Viability of stressed *Mycobacterium tuberculosis* and association with multidrug resistance

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Abstract

This study investigated biological characteristics of recovered stressed *M. tuberculosis* isolates that failed to grow in differential culture media for phenotypic identification and in culture media containing anti-tuberculosis drugs for drug-susceptibility testing, despite of having grown in primary culture. It represents an improvement in the diagnosis of MDR tuberculosis and tuberculosis control.

Key words: *Mycobacterium*, multidrug resistance, culture growth, spoligotyping.

Tuberculosis has never been eradicated from the world and the emergence of multidrug resistant strains (MDR) of *Mycobacterium tuberculosis* has become a worldwide public health problem (Dalcolmo et al., 2007; World Health Organization 2000).

The expansion of MDR strains has been linked to specific genotypes classified by spoligotyping method (Dalla Costa et al., 2009; Gomes et al., 2011; Kamerbeek et al., 1997; Mendes et al., 2011; Von Groll et al., 2010). Isolation (primary culture), species identification (ID) and drug-susceptibility testing (DST) of *M. tuberculosis* are essential procedures for tuberculosis control (Collins et al., 1997). Delay in the diagnosis of MDR tuberculosis leads to patients with chronic disease and continued transmission in the community (Dalcolmo et al., 2007; World Health Organization 2000).

Some researchers showed that bacillary stress due to drug resistance or other stresses may reduce the capacity of in vitro growth of *M. tuberculosis* (Von Groll et al., 2010; Warner and Mizrahi, 2006). On the other hand, as cited by Von Groll et al. (2010), *M. tuberculosis* has the tendency to form clumps when grown in liquid media, this may lead to an unequal distribution of bacilli which may influence the recovery rates of subcultures. These phenomena can affect results of ID and DST and the absence of growth of drug-resistant isolates may delay the correct diagnosis of tuberculosis.

This study aimed to recover and investigate biological characteristics presented by frozen primary cultures of mycobacteria that have showed growth failure in the subcultures for ID and DST tests when they were submitted to a first analysis.

Primary cultures of mycobacteria analyzed at Instituto Adolfo Lutz (IAL), a reference laboratory for the state of São Paulo, Brazil, have been received from local as well as regional laboratories either in solid media as Ogawa or Löwenstein-Jensen or in liquid medium as MB/BacT® or BD BACTEC-MGIT 960 (Collins et al., 1997; Giampaglia et al., 2007; Susemihl et al., 1993).

At IAL, the quality of primary culture has been evaluated by macroscopic and microscopic observation before performing ID and DST tests (Monteiro et al., 2003). During the procedures of ID and DST, an inoculum of each culture with adequate growth was preserved at -70 °C in glass beads humidified with sauton medium containing 10% of glycerol (Giampaglia et al., 2009).

From January 2001 to February 2002, 40 primary cultures classified as adequate for analysis and presumptively identified as *M. tuberculosis* - by cord formation and culture morphology as cited by Monteiro et al. (2003) showed growth failure when subcultured in differential culture me-
dia for phenotypic ID and in culture media containing antituberculosis drugs for DST by resistance ratio method (Collins et al., 1997).

One cryovial of each primary culture was removed from the freezer. Three glass beads were removed from the vial using a sterile loop and each one was inoculated into tubes of Löwenstein-Jensen, Ogawa and Middlebrook 7H9 (7H9) media, which were incubated at 37 °C. If culture growth did not show within 60 days, the procedure were repeated once again to confirm its loss of viability.

The mycobacterial growth was analyzed by morphological characteristics and the average number of colonies in solid media was reported as (+) 20 to 100 colonies, (++) more than 100 colonies (poor growth) and (+++) more than 100 colonies (luxuriant growth). For a better standardization of each inoculum only subcultures from solid media were used in the additional tests.

The recovered primary cultures were submitted to phenotypic identification and to PCR-restriction fragment length polymorphism analysis (PRA) of the hsp65 gene as previously described elsewhere (Collins et al., 1997; Devallois et al., 1997).

The resistance pattern of M. tuberculosis to isoniazid (I), rifampicin (R), ethambutol (E) and streptomycin (S) performed using by MGIT 960 (Giampaglia et al., 2007). Susceptibility to pyrazinamide (P) was determined by pyrazinamidase activity (OPAS-OMS 1986). The spoligotypes obtained by the analysis of spacer oligonucleotide typing (spoligotyping) were compared to Bases de Données: SPOLDB4 - Institute Pasteur de la Guadeloupe (2010). The reference strains of the isolates belonged to Latin-American-Mediterranean (LAM) family - 12 isolates, followed by S - four isolates, Haarlem and modern tuberculosis strains (T) - three isolates each, LAM3/S convergent, Beijing and IS6110 low banding (X) - two isolates each, East-African Indian-EAI1_SOM and Unknown (one isolate each). Seven orphan patterns matched with none at SPOLDB4 - Institute Pasteur de la Guadeloupe (2010). The reference strains were classified as spoligotyping pattern 451(H37Rv) and 482 (M.bovis_BCG).

Identification by phenotypic tests and by PRA hsp65 method showed that all the recovered isolates belonged to the M. tuberculosis complex. Drug susceptibility testing of the 39 isolates showed that 13 (33.3%) were susceptible to all the tested drugs, and 26 (66.7%) were resistant to at least one of the five first-line antituberculosis drugs. Among the 26 resistant isolates, 18 (69.0%) were MDR (Table 2).

Two isolates, susceptible to all the tested drugs (nº 38, nº 39) were unclassified by spoligotyping as they showed persistently no hybridization with the 43 spacer oligonucleotides. The remaining 37 (94.8%) isolates showed 24 patterns, 18 (72.0%) of them were unique pattern and six were clusters (24.0%) consisting of two or three of the 19 remaining isolates (Table 2). Among the isolates included in clusters, 17 (89.5%) were resistant to antituberculosis drugs and 13 (68.4%) were MDR.

Matched patterns with SPOLDB4 showed that most of the isolates belonged to Latin-American-Mediterranean (LAM) family - 12 isolates, followed by S - four isolates, Haarlem and modern tuberculosis strains (T) - three isolates each, LAM3/S convergent, Beijing and IS6110 low banding (X) - two isolates each, East-African Indian-EAI1_SOM and Unknown (one isolate each). Seven orphan patterns matched with none at SPOLDB4 - Institute Pasteur de la Guadeloupe (2010). The reference strains were classified as spoligotyping pattern 451(H37Rv) and 482 (M.bovis_BCG).

The present study showed the recovery of 97.5% of frozen primary cultures of mycobacteria that showed growth failure in the subcultures for ID and DST tests when they were submitted to a first analysis.

Table 1 - Average number of days required to recover primary culture of stressed M. tuberculosis strains – Instituto Adolfo Lutz 2001-2002.

| Growth level | Ogawa medium | Löwenstein-Jensen medium | Middlebrook 7H9 medium |
|--------------|--------------|-------------------------|------------------------|
|              | Susceptible | Resistant | Susceptible | Resistant | Susceptible | Resistant |
| +            | 2 (60.0)    | 1 (60.0)   | 1 (60.0)   | 1 (60.0)   | 3 (16.0)   | 6 (20.0)   |
| ++           | 5 (30.0)    | 13 (34.6)  | 4 (32.5)   | 13 (34.6)  | 6 (17.5)   | 11 (18.0)  |
| +++          | 6 (35.0)    | 11 (31.8)  | 4 (32.5)   | 11 (31.8)  | 4 (15.0)   | 9 (19.0)   |
| Total        | 13 (27.6)   | 25 (34.4)  | 9 (35.5)   | 25 (34.4)  | 13 (16.5)  | 26 (19)    |

Growth level- Solid medium (+) 20/100 colonies, (++) more than 100 colonies (poor growth) and (+++) more than 100 colonies (luxuriant growth); Liquid medium: (+) few clumps, (++) some clumps and (+++) many clumps.
Our already published study about maintenance of *M. tuberculosis* on glass beads (Giampaglia *et al.*, 2009) showed that almost 100.0% of the 730 investigated cultures remained viable after 1 to 5 yr of frozen. The fact that in this study six cultures only grew at the second attempt may be due to the tendency of mycobacteria to form clumps in liquid media (Von Groll *et al.*, 2010). Unequal distribution of bacilli may have occurred in glass beads and may have influenced the recovery rate of mycobacteria.

Others factors that may have influenced the recovery rate of mycobacteria were chromosomal mutations in *M. tuberculosis* that are responsible for resistance to most of the anti-tuberculosis drugs, including rifampin and isoniazid. These mutations have been associated with a fitness cost, seen as a decreased growth rate in vitro (Dalla Costa *et al.*, 2009; Warner and Mizrahi, 2006). Although chromosomal mutations were not investigated in this study our results are consistent with this premise as 66.7% of the stressed *M. tuberculosis* isolates were resistant to at least one of the five first-line antituberculosis drugs and 69.0% of them were MDR.

The average days to recover susceptible strains was 27.6 (range 20-60 days) in Ogawa and 16.5 (range 15-20 days) in 7H9 medium and to recover resistant strains was 34.4 (range 20-60 days) in Ogawa and 19 (range 15-30 days) in 7H9 medium. This result is in agreement with the study of Von Groll *et al.* (2010) that showed that MDR isolate presented a lower reproductive efficiency. No difference was observed between the average days required to recover susceptible and resistant strains of *M. tuberculosis* in Lőwenstein-Jensen.

Spoligotyping allowed the classification of 24 spoligotyping patterns including those representing the major families of tubercle bacilli: Beijing, Haarlem, LAM and EAI. Among the spoligotyping clusters, we found a high proportion of drug-resistant isolates already described in Brazil (Dalla Costa *et al.*, 2009; Gomes *et al.*, 2011; Mendes *et al.*, 2011). This study detected some clusters of drug-resistant and drug-susceptible mycobacteria as those

### Table 2 - Mycobacterium tuberculosis spoligotypes and drug susceptibility testing (DST) patterns – Instituto Adolfo Lutz, Brazil, 2001-2002.

| Type strain | Spoligotyping | Family | DST (isolate number) |
|-------------|---------------|--------|-----------------------|
| 1           |               |        |                       |
| 4           |               |        |                       |
| 17          |               |        |                       |
| 20          |               |        |                       |
| 33          |               |        |                       |
| 34          |               |        |                       |
| 42          |               |        |                       |
| 48          |               |        |                       |
| 50          |               |        |                       |
| 60          |               |        |                       |
| 64          |               |        |                       |
| 119         |               |        |                       |
| 137         |               |        |                       |
| 1905        |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |
| 0           |               |        |                       |

Spoligotyping pattern: black square = hybridizing; empty square = nonhybridizing; Type strain and Family as described by Bases de Données:SPOLDB4 [cited Oct., 2010]. Available from: http://www.pasteurguadeloupe.fr/; DST pattern: I = isoniazid, R = rifampicin, E = ethambutol, S = streptomycin, P = pyrazinamide.
observed by Mendes et al. (2011) in São Paulo city, showing that these strains have been circulating in the São Paulo state.

Our findings emphasize the importance of recovered stressed \textit{M. tuberculosis} isolates that failed to grow in differential culture media for phenotypic ID and in culture media containing antituberculosis drugs for DST, despite of having grown in primary culture. It represents an improvement in the diagnosis of MDR tuberculosis and tuberculosis control.

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