay was initially developed at the Central Tuberculosis Research Institute in Moscow, Russia, as a low-cost DST method that can be employed in areas of limited resources and low technical capacity.[19] This method has the advantage, compared to the Dio-TK colorimetric culture system (Salubris Inc, Woburn, MA, USA), of being based exclusively on conventional methods and materials accessible to any laboratory without reliance on proprietary materials, reagents, and equipment.[20] Briefly, conventional culture broth containing $NaNO_3$ and an antibiotic are inoculated, incubated, and then examined by addition of a reagent that produces a color change in the presence of mycobacterial growth. Susceptibility to first-line drugs can be determined by the Griess method in 8–10 days after obtaining a positive culture (indirect method), or in 21–28 days when applied to a smear-positive sputum sample (direct method). In this report, we describe the implementation and validation of the direct Griess method for rapid DST for isoniazid (INH) and rifampin (RMP) at the National Reference Laboratory (NRL) of the National Institute of Health in Lima, Peru. We view the implementation of the Griess method in the Peruvian National Tuberculosis Program as a two-stage process. The next stage would entail expansion of this capability to the district level laboratories in Lima and to some provinces with higher rates of MDR-TB.

MATERIALS AND METHODS

Specimen processing

Sputum specimens underwent initial processing and fluorochrome acid-fast bacilli (AFB) staining according to standard protocols.[21]

Conventional Löwenstein-Jensen direct method

The conventional method is described in detail elsewhere.[19]

Griess method direct

Principle

The Griess method is based on determination of nitrate reductase (NR) activity in growing *Mycobacterium tuberculosis* cultures. Conventional Löwenstein-Jensen (LJ) medium containing $NaNO_3$ (1 g/l) as a substrate for NR is used for testing. On this medium, by addition of the Griess reagent, mycobacterial growth can be detected sooner than with conventional culture. Addition of this reagent produces a hot pink color, indicative of the conversion of nitrates to nitrites when

NR activity is present. The intensity of this colorimetric reaction is then compared between the tubes with added drugs and drug-free control tubes.

Preparation of Griess reagent

Shortly before use, one part 50% (vol/vol) concentrated hydrochloric acid (HCl) is mixed with two parts 0.2% (wt/vol) sulfanilamide and two parts 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride.

Media preparation

Preparation of conventional LJ medium is described elsewhere in detail.[19] LJ medium for the Griess method is prepared with one modification: 1 g/l $NaNO_3$ is added to LJ medium and completely dissolved by stirring. The antibiotics are then added and the medium is aliquoted and inspissated. Four tubes with modified LJ medium are used for each specimen: one contains INH at critical concentration 0.2 mg/l, one contains RMP at 40 mg/l and two control tubes do not have any drugs added.

Procedure for direct test

The test was performed only on specimens with an AFB smear result of 1+ or higher, corresponding to a mycobacterial concentration of $\geqslant 100\,000$ AFB/ml. Each of the four tubes described above is inoculated with 0.2 ml of the processed sputum sediment. After 28 days of incubation at 37°C, 0.5 ml of freshly made Griess reagent is transferred into one of the growth control tubes, and development of color is observed.* If the color intensity is sufficient, the same amount of Griess reagent is pipetted into the drug-containing tubes. The color intensity in the drug-containing tubes is then compared to the control tube.

Interpretation of results

The results were classified as negative if no color changes or a very pale pink color were observed. Positive results varied from bright pink to deep red or violet. An isolate was considered resistant to a certain drug if there was a positive color change in the antibiotic tube in question and in the drug-free control tube. If no color changes or pale pink color were observed in the control tube, the test was considered to be invalid.

Griess method validation

A total of 232 consecutive clinical specimens from smear-positive TB patients were submitted for DST according to the norms of the National Tuberculosis Program between 24 March and 1 September 2003. Indications for DST included failure of DOT-SCC regimen I (DOT-SCC I),[22] previous treatment history, and history of household contact with an MDR-TB

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* In other settings, readings are initially done after 21 days of incubation, with an additional 7-day reincubation period if little color change is seen in the growth control tube.

patient. All samples were tested for susceptibility to INH and RMP by the INS Laboratory in Lima. Testing was done using the Griess method in parallel with the conventional LJ proportion method, which was used as a reference method.

RESULTS

The Griess test was completed on 192 of 232 specimens. Of the 40 specimens for which testing was not completed, 30 were culture-negative and seven were contaminated according to both methods; in addition, three had invalid Griess test results, with little or no color in the control tube.

Comparison of Griess test results with conventional DST results for 192 specimens is presented in the Table. Among 114 strains that were resistant to INH by the conventional method, 113 (99.1%) were also found to be resistant by the Griess method. Only one isolate was falsely identified as being INH-susceptible. Among 78 strains that were susceptible to INH by the conventional method, 78 (100%) were found to be susceptible by the Griess method. No isolates were falsely identified with INH resistance. Based on these data, sensitivity and specificity of the Griess method for detection of INH resistance in this study were 99.1% and 100%, respectively. Positive and negative predictive values were 100% and 98.7%, respectively, and test accuracy was 99.5%.

Similarly, 101 of 108 (93.5%) isolates that were resistant to RMP by conventional DST were also found to be resistant by the Griess method, with only seven isolates falsely identified as susceptible. Among 84 strains that were susceptible to RMP by the conventional method, 84 (100%) were found to be susceptible by the Griess method. No isolates were falsely identified with RMP resistance. Calculated sensitivity and specificity of this method when applied to testing for RMP resistance were 93.5% and 100%, respectively. Positive and negative predictive values were 100% and 92%, respectively, and test accuracy was 96.4%.

DISCUSSION

In this study, we demonstrate high sensitivity and specificity of the Griess method, relative to conven-tional methods, in the identification of resistance of *M. tuberculosis* to INH and RMP. Sensitivity was 99.1% for INH and 93.5% for RMP, and specificity was 100% for both drugs. In comparison, a smaller study of this method at the Massachusetts State TB Laboratory on 36 isolates from MDR-TB patients from the Tomsk prison in Russia yielded a sensitivity of 75% for INH, 70% for RMP and a specificity of 100% for both drugs (unpublished data). In another report of the performance of the indirect Griess method in Sweden, sensitivity and specificity for INH were 97% and 96%, respectively, and 100% for RMP.[18]

In Peru, patients who have failed previous treatment regimens are treated empirically for MDR-TB. However, earlier identification of MDR-TB cases would minimize the risk of disease progression and amplification of drug resistance due to suboptimal therapy. In addition, other groups of patients—such as health care workers, prisoners, and HIV-positive individuals—would likely benefit from rapid DST which would reduce the opportunity for nosocomial transmission and the morbidity associated with delayed diagnosis of MDR-TB. Rapid screening for MDR-TB in all of these groups of patients would permit expeditious regimen adjustments without unnecessary empiric exposure to second-line drugs.

This study demonstrates the potential usefulness of the Griess method as a susceptible and specific screening tool. Clinicians can be highly confident of a diagnoses of INH or RMP resistance by this method, as resistance to INH and RMP is rarely, if ever, falsely identified in this population. On the other hand, respectively 1% and 8% of INH- and RMP-resistant isolates, respectively, were falsely identified as being susceptible. The results of the Griess test should therefore be considered preliminary and should be subsequently confirmed by conventional methods. An additional consideration in the assessment of the performance of the Griess test is the occasional occurrence of invalid tests. In this study, three (1.7%) of 232 specimens could not be evaluated by the Griess method because they failed to produce the expected color change in a control tube containing no antibiotics. These invalid results can be explained by the fact that about 1% of *M. tuberculosis* strains lack nitrate-

Table   Comparison of Griess test results with conventional DST results in 192 specimens

| Drugs | Conventional method | Griess method | | | | | | |
		Resistant *n*	Susceptible *n*	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %	Accuracy %
INH	Resistant, *n* = 114	113	1					
	Susceptible, *n* = 78	0	78	99.1	100	100	98.7	99.5
RMP	Resistant, *n* = 108	101	7					
	Susceptible, *n* = 84	0	84	93.5	100	100	92	96.4

DST = drug susceptibility testing; INH = isoniazid; RMP = rifampicin.

reductase activity and cannot develop color when treated with the Griess reagent.[19] For patients infected with such isolates, adjustments to empiric treatment regimens must be delayed until the results of conventional DST are available.

For the majority of patients with active TB in Lima, the direct Griess method offers accurate diagnosis of INH and RMP resistance with a substantially shorter turnaround time relative to conventional DST. Conventional DST requires on average 20–40 days for initial culture growth, plus an additional 28 days for DST itself. In contrast, the turnaround time for the direct Griess method in this study was uniformly 28 days, a time savings of 3–6 weeks. Further reductions in turnaround time are possible if the initial colorimetric readings are taken on the 21st day, as suggested in the original protocol.[13] In addition to its rapidity, the Griess method has further obvious benefits that would facilitate its institution in resource-poor settings. Specifically, we confirmed that introduction of the Griess method requires very little training, because the method differs only slightly from the conventional method for DST on LJ slants. Furthermore, the Griess test uses only simple reagents that are inexpensive and easily obtained, does not require maintenance of any specialized equipment, and requires minimal laboratory space and staffing.

CONCLUSION

We demonstrate in this report that capability for accurate and rapid DST for INH and RMP can be readily established in a high MDR-TB prevalence area. Now that the Griess test has been implemented at the Peruvian NRL, the next step will be to expand capability to other sites in the Peruvian TB laboratory network, including district level laboratories in Lima and selected laboratories in other regions of Peru with high rates of MDR-TB. On the basis of our experience at the Peruvian NRL, we are confident that expansion of test capability throughout Peru can be readily achieved, although additional efforts will be needed to assure consistent test quality among multiple test sites. Scale-up of rapid DST capacity will require strengthening of laboratory infrastructure, training and quality control, centralization of procurement and preparation of supplies and media, and ongoing monitoring and supervision of intermediate laboratories by the NRL. For example, given the potential variability of the growth characteristics of media produced at different sites, it may be useful to devise a system for producing media in one location and then distributing it to all testing sites. Expansion of rapid DST capability to multiple laboratory sites will serve at least two important functions: it will free up resources at the NRL, allowing the NRL to focus on more technologically demanding tests such as DST for second-line anti-tuberculosis drugs. More importantly, however, it will provide patients and clinicians with the benefits of greater access to fast and accurate DST results for these two drugs.

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RÉSUMÉ

CONTEXTE : Le traitement de la tuberculose à germes multirésistants (TB-MR) repose fréquemment sur les résultats des tests de sensibilité aux médicaments (DST). Pour cette raison, on est à la recherche de méthodes rapides et simples de DST qui puissent être utilisées dans des pays à faibles revenus. Une de ces méthodes est un test colorimétrique de nitrate réductase connu sous le nom de méthode de Griess. Au Pérou, où le taux d'incidence de la TB est parmi les plus élevés d'Amérique du Sud, l'Institut National de la Santé a entrepris récemment la validation et la mise en œuvre de la méthode directe de Griess.

OBJECTIF : Décrire le processus de validation et la mise en œuvre de la méthode directe de Griess à l'Institut National Péruvien de la Santé.

SCHÉMA : Etude prospective comparant la sensibilité et la spécificité de la méthode directe de Griess à celle de la méthode des proportions de Löwenstein-Jensen pour déterminer la résistance à l'égard de l'isoniazide (INH) et de la rifampicine (RMP) dans les isolats cliniques.

RÉSULTATS : Dans 192 échantillons, la sensibilité et la spécificité de la méthode de Griess pour la détection de la résistance à l'INH ont été respectivement de 99,1% et 100%. Elles ont été respectivement de 93,5% et 100% pour l'identification de la résistance à la RMP.

CONCLUSIONS : Outre ses sensibilité et spécificité élevées et la rapidité d'exécution, la méthode de Griess utilise des réactifs simples et peu coûteux et n'exige qu'un espace de travail réduit et une expérience technique minimale, fournissant donc un outil idéal de dépistage pour les contextes à faibles revenus où les taux de TB-MR sont élevés.

RESUMEN

SITIO : El tratamiento de tuberculosis multidrogo-resistente (TB-MDR) suele basarse sobre los resultados de preubas de sensibilidad (PS), por lo cual se busca metodos de PS rápidos y simples que se podría aplicar en paises de bajos recursos. Un método con tales caracteristicas es un examen colorimetrico de nitrata reductasa, el método Griess. En el Perú, donde la tasa de incidencia de TB es uno de las mas altas en America del Sur, el Instituto Nacional de Salud recien cumplió la validación del método Griess.

OBJECTIVO : Describir el proceso de validación del método directo Griess en el Instituto Nacional de Salud de Perú.

DESIGNO : Estudio prospectivo comparando la sensibilidad y especificidad del método directo Griess con el método de proporciones Löwenstein-Jensen para determinar la resistencia a isoniacida (INH) y rifampicina (RMP) en cepas clinicas.

RESULTADOS : Entre 192 cepas, la sensibilidad y especificidad del método Greiss para detectar resistencia a INH fue 99,1% y 100%, respectivamente. Para la identificación de resistencia a RMP, la sensibilidad y especificidad fue 93,5% y 100%, respectivamente.

CONCLUSIONES : El método Griess tiene una alta sensibilidad y especificidad y una demora corta en tener resultados ; ademas, utiliza reactivos simples y baratos y requiere poco espacio del laboratorio y habilidades tecnicas mínimas. Asi que el método Griess es un herramiento ideal para sitios con bajos recursos con tasas altas de TB-MDR.