Original Article

Surveillance and Characterization of Drug-Resistant *Mycobacterium tuberculosis* Isolated in a Reference Hospital from Argentina during 8 Years' Period

Belén Rocío Imperiale[1], Ángela Beatríz Di Giulio[2], María Belén Mancino[3], Martín José Zumárraga[4], Nora Susana Morcillo[3]

[1]Laboratory of Immunology of Respiratory Diseases, Institute of Experimental Medicine-CONICET, National Academy of Medicine, [2]Mycobacteriology Laboratory, Petrona V. de Cordero Hospital, San Fernando, [3]Tuberculosis and Mycobacterioses Laboratory, Dr. Cetrángolo Hospital, Vicente López, [4]Institute of Agrobiotechnology and Molecular Biology (IABIMO)-CONICET-INTA, Hurlingham, Buenos Aires, Argentina

Abstract

Background: Argentina is considered a country with a middle tuberculosis (TB) incidence. However, according to the last national epidemiological report released in 2018, since 2013, the trends are steadily increasing. The aims of this study were to determine the drug-resistance (DR), multi-DR and extensively DR (MDR/XDR-TB), and rifampicin resistance (RIF-R) burden as a part of the local TB diagnosis (June 2010–August 2018); to detect the mutations associated to isoniazid (INH) and RIF-R and their geographical distribution; and to analyze the lineage relationship among the genetic patterns of the isolates circulating in the community. **Methods:** Respiratory and extrapulmonary specimens were processed by Ziehl–Neelsen stain and cultured on specific media. Drug-susceptibility testing of isolates was performed by the MGIT 960 and a colorimetric micro-method. Mutations conferring DR were detected by Genotype and DNA sequencing. **Results:** The study showed a DR-TB prevalence of approximately 20% of the isolated strains, while M/XDR-TB-and particularly RIF-R-affected more than 5.0% of the total amount of cases. DR geographical distribution revealed isolates carrying mutations in the *inhA* gene promoter region only constrained to three districts where it was also registered two same family relatives' cases with the infrequent *rpoB* S522 L/Q mutation. The fact that most DR/MDR-TB isolates were not grouped in genetic clusters suggested that these cases may mostly have occurred due to endogenous reactivation rather than recently transmission. **Conclusion:** According to the obtained results, it would be convenient, in highly MDR-TB suspected individuals, to confirm phenotypically, the INH and RIF susceptibility detected by molecular tests.

Keywords: Drug-resistance, genotyping, mutations, tuberculosis

Introduction

The World Health Organization End Tuberculosis (WHO End TB) Strategy, approved by the World Health Assembly in 2014, calls for an action driving to an 80% reduction in the TB incidence rate and 90% reduction in TB deaths by 2030 compared with the 2015 ciphers. In this last year, the Sustainable Development Goals for 2030 were adopted by the United Nations.[1]

According to 2017 WHO report, an estimated 10.4 million people fell ill with TB in 2016.[2] The decline rate in TB incidence worldwide was only 1.5% from 2014 to 2015.[1] Therefore, to reach the first milestones of the End TB Strategy, it will be necessary to accelerate the decline to a 4%–5% annually by 2020.[1,2] In addition, TB control is mainly threatened among other factors by the rise of immunosuppressive conditions in the hosts and the appearance of drug-resistant (DR-TB) cases particularly by the emergence of extensively DR-TB (XDR-TB) strains.[3-5] Contributing to worsening this situation, the WHO 2017 reported 129,689 people starting treatment for DR-TB, a small increase from 125,629 in 2015 but only 22% of the whole estimated incidence. Besides, the treatment success remains low at only 54% globally.[2]

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_94_19

Address for correspondence: Dr. Belén Rocío Imperiale, Pacheco de Melo 3081 (1425), CABA, Buenos Aires, Argentina.
E-mail: belen_imperiale@yahoo.com.ar

ORCID: http://orcid.org/0000-0003-0198-8746

How to cite this article: Imperiale BR, Di Giulio ÁB, Mancino MB, Zumárraga MJ, Morcillo NS. Surveillance and characterization of drug-resistant *Mycobacterium tuberculosis* isolated in a reference hospital from Argentina during 8 years' period. Int J Mycobacteriol 2019;8:223-8.

Mycobacterium tuberculosis – the main pathogen of the *M. tuberculosis* complex – can acquire several resistances mainly to the first-line and eventually to second-line anti-TB antibiotics. Based on the genetic changes in the *M. tuberculosis* genome and with epidemiological and clinical purposes, DR-TB can be classified as rifampicin (RIF)-resistant (RR-TB); multi-DR (MDR-TB), caused by strains simultaneously resistant to isoniazid (INH) and RIF; and XDR-TB, caused by MDR strains with the additional resistance to a fluoroquinolone (FQ) and an injectable second-line drug.[3,6]

In 2016, there were estimated 600,000 new RR-TB cases, the most effective first-line anti-TB drug, being 490,000 of them caused by MDR-TB strains.[2]

In Argentina, a total of 11,560 TB cases were reported to the National TB Control Program in 2016, with an incidence rate of 26.5/100,000 inhabitants and 6.4% increment comparing with 2014.[7,8] During 2015–2016, MDR-TB accounted for 174 cases, 5.3% had not had a previous treatment history, while 12 patients accomplished with the XDR-TB criteria.[7,8]

Dr. Cetrángolo Hospital (CH) is a reference center for diagnosis and treatment of lung diseases, including TB and mycobacterioses for the Northern Sanitary Region of Buenos Aires Province (NSRBA). It comprises an area of 9227.13 km^2 with around 4 million inhabitants (calculated population density: 433.5 inhabitants/km^2).[9]

As Argentina itself, CH has kept DR-TB surveillance, and the prevalence of MDR-TB cases records for more than 20 years.[7] In fact, CH has contributed with 27 RR-TB, 21 MDR-TB (21/174, 12.1%) cases, for the above-mentioned 2015–2016 period of the national DR surveillance. At the same time and also among patients receiving medical attention at CH, there were diagnosed 3 XDR-TB cases in no previously treated children between 10 and 14 years old (NM personal communication).[7,8] These last figures confirm the spreading and transmission of DR bacilli in the community.

Therefore, the aims of this study were to determine the clinical burden of M/XDR-TB and RR-TB as a part of the overall TB diagnosis in CH from June 2010 to August 2018; to detect the mutations associated to INH and RIF resistance and their geographical distribution; and to analyze the lineage relationship among the genetic patterns of the isolates that are being actively transmitted in this community.

Methods

Clinical and epidemiological information of the cases

During the study (June 2010 to August 2018), the following information was collected: age, gender, HIV infection, residence district, localization of the disease, HIV coinfection, and comorbidities. The cases were referred to the residence geographical localization in NSRBA.

Microbiologic diagnostic

Respiratory and extrapulmonary specimens but blood were processed by Ziehl–Neelsen stain and cultured on solid egg-based media and the BACTEC MGIT 960™ system. Myco-F-Lytic BACTEC 9050™ system was used to culture blood samples.[10,11]

First-line drug-susceptibility testing (DST) was performed by the commercial kit SIRE MGIT 960™[12] and the second-line DST by a colorimetric micro-method (resazurin microassay, REMA) that uses resazurin as a vital dye that indicates bacterial growth or the absence of it. This method was also used to determine the DR level of M/XDR-TB, RR, INH-R, and FQ-R isolates.[13]

Molecular studies on *M. tuberculosis*

M. tuberculosis Complex strains were identified by the commercial technique GenoTypeCM Mycobacterium Assay™ (GT-CM).[14]

For MDR-TB detection, it was used the GenoType MTBDR*Plus*™ (GTTBMDR) system which simultaneously identifies mutations in *inhA* and *katG* genes related to INH-R, and mutations located in the "hot spot region" of *rpo*B gene associated to RR.[14,15]

DNA sequencing for detecting mutations related to RR, INH-R, and FQ-R was also performed as previously published.[15] Briefly, isolates INH-R was investigated by sequencing a segment of 435 bp of *kat*G gene, another of 648 bp of *inh*A promoter region and the whole *inh*A gene. A segment of 250 bp of *rpo*B gene was also sequenced searching mutations related to RR. The 320 bp of the "Quinolone resistant determining region" in the *gyr*A and the 375 bp in *gyr*B were also sequenced looking for mutations related to FQ-R.[16-18]

Two molecular genotyping methods used to determine genetic diversity of the *M. tuberculosis* strains were used in sequential steps: (a) the spacer oligonucleotide typing or spoligotyping, which is based on a polymerase chain reaction amplification of the clustered regularly interspaced short palindromic repeats locus and detection of the presence of different spacers between the repeats by reverse hybridization on a membrane; (b) genetic patterns analysis obtained by restriction fragment length polymorphism (RFLP) using the insertion sequence *6110* (IS*6110*) as probe (IS*6110*-RFLP).[19,20]

Statistical analysis

Epidemiological, clinical, and microbiological data were collected in an Excel file designed to collect the involved variables. The Microsoft MedCalc 16.4 (MedCalc Software, Mariakerke, Belgium) was used to analyze results.[12,15]

The analysis of the molecular patterns of the isolates was performed by the BioNumerics™ software (Applied Maths NV, Sint-Marten-Latem, Belgium) while octal codes obtained by *spoligotyping* were determined and compared using the international database, SITVITWEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/contact.jsp).[21-23]

Results

Mycobacterial isolates

During the study, mycobacteria as etiological agents of the disease were isolated from 3,014 patients: 2758 (91.5%) were confirmed as TB, and in 256 (8.5%) cases, a nontuberculous mycobacteria (NTM) was considered the pathogenic agent. Men accounted for 61.0% of the whole cases. The global HIV coinfection reached 6.1% (n: 184) of the cases, 149 (5.4%) in TB, and 19 (7.4%) in NTM-infected individuals; 407 (13.5%) cases had an extrapulmonary localization of the disease and 347 (11.5%) were isolates submitted to the laboratory for identification and/or DST. A total of 2206 (80.0%) TB cases had pulmonary localization of the disease and 1482 (67.2%) with acid-fast bacilli demonstrable by Ziehl–Neelsen stain. Besides, the sanitary system identified 822 (29.8%) as previously treated cases.

A total of 1582 *M. tuberculosis* isolates, 427 (27%) from previously treated cases, were processed by DST and 722 (45.6%), suspected of being M/XDR-TB, were selected to be studied by molecular methods.

Drug susceptibility testing

Phenotypic DST results are shown in Table 1. During the study, a total of 340 (21.5%) out of 1582 cases with DST results had a disease caused by an isolate resistant to at least one anti-TB agent (DR-TB): 96 (28.2%) were globally RR, 18 (5.3%) of them mono RR; 166 (48.8%) were globally INH-R while M/XDR plus Pre-XDR accounted for 78 (22.9%) of the tested organisms.

Global DR was found in 90 out of 427 (21.8%) previously treated patients being RR-TB detected in 38 (8.9%) and 58 cases out of 1155 (5.0%) with and without treatment background, respectively.

Table 1: Predominant drug-resistance patterns found by phenotypic drug-susceptibility testing methods

Year	Patient	*n*° (%)						
		DST/Pt	DRt	RRt	INHt	MDR	P-XDR	XDR
2017	345	200	45	14	22	11	1	
2016	335	191	43	10	22	8	-	
2015	265	160	46	13	26	8	3	
2014	327	183	40	13	19	11	1	1
2013	387	210	44	14	24	12	1	
2012	337	202	38	12	14	5	1	
2011	375	217	47	13	26	9	-	1
2010	387	219	37	7	13	5	-	
Total *n*°	2758	1582	340	96	166	69	7	2
Percentage	100	57.4	21.5	6.1	10.5		4.9	

DST/Pt: Drug-susceptibility testing per patient, *t*: Total figures, DRt: Total drug resistance, RRt: Total resistance to RIF, INHt: Total resistance to INH, MDR: Strain resistant simultaneously at least to INH and RIF, P-XDR: Multidrug-resistant strain also resistant to either an aminoglycoside tested (capreomycin, kanamycin, or amikacin) or a fluoroquinolone (levofloxacin [LX], ofloxacin, and moxifloxacin), XDR: TB caused by a strain resistant also to isoniazid and rifampicin

Regarding the global incidence calculated on the 1582 individuals tested, RR and INH-R accounted for 6.1% and 10.5%, respectively, while M/XDR-TB and Pre-XDR-TB occurred in 4.9% of the cases.

Resistance to aminoglycosides (amikacin and/or kanamycin) was found in 9 cases, 7 with a Pre-XDR-TB pattern while FQs (levofloxacin and moxifloxacin) resistance was verified in 2x DR-TB and 2 Pre-XDR cases.

Molecular drug resistance

Genotypic valid results were obtained for 760 out of 772 (98.4%) analyzed isolates. A total of 236 genomic analyses of the *rpo*B, *kat*G, *inh*A, and gyrA/gyrB genes were performed on 186 out of the 193 (96.4%) phenotypically characterized DR cases. Only those isolates showing also resistance to aminoglycosides were not molecularly investigated.

Table 2 shows mutations or deletions related to RR and INH-R. The comparison of GTTBMDR results and sequencing is also shown in Table 2. The overall concordance for RR and INH-R detection between the two methods was 97.0%.

RR was detected in 77 isolates simultaneously by GTTBMDR and sequencing and 13 (16.9%) showed discordant results. In 6 (8%) RR cases, a wild type GTTBMDR was found while sequencing revealed 1: case with deletion at codon position 537 of the *rpo*B gene, 3 (4.0%) with a point mutation ATG > ACG at codon 515, 1 mutated at codon 531, and 1 mutated at position 572 (ATC>TTC), [Table 2].

The followings were the rest of discrepancies found: 1 strain presented a TCG531TTG mutation detected by GTTBMDR while sequencing showed GAC516GTC mutation; 3 strains mutated at GAC517GGC by GTTBMDR showed by sequencing a deletion affecting codons 510–517 of *rpo*B gene; in 3 strains, GTTBMDR detected a CAC526TAC mutation but sequencing showed 2 strains with CAA513CCA and 1 with GAC565CAG mutations [Table 2]. Besides, in one case, a multiple mutation comprising codons 513–566 were found.

INH-R was correctly detected by GTTBMDR in fully agreement with sequencing in 95.5% (150/157) of the tested isolates. In 5 (3.2%) cases, the GTTBMDR gave wild type patterns for *inh*A promoter region and *kat*G gene, but mutations at codons 321, 107, 315 and the open reading frame of the *inh*A gene were detected by sequencing [Table 2].

Analyzing the discrepancies found in detecting mutations leading to RR and INH-R by GTTBMDR and sequencing considered as the gold standard, the agreement between both methods was 91.5% (214/234).

Relations between mutations and DR levels reveal that 118 out of 125 (94.4%) of INH-R strains mutated in *kat*G gene had INH MIC values ranged between 16.0 and ≥32.0 μg/mL while 75.0% (24/32) of the *inh*A mutated strains presented MIC values between 1.0 and 0.50 μg/mL. Almost 90% of the RR strains showed resistance levels ranged from 8.0 to ≥64.0 μg/mL regardless the *rpo*B mutation. Both FQ-R

Table 2: Mutations of drug-resistant strains found by GenoType MTBDR*Plus* and DNA sequencing related to the predominant *spoligotyping* patterns family

Gene position	GenoType MTBDR*Plus*			Sequencing			Spoligotyping	
	n°	Sequence mutation	aa changed	*n*°	Sequence mutation	aa changed	Family	Percentage
*rpo*B (N: 77)								
531	45	TCG531TTG	Ser>Leu	44	TCG531TTG	Ser>Leu	LAM3, 5, 9	48.3
				1	GAC516GTC	Asp>Val	T1	24.1
							H2	13.8
							OP	-
531	1	TCG531CCG	Ser>Pro	1	TCG531TTG	Ser>Leu	T	1/1
516	4	GAC516GTC	Asp>Val	4	GAC516GTC	Asp>Val	T	3/4
526	8	CAC526TAC	His>Tyr	8	CAC526TAC	His>Tyr	T	8/8
526	2	CAC526TAC	His>Tyr	2	CAA513CCA/ GAC565CAG	Gln>Pro/ Asp/Gln	T5	2/2
526	1	CAC526TAC	Gln>Tyr	1	CAA513CCA	Gln>Pro	T	1/1
526	1	CAC526GAC	His>Asp	1	CAC526GAC	His>Asp		
	2	CAC526GAC	His>Asp	2	CAC526CTC	His>Leu	U	2/2
522	1	TCG522TTG	Ser>Leu	1	TCG522CAG	Ser>Gln	T	3/3
522	2	TCG522CAG	Ser>Gln	2	TCG522CAG	Ser>Gln		
516	3	GAC517GGC	-	3	DEL 510-517	-	T1	2/3
							LAM3	1/3
	1	Not defined		1	Multiple mutations***		LAM5	1/1
WT	1	-	-	1	TCG531TTG	Ser>Leu	ND	-
WT	1	-	-	1	DEL537	-	LAM	1/1
WT	3	-	-	3	ATG515ACG	Met>Thr	T5	3/3
WT	1			1	ATC572TTC	Ile>Phe	H3	1/1
*Kat*G (N: 125)								
315	119	AGC315AAC	Ser>Thr	119	AGC315AAC	Ser>Thr	LAM3, 5, 9	19.8
							H2	2.8
							T	7.5
WT	1	-	-	1	TGG321TGC	Trp>Cys	T1	1/1
WT	1	-	-	1	AAA107AAA	Lys>Lys	LAM3	1/1
315	2	AGC315AAC	Ser>Thr	1	ATC321ACC	Lys>Asn	ND	-
				1	ATC317ACC	Ile>Thr	ND	-
WT	2	-	-	2	AGC315AGA	Lys>Arg	LAM3	1/1
							H2	1/1
InhA (N: 32)								
-15	29	C-15T		28	C-15T		H3	18.8
				1	C-17		LAM3, 9	21.9
							T1	28.1
							Beijing	3.1
							T1	1/1
	2	C-8T		2	C-8T		LAM9	2/2
	1	WT		1	GGG83AGG	Gly>Arg	LAM9	1/1
Total		234						

***Mutations at positions: 542, 544, 548, 550, 552, 562, 566 of the *rpo*B gene; OP: Orphan pattern, WT: Wild type, aa: Amino acid

strains mutated at *gyr*A GAC94TAC (D > Y) showed MIC ≥16.0 μg/mL.

Genotyping

Table 2 also shows the predominant *spoligotyping* families – expressed in percentages of the total patterns found for each one of the strains – in which the DR isolates could be grouped. Latin-American and Mediterranean family, T (Tuscany), and H (Haarlem) lineages were the more representative ones.

In one patient with a first drug-susceptible isolate, genetic patterns allowed the confirmation of MDR generation in the original strain discarding the reinfection from an external contagious source. S*poligotyping* was also used to analyze seven isolates from health-care workers from two different hospitals.

Three cases showed an infrequent deletion in *rpo*B gene with GAA base-sequence located between 2 contiguous codons: G, last base of 517, and AA 2 first bases of codon 518. Two of these isolates showed identical *spoligotyping* pattern.

Geographical distribution of the resistant isolates revealed isolates carrying mutations in the promoter region of the *inhA* gene that were exclusively found and constrained to three neighborhoods districts of the NSRBA region, where it was also registered two cases, in the same family relatives, with the infrequent *rpoB* S522 L/Q mutation.

Discussion

Among the whole landscape of TB worldwide, Argentina is considered as a middle TB incidence country. However, according to the last national epidemiological report released in 2018, since 2013, the trends are showing an increment in the total amount of cases. This increment was particularly produced between the years 2017 and 2018.[7,24]

This study has shown a DR-TB prevalence of approximately 20% of the circulating *M. tuberculosis* strains and an incidence of M/XDR-TB, and particularly RR affecting more than 5.0% of the total amount of cases registered in CH during the study. Regarding the global incidence calculated on the 1582 individuals tested, RR-TB and INH-R accounted for 6.1% and 10.5%, respectively, while M/XDR-TB and Pre-XDR-TB occurred in 4.9% of the cases. These results focus on a higher risk of being infected by a DR-TB when residing in NSRBA area.

On the other hand and according with the obtained results, it would be convenient to confirm by phenotypic methods, the susceptibility to INH and RIF detected by commercial tests especially in highly DR-TB suspected individuals. This concept is based on discordances found in some of the isolates between phenotypic and molecular methods on DR detection, elucidated later by gene sequencing.[16,25] Besides, the knowledge of DR levels related to the mutated codons leading to INH and RIF resistance could help to orientate the treatment particularly in the replacement of INH-structural analog, ethionamide instead of INH, in those INH-R isolates carrying *kat*G and no *inh*A mutation.[26]

A practical subproduct of this work was obtained by analyzing the *spoligotyping* patterns from 2 supposed nosocomial outbreaks apparently occurred during the year 2016 among the health-care workers staffs from two different hospitals. The prime idea of being nosocomial outbreaks could be discarded on the basis of the different orphan *spoligotyping* patterns found. This fact shows no connection among the involved people.

As it was previously observed and reported, the genotyping analysis of DR isolates can contribute to surveillance activities on the clonal dispersion of the community transmission of the main lineages. This surveillance can help to estimate the proportions of endogenous reactivation and active transmission of DR strains in the community.[27] The fact that most DR/MDR-TB isolates were not grouped in genetic clusters suggests that these cases may mostly have occurred due to endogenous reactivation than for recent transmission.[27,28]

Geographical distribution of the DR isolates revealed mutations in the promoter region of the *inh*A gene circulating so far in three neighborhoods districts of the NSRBA. At the same time and in the same region, two cases from family relatives with the infrequent *rpoB*S522 L/Q mutation were also registered. An exhaustive contact tracing of these cases, as well as prevention measures, should be maintained over the time to prevent the spreading of these strains in the community.

Conclusions

Results of this study also emphasized the need of a properly detection of DR cases. Implementing DST to all new diagnosed cases as well as those previously treated. Resources to accomplish with this WHO recommendation should be a priority.

On the other hand, the development and standardization of DST methodology to second-line and alternative anti-TB compounds (e.g., clofazimine, linezolid, and new generation FQs) are urgently needed to monitor the emergence of resistance and should be a higher priority when such a new drugs are introduced.[29]

Acknowledgment

We would lie to acknowledge Miss. Bibiana Zingoni, Mr. Marcelo Mazza and Mr. Guillermo Alonso for their technical work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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