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Detection of Drug Resistant Mycobacterium Tuberculosis Strains Using Kit SIRE Nitratase®: a Multicenter Study

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HIGHLIGHTS

- The Kit SIRE Nitratase® is the only commercial test using the Nitrate Reductase Assay
- It is a know-how transference from the public university to the national industry.
- It can be implanted in the health system in countries with high TB burden
- Highlighting the good accuracy, less time to results and less laborious.

Abstract: (1) Background: The Commercial Kit SIRE Nitratase® PlastLabor, is a drug susceptibility test kit used to detect *Mycobacterium tuberculosis* resistance to first-line TB treatment drugs. The present study aimed at evaluating its performance in a multicenter study. (2) Methods: To determine its accuracy, the proportion methods in Lowenstein Jensen medium or the BACTEC™MGIT™960 system was used as a gold standard. (3) Results: The study revealed that the respective accuracies of the kit with 190 *M. tuberculosis* clinical isolates, using the proportion methods in Lowenstein Jensen medium or BACTEC™MGIT™960 system as a gold standard, were 93.9% and 94.6%, 96.9% and 94.6%, 98.0% and 97.8%, and 98.0% and 98.9%, for streptomycin, isoniazid, rifampicin, and ethambutol, respectively. (4) Conclusion: Thus, the kit can rapidly screen resistance to streptomycin, isoniazid, rifampicin, and ethambutol. Additionally, it does not require sophisticated equipment; hence, it can be easily used in the laboratories of low and middle income countries.

Keywords: tuberculosis, MDR, nitrate reductase, technology

INTRODUCTION

Multidrug resistant tuberculosis (MDR-TB) is a threat to TB control worldwide. In 2017 the total number of MDR-TB cases recorded in Brazil was 86.858, the incidence of TB associated with HIV was 44/100.000, the estimated number of MDR/RR-TB (Rifampicin Resistant Tuberculosis) cases was 2000 (among all notified pulmonary TB cases), and the number of laboratory confirmed Extensively Drug Resistant Tuberculosis (XDR-TB) cases was 16 [1]. Therefore, the accurate identification and drug susceptibility testing (DST) of all *Mycobacterium tuberculosis* (*M. tuberculosis*) strains is crucial in choosing the proper therapy, achieving drug resistance surveillance, and reducing disease transmission [1].

Although DST methods based on liquid mediums are very efficient and effective in detecting *M. tuberculosis* in clinical samples, their high cost hinders their widespread implementation [1]. In 2009, the World Health Organization (WHO) recommended the use of the nitrate reductase assay (NRA), which is a fast DST methodology based on the capacity of *M. tuberculosis* to reduce nitrates to nitrites that can be detected using the Griess reagent, for rifampicin and isoniazid resistance screening in patients with suspected MDR-TB, under clearly defined programmed and operational conditions [2-4]

In 2012, a preliminary study on NRA-DST was conducted in the Mycobacteria Research Laboratory of the Federal University of Minas Gerais (FUMG), and the results showed that it had an excellent performance, which was consistent with the results of other studies [5-7]. After this study, a consortium was formed between the Brazilian Tuberculosis Research Network (REDE-TB) at FUMG and PlastLabor® (Rio de Janeiro, Brazil),

which aimed to develop a commercial test kit called Kit SIRE Nitratase®, for the DST of first-line TB drugs against *M. tuberculosis* strains, based on the NRA.

The kit is a commercial test tool containing the reagents required to perform NRA-DST, in order to determine bacterial susceptibility to streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB). It comprises three glass tubes containing a solid Lowenstein Jensen (LJ) medium incorporated with potassium nitrate (KNO₃) for growth control, four tubes containing LJ + KNO₃ and the incorporated SIRE drugs, and Griess reagents for the development of results [8,9].

The kit is manufactured following good manufacturing practice standards and quality control parameters, in accordance with ISO 9001-2015, and is duly registered with the National Health Surveillance Agency of Brazil (ANVISA – Registration number: 80035670010) [8,9].

Therefore, this study aimed at evaluating its performance in a multicenter study.

MATERIAL AND METHODS

Participating laboratories

This study was conducted at the following laboratories in Brazil: *Instituto Adolfo Lutz, São Paulo; Centro de Referência Hélio Fraga, Fundação Oswaldo Cruz, Rio de Janeiro; Instituto de Doença do Tórax da Universidade Federal do Rio de Janeiro, Rio de Janeiro; Instituto Nacional de Doenças Infecciosas, Fundação Oswaldo Cruz, Rio de Janeiro; Instituto Evandro Chagas, Pará; Fundação de Medicina Tropical, Amazonas; Centro de Desenvolvimento Científico e Tecnológico, Centro Estadual de Vigilância em Saúde (CEVS), Secretaria Estadual da Saúde do Rio Grande do Sul (SESRS), Rio Grande do Sul; Laboratório de Pesquisa em Ciências da Saúde, Faculdade de Medicina da Universidade Federal da Grande Dourados, Mato Grosso do Sul. In Portugal, the kit was tested at the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa.*

Clinical Isolates

Each site tested its own panel of *M. tuberculosis* clinical isolates (only clinically confirmed cases of pulmonary TB), including both susceptible and resistant isolates. This resulted in the inclusion of a total of 258 isolate samples in the study, of which 66, 83, 65, and 44 were resistant to STR, INH, RIF, and EMB, respectively. Additionally, among these isolates, 61 were MDR, and the H37Rv reference strain was tested as a positive control at all sites.

Drug Susceptibility Test

Based on the method used at each site, the proportion methods (PM) using LJ or the BACTEC™ MGIT™ 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) (MGIT), were employed as gold standards. The proportion method in LJ medium was performed using the recommended critical concentrations: 4, 0.2, 40, and 2 µg/mL for STR, INH, RIF, and EMB, respectively [10,11], while the MGIT method proportion method used the critical concentrations: 1, 0.1, 1 and 5 µg/mL for STR, INH, RIF, and EMB, respectively [12].

Drug susceptibility testing using Kit SIRE Nitratase®

DST using Kit SIRE Nitratase®, was performed according to manufacturer's instructions [8]. The isolates to be tested were subcultured in an LJ medium for two to three weeks at 37 °C. The inoculum was prepared from LJ by scraping a loop full of colonies into a test tube containing sterile deionized water and glass beads. The bacterial suspension was vortexed and allowed to sediment for approximately 10 min. The supernatant was transferred to another tube, and the concentration of the inoculum was adjusted to a N° 1 McFarland tube, which corresponded to the undiluted inoculum. The suspension was further diluted (1:10) in sterile distilled water, resulting in a 1:10 inoculum. To perform the test, 200 µL of the undiluted inoculum was inoculated into antibiotic tubes containing KNO₃. The critical antibiotic concentrations used were 4, 0.2, 40, and 2 µg/mL for STR, INH, RIF, and EMB, respectively.

Also, three drug-free growth control tubes were inoculated with 200 µL of the 1:10 inoculum. All tubes were incubated at 37 °C for seven days, after which, 500 µL of the reagent mix (1 part, 50% (vol/vol) concentrated hydrochloric acid (HCl); two parts, 0.2% (wt/vol) sulfanilamide; and two parts, 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride) was added to one growth control tube.

If a color change (varying from pink to dark red) was observed, it implied that nitrates had been reduced to nitrites, and that the test was positive. Thus, all the remaining tubes containing isolates were identified using the reagent mixture. If no color appeared in the growth control tube at day seven, the remaining tubes were re-incubated, and the identification test repeated at day 10 and 14. An isolate was considered resistant to the antibiotic at its critical concentration if the color change in the tube containing the drug was dark red, and was similar to the color observed in the 1:10 inoculum growth control tube [8,9]. All the Kit SIRE Nitratase[®] components are shown in Figure 1.



Figure 1. The Kit SIRE Nitratase[®] components

Legend: (a) = Kit SIRE Nitratase[®] complete; (b) = Control tubes (without drug); (c) = SIRE tubes: a LJ+KNO₃ medium with S (STR): streptomycin, I (INH): isoniazid, R (RIF): rifampicin, E (EMB): ethambutol, respectively.

Statistical Analyses

The specificity, sensitivity, accuracy, as well as the kappa values of the results obtained at the different study centers were calculated using Godoy and Braile, version 1999 [13]. To compare the LJ and the MGIT PM accuracy of Kit SIRE Nitratase[®], STATA/MF software v12 (Copyright 1985-2015; StataCorpLP[®], USA) was used.

Repeatability of Kit SIRE Nitratase[®] with proficiency strains

The repeatability of Kit SIRE Nitratase[®] was evaluated using 24 proficiency *M. tuberculosis* strains, in accordance with international standards [14,15]. Of these 24 strains, 10 were resistant to STR, 10 to INH, 14 to RIF, and three to EMB; one presented the H37Rv reference strain (positive control). The evaluation was performed at the Hélio Fraga Reference Center, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The same technician performed these tests on three consecutive days, using three different kits. Agreement between the tests were determined using the triple Kappa test application of the STATA/MF software v12 (Copyright 1985-2015; StataCorpLP[®], USA).

Ethical approval

This study was approved by the Research Ethics Committee of the Minas Gerais Hospital Foundation (technical report number, 018B/20; UFMG Ethics Committee protocol numbers, CAAE-11821913.6.000.5257 and CAAE 0223.2412.7.1001.5149; and DEPE/HC protocol number, 139/12).

RESULTS

For a total of 190 *M. tuberculosis* clinical isolates, out of the 258 that were included in the study, valid results were obtained using Kit SIRE Nitratase®. The panel was then split in two, with 98 and 92 clinical isolates evaluated using the LJ (Table 1) and the MGIT method proportion methods (Table 2) as gold standards, respectively.

Table 1. Drug susceptibility testing using Kit SIRE Nitratase®; sensitivity, specificity, and accuracy using the Lowenstein Jensen medium as a standard (n = 98)

Drug	Drug susceptibility profile by NRA	Proportion Method using Lowenstein Jensen		Sensitivity (%)	Specificity (%)	Accuracy (%)
		Resistant	Susceptible			
Streptomycin ^a	Resistant	33	6	100	90.8	93.9
	Susceptible	0	59			
Isoniazid ^b	Resistant	41	3	100	94.7	96.9
	Susceptible	0	54			
Rifampicin ^c	Resistant	28	0	93.3	100	98
	Susceptible	2	68			
Ethambutol ^d	Resistant	17	0	89.5	100	98
	Susceptible	2	79			

Legend: a: Kappa = 0.87 (very good); b: Kappa = 0.94 (very good); c: Kappa = 0.95 (very good); d: Kappa = 0.93 (very good). Included sites: Pará/IEC, Manaus/FMT, IPEC/Fiocruz, IDT/UFRJ, and CDCT/RS.

Table 2. Drug susceptibility testing using Kit SIRE Nitratase®; sensitivity, specificity, and accuracy using the BACTEC™ MGIT™ 960 system as a standard (n = 92)

Drug	Drug susceptibility profile by NRA	BACTEC MGIT 960		Sensitivity (%)	Specificity (%)	Accuracy (%)
		Resistant	Susceptible			
Streptomycin ^a	Resistant	33	5	100	91.5	94.6
	Susceptible	0	54			
Isoniazid ^b	Resistant	38	0	88.4	100	94.6
	Susceptible	5	49			
Rifampicin ^c	Resistant	33	0	94.3	100	97.8
	Susceptible	2	57			
Ethambutol ^d	Resistant	25	1	100	98.5	98.9
	Susceptible	0	66			

Legend: a: Kappa = 0.89 (very good); b: Kappa = 0.89 (very good); c: Kappa = 0.95 (very good); d: Kappa = 0.97 (very good). Included sites: Adolfo Lutz/SP, CRPHF/Fiocruz, Dourados/UFGD, and IHMT/Lisboa.

No statistically significant differences were observed when the accuracy of Kit SIRE Nitratase® obtained using the LJ or the MGIT PM systems was compared: STR, $p = 0.8393$; INH, $p = 0.4156$; RIF, $p = 0.9491$; and EMB, $p = 0.5981$. When evaluating the agreement of each drug at each site, the kappa values ranged from 0.71 (good) to 1.00 (very good) for STR; 0.76 (good) to 1.00 (very good) for INH; 0.64 (good) to 1.00 (very good) for RIF; and 0.51 (weak) to 0.86 (very good) for EMB, as shown in Table 3.

Table 3. The agreement rates according to the site of Kit SIRE Nitratase® use

Kappa Agreement values*				
Standard Method - Proportion Method using the BACTEC™MGIT™960 system				
Site	S	I	R	E
IAL/SP	0.82	1.00	1.00	0.83
CRHF/RJ	0.80	0.92	0.91	0.78
LPCS/MS	0.69	0.89	0.64	----- ^a
IHMT/PT	0.83	1.00	1.00	0.82

Kappa Agreement values*				
Standard Method - Proportion Method using the Lowenstein Jensen medium				
Site	S	I	R	E
IEC/PA	1.00	1.00	0.83	0.51
IDT/RJ	0.91	1.00	1.00	0.78
IPEC/RJ	0.69	0.74	1.00	0.86
FMT/AM	0.73	0.76	1.00	----- ^a
CDCT/RS	0.71	----- ^b	1.00	0.53

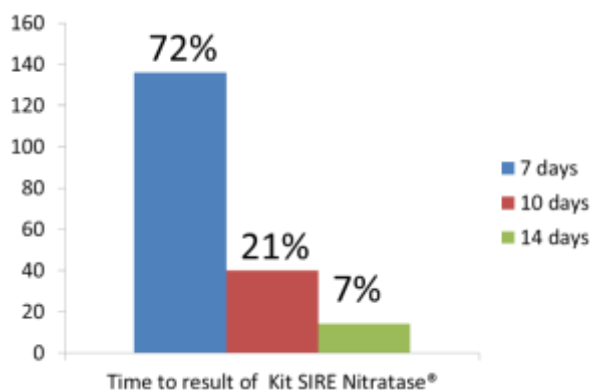
Legend: IAL/SP, Instituto Adolfo Lutz, São Paulo; CRHF/RJ, Centro de Referência Hélio Fraga, Fundação Oswaldo Cruz, Rio de Janeiro; IDT/RJ, Instituto de Doença do Tórax da Universidade Federal do Rio de Janeiro, Rio de Janeiro; IPEC/RJ, Instituto Nacional de Doenças Infecciosas, Fundação Oswaldo Cruz, Rio de Janeiro; IEC/PA, Instituto Evandro Chagas, Fundação Oswaldo Cruz, Pará; FMT/AM, Fundação de Medicina Tropical, Amazonas; CDCT/RS, Centro de Desenvolvimento Científico e Tecnológico, Centro Estadual de Vigilância em Saúde, Secretaria Estadual da Saúde do Rio Grande do Sul, Rio Grande do Sul; LPCS/MS, Laboratório de Pesquisa em Ciências da Saúde, Faculdade de Medicina da Universidade Federal da Grande Dourados, Mato Grosso do Sul. IHMT/PT, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal.

*Kappa <0.20 (poor); 0.21-0.40 (weak); 0.41-0.60 (moderate); 0.61-0.80 (good); > 0.80 (very good). **S (STR)**, streptomycin; **I (INH)**, isoniazid; **R (RIF)**, rifampicin; **E (EMB)**, ethambutol.

^a, Kappa not calculated (strains susceptible only to ethambutol).

^b, Kappa not calculated (strains resistant only to isoniazid).

It took approximately seven to 10 days to obtain results. At six sites, most of the results were obtained at day 7 of incubation, and of the 190 isolates, results were obtained for 136, 40, and 14 at day 7, 10, and 14 of incubation, respectively (Figure 2). Result interpretation based on color change is shown in Figure 3.

**Figure 2.** The time to obtain results by Kit SIRE Nitratase®

Legend: In 72% stands for 136 of 190 clinical isolates of *M. tuberculosis*; 21% stands for 40 of 190 clinical isolates of *M. tuberculosis*; 7% stands for 14 of 190 clinical isolates of *M. tuberculosis*.

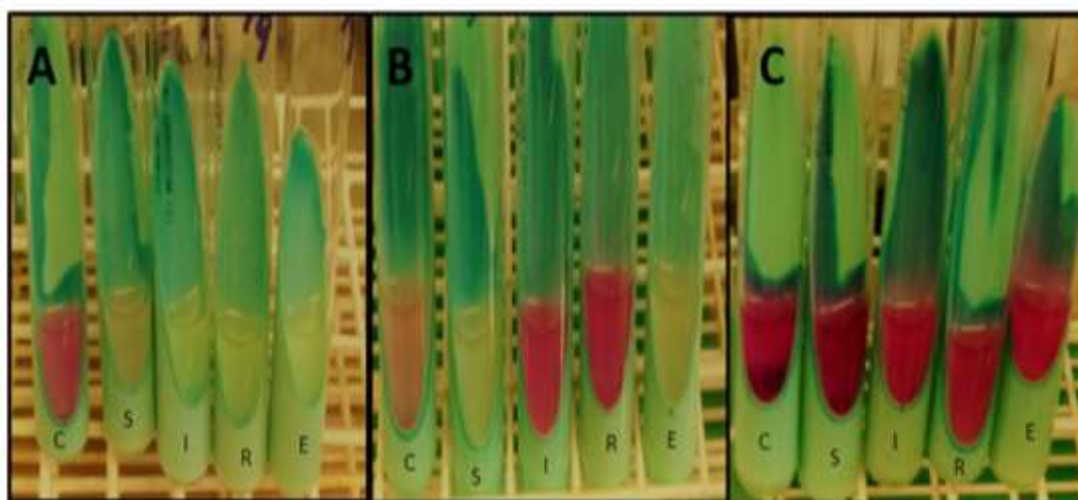


Figure 3. Interpretation of results of DST by Kit SIRE Nitratase®

Legend: A = Test of a susceptible strain. B = Test of a strain resistant to isoniazid and rifampicin. C = Test of a strain resistant to all tested drugs. C*: control tube (without drug); S (STR): streptomycin, I (INH): isoniazid, R (RIF): rifampicin, E (EMB): ethambutol. An isolate was considered resistant to the antibiotic at the critical concentration if the color change in the tube containing the drug was darker or equal to the color developed in the 1:10 growth control tube.

With the exception of EMB, Kit SIRE Nitratase® repeatability test with the proficiency strains showed good agreement for all drugs (Table 4).

Table 4. Kit SIRE Nitratase® repeatability test results with proficiency strains.

Drug	Triple Kappa	Interpretation of triple Kappa*
S	0.83	very good
I	0.81	very good
R	0.88	very good
E	0.30	weak

Legend:*Kappa < 0.20 (poor); 0.21-0.40 (weak); 0.41-0.60 (moderate); 0.61-0.80 (good); >0.80 (very good); S (STR), streptomycin; I (INH), isoniazid; R (RIF), rifampicin; E (EMB), ethambutol.

DISCUSSION

Kit SIRE Nitratase® showed good accuracy compared with the gold standard, and the results of this study are similar to the values established by the Supranational Laboratory Network/World Health Organization (WHO)/International Union Against Tuberculosis and Lung Diseases (IUATLD), which obtained accuracy levels of 99.0% and 97.0% for RIF and INH, respectively, and 92.0% for EMB and STR, indicating reasonable goals for reference laboratories [16].

Used in different settings, the in-house NRA kit has also been evaluated in other studies to determine the susceptibility of *M. tuberculosis* to first-line anti-tuberculosis drugs [17,19]. The accuracy obtained was higher than 96.6% for INH and RIF, and lower than 85.3% for STR [19,20]. A possible explanation for the low accuracy detected for STR in other studies, and for EMB in this study, could be the degradation of these antibiotics in an LJ medium [21].

Drug susceptibility phenotypic tests for EMB and STR are less reliable compared with those for INH and RIF, whose resistance defines MDR and XDR tuberculosis [22]. Similar findings were obtained using rapid growth detection systems [23]. The results obtained in this study are close to those obtained in a pilot study carried out with Kit SIRE Nitratase® by our team, which demonstrated that the kit is a stable product, has good accuracy, and can be incorporated into the routine of laboratories that perform DST [9].

In addition to its high accuracy, its main advantages are: it is used in a classic LJ medium, with which TB laboratories are already familiar; no additional equipment is required; and it is faster than PM using LJ. In this study, most of the technicians who performed the tests found the kit less laborious than PM using LJ, and its results easier to interpret, based on color change, which is another positive aspect of the method [24,19]. These features make it suitable for use in laboratories in low and middle income countries; however, biosafety procedures need to be maintained due to the risk of aerosol production during handling.

With the exception of EMB, which had the lowest kappa value and has shown poor repeatability performance in other studies, repeatability evaluation showed very good agreement for all drugs [16,20]. Thus, test results for EMB should be interpreted with caution, as concluded in other studies [7,25]. Our team emphasizes that the use of streptomycin and ethambutol should be reconsidered, not only when using Kit SIRE Nitratase[®], but also when using the other tests mentioned here as gold standard. Although the use of internal Nitratase STD methods has been shown to be highly accurate, the SIRE Nitratase[®] Kit ensures good handling and manufacturing practices, as non-commercial STD methods are prone to errors due to lack of standardization and local variations in methodology [26]. Given that the kit has a validity of four weeks [27], it is necessary to plan the logistics necessary for on-demand delivery to laboratories, especially in settings outside Brazil.

The study has some limitations. The kit does not have a para-nitrobenzoic acid (PNB) tube, which inhibits the growth of the *M. tuberculosis* complex, and rather allows the growth of non-tuberculous mycobacteria, mixed cultures, or contamination. The sample size was small; however, the same results were obtained by other authors who used a similar number of samples. Additionally, a negative control group was not included and genotypic methods were not performed.

CONCLUSION

The commercial Kit SIRE Nitratase[®] can be used in the rapid screening of drug resistance. It does not require sophisticated equipment and can be used in laboratories in low and middle income countries.

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Conflicts of Interest: The Kit SIRE Nitratase[®] is a commercial test developed after a consortium formed between the Brazilian Tuberculosis Research Network (REDE-TB) at UFMG and PlastLabor[®]. The company Plast labor the amount of R\$ 9.684,44 for the transfer of distributed technology as follows: R\$ 3.000 of extension bag (Silvana and Maria de Fátima); R\$ 3.300,00 of Autonomous; R\$ 1.500,00 of consumables; R\$ 193,79 UFMG 2%, R\$ 484,47 Unity 5%; R\$ 484,47 of Department 5%; R\$ 726,71 of Fundep 7,5%. During the multicenter validation, Isabela Neves de Almeida has received R\$ 10.000 in order to financial support for travel to meetings relevant to the study or other reasons.

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