INT J TUBERC LUNG DIS 15(9):1206–1210

http://dx.doi.org/10.5588/ijtld.10.0697

Field assessment of the direct nitrate reductase assay for rapid detection of multidrug-resistant tuberculosis in Honduras

S. Rosales,*†‡ N. Almendarez,§ H. Membreño,§ S. E. Hoffner,‡¶ L. Pineda-Garcia*

***Escuela de Microbiología, Universidad Nacional Autónoma de Honduras, Tegucigalpa, Honduras; †Division of Global Health, Department of Public Health Sciences, Karolinska Institutet, Stockholm, ‡Department of Preparedness, Swedish Institute for Communicable Disease Control (Smittskyddsinstitutet), Solna, Sweden; §Laboratorio Nacional de Referencia de Tuberculosis, Secretaria de Salud, Tegucigalpa, Honduras; ¶Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden**

SUMMARY

SETTING: The national TB reference laboratory and four health care units connected to the national laboratory network in Honduras, Central America.

OBJECTIVE: To evaluate the performance of the direct nitrate reductase assay (NRA) for rapid, low-cost detection of multidrug-resistant tuberculosis (MDR-TB) in a resource-limited setting.

DESIGN: Consecutive smear-positive samples ($n = 185$) were prospectively analysed with NRA and compared to the proportion method on Löwenstein Jensen medium (PM-LJ) to detect resistance to isoniazid (INH) and rifampicin (RMP).

RESULTS: The NRA sensitivity, specificity, positive and negative predictive values for INH and RMP were respectively 100%, 99%, 91%, 100% and 80%, 100%, 100%, 99%. Good agreement was observed between NRA and PM-LJ ($\kappa > 0.8$).

CONCLUSION: The direct NRA is a reliable alternative for rapid and low-cost identification of MDR-TB cases in resource-limited settings.

KEY WORDS: *Mycobacterium tuberculosis*; multidrug-resistant tuberculosis; NRA; drug susceptibility testing

NEW DIAGNOSTICS for a more rapid detection of drug-resistant tuberculosis (TB) are needed. The impact of rapid TB tests on the clinical outcome of patients, especially when multidrug-resistant TB (MDR-TB) is suspected, is undeniable. The timely detection of MDR-TB is of importance also from the viewpoint of public health as it allows the prompt implementation of adequate treatment and infection control measures to reduce the risk of transmission.

In November 2009, as a result of an evidence-based assessment, the World Health Organization decided to endorse three non-commercial culture and drug susceptibility testing (DST)[1] methods for implementation, mainly in low-/middle-income countries. One of these, the nitrate reductase assay (NRA), is based on the ability of *Mycobacterium tuberculosis* to reduce nitrate. Nitrate reduction is used as a growth indicator and is detected by a colour reaction.[2] The indirect NRA has repeatedly been proven to be highly sensitive (≥95%) and specific (≥99%) for the detection of resistance to isoniazid (INH) and rifampicin (RMP).[3] However, only limited information is available on direct NRA.[4–7]

Our objective was to evaluate direct NRA in the rapid detection of INH and RMP resistance under field conditions. The study was conducted in Honduras, a lower-middle-income country with moderate TB incidence (37 per 100 000 population in 2008), which uses passive case finding based on acid-fast microscopy. DST is only performed at the national reference laboratory (NRL) and only in patients at increased risk of drug-resistant TB (defaulters, retreatment and failure cases).[8]

MATERIALS AND METHODS

Study sites

Specimens were collected in coordination with the Microbiology School at the Universidad Nacional Autonoma de Honduras (UNAH) and the Honduran National TB NRL from March 2008 to June 2009. Four health care units in the main metropolitan areas of Tegucigalpa and San Pedro Sula, which have the highest numbers of TB cases nationwide, were selected for the study: the Instituto Nacional Cardiopulmonar (INCP) in Tegucigalpa, the Regional Laboratory of Cortes (RLC), the Mario Catarino Rivas

Correspondence to: Senia Rosales, Division of Global Health, Division of International Health (IHCAR), Department of Public Health Sciences, Karolinska Institutet, Nobels väg 9, SE 171 77, Stockholm, Sweden. Tel: (+46) 8 524 833 69. Fax: (+46) 8 31 15 90. e-mail: seniarosales@gmail.com

Article submitted 4 November 2010. Final version accepted 18 February 2011.

Hospital and the Miguel Paz Barahona Healthcare Center in San Pedro Sula.

The study was approved by the Ethics Committees of UNAH and INCP.

Sample size

Based on the number of smear-positive TB patients reported in Tegucigalpa and San Pedro Sula in 2004–2006, the total study population was estimated at 680 cases. The proportion of MDR-TB among new cases was estimated to be 2%.[9] A sample size of 148 sputum samples was calculated to achieve a 95% confidence level, with an error of 2%. The sample size was increased to 185 to compensate for contamination or lack of growth.

Specimens

Consecutive smear-positive sputum samples were included in the study. Study samples were provided by new or previously treated TB patients for routine examination. Only one sample per patient was included and no personal identifiers were registered.

Specimen processing

All sputum samples were examined by Ziehl-Neelsen (ZN) microscopy. Those with a positive smear were selected and processed by the modified Petroff method.[10] The specimens collected in San Pedro Sula were decontaminated in situ and stored at −20°C. Decontaminated sediments were subsequently transported in batches, typically every second week, to the NRL in Tegucigalpa, where NRA and conventional DST with the proportion method (PM) on Löwenstein-Jensen (LJ) medium were performed. Samples collected at the INCP were decontaminated and stored at −20°C until culture on LJ and NRA media.

The sediments was resuspended in 2 ml sterile distilled water and used for culture inoculation as well as for preparation of new smears. All smears were graded according to the scoring scale recommended by the Pan American Health Organization for direct smear testing.[11]

NRA

Direct NRA was performed based on the protocol described by Musa et al.,[5] with an increased critical concentration of RMP and a slight modification in reading time points. The LJ medium containing potassium nitrate (1000 mg KNO_3/l) was prepared at the NRL, with critical concentrations of 0.2 mg/l for INH and 40.0 mg/l for RMP. A 1:10 dilution of the resuspended sediment was prepared using sterile distilled water. Three drug-free LJ-KNO_3 growth control tubes were inoculated with 0.2 ml of the 1:10 dilution. Drug-containing tubes were inoculated with 0.2 ml of the undiluted suspension. All tubes were incubated at 37°C. After 10 days, 0.5 ml of freshly prepared Griess reagent consisting of 1 part 50% (vol/vol) hydrochloric acid, 2 parts 0.2% (wt/vol) sulfanilamide and 2 parts 0.1% (wt/vol) n-1-naphtylethylenediamine dihydrochloride were added to one of the growth controls. If a colour change was observed, the Griess reagent was also added to the LJ slopes containing drugs. If not, the tubes were re-incubated, and the procedure repeated at day 14 and, if necessary, also at day 28.

An isolate was considered resistant if any colour change was observed in the drug-containing tube. If the control was negative at day 28, the NRA was considered invalid. The NRA results were interpreted without the knowledge of the PM results.

M. tuberculosis *isolation and identification*

Two LJ slopes without KNO_3 or drugs were inoculated with 0.2 ml of the undiluted, suspended sediment and incubated at 37°C up to 8 weeks or until visible growth was observed. The isolates were confirmed to belong to the *M. tuberculosis* complex by biochemical tests (niacin production, catalase activity and nitrate reduction).[10]

Reference technique

Indirect PM on LJ[12] performed at the NRL was used as reference DST. Critical concentrations used were respectively 0.2 and 40.0 mg/l for INH and RMP. If no growth was observed in the control tubes after 42 days of incubation, the test was invalid. PM results were read without the knowledge of NRA results.

Quality assurance

Each batch of LJ medium (with or without KNO_3 and/or drugs) was tested for sterility and for the detection of resistance using the drug-susceptible *M. tuberculosis* H37Rv strain and a clinical MDR-TB isolate.

Discrepant results

For the resolution of discrepant results, three *M. tuberculosis* isolates (1 false INH-resistant, 1 false RMP-susceptible and 1 MDR-TB without PM result) were sent to the Smittskyddsinstitutet (SMI), where DNA extraction was performed according to a standard protocol.[13] Sequencing of the *rpo*B and *kat*G genes, as well as the regulatory region of the *inh*A gene, was performed.

Data analysis

Data were compiled in a Microsoft Office Excel® 2007 (Microsoft, Redmond, WA, USA) spreadsheet and analysed using Epi Info™ version 3.5.1 (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA) and the open software Open Epi version 2.3 (CDC).[14] The diagnostic parameters used to evaluate NRA performance were sensitivity (ability to detect true resistance), specificity (ability to detect true susceptibility), and positive and negative predictive

values (proportion of resistant and susceptible results correctly diagnosed). The 95% confidence intervals (95%CIs) were calculated for each outcome measure.[15]

The level of agreement between NRA and the PM was determined by McNemar's χ^2 test and the kappa coefficient, with the following scale to interpret κ values: <0, less than chance agreement; 0.01–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; ⩾0.81, very good.[15] The contamination proportions of NRA and LJ cultures and the NRA time to DST results were also analysed.

RESULTS

A total of 185 samples were tested using NRA; of these, 141 had interpretable results. The description of samples included in the final analysis is shown in Figure 1. The majority of the samples (54%) were provided by TB patients diagnosed in San Pedro Sula. A total of 41 smear-positive samples came from previously treated patients. Of these, 12 had invalid NRA results, 1 was contaminated, 25 yielded susceptible NRA results and 3 were NRA-resistant (2 MDR-TB, 1 INH-resistant).

Three non-tuberculous mycobacteria (NTM) were identified in this study. One was a niacin-negative strain with nitrate reductase activity; the remainder were nitrate reductase-negative. All of the isolates confirmed as belonging to the *M. tuberculosis* complex were nitrate reductase-positive.

The proportion of contaminated tests observed was low. In total, we observed two contaminated samples for NRA (1.1%) and five for conventional LJ cultures (2.7%). Overall agreement between direct NRA and indirect PM was very good. For both drugs tested, the κ coefficient was >0.81, with no statistically significant difference between the methods used (McNemar's, $P > 0.05$). Compared with PM, one isolate was false-resistant for INH and another false-susceptible for RMP using NRA (Table). No mutations in the *kat*G gene or in the *inh*A gene regulatory region were found in the false INH-resistant isolate. Although the lack of these mutations does not prove susceptibility, this finding supports the classification as INH-susceptible. Sequencing testing could not be performed on the false RMP-susceptible strain. One of the five isolates found to be MDR-TB using NRA did not yield interpretable results with PM due to insufficient growth. However, the sequencing analysis detected mutations in the *rpo*B (Ser531Phe) and *kat*G (Ser 315 Thr) genes. This isolate was therefore considered confirmed as MDR-TB and included in the final analysis.

Among the 141 samples with interpretable NRA results, 33 had results available in 10 days (23%) and 47 at day 14 (33%). Results for the remaining 61 samples were available at day 28. The delay in NRA testing of most of the samples influenced the time to results. As shown in Figure 2, samples processed

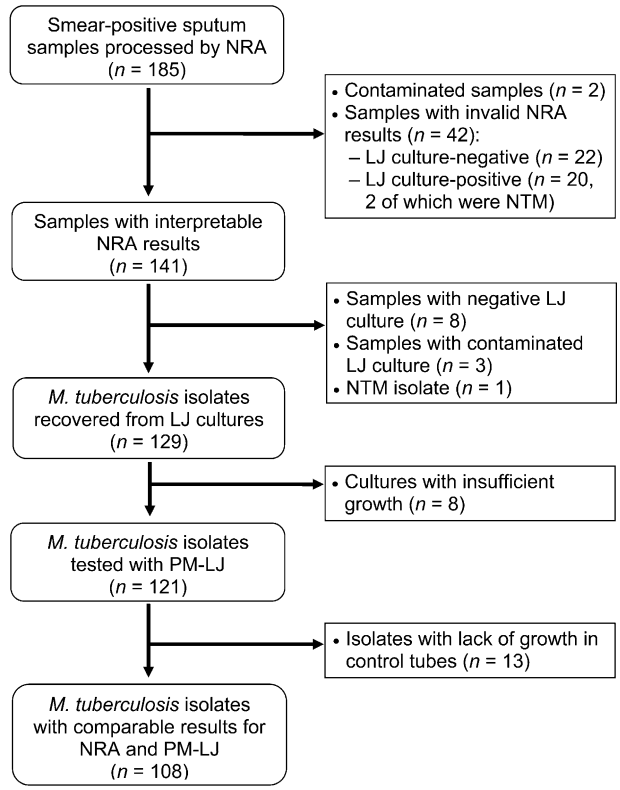

Figure 1 Flow diagram of the samples included in the study. NRA = nitrate reductase assay; LJ = Löwenstein Jensen; NTM = non-tuberculous mycobacteria; PM = proportion method.

Table Detection of resistance to INH and RMP by NRA and PM-LJ (n = 108)

				NRA performance				
		PM-LJ results		Sensitivity %	Specificity %	PPV %	NPV %	κ
Drug	NRA results	Resistant	Susceptible	(95%CI)	(95%CI)	(95%CI)	(95%CI)	(95%CI)
INH	Resistant	10	1	100	99	91	100	0.95
	Susceptible	0	97	(72–100)	(94–100)	(62–98)	(96–100)	(0.76–1.14)
RMP	Resistant	4	0	80	100	100	99	0.88
	Susceptible	1	103	(38–96)	(96–100)	(51–100)	(95–100)	(0.69–1.07)

INH = isoniazid; RMP = rifampicin; NRA = nitrate reductase assay; PM-LJ = proportion method on Löwenstein-Jensen; PPV = positive predictive value; NPV = negative predictive value; CI = confidence interval.

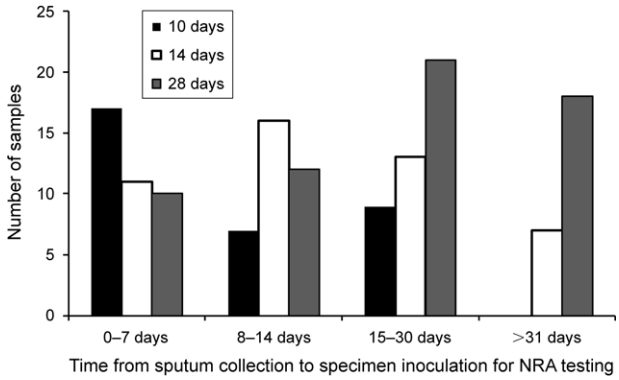

Figure 2 Effect of storage time before sample processing. NRA = nitrate reductase assay.

during the first 7 days after collection were more likely to have NRA results at day 10.

Results of samples stored for up to 15 days differed from those stored for a longer period. As expected, lower culture positivity was seen after long storage. This effect was even more pronounced for samples with scanty positive smear results. Most samples with more than 15 days storage had results available only by day 28.

DISCUSSION

In most evaluations of new techniques for improved laboratory detection of, for example, drug-resistant TB, the test protocol and conditions are optimised; results obtained might be hard to repeat and maintain in routine diagnostic conditions. In this study, on the contrary, field conditions in sampling and logistics significantly delayed transportation of specimens to the NRL. Conditions were clearly not suitable for demonstrating the potential of the rapid test. Both the proportion of interpretable tests and the time to DST results were not as good as they could have been in optimal settings. Given that, in most evaluations, good test results could be difficult to repeat and sustain, in our opinion this test would give better results if implemented after necessary infrastructure improvements.

The main aim of the field assessment was to determine the performance of direct NRA in detecting resistance to INH and RMP in smear-positive sputum samples within the framework of a laboratory system in a lower-middle-income country. Despite the limitations discussed above, in terms of diagnostic performance, the direct NRA demonstrated very good agreement with conventional indirect DST, and offered more rapid detection of drug resistance.

Few evaluations of direct NRA have been reported.[4–6,16] For RMP, sensitivity has ranged from 88% to 100% (pooled sensitivity >94%), whereas for INH the sensitivity has been between 93% and 100%. The specificity for both drugs has been excellent, >99%.[3,17,18] Our results for INH are in line with earlier studies. Regarding the relatively low sensitivity for RMP (80%), it must be taken into consideration that our study only included five RMP-resistant isolates, of which one was misclassified.

In this study, 56% of the NRA results were available at day 14 after inoculation. Similar results have been reported elsewhere.[5,6] In contrast, a study from Brazil reported 87% of results available at day 15. This difference may be explained by differences in the bacillary load of the inoculum. In samples with concentrated smears 1+ and 2+, we observed that respectively 30% and 48% had an interpretable NRA result at day 14. In the Brazilian study, the percentages of NRA results obtained at day 15 were slightly higher, respectively 69% and 79%, for smear positive 1+ and 2+. The type of samples included (high vs. low bacillary load), the final volume of the sediment (1–3 ml) and the number and time points for readings may have influenced the results. Nevertheless, a noteworthy feature of NRA is its capacity to provide reliable DST results in samples with low bacillary load.

NRA proved to be highly specific for *M. tuberculosis* complex. However, detection of nitrate-reductase positive NTM cannot be excluded, as was observed in one case. *P*-nitrobenzoic acid could be added to the NRA medium using growth inhibition as a way of confirming the presence of *M. tuberculosis* complex in the samples.[19] This possibility requires further assessment.

As already stated, we believe that some limitations observed in our study are related to weaknesses in the infrastructure. For example, the high proportion of NRA results obtained only at day 28 and the invalid results were most likely related to time delays in specimen processing. Typically, the transportation of samples from San Pedro Sula, located approximately 250 km from the NRL in Tegucigalpa, was for practical reasons delayed for more than 2 weeks. It should be remembered that the technique is not aimed at studying frozen samples, but should be carried out directly on fresh clinical specimens to ensure rapidity.

We cannot fully rule out other shortcomings, such as the decontamination procedure. Even at the NRL, it was challenging to cope with the increased demand for DST. The need to consider infrastructure improvements before the roll-out of a new DST method was clearly felt.

Experiences from Peru have been carefully documented[20,21] to demonstrate that the impact of a new technique relies not only in the proof-of-principle of the test or the time to deliver results: the overall turnaround time is a combination of technical procedures and operational delays found in health service systems with weak infrastructures.[22] Strengthening the laboratory network should therefore be a priority. The political commitment to ensure appropriate funding is crucial in adapting improved laboratory

techniques.[20] Clear guidelines and procurement, timely delivery of supplies, personnel training[21,22] and innovative solutions to ensure efficient specimen transportation[23] are key factors in optimising the benefits of any new, rapid and low-cost DST techniques.

CONCLUSIONS

Direct NRA proved an interesting alternative for rapid identification of MDR-TB. Both the specificity and sensitivity were in line with previous reports for the indirect, and more time-consuming, NRA. An important conclusion for broad implementation of this and other rapid tests in low- or middle-income countries was the necessity to adapt the infrastructure and logistical conditions needed for the test to show its full potential. This must be considered also in Honduras if and when direct NRA is considered for implementation in the laboratory network.

Acknowledgements

The authors thank P Castro (Instituto Nacional Cardiopulmonar), W Sosa (national reference laboratory), C A Rodriguez and C Lanza (Universidad Nacional Autonoma de Honduras) for their technical assistance; N Amador, M Talavera, E Vargas, A X Carbajal, V Rostran, A Andara, F Antunez, M Osorio and the staff of the Honduran National TB Laboratory Network for their collaboration. This study was supported by the Swedish International Development Cooperation Agency, contribution no. 75007345.

References

1 World Health Organization. Strategic and technical advisory group for tuberculosis. Report of the ninth meeting. Geneva, Switzerland: WHO, 2009.
2 Angeby K A, Klintz L, Hoffner S E. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. J Clin Microbiol 2002; 40: 553–555.
3 Martin A, Panaiotov S, Portaels F, et al. The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J Antimicrob Chemother 2008; 62: 56–64.
4 Solis L A, Shin S S, Han L L, et al. Validation of a rapid method for detection of *M. tuberculosis* resistance to isoniazid and rifampin in Lima, Peru. Int J Tuberc Lung Dis 2005; 9: 760–764.
5 Musa H R, Ambroggi M, Souto A, Angeby K A. Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. J Clin Microbiol 2005; 43: 3159–3161.
6 Affolabi D, Odoun M, Martin A, et al. Evaluation of direct detection of *Mycobacterium tuberculosis* rifampin resistance by a nitrate reductase assay applied to sputum samples in Cotonou, Benin. J Clin Microbiol 2007; 45: 2123–2125.
7 Shikama Mde L, Silva R R, Martins M C, et al. Rapid detection of resistant tuberculosis by nitrate reductase assay performed in three settings in Brazil. J Antimicrob Chemother 2009; 64: 794–796.
8 Varela Martinez C. Plan estrategico nacional para el control de la tuberculosis. PENTB 2009–2015. Resumen. Rev Med Hondur 2010; 78: 39–48. [Spanish]
9 World Health Organization. Anti-tuberculosis drug resistance in the world. Report no 3. WHO/HTM/TB/2004.343. Geneva, Switzerland. WHO, 2004.
10 Kent P T, Kubica G P. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, USA: US Department of Health and Human Services, Centers for Disease Control and Prevention, 1985.
11 Organización Panamericana de la Salud. Manual para el diagnóstico bacteriológico de la tuberculosis. Parte 1: baciloscopia. Washington DC, USA: OPS, 2008. [Spanish]
12 Canetti G, Froman S, Grosset J, Hauduroy P. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ 1963; 29: 565–568.
13 Jureen P, Werngren J, Hoffner S E. Evaluation of the line probe assay (LiPA) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2004; 84: 311–316.
14 Dean A G, Sullivan K M, Soe M M. Open source epidemiologic statistics for public health 2010. Atlanta, GA, USA: Centers for Disease Control and Prevention, 2009. www.OpenEpi.com Accessed May 2011.
15 Elston R C, Johnson W D. Basic biostatistics for geneticists and epidemiologists: a practical approach. Chichester, UK: John Wiley and Sons, 2008.
16 Shikama M L, Ferro e Silva R, Villela G, et al. Multicentre study of nitrate reductase assay for rapid detection of rifampicin-resistant *M. tuberculosis*. Int J Tuberc Lung Dis 2009; 13: 377–380.
17 Bwanga F, Hoffner S, Haile M, Joloba M L. Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. BMC Infect Dis 2009; 9: 67.
18 Chang K C, Yew W W, Zhang Y. A systematic review of rapid drug susceptibility tests for multidrug-resistant tuberculosis using rifampin resistance as a surrogate. Expert Opin Med Diagn 2009; 3: 24.
19 Giampaglia C M S, Martins M C, Inumaru V T G, Butuem I V, Telles M A S. Evaluation of a rapid differentiation test for the *Mycobacterium tuberculosis* complex by selective inhibition with ρ-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. Int J Tuberc Lung Dis 2005; 9: 206–209.
20 Shin S S, Yagui M, Ascencios L, et al. Scale-up of multidrug-resistant tuberculosis laboratory services, Peru. Emerg Infect Dis 2008; 14: 701–708.
21 Griffin A M, Caviedes L, Gilman R, et al. Field and laboratory preparedness: challenges to rolling out new multidrug-resistant tuberculosis diagnostics. Rev Panam Salud Pública 2009; 26: 120–127.
22 Yagui M, Perales M T, Asencios L, et al. Timely diagnosis of MDR-TB under program conditions: is rapid drug susceptibility testing sufficient? Int J Tuberc Lung Dis 2006; 10: 838–843.
23 Harries A D, Michongwe J, Nyirenda T E, et al. Using a bus service for transporting sputum specimens to the Central Reference Laboratory: effect on the routine TB culture service in Malawi. Int J Tuberc Lung Dis 2004; 8: 204–210.

RÉSUMÉ

CONTEXTE : Le Laboratoire National de Référence de la Tuberculose (TB) ainsi que quatre unités de Soins de Santé connectées au Réseau National de Laboratoires au Honduras, Amérique Centrale.

OBJECTIF : Evaluer dans un contexte à ressources limitées la performance du test direct de nitrate réductase (NRA) pour la détection rapide et peu coûteuse de la TB à germes multirésistants (TB-MDR).

SCHÉMA : On a analysé de manière prospective 185 échantillons consécutifs à bacilloscopie positive des frottis au moyen de la NRA et on en a comparé les résultats avec ceux de la méthode des proportions sur milieu de Löwenstein-Jensen (PM-LJ) pour la détection de la résistance à l'égard de l'isoniazide (INH) et de la rifampicine (RMP).

RÉSULTATS : La sensibilité de la NRA a été de 100% pour l'INH et de 80% pour la RMP, la spécificité étant de 99% et de 100% et la valeur prédictive négative de 100% et de 99%. On a observé une bonne concordance entre NRA et PM-LJ ($\kappa > 0,8$).

CONCLUSION : Dans des contextes à ressources limitées, la NRA directe constitue une alternative fiable pour une identification rapide et peu coûteuse des cas de TB-MDR.

RESUMEN

MARCO DE REFERENCIA: El laboratorio Nacional de Referencia de Tuberculosis y cuatro centros de salud integrantes de la Red Nacional de Laboratorios en Honduras, América Central.

OBJETIVO: Evaluar la prueba de nitrato reductasa (NRA) directa para la detección rápida y de bajo costo de tuberculosis multirresistente (TB-MDR) en un establecimiento de escasos recursos.

DISEÑO: Muestras consecutivas de esputo con baciloscopía positiva ($n = 185$) fueron analizadas prospectivamente con NRA y comparadas con el método de las proporciones en medio Löwenstein Jensen (PM-LJ) para la detección de la resistencia a isoniazida (INH) y rifampicina (RMP).

RESULTADOS: La sensibilidad, especificidad, valores predictivos positivo y negativo de NRA para INH y RMP fueron 100%, 99%, 91%, 100% y 80%, 100%, 100%, 99%, respectivamente. Se observó una buena concordancia entre NRA y PM-LJ ($\kappa > 0.8$).

CONCLUSION: La NRA directa es una alternativa confiable para la identificación rápida y de bajo costo de casos de TB-MDR en lugares de escasos recursos.