

## Molecular characterization of *Mycobacterium tuberculosis* and *Mycobacterium bovis* isolates by *Enterobacterial Repetitive Intergenic Consensus-PCR*

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Tuberculosis (TB) is an infectious disease in which the molecular typing methods allow to have important information about the dynamics of transmission and to assist properly in disease control. Although the ERIC-PCR (Enterobacterial repetitive intergenic consensus-PCR) assay is fast and easy to perform, scarce studies have reported its use in epidemiological studies in TB outbreaks. In this study, we aimed to genotype *Mycobacterium tuberculosis* and *M. bovis* isolates by ERIC-PCR and compare its discriminatory power with two other classically used methods: 12 loci-MIRU (Mycobacterial Interspersed Repetitive Units) and Spoligotyping. The *M. tuberculosis* isolates studied were from northwestern and southwestern and *M. bovis* from northwestern Parana, Brazil. ERIC-PCR rendered banding patterns with great diversity (1 to 12 bands) of molecular sizes, ranging from 100 to 1600 bp. ERIC-PCR showed to be fast, simple and affordable to differentiate isolates. ERIC-PCR would be an important tool in the epidemiology of TB as screening in case of outbreak, which demands rapid intervention. However if any doubt persist, as it may occur with the application of only one genotypic method, other genotyping methods should be applied and carefully interpreted, always with additional epidemiological information.

**Keywords:** Tuberculosis. Molecular characterization. ERIC-PCR. Epidemiology.

### INTRODUCTION

Tuberculosis (TB) is an infectious disease caused mainly by *Mycobacterium tuberculosis* and it has been known for centuries. In Brazil, it is estimated an incidence of 84/100.000 in the population, in 2016, with a total of 81,137 cases notified (WHO, 2017).

Molecular differentiation of isolates is useful in epidemiological investigations as it is a means to better understand the mechanisms that influence the dynamics of transmission, identification of risk factors in a specific community and assist properly in disease control. The

genetic differentiation of *M. tuberculosis* complex isolates can determine the source of outbreaks and the relation between TB in domestic and wild animals, and identify the source of human infections (Mears *et al.*, 2015).

A highly discriminatory typing method based on variations of the IS6110 specific sequences in the *M. tuberculosis* genome, the Restriction Fragment Length Polymorphism (RFLP) has been widely used in epidemiological studies of TB. However, despite the promising results, the RFLP is laborious, requiring highly skilled personnel to carry out the trial.

Spoligotyping, which is based on the amplification of a region containing multiple direct repeats (DR) in a highly polymorphic locus in the genome of bacillus belonging to the *M. tuberculosis* complex, brought valuable information on the evolution of the bacillus. At present, many studies have used the Mycobacterial

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Interspersed Repetitive Units (MIRU), such as the 12 loci-MIRU, 15 loci-MIRU and 24 loci-MIRU, a PCR technique based on the study of Variable Number of Tandem Repeats sequences (VNTRs) that are repeated hundreds of times in the mycobacterial genome (Supply *et al.*, 2000; Supply *et al.*, 2001; Pandolfi *et al.*, 2007; Machado *et al.*, 2014).

The Enterobacterial Repetitive Intergenic Consensus sequence (ERIC) are repetitive elements of 126 bp and appear to be restricted to transcribed regions of the bacterial chromosome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. ERIC sequence was initially well-characterized in Gram negative bacillus belonging to the family *Enterobacteriaceae* and has been used to characterize clonal difference in species from this family. The presence of these sequences in mycobacteria was previously detected by some authors (Versalovic, Koeuth, Lupski, 1991; Englund, 2003; Sampaio *et al.*, 2006a; Sampaio *et al.*, 2006b), including in *M. tuberculosis* (Sechi *et al.*, 1998). According to Sechi *et al.* (1998) and Sampaio *et al.* (2006a; 2006b), ERIC-PCR has sensitivity and is easy to be applied in epidemiological studies of TB outbreaks, especially when the IS6110 RFLP cannot help the differentiation or when the study involves other mycobacterial species.

Although ERIC-PCR is fast and easy to perform, scarce studies have reported its use in epidemiological studies in TB outbreaks. In this study, we aimed to

characterize molecularly *M. tuberculosis* isolates by ERIC-PCR and compare its sensitivity with two other very used classical methods, MIRU and Spoligotyping.

## MATERIAL AND METHODS

### *Mycobacterium tuberculosis* Complex isolates

The *M. tuberculosis* clinical isolates used in this study were from patients attended at two independent laboratories at northwestern and southwestern Parana, Brazil. Isolates were previously biochemically identified (Kent, Kubica, 1985) and genotyped by MIRU and Spoligotyping (Machado *et al.*, 2014; Noguti *et al.*, 2010). Of the 93 isolates from northwestern Paraná, Brazil, which were previously typed by Spoligotyping and/or 12 loci-MIRU in our laboratory (Noguti *et al.*, 2010), 73 were typed by Spoligotyping plus ERIC-PCR and 63 by 12 loci-MIRU plus ERIC-PCR (Table I). Spoligotyping of 73 isolates from the northwestern rendered 29 patterns, where 16 were unique and 13 clusters (57 isolates) with 2-10 isolates each one. The 12 loci-MIRU of 63 isolates rendered 52 patterns, where 47 were unique and 5 clusters (15 isolates) with 2-6 isolates each one. Spoligotyping of 83 isolates from the southwestern study (Machado *et al.*, 2014) rendered 27 profiles, in which 16 were unique and 11 clusters (67 isolates) with 2-13 isolates each one. The 12 loci-MIRU

**TABLE I** - Discriminatory power of Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR), Spoligotyping and 12 loci-MIRU-VNTR typing, alone and in association in *Mycobacterium tuberculosis*

Methods / n° isolates	n° patterns	n° unique	n° clusters	n° clustered isolates	HGI <sup>a</sup>
<b>Northwestern</b>					
Spoligotyping / 73	29	16	13	57	0.953
12 loci-MIRU / 63	52	47	5	15	0.989
ERIC-PCR / 73	59	48	11	24	0.994
Spoligotyping + MIRU / 63	57	53	4	10	0.996
ERIC-PCR + Spoligotyping / 73	72	71	1	2	0.999
ERIC-PCR + 12 loci-MIRU / 63	63	63	0	0	1.0
<b>Southwestern</b>					
Spoligotyping / 83	27	16	11	67	0.993
12 loci-MIRU / 83	75	69	6	14	0.997
ERIC-PCR / 83	41	25	16	58	0.969
Spoligotyping + MIRU / 83	81	79	2	4	0.999
ERIC-PCR + Spoligotyping / 83	68	63	5	20	0.990
ERIC-PCR + 12 loci-MIRU / 83	82	81	1	2	0.999

a: HGI: Hunter-Gaston Index.

of 83 isolates rendered 75 patterns, in which 69 were unique and 6 clusters (14 isolates) with 2-3 isolates each one.

The retrospective demographic and epidemiological data were collected for all patients in previous studies (Kent, Kubica, 1985; Noguti *et al.*, 2010), by review of a national TB notification database (SINAN – National Diseases Notification System), being asked information such as: city and zip code of residence at the time of diagnosis, age, sex, ethnicity, HIV status, sample susceptibility profile, alcoholism and the occurrence of other diseases. The previous studies were approved by the Ethics Committee of the State University of Maringa, Parana (protocol n° 018/2011, CAAE n° 0375.0.093.000-10).

The 17 *M. bovis* isolates, another species from the *M. tuberculosis* complex, were obtained from lymph node samples collected from bovines with macroscopic tuberculous-like lesions or positive skin test. Animals were selected during postmortem inspection in an abattoir in the northwestern Paraná, Brazil. The isolates were previously identified biochemically (Kent, Kubica, 1985) and genotyped by Spoligotyping (Figure 1). The 17 *M. bovis* isolates rendered 5 spoligopatterns, in which one was unique and 4 clusters (16 isolates) with 2, 2, 3 and 9 isolates.

## DNA extraction

*M. tuberculosis* and *M. bovis* DNA were extracted from subcultures on Difco™ Lowenstein and Stonebrink media, respectively. Briefly, a loopful of bacterial growth was suspended in 6 M guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo, USA) and bacilli were lysed by freezing at -20 °C for 30 min, followed by heating at 100 °C for 10 min. This procedure was repeated twice. DNA was further extracted by 2 volumes of phenol-

chloroform-isoamyl alcohol (25:24:1, v/v), followed by two steps of extractions with chloroform-isoamyl alcohol (24:1, v/v). DNA was purified by ethanol precipitation, dissolved in 50 µL of Tris-EDTA, pH 8.0 (TE buffer) and stored at -20 °C until use (González-Y-Merchand *et al.*, 1996). DNA concentration was determined by ultraviolet spectrophotometry.

## ERIC-PCR

ERIC-PCR was performed based on the analysis of *Enterobacterial Repetitive Intergenic Consensus* sequence according to Sechi *et al.* (1998) in *M. tuberculosis* clinical isolates from northwestern (Noguti *et al.*, 2010) and southwestern (Machado *et al.*, 2014), and in *M. bovis* isolates from northwestern, Paraná, Brazil. The amplification of the sequence in *M. tuberculosis* and *M. bovis* isolates and *M. tuberculosis* H<sub>37</sub>Rv (control) were carried out with 1 µL of each mycobacterial DNA (10 ng) in 24 µL of a reaction mixture containing 2.5 µM of each primer ERIC1R (ATGTAAGCTCCTGGGGATTAC) and ERIC2F AAGTAAGTGAAGCTGGGGTGAAGCG) and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA) according to manufacturer's instructions. The amplification was carried out in a thermocycler (Applied Biosystems® Veriti® Thermal Cycler) programmed to 94 °C for 2 min and 35 cycles of 94 °C for 45 sec, 52 °C for 1 min, 70 °C for 10 min and finally 70 °C for 20 min.

The PCR amplicons were subjected to electrophoresis in 2.0% w/v agarose gel (Invitrogen Life Technologies, São Paulo, Brazil). The 100-bp DNA Ladder (Invitrogen Life Technologies, São Paulo, Brazil) was used as molecular marker. Gel was stained with ethidium bromide 0.5 µg/µL, visualized under an ultraviolet light Macrovue (Pharmacia Bioscience, Upsala, Sweden) and photodocumented digitally by Power Shot S215 (Cannon, NY, USA).

Binary spoligotyping	n° isolates
■ ■ ■ ■ ■ ■ □ □ □ □ □ ■ □ □ □ □	2
■ ■ ■ ■ ■ ■ ■ ■ ■ □ □ ■ □ □ □ □	3
■ ■ ■ ■ ■ ■ ■ ■ ■ □ ■ □ □ □ □	9
■ ■ ■ ■ ■ ■ ■ ■ ■ □ ■ □ □ □ □	1
■ ■ □ □ □ □ □ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ □ □ □ □ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ □ □ □ □	2

**FIGURE 1** - Binary spoligotyping data of 17 *Mycobacterium bovis* isolates from lymph node samples collected from bovines with macroscopic tuberculous-like lesions or positive skin test during postmortem inspection in an abattoir in the northwestern region of Paraná State, Brazil.

## Analysis

BioNumerics software (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of multi-band patterns in ERIC-PCR and compared to the Spoligotyping and 12 loci-MIRU, previously performed (Machado *et al.*, 2014; Noguti *et al.*, 2010). Dendrograms were constructed and the genetic distance was built employing the UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Mean) based on 1% optimization and position tolerance for the three methods (Sneath, Sokal, 1973). Only isolates with 100% similarity were considered closely related.

The evaluation of the discriminative power of each typing method, separately as well as, in combination was undertaken by using the Hunter-Gaston index (HGI) (Hunter, Gaston, 1988), which is based on the probability that two unrelated strains sampled from the population test will be placed into different typing groups.

## RESULTS

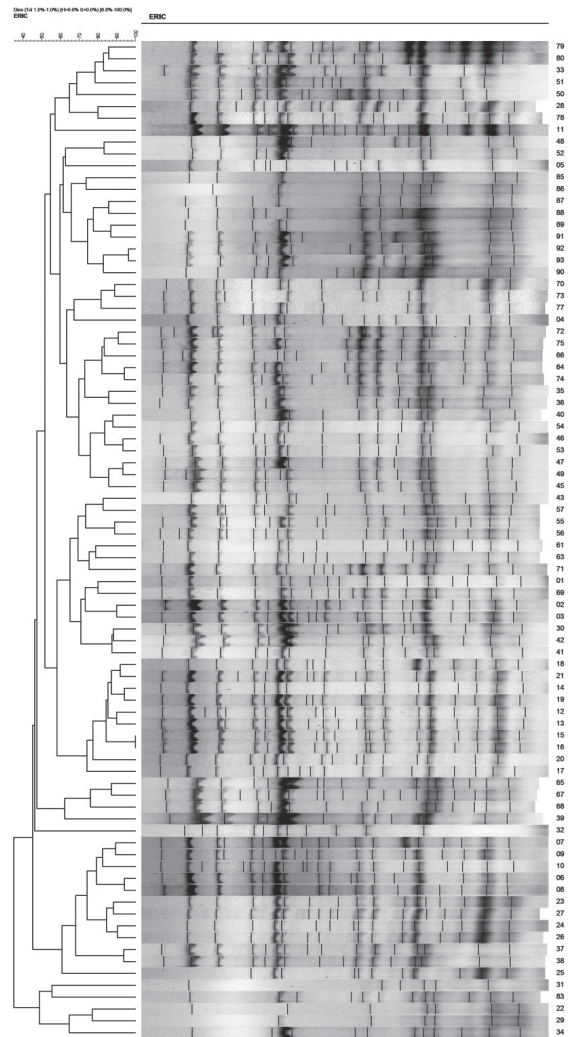
A comparison of the discriminatory power of ERIC-PCR to Spoligotyping and MIRU can be seen at Table I. ERIC-PCR, of 73 isolates from northwestern Paraná previously typed by Spoligotyping, rendered 59 patterns, in which 48 were unique and 11 clusters (24 isolates) with 2-4 isolates each one. The application of Spoligotyping plus ERIC-PCR in 73 isolates could not differentiate only 2 clustered isolates. The analysis of 63 isolates genotyped by 12 loci-MIRU plus ERIC-PCR showed 100 % of differentiation (Figure 2). The three methods rendered 100 % of differentiation of the isolates.

ERIC-PCR, carried out in 83 isolates from southwestern Parana, rendered 41 ERIC patterns. From these, 25 were unique and 16 clusters (58 isolates) with 2-9 isolates each one.

The analyses of 83 isolates typed by 12 loci-MIRU plus Spoligotyping could not differentiate only 4 clustered isolates in 2 clusters. Spoligotyping plus ERIC-PCR showed total differentiation of isolates in 6 of 11 clusters by Spoligotyping. The other isolates in the five clusters had partially differentiation of isolates. The application of 12 loci-MIRU plus ERIC-PCR could not differentiate only 2 clustered isolates (Figure 3). The three methods rendered 100% of differentiation of the isolates. ERIC-PCR, of 17 *M. bovis* isolates, rendered 100 % of differentiation.

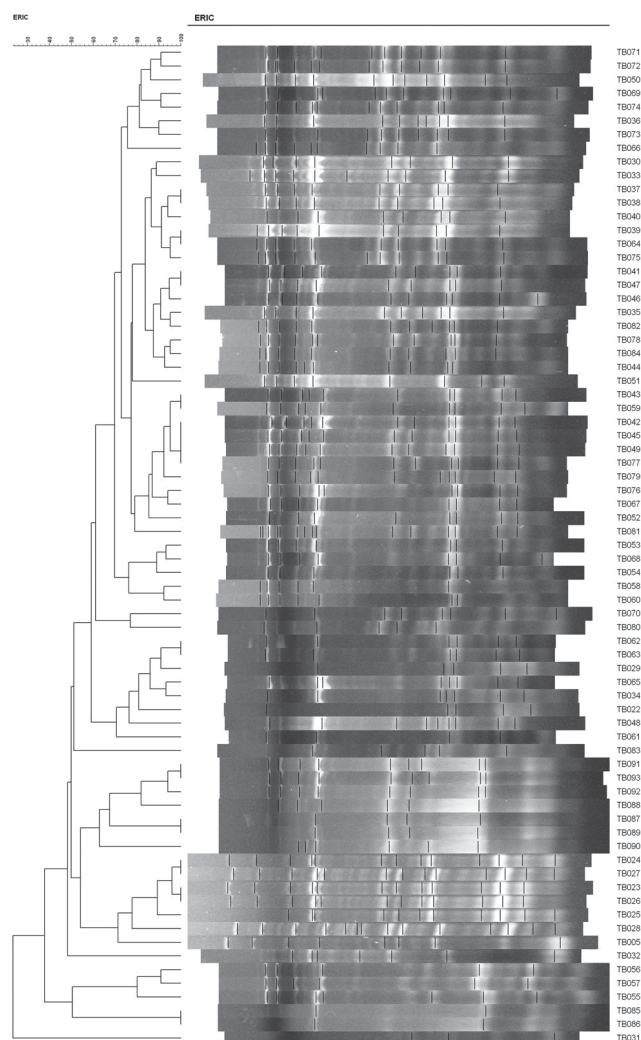
## DISCUSSION

The present study was designed to compare



**FIGURE 2** - Combined numerical analysis of *Mycobacterium tuberculosis* clinical isolates from northwestern, Paraná, Brazil, using Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR). The dendrogram was obtained using the BioNumerics software (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium).

a very simple and fast genotyping method, ERIC-PCR, with Spoligotyping and MIRU to differentiate epidemiologically related from unrelated *M. tuberculosis* isolates. We feel this need since ERIC-PCR was a genotyping method that had been used previously, 10 years ago, in *Mycobacterium tuberculosis* and mycobacteria other than tuberculosis. From that, no study was carried out with ERIC-PCR in *M. tuberculosis* once other very used and established genotyping methods for epidemiological studies in tuberculosis showed to be useful, such as Spoligotyping and MIRU. At the time, ERIC-PCR has been used for genotyping different Gram negative bacillus. As ERIC-PCR is fast and an inexpensive method, it could be used as a screening tool in low-income laboratories to



**FIGURE 3** - Combined numerical analysis of *Mycobacterium tuberculosis* clinical isolates from southwestern, Paraná, Brazil, using Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR). The dendrogram was obtained using the BioNumerics software (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium).

differentiate isolates from a suspected outbreak in little time.

The *M. tuberculosis* isolates studied were from two different settings, northwestern and southwestern Parana, Brazil. In the northwestern, patients belonged to eight cities (Maringá and seven small cities surrounding) which is considered a low-endemic area of TB in Parana, with a TB incidence rate of 22.62/100.000 (Noguti *et al.*, 2010). In the southwestern, which borders the Paraguay and Argentina countries, patients were from two major cities, Foz do Iguaçu and Cascavel, and other eight small surrounding cities at the Brazilian border, with a TB incidence rate of 41.8/100.000 and 25.5/100.000, respectively (Machado *et al.*, 2014).

In our study, comparing the performance of the three methods used separately and in combination as genotyping tools in *M. tuberculosis*, the classical 12 loci-MIRU showed better performance for genotypic differentiation of isolates from southwestern setting (HGI 0.997), which had higher incidence of TB compared with the northwestern setting. Nonetheless, ERIC-PCR was the best method for differentiating isolates from the northwestern setting (HGI 0.994).

The ERIC-PCR Electrophoretic analysis of the *M. tuberculosis* generated multiple bands with variable intensities. We observed banding patterns that had 1 to 12 bands with molecular sizes ranging from 100 to 1600 bp (Figure 2). Our analyses were carried out only with fragments below 1600 bp, which showed better resolution. The distribution of the fragments occurred evenly, but the majority was concentrated at 600 bp region. As there are no established criteria for interpreting ERIC-PCR, what could cause difficulty to interpret the band patterns, we considered isolates to be different, according to Pellegrino *et al.* (2002), based on a single band difference and additional support analysis by the other two used genotyping methods (Spoligotyping and MIRU).

ERIC-PCR had the ability to differentiate isolates that were clustered by 12 loci-MIRU and Spoligotyping separately or combined. The combination of ERIC-PCR with 12 loci-MIRU showed to be equal to 12 loci-MIRU plus Spoligotyping (HGI 0.999) to differentiate isolates from southwestern setting. In isolates from the northwestern, ERIC-PCR with 12 loci-MIRU (HGI 1.0) as well as ERIC-PCR with Spoligotyping (HGI 0.999) showed good discrimination of isolates, which suggests the high value in using two or more methods for genotypic studies in TB. Our results came in agreement with Sechi *et al.* (1998) and indicate that the ERIC sequence could be used as an independent marker, once the relationship among the profiles obtained by ERIC-PCR and those obtained by the available genotyping method for *M. tuberculosis* were not related. In the study conducted by Noguti *et al.* (2010), there were two isolates.

Cangelosi *et al.* (2004), Ashworth *et al.* (2008) and Jang *et al.* (2011) evaluated the utility of a commercially available rep-PCR System, initially developed for genotyping *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterobacteriaceae* and others microorganism, to genotype *M. tuberculosis* and *Mycobacterium avium* Complex and found the system to be highly discriminative.

In contrast to the 12 loci-MIRU and Spoligotyping methods tested in our study, ERIC-PCR requires only one PCR per sample. 12 loci-MIRU requires 12 separately PCR and Spoligotyping one PCR followed by

hybridization reaction. Thus, in terms of time spent, ERIC-PCR is very fast compared to the others. The same analysis can be made about the cost of the three methods, in which ERIC-PCR proved to be the less expensive.

Also, in our study, ERIC-PCR showed to be useful in differentiating *M. bovis*, species from the *M. tuberculosis* complex, which showed 100% of differentiation of isolates clustered by Spoligotyping. Although, our results came in agreement with Sechi *et al.* (1999), a study with a larger number of *M. bovis* isolates would be of great interest to understand the real utility of ERIC-PCR as genotypic tool for this species.

One limitation of our study is that none of the genotyped isolates were members of the Beijing family, which is not common in our region. In this sense, additional studies would contribute to know if ERIC-PCR has the ability in discriminating members of this family and also among them, which is very common in Asian countries with high incidence of TB.

As for any laboratory methodology, the reproducibility of the results depends on the standardization of each step during its execution. In the relevant literature, with other bacteria, no standardization on the DNA concentration was used to perform ERIC-PCR in most studies. In our experience with ERIC-PCR, standardization of the mycobacterial DNA concentration should be carried out to have more constant intensities of the DNA bands in the gel analyzes. Also, reagents and equipment should also be of the same manufacturer. In our study, we did not standardize the concentration of DNA from isolates to perform ERIC-PCR inasmuch as they were from previous studies (Machado *et al.*, 2014; Noguti *et al.*, 2010) and were stored at -20 °C. As the ERIC-PCR were performed in duplicate by independent assays for the same isolates in different days, to assure its reproducibility, we could observe that no problem to read the gels occurred with the DNA bands with the most intensity. However, in a few cases, the bands with low intensity were difficult to be observed in the gel.

As the ERIC sequence, is not a proprietary technology and the primers and PCR conditions are commercially available, additional studies with isolates from different countries would help to elucidate the applicability of ERIC-PCR in outbreaks, which require fast resolution, and could after be completed with MIRU and/or Spoligotyping methods.

The molecular typing method has been used for a variety of epidemiologic investigations, such as those to confirm outbreaks in institutions and to identify them in situations that appear to be sporadic cases of TB, risk factors for recent infections or rapid progression diseases, tracking the geographical distribution of *M. tuberculosis*

clusters of importance to public health and cross-laboratory contamination assessment. An ideal genotyping method should generate conclusive information in a fashion time for allowing direct interventions. However, to date, there is no method that fully meets qualities as to be simple, reproducible and affordable to be applied in many laboratories. In this context, ERIC-PCR could, maybe, fill these qualities. In conclusion, we observed that ERIC-PCR would be an important tool in the epidemiology of TB as a screening in outbreaks, which demand rapid intervention. However if any doubt persists, as it may occur with the application of only one genotypic method, other disposable genotyping methods for this purpose should be applied and carefully interpreted always with additional epidemiological information.

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## CONFLICT OF INTEREST

No conflict of interest declared.

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