

Phenotypic and Genotypic Drug Susceptibility Assessment of *Mycobacterium bovis* Bacillus Calmette-Guérin Clinical Strains

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Purpose: *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is the only vaccine licensed against tuberculosis. Despite the protection offered by the vaccine, in some circumstances children and immunocompromised individuals can develop associated infections, known as BCGitis. Drug susceptibility patterns of BCG clinical strains have rarely been described. We aimed to describe the susceptibility pattern of BCG clinical strains isolated in two different countries.

Methods: We performed culture-based drug susceptibility testing of thirty one BCG strains isolated from patients in Brazil (n=5, 16%) and Argentina (n=26, 84%) using the broth micro-dilution method (phenotypic method). Final antibiotic concentrations for susceptibility testing ranged from 0.5 to 16 mg/L for amikacin, 0.3125 to 10 mg/L for ethambutol, 0.05 to 1.6 mg/L for isoniazid and 0.25 to 8 mg/L for rifampicin, streptomycin and ofloxacin. Additionally, we compared the results with genetic data obtained by whole genome sequencing.

Results: By using the phenotypic method we detected one strain resistant to ethambutol, three strains resistant to rifampicin and one resistant to isoniazid. Additionally, two strains that were phenotypically resistant to both isoniazid and rifampicin carried mutations in the *katG* and *rpoB* genes simultaneously.

Conclusion: There is evidence of the emergence of BCG-resistant strains isolated from vaccine-related complications. We recommend drug susceptibility testing of the BCG strain causing the infection in order to prevent treatment failure.

Keywords: BCG, mycobacteria, resistance, vaccine

Introduction

Tuberculosis (TB) remains one of the major causes of mortality worldwide, causing approximately 1.2 million of deaths, with an additional 251,000 deaths among HIV-positive patients.¹ The BCG vaccine is the only one licensed against tuberculosis and was obtained through continuous passages of a *Mycobacterium bovis* strain in potato-bile media until attenuation was achieved.² Following its successful application to prevent severe tuberculosis in infants, the strain was sent to multiple laboratories around the world for local production of the vaccine, generating genetically divergent strains, product of the different conditions in which the strains were maintained and processed.³⁻⁵ Today, BCG vaccines are a family of related strains with described genetic and immunogenic differences⁶ but no superiority regarding protective effect has been demonstrated of one vaccine over the other.⁷

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Although considered safe, adverse reactions such as local or disseminated infections after vaccination might happen.⁸ At present, there are no standardized guidelines for the management of BCG disease and cases are treated with first-line anti-tuberculosis drugs as isoniazid, ethambutol, rifampicin and streptomycin. However, data on the existence of drug-resistant BCG strains have been reported and demands further investigation of the dimension of this problem by drug susceptibility testing.^{9,10} We therefore verified drug susceptibility of a group of BCG clinical isolates recovered from patients in Brazil and Argentina against the major antibiotics used for treatment of tuberculosis and BCGitis, and compared the results obtained by phenotypical drug susceptibility testing (DST) with data from whole genome sequencing (WGS).

Materials and Methods

Bacterial Strains and Growth Conditions

Thirty one BCG clinical isolates were obtained from the bacterial collection of two different laboratories. Five strains (16%) were sent by the Laboratório de Referência Nacional de Tuberculose e Micobacterioses Angela Maria Werneck, Centro de Referência Professor Hélio Fraga (CRPHF) in Rio de Janeiro, Brazil. At the time of collection of the strains, the vaccine in use in Brazil was BCG Moreau RDJ. We also received 26 isolates (84%) from the Instituto Nacional de Enfermedades Infecciosas Dr. Carlos G. Malbrán from Buenos Aires, Argentina, where two vaccines are licensed: BCG Pasteur and BCG Sofia SL222. The isolates had been collected for a period of 15 years (2001–2016) and were maintained in Middlebrook 7H9 broth (BD Difco) plus 10% glycerol at -20°C . The clinical information of the patients was obtained (when possible) through medical records maintained in each laboratory. Table 1 shows the clinical characteristics of the patients. BCG Moreau RDJ was supplied by Dr. Rafael Silva Duarte of the Laboratory of Mycobacteria at the Federal University of Rio de Janeiro. BCG Pasteur and BCG Sofa SL222 were supplied by Dr. Claudia Argüelles of the National Institute of Biologicals Production of Argentina.

Preparation of Antibiotics and Reagents

Amikacin, ethambutol, isoniazid, ofloxacin, rifampicin and streptomycin (Sigma-Aldrich) were each dissolved and used according to the manufacturer's instructions. Stock

solutions were conserved at -20°C . Resazurin sodium salt (Sigma-Aldrich) was prepared as a 0.02% filtered solution and kept at 4°C . Final antibiotic concentrations for susceptibility testing ranged from 0.5 to 16 mg/L for amikacin, 0.3125 to 10 mg/L for ethambutol, 0.05 to 1.6 mg/L for isoniazid and 0.25 to 8 mg/L for rifampicin, streptomycin and ofloxacin.

Drug Susceptibility Assay

All clinical isolates and the three vaccine strains were submitted to drug susceptibility assays. Quality control of the assays was done by including the strain *Mycobacterium tuberculosis* H37Rv ATCC 27294. All strains were grown on Middlebrook 7H10 (BD Difco) and incubated at 37°C for at least three weeks.

Two fold-serial dilutions of each drug were prepared in 0.1 mL of Middlebrook 7H9 broth (BD Difco) supplemented with 10% oleic acid-albumin-dextrose complex (OADC), casitone, glycerol (0.05%) and tween 80 (0.05%). To each well, 0.1 mL of a bacterial suspension adjusted to the density of a McFarland 1 standard was added; then, the plate was incubated at 37°C ; an antibiotic-free micro-plate for growth control was also prepared from the bacterial suspension. The bacterial suspension was added in triplicate to the assay plate on the same day as it was prepared. Bacterial growth was confirmed by adding 30 μL of resazurin to the control and assay plates; a change of color from blue to pink after 24 hours indicated the reduction of the resazurin and, therefore, cell viability. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug that inhibited growth of the microorganism, evidenced by a no reduction of the resazurin (blue color).

Quality Control Assays

Loss of activity of anti-mycobacterial agents during incubation can lead to false interpretation of results and, in the worst case, cause failures in patient treatment based on non-precise results. We therefore evaluated the stability of anti-mycobacterial drugs during the resazurin micro-plate assay over a period of five weeks. Briefly, 0.1 mL of a bacterial suspension of *M. tuberculosis* H37Rv ATCC 27294 was added in triplicate to an antibiotic-containing plate prepared as described before and incubated at 37°C . Then, new suspensions with the same density were added at seven, 14, 21 and 28 days after the plate was prepared

Table I Clinical Characteristics of the Patients

Country	Strain	Clinical Sample	Age	Comorbidity	Year
Brazil	4BR	Lymph node	2	–	2016
	5BR	Lymph node	2	–	2016
	6BR	Blood culture	5 months	Miliary TB	2015
	7BR	Tracheal aspirate	5 months	Miliary TB	2015
	8BR	Abscess at the inoculation site	8 months	–	2015
Argentina	1AR	Lymph node	1	–	2015
	2AR	Lymph node	1	HIV+	2013
	3AR	Blood culture	–	HIV+	2005
	4AR	Bone biopsy	1	–	2007
	5AR	Gastric juice aspirate	2	–	2014
	6AR	Gastric juice aspirate	3	–	2016
	7AR	Lymph node	1	HIV+	2001
	8AR	Bone marrow	1	HIV+	2001
	9AR	Blood culture	1	HIV+	2001
	10AR	Lymph node	8	–	2003
	11AR	Lymph node	–	HIV+	2005
	12AR	Lymph node	4	HIV-	2006
	13AR	Abscess at the inoculation site	1	-	2009
	14AR	Lymph node	-	-	2012
	15AR	Lymph node	1	HIV+	2014
	16AR	Skin biopsy	-	-	2014
	17AR	Abscess at the inoculation site	2	-	2010
	18AR	Lymph node	1	HIV+	2004
	19AR	Blood culture	-	HIV+	2002
	20AR	Blood culture	-	HIV+	2003
	21AR	Blood culture	3	HIV+	2005
	22AR	Non identified	1	HIV-	2005
	23AR	Bone biopsy	1	HIV-	2006
	24AR	Blood culture	1	HIV+	2007
	25AR	Bone biopsy	-	-	2013
	26AR	Abscess at thumb	1	-	2015

Note: (-) indicates that related information was not available.

Abbreviations: HIV, human immunodeficiency virus; TB, tuberculosis.

and left incubated at 37°C. Resazurin was added seven days after each inoculum was added to the plate.

Genome Analysis

WGS of the 31 strains was performed using the Nextera XT DNA Library preparation kit on an Illumina HiSeq 2500 platform. Quality control of the reads obtained was performed with FastQC program. In order to undercover drug-resistance associated mutations, we performed variant call analysis with the software Snippy v. 4.4.0 by aligning the reads obtained to reference assemblies of each vaccine obtained from the NCBI.^{4,11} For the analysis

of the Brazilian strains, we used the assembly of the Vaccine Moreau RDJ (access GCF_000967285.1) published by Gómes et al.¹¹

For the analysis of the Argentinean strains related to patients vaccinated with the Pasteur vaccine, we used the assembly of vaccine Pasteur 1173P2 (access GCF_000009445.1) published by Brosch et al.⁴ For the analysis of the strains from patients vaccinated with BCG Sofia we decided to use the assembly of the vaccine BCG-1 Russia¹² from which BCG Sofia was delivered, since there is not a whole genome assembly available of this vaccine. The Illumina reads generated were deposited in the Sequence

Read Archive (SRA) of the NCBI under the Bioproject PRJNA575846.

Results

Quality Control and Drug Stability Assays

All drugs maintained the same MIC up until 35 days of incubation, except for ethambutol, that had its value increased two-fold from the fourth week on, reaching a MIC value of five mg/L after its fifth week of incubation (Table 2).

Drug Susceptibility Testing of BCG Moreau, BCG Pasteur and BCG Sofia Vaccine Strains

Recently, broth micro-dilution breakpoints for ethambutol, isoniazid and rifampicin were published by the Clinical and Laboratory Standards Institute (CLSI) for organisms belonging to the *Mycobacterium tuberculosis* complex. It was stated that a MIC of 4 mg/L for ethambutol does not correlate with either a susceptible or resistant result in commercial, automated, short incubation broth systems and has not been correlated with clinical response data (Table 3).

For ethambutol, BCG Sofia had the lowest MIC (0.625 mg/L); however, the control plate presented growth only at 28 days. BCG Moreau had a MIC of 1.25 mg/L, being classified as susceptible. BCG Pasteur had a MIC of 2.5 mg/L and was classified as inconclusive by the CLSI criteria. Considering isoniazid and rifampicin, the three vaccines had MIC values lower than the CLSI breakpoint. Broth micro-dilution breakpoints for second-line drugs like amikacin, ofloxacin and streptomycin have not been established yet. For amikacin and streptomycin, the three vaccines had MICs of 0.5 mg/L and 0.25 mg/L, respectively. For ofloxacin, BCG Moreau and Pasteur had a MIC that was two-fold higher than that of BCG Sofia (Table 4).

Table 2 Minimum Inhibitory Concentration of the Antibiotics Tested and Stability Over Time

	Incubation Time (Days)				
	7	14	21	28	35
Amikacin	0.5	0.5	0.5	0.5	0.5
Ethambutol	0.625	0.625	0.625	1.25	5
Isoniazid	0.05	0.05	0.05	0.05	0.05
Ofloxacin	0.25	0.25	0.25	0.25	0.25
Rifampicin	0.25	0.25	0.25	0.25	0.25
Streptomycin	0.25	0.25	0.25	0.25	0.25

Note: MIC values are expressed in mg/L.

Table 3 Breakpoints for Broth Micro-Dilution Method for *Mycobacterium tuberculosis* Complex Published by the CLSI

	Breakpoints (mg/L)		
	Susceptible	Inconclusive	Resistant
Ethambutol	≤2	4	≥8
Isoniazid	≤0.12	–	≥0.25
Rifampicin	≤1	–	≥2

Table 4 Drug Susceptibility Testing of BCG Vaccines to First and Second-Line Antituberculosis Drugs

Vaccine	AMK	EMB	INH	OFX	RIF	STR
BCG Moreau	0.5	1.25	0.05	0.5	0.25	0.25
BCG Sofia	0.5	0.625	0.05	0.25	0.25	0.25
BCG Pasteur	0.5	2.5	0.1	0.5	0.25	0.25

Note: MIC values are expressed in mg/L.

Abbreviations: AMK, amikacin; EMB, ethambutol; INH, isoniazid; OFX, ofloxacin; RIF, rifampicin; STR, streptomycin.

Drug Susceptibility Testing of BCG Clinical Strains

For ethambutol, we detected one strain related to BCG Sofia to be resistant against this antibiotic. We found two strains resistant to isoniazid in the group of Brazilian strains. In the case of rifampicin, we observed resistant strains in the group related to both the Moreau and the Pasteur vaccine. Figure 1 shows the MIC distribution for all the drugs tested.

Among BCG clinical strains from Brazil, three strains had a MIC for ethambutol two-fold higher than the BCG Moreau vaccine while another strain had a MIC four-fold higher; these strains had MIC values between 2 and 8 mg/L and could be classified as having intermediate or inconclusive susceptibility. For isoniazid and rifampicin, two strains had a MIC higher than 1.6 mg/L and 8 mg/L, respectively, being classified as resistant to both antibiotics. One strain had a MIC for amikacin eight-fold higher than the Moreau vaccine (4 mg/L vs 0.5 mg/L). For ofloxacin, two strains had a MIC lower than the vaccine (0.25 mg/L vs 0.5 mg/L). For streptomycin, all the strains had the same MIC as the vaccine.

In the group of Argentinean strains identified as BCG Sofia, five strains had a MIC for ethambutol higher than the vaccine; one of them had a MIC of 10 mg/L and was

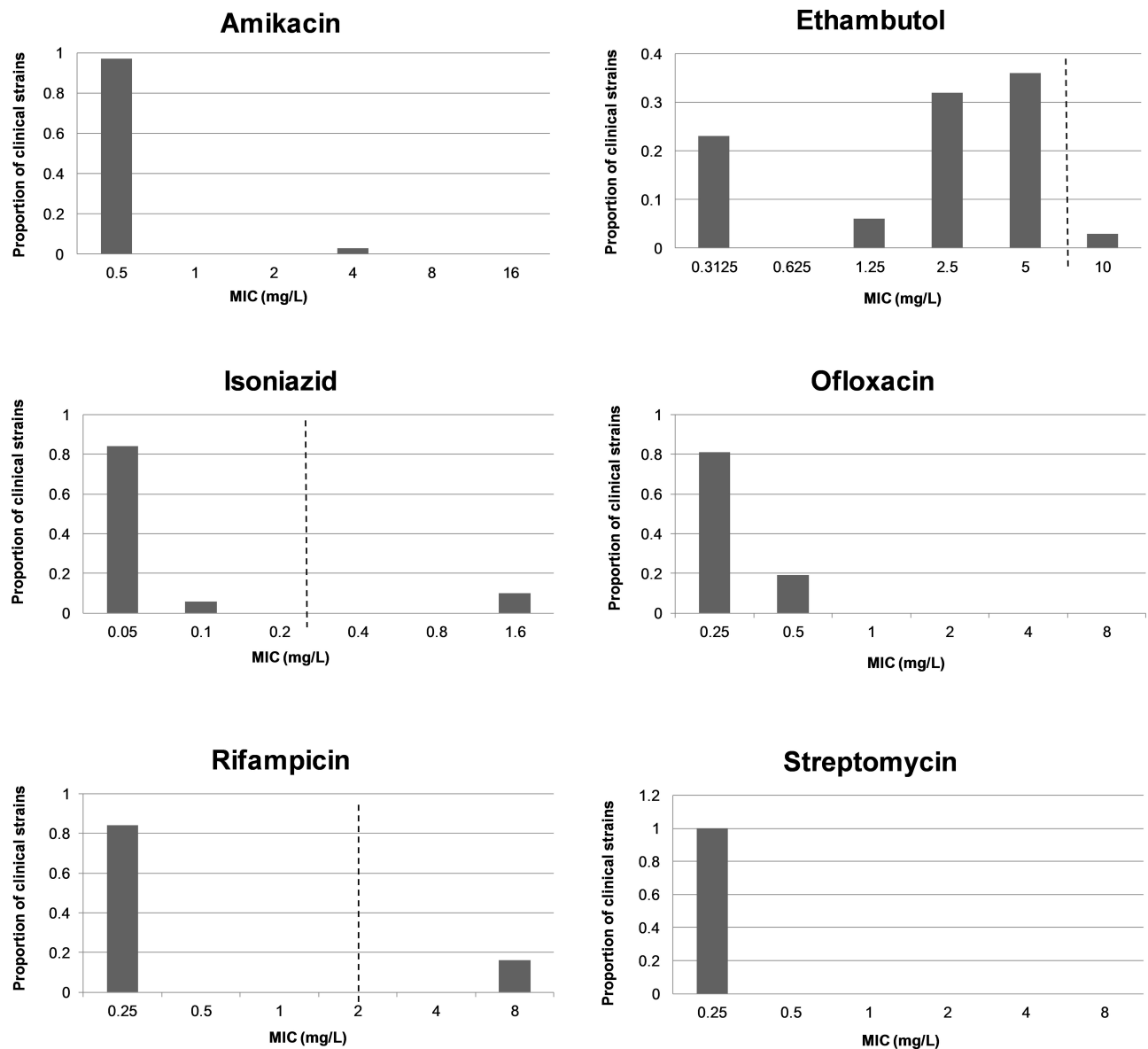


Figure 1 Minimum inhibitory concentration of BCG clinical strains for all the drugs tested.

Notes: Pointed lines indicate the breakpoint for resistance according to CLSI. For streptomycin, amikacin and ofloxacin the breakpoints for this method have not yet been determined. Abbreviations: MIC, Minimum inhibitory concentration.

considered as resistant. Three strains had a MIC for ofloxacin two-fold higher than the vaccine. For isoniazid, rifampicin and amikacin all the strains had the same MICs as the Sofia vaccine strain.

Finally, in the group of strains from Argentina identified as BCG Pasteur, all the strains had the same MIC value as the vaccine strain for amikacin and streptomycin. Fourteen strains had a MIC for ethambutol between 2.5 and 5 mg/L, hence being classified as having intermediate or inconclusive susceptibility by the CLSI guideline. One strain had a MIC for isoniazid higher than 1.6 mg/L and was considered resistant. Three

strains had a MIC for rifampicin higher than 8 mg/L and were classified as resistant. All the strains had a MIC for ofloxacin two-fold lower than the vaccine Pasteur.

Table 5 shows the minimal inhibitory concentration of the BCG clinical strains with the MIC₅₀ and MIC₉₀ statistics meaning the minimum inhibitory concentration at which 50% and 90% of the strains are inhibited.

Analyzing the genomes of the BCG clinical strains, we found non-synonymous SNPs in genes associated with resistance to antibiotics in the two Brazilian strains resistant to isoniazid and rifampicin, both strains had

Table 5 Minimum Inhibitory Concentrations of BCG Clinical Strains Against the Antibiotics Tested

BCG Moreau-Related Clinical Strains				
	Range	MIC ₅₀	MIC ₉₀	Mode
Amikacin	0.5–4	0.5	0.5	0.5
Ethambutol	1.25–5	2.5	2.5	2.5
Isoniazid	0.05–1.6	0.1	1.6	0.1–1.6
Ofloxacin	0.25–0.5	0.5	0.5	0.5
Rifampicin	0.25–8	0.25	8	0.25
Streptomycin	0.25	0.25	0.25	0.25
BCG Sofia-Related Clinical Strains				
	Range	MIC ₅₀	MIC ₉₀	Mode
Amikacin	0.5	0.5	0.5	0.5
Ethambutol	0.3125–10	2.5	5	2.5–5
Isoniazid	0.05	0.05	0.05	0.05
Ofloxacin	0.25–0.5	0.25	0.5	0.25–0.5
Rifampicin	0.25	0.25	0.25	0.25
Streptomycin	0.25	0.25	0.25	0.25
BCG Pasteur-Related Clinical Strains				
	Range	MIC ₅₀	MIC ₉₀	Mode
Amikacin	0.5	0.5	0.5	0.5
Ethambutol	0.3125–5	2.5	5	5
Isoniazid	0.05–1.6	0.05	0.05	0.05
Ofloxacin	0.25	0.25	0.25	0.25
Rifampicin	0.25–8	0.25	8	0.25
Streptomycin	0.25	0.25	0.25	0.25

Note: MIC values are expressed in mg/L.

Abbreviation: MIC₅₀ and MIC₉₀, minimum inhibitory concentration at which 50% and 90% of the strains are inhibited.

nsSNP in the *katG* and *rpoB* genes simultaneously (*katG* S293T and *rpoB* D359V). Another strain in this group had a deletion of three nucleotides in *rpoB* but was sensitive to rifampicin by the phenotypical method. Mutations associated with resistance to amikacin, ethambutol, ofloxacin and streptomycin were not found during the analysis.

In the phenotypically resistant strains found in the Argentinean group, no mutations associated with any antibiotic were detected.

Discussion

Loss of activity of anti-mycobacterial agents during incubation can lead to false interpretation of results and, in the worst case, cause failures in patient treatment based on non-precise results. We therefore evaluated the

stability of anti-mycobacterial drugs during the resazurin micro-plate assay over a period of five weeks. All drugs maintained the same MIC up until 35 days of incubation, except for ethambutol. An extensive evaluation of stability of several anti-mycobacterial drugs was recently reported by Schoutrop et al,¹³ observing at least a four-fold increase in the MIC for rifampicin and streptomycin when using commercial micro-plate assay in cation adjusted Mueller Hinton Broth (CAMHB), incubated at 37°C for fourteen days and using the reference strain *Mycobacterium avium* subsp. *hominissuis* IWGMT49. Different from their results, we observed that stability of these drugs is maintained when using 7H9 Middlebrook Broth supplemented with OADC. Schoutrop et al also demonstrated that dilution of the drugs in 7H9 Broth with OADC and incubation at 37°C reduced concentration of isoniazid and rifampicin in 7H9 media (54% and 92% respectively) after seven days, while our results show that the activity of both drugs remains unaffected for at least five weeks. Perhaps, these discrepancies are due to the presence of some residual anti-mycobacterial activity of secondary products during degradation of the drug, the latter suggested by the authors. Griffith and Bodily,¹⁴ described the loss of half of the activity of ethambutol dispensed on solid agar 7H10 media and maintained at 37°C after two to four days of incubation; these results are in agreement with our present observations. Our results show that, using 7H9 broth media, the activity of ethambutol remains unaffected until 21 days but that for *Mycobacterium* species that require longer incubation times, interpretation of results using this drug needs caution. Under our experimental conditions, amikacin, isoniazid, ofloxacin, rifampicin and streptomycin were stable for at least 35 days.

We observed a lack of agreement between drug resistance as determined by phenotypical DST and the presence of genetic modifications that have been associated with drug resistance. This inconsistency has been reported before and is a major problem for patient treatment using genetic-based procedures for drug resistance without confirmation by conventional DST.^{15–17} This phenomenon could be related to the existence of unknown resistance mechanisms like non described mutations in unknown drug-associated genes and contribution of efflux pumps.¹⁸

Strains harboring drug-associated mutations but being phenotypically sensitive have been described for *M. tuberculosis* strains in which MICs for streptomycin, ethambutol or rifampicin were increased only slightly,¹⁵ this might be due to difficulties inherent to the broth micro-dilution technique and could be more evident in front of higher antibiotic concentrations during the phenotypic assay.

Another factor to consider is that almost all drug susceptibility testing procedures, either based on culture or genotyping-based procedures were performed on strains of *M. tuberculosis* and that few data exist on DST in BCG clinical strains. Resistance to both isoniazid and ethionamide was previously observed in strains of BCG Danish and BCG Connaught.^{19,20} Intermediate susceptibility of BCG Danish had been addressed by the Statens Serum Institut and is now specified in the leaflet of the vaccine; nonetheless, the WHO states that there is no need of a change of vaccine policy.²¹ Intrinsic resistance to isoniazid was observed by Hesselting et al in an isolate from a six-month-old HIV-positive child with axillar adenitis that had been vaccinated with BCG Danish vaccine; the strain later acquired resistance to rifampicin during the child's treatment.⁹ In a study performed in the Netherlands, five strains isolated in children with lymphadenitis had a MIC higher (0.5 µg/mL) than that observed for the vaccine (0.1–0.2 µg/mL).²² Finally, in Brazil, a BCG strain isolated from an immunocompromised child with lymphadenitis was intrinsically resistant to isoniazid and later became resistant to rifampicin, sequence analysis demonstrated the S315T mutation in *katG* gene and D516V mutation in *rpoB* gene.¹⁰

Conclusion

Infections caused by vaccination with BCG are scarce and when diagnosed and treated early, there is a high probability of positive outcome. However, our report adds information on cases with intrinsic or acquired resistance to antibiotics and highlights the need for drug susceptibility screening of the strain before or during patient treatment. Also, we emphasize the urgency for standardization of second-line drugs' breakpoints for broth micro-dilution method in view of the emergence of first-line drug-resistant strains.

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Disclosure

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