COMUNICACIÓN BREVE

Utility of nitrate reductase assay for detection of multidrug-resistant *Mycobacterium tuberculosis* in a low resource setting

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Introduction. The performance of a drug susceptibility test may change when moving from the research stage to implementation on a population level in actual public health practice.

Objective. The performance of a rapid drug susceptibility test was described for detecting multidrug-resistant *Mycobacterium tuberculosis* when implemented in the routine workflow of a low-resource reference laboratory.

Materials and methods. A prospective study was done comparing the performance of the nitrate reductase assay with the conventional proportion method for rifampicin and isoniazid on 364 isolates were obtained from multidrug-resistant tuberculosis risk patients referred from diffrent Colombian laboratories.

Results. When compared with the proportion method, the nitrate reductase assay sensitivity was 86.8% and 84.9% for rifampicin and isoniazid, respectively, whereas nitrate reductase assay specificity was 100% for isoniazid and rifampicin. Nitrate reductase assay sensitivity was significantly higher when the age of isolate was less than 70 days. A sensitivity of 94.4% dropped to 78.1% for rifampicin resistance for fresh and old isolates, respectively (Fisher exact test, p=0.05). For isoniazid resistance using fresh and old isolates, 94.7% vs.74.3% sensitivities, were achieved (chi square test, p=0.03). The proportion of nitrate reductase assay ambiguous results was significantly higher in multidrug-resistant than in non-multidrug-resistant isolates (17.6% vs. 4.0%, chi square test, p<0.005).

Conclusions. The nitrate reductase assay demonstrated provided reliable results for antibiotic resistance. However, using old cultures leds to a higher proportion of false sensitive results; furthermore, the nitrate reductase assay capability to detect multidrug-resistant tuberculosis decreased due to a higher proportion of non-interpretable results.

Key words: *Mycobacterium tuberculosis*, drug resistance, microbial sensitivity tests, methods, nitrate reductase.

Utilidad del ensayo de nitrato reductasa en la detección de *Mycobacterium tuberculosis* multirresistente en caso de recursos limitados

Introducción. El rendimiento de cualquier prueba rápida de sensibilidad a medicamentos para *Mycobacterium tuberculosis* puede cambiar cuando se pasa de la etapa de investigación a su implementación en la práctica de salud pública.

Objetivo. Describir el rendimiento del ensayo de nitrato reductasa para detectar tuberculosis multirresistente en la rutina de trabajo de un laboratorio de referencia con recursos limitados.

Materiales y métodos. Se comparó prospectivamente la utilidad del ensayo de nitrato reductasa con el método de las proporciones para la detección de resistencia a isoniacida y rifampicina en aislamientos de pacientes con riesgo de multirresistencia.

Resultados. Comparando con el método de las proporciones, la sensibilidad del ensayo de nitrato reductasa fue de 86,8 % y 84,9 % para rifampicina e isoniacida, respectivamente; la especificidad fue 100 % para rifampicina e isoniacida. La sensibilidad del ensayo de nitrato reductasa fue significativamente mayor cuando se emplearon aislamientos en los cuales el tiempo entre la realización del cultivo y la inoculación del ensayo de nitrato reductasa no superaba 70 días: 94,3 % *Vs.* 78,1 % para resistencia a rifampicina usando aislamientos frescos o envejecidos, respectivamente; prueba exacta de Fisher, p=0,05; 94,4 % *Vs.* 74,3 % para resistencia a isoniacida usando aislamientos frescos o envejecidos, respectivamente, y prueba de ji al cuadrado, p=0,03. La proporción de resultados no interpretables en el ensayo de nitrato reductasa fue mayor en los aislamientos multirresistentes que en los otros

Conclusiones. La experiencia con el ensayo de nitrato reductasa demuestra que produce resultados resistentes confiables. El uso de cultivos envejecidos conduciría a una mayor proporción de resultados falsos sensibles, mientras que su capacidad para detectar multirresistencia disminuye a consecuencia de una mayor proporción de resultados no interpretables.

The spread of multidrug-resistant strains of *Mycobacterium tuberculosis* has become a major public health concern and poses a formidable challenge to tuberculosis control due to its complex diagnostic and treatment challenges. Conventional methods for drug susceptibility testing of *Mycobacterium tuberculosis*, such as the proportion method, the absolute-concentration method, and the resistance ratio method, are used globally but have a long turnaround time (1). The time lag poses a significant threat to the patient, the community and health care workers. The commercial liquid culture BACTEC MGIT 960 method is faster but requires costly equipment and substrate, and is not feasible in most resource-poor settings. Several other promising techniques, genetic as well as phenotypic, have been recently reported (2,3). One affordable option for rapid drug resistance detection is the nitrate reductase assay (NRA) that uses colorimetric detection of nitrite as an indication of growth. It requires neither elaborate equipment nor expensive substrates or reagents. A recent review (4) pointed out that although nitrate reductase assay has been shown to be highly sensitive and specific in the detection of rifampicin and isoniazid resistance, additional studies are required to determine its performance in the target population, i.e. a population in which multidrug-resistance tuberculosis is suspected. In a previous study carried out in our laboratory (5) , this technique was compared with other rapid drug susceptibility testing methods using a panel of 64 strains. The NRA appears as rapid, inexpensive, and easy to perform (5). Nevertheless, the performance characteristics of drug susceptibility tests may change when moving from the research stage to implementation at a population level in actual public health practice (6). The current study was designed to test the performance of nitrate reductase assay for rifampicin and isoniazid resistance detection in the routine workflow of a reference laboratory with limited resources. This laboratory routinely receives isolates from groups targeted for drug susceptibility testing, as defined by the National Tuberculosis Program norms.

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Materials and methods

Strains and inoculum preparation

The nitrate reductase assay was evaluated in 364 *Mycobacterium tuberculosis* complex isolates referred to the laboratory for drug susceptibility testing. They were obtained from patients who were at high risk of multidrug-resistant tuberculosis, as specified the National Tuberculosis Program norms, with the following critera: (1) contacts with multidrug-resistant tuberculosis, (2) tuberculosis patients co-infected with HIV, (3) exposure in institutions that have multidrug-resistant tuberculosis outbreaks or a high multidrug-resistant tuberculosis prevalence, (4) patients with history of previous tuberculosis treatment and (5) those who remain positive after two o more months of treatment. All clinical isolates were tested prospectively in a blind manner for both nitrate reductase assay and the proportion method on Lowenstein Jensen, which served as the reference standard. Colonies from Lowenstein Jensen were transferred to a tube containing 6-9 sterile glass beads and 3-4 ml of 7H9-S broth [(Middlebrook 7H9 broth base (Difco, Sparks, MD, USA; 4.7g per liter), with 0.2% glycerol, supplemented with 10% oleic-albumin-dextrose-catalase (OADC; Becton Dickinson, Sparks, MD, USA)]. Tubes were vigorously agitated and clumps were allowed to settle for 30 min. The supernatants were then adjusted with distilled water to equal the density of 1.0 Mc Farland standard for use in nitrate reductase and proportion method assays. The reference strain H37Rv was tested in parallel.

Antituberculous drugs

Rifampicin, isoniazid, ethambutol and streptomycin were obtained in a powdered formulation from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of isoniazid, ethambutol and streptomycin were prepared in deionized water at 10 g/L and rifampicin was prepared in dimethylsulfoxide at 20 g/L. Stock solutions were kept at $-20°C$ for no more than one month.

Proportion method

The proportion method was performed on Lowenstein Jensen medium according to Canetti *et al.* (1) with the recommended critical concentrations of 0.2 µg/ml for isoniazid, 40 µg/ml for rifampicin, 2 µg/ml for ethambutol and 4 µg/ml for streptomycin. The results were read for the first time on 28th

day. If this reading demonstrated resistance, no further readings were required. If the result of the first reading was susceptible, a second and final reading was made on day 42. The results from the proportion method served as the reference standard.

Nitrate reductase assay

The nitrate reductase assay was performed as described Angeby *et al.* (7). Briefly, the antibiotic was included in the Lowenstein Jensen medium at a concentration of: 0.2 μg/ml for isoniazid, 40 μg/ml for rifampicin, 2 μg/ml for ethambutol and 4 μg/ml for streptomycin; 1000 mg/L of KNO_3 was also added. Part of the inoculum was adjusted to equal the density of the No 1.0 McFarland standard and diluted 1:10 in distilled water. For each isolate, 0.2 ml of the undiluted inoculum was added into the tubes containing Lowenstein Jensen medium with KNO_3 and the anti-tuberculosis drugs; and 0.2 ml of the 1:10 dilution was inoculated into drug-free media containing KNO_3 (tubes in triplicate), which served as the controls. Tubes were incubated at 37°C for 14 days, and 0.5 ml of a mixture of three reagents (1 part 50% HCl, 2 parts 0.2% sulfanilamide and 2 parts 0.1% N-1-naphthylenthylenediamine dihydrochloride) was added to one drug-free control tube after 7 days of incubation. If the color changed to pink, then tubes with drugs were tested. An isolate was considered resistant if the color change in the drug-tube was greater than in the 1:10 diluted growth control on the same day. Drug-free control tubes that did not show any color change were further incubated and the procedure repeated at day 10 and day 14. Non-interpretable results were defined as those obtained when isolates failed to show any color change in the drug-free control tube even at day 14.

Data analysis

Statistical analyses were carried out with Epi Info version 6.04. The nitrate reductase assay/proportion method comparisons were evaluated in terms of sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility). To demonstrate the effect of the time period from specimen culture to nitrate reductase assay inoculation on the performance of this assay, the assays were separated in two groups, ≤70 and >70 days and the percent sensitivity compared. chi square or Fisher´s exact tests provided significance tests for between-groups distribution of discontinuous variables. Factors associated with non-interpretable nitrate reductase assay results

were also examined. Variables analyzed included multidrug-resistant status and time (in days) from the specimen culture to nitrate reductase assay inoculation (2 groups: ≤70 and >70 days). The odds ratios and 95% confidence intervals were estimated using binary logistic regression, with "interpretable nitrate reductase assay results" status as the outcome.

Results

A total of 364 specimens were received from 344 patients. Results of 39 isolates (10.7%) were unavailable using the conventional method because of failure to grow. Among the remaining 325 isolates, 81 (24.9%) were rifampicin-resistant and 88 (27.1%) isoniazid-resistant. Of the rifampicin-resistant strains, 91.4 % (74/81) were also resistant to isoniazid (multidrug-resistant); 14 strains were isoniazid-resistant and rifampicin-susceptible. Of the 325 isolates that had available results by the proportion method, 302 (92.9%) were interpretable by the nitrate reductase assay. The comparison data comparing the nitrate reductase assay with the conventional proportion method are shown in table 1. Nitrate reductase assay results in detecting multidrug-resistant and non-multidrug resistant strains were available in a median of 14 and 10 days from date of inoculation, respectively. Overall, the median time for the availability of the results was 10 days.

The sensitivity of the nitrate reductase assay was as follows: isoniazid (84.9%), streptomycin (58.8%), ethambutol (54.5%) and rifampicin (86.8%). The specificity was 100% for isoniazid and rifampicin, and higher than 99% for streptomycin and ethambutol (table 1). The overall agreement between the nitrate reductase assay and the proportion method was 96.5%.

Rifampicin and isoniazid sensitivities were much lower than anticipated from previous studies (4). Upon nitrate reductase assay repetition of those isolates formerly classified as false-susceptible, we found that using fresh subcultures of the 9 isolates classified as false-rifampicin susceptible strains, 4 could be classified as resistant. Similarly, 5 of the 11 false-isoniazid susceptible strains were re-classified as resistant. These data gave an new overall sensitivity of 92.6% (63/68) and 91.8% (67/73) for rifampicin and isoniazid, respectively. All of the re-classified false-susceptible results were formerly obtained using isolates more than 70 days intervened between specimen culture to assay inoculation. This suggested that the low

Table 1. Drug susceptibility testing of 302 *Mycobacterium tuberculosis* strains by the nitrate reductase assay and the proportion method.

Drug	Nitrate reductase assay	Proportion method		Agreement (%)
		Resistant	**Susceptible**	
Isoniazid	Resistant	62	0	96.4
	Susceptible	11	229	
Streptomycin	Resistant	20	2	94.7
	Susceptible	14	266	
Ethambutol	Resistant	6	1	98.0
	Susceptible	5	290	
Rifampicin	Resistant	59	0	97.0
	Susceptible	9	234	

levels of sensitivity previously obtained using the *Mycobacterium tuberculosis* isolates as they arrived (regardless of previous manipulation or culture maintenance conditions), appeared to be related to the the period from specimen culture to nitrate reductase assay inoculation. In summary, the rifampicin sensitivities were significantly higher (Fisher exact test, p=0.05) when analyzing isolates in which the days from specimen cultures to nitrate reductase assay inoculation were less than 70 [94.4% (34/36)] in comparison with those of more than 70 days [78.1% (25/32)]. For isoniazid resistance a similar data trend was seen [94.7% (36/38) vs.74.3% (26/35), chi square test, p=0.03)].

Overall, 23 of the 325 (7.1%) isolates gave nitrate reductase assay non-interpretable results. These non-interpretable results were more common among multidrug-resistant strains (13 of 74; 17.6%) than among non multidrug-resistant strains (10 of 251; 4.0%) (OR=4.7, 95%CI: 1.8-12.6). Furthermore, no associations were discerned between the time (in days) from the specimen culture to nitrate reductase assay inoculation (2 groups: ≤70 and >70 days) and the proportion of interpretable results (OR=0.8, 95%CI: 0.3-2.2).

Discussion

This study assessed the performance of the nitrate reductase assay on a population at risk of multidrug-resistant tuberculosis (prevalence of multidrug-resistant, 22.8%) in the routine workflow of a low-resource reference laboratory. It provided support for the findings of earlier studies made on isolates maintained at low temperatures (7-12), as the evaluation was made under more controlled methodological conditions. These conditions were specified as follows: (1) the study was performed in an appropriately broad group of patients with and without multidrug-resistant tuberculosis and in pertinent patients groups (without selection bias); (2) the nitrate reductase assay and the proportion method were made in all patients simultaneously (preventing verification bias); (3) all nitrate reductase assay results were interpreted by staff members who were unaware of the other test results, using an appropriate reference standard for comparison; and (4) in contrast with previous evaluations, in which bacilli to be tested were obtained from colonies from fresh subcultures in solid medium (5,7,8,11,12), the present study analyzed the isolates, as they arrived, regardless of previous manipulation or culture maintenance conditions.

Some isolates had been submitted from as far as Argentina and may have spent several days in transit. In other laboratories, isolates obtained at the time of diagnosis are sent for rapid identification of multidrug-resistant statis during the waiting period for incubation of sputa obtained from patients who remain positive after month 2 or more of treatment.. These factors explain, at least in part, the high proportion of old isolates included in the current study. No relationship was observed between the degree of positivity obtained by direct smear or culture, and isolate arrival time (data not shown). Recommendations have been made for inoculating drug susceptibility testing media by using dilutions of a standard inoculum prepared by scraping freshly grown colonies (of no more than four to five weeks old); however, in case of using older cultures, it have been deemed acceptable to prepare lower dilutions of the standard inoculum (13).

At the programmatic level, the most important susceptibility data that are likely to affect a change in therapy are those for the detection of multidrug-resistant tuberculosis. Delays in initiating

multidrug-resistant tuberculosis treatment where appropriate has serious consequences, especially if patients are attending the health facility every day to receive an ineffective treatment. During this period, multidrug-resistant tuberculosis may be transmitted to household contacts, other patients and health care personnel.

In the current study, the sensitivity of the nitrate reductase assay was 86.8% and 84.9% for rifampicin and isoniazid, respectively. These values are outside the published range of rifampicin and isoniazid sensitivities in a recent meta-analysis (88-100% for rifampicin sensitivity and 87-100% for isoniazid sensitivity); these low levels of sensitivities appear related to the period from specimen culture to nitrate reductase assay inoculation. Martin *et al.* (10), in their multicenter evaluation of this technique using a set of 30 isolates, stated that to perform nitrate reductase assay it is important to use fresh cultures. In this way, upon nitrate reductase assay repetition of all false rifampicin- and isoniazid-susceptible strains using fresh subcultures, the values of sensitivity increased up to 93% and 92% for rifampicin and isoniazid, respectively.

Overall, 7.1% isolates that had available results by the proportion method gave nitrate reductase assay non-interpretable results. After evaluating the factors related with nitrate reductase assay non-interpretable results, the multidrug-resistant strains were found to be associated with a higher proportion of nitrate reductase assay non-interpretable results, and these results were not related with the days from specimen cultures to nitrate reductase assay inoculation. This is not surprising, considering that the results of the nitrate reductase assay for multidrug-resistant isolates were available later than non- multidrug-resistant isolates. Isoniazid resistant strains have shown lower rates of multiplication than pan-sensitive ones and different requirements for optimal growth have also been reported for isoniazid-resistant isolates (14-16). This possibly explains, in part, their failure to grow after the 14-day period of nitrate reductase assay incubation. Moreover, the proportion method gave interpretable results; this excluded the possibility that the non-interpretable nitrate reductase assay results were associated to the use of a low bacilli inoculum, as both methods were made at the same time by diluting the same 1.0 McFarland standard inoculum.

The nitrate reductase assay provided reliable isoniazid and rifampicin positive (resistant) results within a mean of 14 days after receiving the patient isolate in the reference laboratory. In accordance with previous studies (5,7-12,17-19), its 100% specificity for rifampicin and isoniazid, avoids in a non-multidrug-resistant patient which is conducting a first-line drug treatment, any change to a treatment with second line drugs to that is more toxic, less effective and very expensive.

Susceptibility testing for *Mycobacterium tuberculosis* is complex and concordance among even regional laboratories performing reliable standard testing is particularly variable for ethambutol and streptomycin (20). The current findings with these two drugs agreed with previous and recent data for nitrate reductase assay (5,21,22), demonstrating insufficient concordance of the assay with the normally recommended standards to recommend them for routine use. Nonetheless, Rosales *et al.* (23) have recently evaluated the nitrate reductase assay for the rapid detection of resistance to second-line drugs such as ofloxacin and kanamycin. They showed a clear potential of nitrate reductase assay for prompt detection of extensively drug-resistant tuberculosis cases, although they stated the need of further studies to optimize the testing of second-line drugs.

In November 2009, the WHO Strategic and Technical Advisory Group for Tuberculosis stated that, based on the published evidence, the performance of non-commercial drug susceptibility tests were acceptable under stringent laboratory protocols when applied in reference/national laboratories in selected settings. Furthermore, they endorsed the selective use nitrate reductase assay for screening of patients suspected of having multidrug-resistant tuberculosis, again under clearly defined programmatic and operation conditions (24). As demonstrated herein, experience with this technique under field conditions confirms previous observations about nitrate reductase assay ability to provide reliable isoniazid and rifampicin resistant results. Nevertheless, when using it in routine laboratory diagnosis, the following factors influence the nitrate reductase assay capability to detect multidrug-resistant tuberculosis: (1) fresh cultures must be used (at least <70 days from specimen culture to nitrate reductase assay inoculation), and (2) multidrug-resistant isolates may be associated with a higher proportion of nitrate reductase assay non-interpretable results, thus resulting in a reduction of its capacity to detect multidrug-resistant tuberculosis.

Conflict of interests

The authors state that they have no conflict of interests.

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