Detection of multidrug-resistant tuberculosis from stored DNA Samples: A multicenter study

Marie Sylvianne Rabodoarivelo, A Brandao, M Cergole Novella, A C. Bombonatte, B Imperiale, N Rakotosamimanana, N Morcillo, V. Rasolofo, J Palomino, A. Martin

Original Article

Detection of Multidrug-Resistant Tuberculosis from Stored DNA Samples: A Multicenter Study

Marie Sylvianne Rabodoarivelo[1,2], A. Brandao[3,4], M. C. Cergole Novella[5], A. G. C. Bombonatte[6], B. Imperiale[7], N. Rakotosamimanana[1], N. Morcillo[8], V. Rasolofo[1,2], J. C. Palomino[9], A. Martin[10]

[1]Mycobacteria Unit, Institut Pasteur de Madagascar, Antananarivo, [2]Ecole Doctorale Sciences de la Vie et de l'Environnement, Université d'Antananarivo, Antananarivo, Madagascar, [3]Instituto Adolfo Lutz Núcleo de Tuberculose e Micobacteriosis Sao Paulo, Sao Paulo, [4]Fundação Oswaldo Cruz, Rio de Janeiro, [5]Instituto Adolfo Lutz, CRL-Santo Andre, [6]Instituto Adolfo Lutz, CRL-Santos, Brazil, [7]Institute of Experimental Medicine-CONICET, National Academy of Medicine, Buenos Aires, [8]Dr. Cetrangolo Hospital, Reference Laboratory of Tuberculosis Control Program, Buenos Aires, Argentina, [9]Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, Gent, [10]Université catholique de Louvain (UCL), Institute of Experimental and Clinical Research (IREC), Laboratory of Medical Microbiology (MBLG), Brussels, Belgium

Abstract

Background: In low-income countries, rapid detection of tuberculosis (TB) drug resistance is often restricted by the difficulties of transporting and storing sputum samples from remote health centers to the reference laboratories where molecular tests are available. The aim of this study was to evaluate the performance of four transport and storage systems for molecular detection of rifampicin (RIF) and isoniazid (INH) resistance. **Methods:** This was a multicenter study. Molecular detection of RIF and INH resistance was performed directly from smear-positive TB sputa spotted on a slide, FTA card, GenoCard, and ethanol using the Genotype MTBDRplus assay. The performance of the DNA extraction method from each storage support to detect drug resistance was assessed by calculating their sensitivity and specificity compared to the phenotypic method. **Results:** From all sites, the overall sensitivity and specificity for RIF-resistance detection was 88% and 85%, respectively, for slides, 86% and 92%, respectively, for GenoCard, 87% and 89%, respectively, for FTA card, and 88% and 92%, respectively, for ethanol. For INH-resistance detection, the overall sensitivity and specificity was 82% and 90%, respectively, for slides, 85% and 96%, respectively, for GenoCard, 86% and 92%, respectively, for FTA card, and 86% and 94%, respectively, for ethanol. **Conclusion:** Smear slides and filter cards showed to be very useful tools to facilitate DNA extraction from sputum samples with the potential to accelerate the detection of drug resistance in remote areas.

Keywords: Filter cards, *Mycobacterium tuberculosis*, molecular diagnosis, multidrug-resistant detection, smear slides

Introduction

Tuberculosis (TB) remains a serious public health concern worldwide, especially in developing countries. Worse, the TB situation is compounded by the emergence of multidrug-resistant (MDR) and extensively drug-resistant TB, which constitute a major challenge for TB control programs.[1] According to the 2016 WHO Global TB Report, there were an estimated 10.4 million new TB cases worldwide and 1.8 million TB deaths in 2015.[2] Rapid and accurate diagnosis of TB and MDR-TB is quite important for TB treatment and control and helps to prevent the spread of resistant *Mycobacterium tuberculosis* (MTB) strains.

In low- and middle-income countries, sputum smear microscopy examination is routinely used for pulmonary TB diagnosis;[3] however, it does have limits as it cannot identify the TB bacillus or detect the drug resistance. In most cases, sputum smear microscopy is the only diagnostic test available in peripheral-level laboratories and health centers. Culture and drug susceptibility testing (DST) is mostly done in reference laboratories since they require a biosafety level 3 facility with adequate equipment.[4] Under these circumstances, treatment is, thus, directly administered based on smear microscopy examination at peripheral-level

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_193_17

Address for correspondence: Dr. Marie Sylvianne Rabodoarivelo, Institut Pasteur De Madagascar, Antananarivo, Madagascar. E-mail: msylvianne@pasteur.mg

How to cite this article: Rabodoarivelo MS, Brandao A, Novella MC, Bombonatte AG, Imperiale B, Rakotosamimanana N, *et al*. Detection of multidrug-resistant tuberculosis from stored DNA samples: A multicenter study. Int J Mycobacteriol 2018;7:40-4.

laboratories, without knowing the results of culture or DST. Resistance to anti-TB drugs is only suspected after treatment failure or relapse. Indeed, a risk of inadequate treatment and spread of resistant MTB strains among patient's contacts may occur, which could negatively influence the success of TB control programs.[5]

Over the last decade, novel molecular tests have been developed and are now available for rapid detection of TB drug resistance. Some of these methods have already been approved and recommended by the WHO for rapid molecular detection of MDR-TB, such us the Genotype MTBDRplus line probe assay and GeneXpert MTB/rifampicin (RIF).[6] However, these tests are not easily available at peripheral laboratories, which have to rapidly refer the sputum samples to reference laboratories for the detection of resistance to anti-TB drugs. This is often arduous and expensive since the procedures for (potentially infectious) sputum sample transportation from remote areas require specific conditions and logistics.[7,8] Therefore, rapid diagnosis of drug resistance is often limited by constraints of both sputum storage and safe transport from remote health centers to reference laboratories, which may represent a challenge in terms of safety, logistics, sample management, delays, and contamination.

To overcome these difficulties of transporting and storing samples, we recently conducted a retrospective field study to evaluate the performance of four transport and storage systems allowing specimens to be collected and shipped in a safe manner for the molecular detection of MDR-TB.[9] In that preliminary study, 200 MTB strains were analyzed, spotted on the transport system, slides, and filter cards, where the DNA was extracted for detection of resistance to RIF and isoniazid (INH) using the GenoType MTBDRplus. The study showed that detection of drug resistance from slides and filter cards was feasible, with a good sensitivity and specificity from 95% to 100%.[9] In the present evaluation, unlike in the previous study where MTB strains were used, we evaluated the performance of slide and filter cards to detect MDR-TB directly from sputum samples spotted on the storage supports, in a multicenter study in three countries with high and middle burden of TB. Sensitivity and specificity of the GenoType MTBDRplus to detect resistance to RIF and INH using DNA extracted from each storage support were investigated.

Methods

Study sites and sputum samples

This study was conducted at three participating sites: Mycobacteriology Unit, Institute Pasteur of Madagascar, Antananarivo, Madagascar; Mycobacteria Reference Laboratory of Buenos Aires Tuberculosis Control Program Hospital Dr. Cetrángolo, Buenos Aires, Argentina; and Núcleo de Tuberculose e Micobacteriosis, Instituto Adolfo Lutz, Sao Paulo, Brazil. In total, 134 smear-positive sputum samples received between January 2014 and June 2015 in the selected health-care centers were analyzed. DST of all positive samples was performed using conventional methods used by each laboratory. Four storage supports were evaluated: slides, FTA cards, GenoCard, and ethanol. After decontamination, 100 µl of the decontaminated sputum sample was spotted on a slide, FTA card, and ethanol, whereas 60 µl on a GenoCard according to the optimized protocol from our previous study.[9] All support materials were stored at room temperature until DNA extraction. All tests were performed blind without knowing the resistance profile of the samples, and the results were compared to those obtained by the conventional DST method. The three participating sites were randomly numbered from 1 to 3 for the purpose of this publication. No ethical approval was required (the study was done with the leftover samples).

DNA extraction from each support

DNA extraction from each support was performed according to the optimized protocol described before using Chelex.[9] Briefly, from smear slide, the oil immersion after the smear microscopy was first removed with xylene. The smear was then scraped off and transferred to a new microtube. Seventy-five microliters of 10% Chelex-100 was added and incubated at 95°C for 30 min. After centrifugation at 13,000 rpm for 10 min, the supernatant containing the DNA was harvested for the molecular test. From GenoCard filter cards, eight small discs were initially punched, and DNA was extracted by adding 60 µl of 10% Chelex-100 into the discs followed by incubation at 95°C for 30 min. After centrifuged at 13,000 rpm for 10 min, the supernatant containing DNA was harvested for the molecular test. From the FTA card, four small discs were punched, rinsed three times with 200 µl of Whatman FTA purification reagent (GE Healthcare Life Sciences: United States of America) and twice with 200 µl of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8) according to the manufacturer's instruction. DNA was extracted by adding 75 µl of 10% Chelex-100 into the discs, mixed, and incubated at 95°C for 30 min. After centrifugation at 13,000 rpm for 10 min, the supernatant containing DNA was harvested for the molecular test. From the sputum stored in absolute ethanol, the sample was first harvested by centrifugation at 12,000 ×*g* for 5 min. The pellet was washed with 200 µl of TE buffer and centrifuged at 12,000 ×*g* for 10 min. Seventy-five microliters of 10% Chelex-100 was added to the pellet, mixed, and incubated at 95°C for 30 min. After centrifuged at 13,000 rpm for 10 min, the supernatant containing DNA was harvested for the molecular test.

GenoType MTBDRplus

GenoType MTBDRplus V2 (Hain Lifescience, Germany) was carried out according to the manufacturer's instructions. Briefly, for the amplification process, a total of 5 µl extracted DNA was added to 45 µl of PCR mixture containing 10 µl of amplification Mix A and 35 µl of amplification Mix B provided with the kit. The amplification protocol consisted of 15 min of denaturing at 95°C, followed by 10 cycles comprising 30s at 95°C and 2 min at 65°C, an additional 20 cycles comprising 25s at 95°C, 40s at 50°C, and 40s at 70°C, and a final extension at 70°C for 8 min. Hybridization, detection steps,

and interpretation of results were performed according to the manufacturer's instructions.

Data analysis

The performance of the DNA extraction method from each storage support to detect RIF and INH resistance using GenoType MTBDRplus was assessed separately for each participating site by calculating sensitivity and specificity using a 2 × 2 table.

Results

The overall results obtained with the GenoType MTBDRplus on DNA extracted from the four storage supports – Slides, FTA cards, GenoCard, and ethanol – are summarized in Table 1. Results were compared to the phenotypic method (MGIT 960 or proportion method on solid medium) used routinely. For the slides, the overall sensitivity and specificity (all sites) for RIF resistance detection was 88% (75%–100%) and 85% (74%–100%), respectively. For INH resistance, the overall sensitivity was 82% (68%–100%) and the specificity 90% (74%–100%). With the GenoCard, the overall sensitivity for all sites was 86% for RIF resistance (58%–100%) and the specificity 92% (83%–100%). For INH-resistance detection, the overall sensitivity was 85% (75%–100%) and the specificity 96% (88%–100%). For the FTA card, the overall sensitivity for all sites was 87% for RIF-resistance detection (71%–100%) and the specificity was 89% (79%–100%). For INH resistance, the overall sensitivity was 86% (75%–100%) and the specificity was 92% (75%–100%). Finally, for ethanol, the overall sensitivity was 88% for RIF (63%–100%) and the specificity was 92% (85%–100%). For INH resistance, the overall sensitivity was 86% (79%–100%) and the specificity was 94% (83%–100%). Site 1 found invalid results in GenoType MTBDRplus due to the absence or weak reaction for >1 WT line (corresponding to wild-type probe) for one RIF-resistant, two RIF-susceptible, six INH-resistant, and eight INH-susceptible samples using the GenoCard as support. Furthermore, 10 INH-resistant and 9 INH-susceptible samples gave invalid results using the FTA filter card support. Finally, the ethanol system gave invalid results for three INH-resistant and three INH-susceptible samples. Site 2 found invalid results only in ethanol for one RIF-resistant, one RIF-susceptible, and one INH-susceptible samples. For site 3 and for all the other supports, all the results could be correctly interpreted.

Discussion

Our study shows that the possibility of extracting DNA from sputum smears or from sputum samples spotted on filter cards could be a good alternative for the rapid detection of drug resistance in remote areas, where storage and transportation of clinical specimens are limited. A pilot study using dried culture spots (Whatman filter cards) of inactivated MTB was proposed by Scott *et al*. in South Africa for the external quality assessment of the GenXpert MTB/RIF.[10] Furthermore, for the implementation of the GeneXpert in South Africa, Gous *et al*. investigated the performance of MTB dried culture spots in remote nonlaboratory settings, such as clinics, by nonlaboratory-trained personnel.[11] They showed the stability of intact mycobacterial cells on filter paper for up to 6 months at all temperatures (4°, RT, and 37°C) with an overall good performance of the Xpert MTB/RIF assay. The major advantage of this approach is that the inactivated mycobacterial strains can be safely transported without the requirement of cold-chain transport. The spots are easy to use and robust. As the dried culture spots material is not platform specific, the same group determined the potential application of this system for the molecular line probe assay (Genotype MTBDRplus).[12] Overall, the dried culture spots appear to be also suitable for MTBDRplus assay due to their similar sensitivities compared to their previous study using GenXpert. In 2012, the group of Gomgnimbou *et al*. collected Ziehl–Neelsen-positive slides from TB diagnostic centers in Burkina Faso and extracted the DNA using the Chelex method.[13] DNA was successfully PCR amplified and produced a full spoligotyping pattern. In 2013, de Almeida *et al*. evaluated six different DNA extraction methods for the detection of MTB by PCR-IS*6110* in DNA extracted from microscope slides. The results of this work lead to the conclusion that *Chelex + NP-40* method was able to provide a good quantity of DNA for molecular diagnosis of TB.[14] Rakotosamimanana *et al*. published a good review on the usefulness of the molecular test applied on DNA isolated from smear microscopy slides for molecular diagnostics, drug-resistance testing, and genotyping.[15]

From our experience, we would recommend the use of smear slides, which is easily available in almost all TB diagnostic laboratories and is easy to transport for further molecular testing. Furthermore, additional transport systems such as filter cards (FTA and GenoCard) now available for biological sample storage and long-term preservation of DNA facilitate transportation at room temperature. They represent good alternatives to avoid the transportation of vials containing infectious samples and address the biosafety risk related to a possible accident. On the other hand, using FTA cards are more expensive since they need an expensive purification reagent to be added during the DNA extraction. This is not a requirement when using GenoCards. We also evaluated the usefulness of vials containing ethanol to be transported to the reference laboratory, but this system was less friendly and can leak during transportation.

CONCLUSION

In summary, we can conclude that smear slides and filter cards showed to be very useful tools to facilitate DNA extraction from sputum samples. We would encourage researchers and diagnostic laboratories in other settings to adopt the use of slides and filter cards as transport and storage systems due to the simplicity of DNA extraction for molecular detection of drug resistance as was the case in our study performed in Brazil, Madagascar, and Argentina.

Table 1: Susceptibility profiles of clinical isolates and specificity and sensitivity of DNA extraction from each support system to detect rifampicin and isoniazid resistance using the genotype MTBDRplus in each site

Sputum transport matrix	Genotype MTBDRplus	Site 1				Site 2				Site 3			
		Phenotypic DST				Phenotypic DST				Phenotypic DST			
		RIF		INH		RIF		INH		RIF		INH	
		R (n=10)	S (n=26)	R (n=22)	S (n=14)	R (n=24)	S (n=24)	R (n=24)	S (n=24)	R (n=1)	S (n=49)	R (n=3)	S (n=47)
Slide	R	9	5	15	0	18	7	19	7	1	0	3	0
	S	1	21	7	14	6	17	5	16	0	49	0	47
	Invalid												
	Sensitivity (%)	90		68		75		79		100		100	
	Specificity (%)	81		100		74		71		100		100	
GenoCard	R	9	4	12	0	14	2	19	3	1	0	3	0
	S	0	20	4	6	10	22	5	21	0	49	0	47
	Invalid	1	2	6	8								
	Sensitivity (%)	100		75		58		79		100		100	
	Specificity (%)	83		100		92		88		100		100	
FTA card	R	9	4	11	0	17	3	20	6	1	0	3	0
	S	1	15	1	5	7	21	4	18	0	49	0	47
	Invalid		7	10	9								
	Sensitivity (%)	90		75		71		83		100		100	
	Specificity (%)	79		100		88		75		100		100	
Ethanol	R	10	4	15	0	14	2	19	5	1	0	3	0
	S	0	22	4	11	9	21	4	19	0	49	0	47
	Invalid			3	3	1	1	1					
	Sensitivity (%)	100		79		63		80		100		100	
	Specificity (%)	85		100		92		83		100		100	

RIF: Rifampicin, INH: Isoniazid, Invalid: Absence/weak reaction for more than one WT line. DST: Drug susceptibility testing, *R: Resistant, *S: Susceptible, *WT: Wild-type

Financial support and sponsorship

This study was partially funded by Damien foundation (Belgique).

Conflicts of interest

There are no conflicts of interest.

References

1. Zignol M, van Gemert W, Falzon D, Sismanidis C, Glaziou P, Floyd K, *et al.* Surveillance of anti-tuberculosis drug resistance in the world: An updated analysis, 2007-2010. Bull World Health Organ 2012;90:111-9D.
2. World Health Organisation. Global Tuberculosis Report 2017. Geneva; 2017. Available from: http://www.who.int/tb/publications/global_report/en/. [Last accessed on 2017 Nov 03].
3. World Health Organisation. International Standards for Tuberculosis Care 2006. Geneva; 2006. Available from: http://www.who.int/tb/publications/2006/istc/en/. [Last accessed on 2017 Jun 30].
4. Diagnostic standards and classification of tuberculosis in adults and children. This official statement of the American thoracic society and the centers for disease control and prevention was adopted by the ATS board of directors, July 1999. This statement was endorsed by the council of the infectious disease society of America, September 1999. Am J Respir Crit Care Med 2000;161:1376-95.
5. Domingo S. DOTS plus strategy in resource-poor countries. Int J Tuberc Lung Dis 1999;3:843-6.
6. Palomino JC, Vandamme P, Martin A. Classical and new assays for detecting drug resistance in tuberculosis. Biomark Med 2014;8:1105-14.
7. Espinal MA, Laszlo A, Simonsen L, Boulahbal F, Kim SJ, Reniero A, *et al.* Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N Engl J Med 2001;344:1294-303.
8. World Health Organization. Guidelines for Surveillance of Drug Resistance. 5th ed. Geneva; 2015. Available from: http://www.who.int/tb/publications/2015/drs_guidelines/en/. [Last accessed on 2017 Jun 30].
9. Rabodoarivelo MS, Imperiale B, Andrianiavomikotroka R, Brandao A, Kumar P, Singh S, *et al.* Performance of four transport and storage systems for molecular detection of multidrug-resistant tuberculosis. PLoS One 2015;10:e0139382.
10. Scott LE, Gous N, Cunningham BE, Kana BD, Perovic O, Erasmus L, *et al.* Dried culture spots for Xpert MTB/RIF external quality assessment: Results of a phase 1 pilot study in South Africa. J Clin Microbiol 2011;49:4356-60.
11. Gous N, Cunningham B, Kana B, Stevens W, Scott LE. Performance monitoring of *Mycobacterium tuberculosis* dried culture spots for use with the geneXpert system within a national program in South Africa. J Clin Microbiol 2013;51:4018-21.
12. Gous N, Isherwood LE, David A, Stevens W, Scott LE. A pilot evaluation of external quality assessment of GenoType MTBDRplus versions 1 and 2 using dried culture spot material. J Clin Microbiol 2015;53:1365-7.
13. Gomgnimbou MK, Refrégier G, Diagbouga SP, Adama S, Kaboré A, Ouiminga A, *et al.* Spoligotyping of *Mycobacterium africanum*, Burkina Faso. Emerg Infect Dis 2012;18:117-9.
14. de Almeida IN, Da Silva Carvalho W, Rossetti ML, Costa ER, De Miranda SS. Evaluation of six different DNA extraction methods for detection of *Mycobacterium tuberculosis* by means of PCR-IS6110: Preliminary study. BMC Res Notes 2013;6:561.
15. Rakotosamimanana N, Rabodoarivelo MS, Palomino JC, Martin A, Razanamparany VR. Exploring tuberculosis by molecular tests on DNA isolated from smear microscopy slides. Int J Infect Dis 2017;56:248-52.