SHORT COMMUNICATION

Mycobacterium tuberculosis Complex Differentiation Using *gyr*B-Restriction Fragment Length Polymorphism Analysis

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Mycobacterium tuberculosis *complex (MTBC) members are causative agents of human and animal tuberculosis. Differentiation of MTBC members is required for appropriate treatment of individual patients and for epidemiological purposes. Strains from six MTBC species* – M. tuberculosis, M. bovis *subsp*. bovis, M. bovis BCG, M. africanum, M. pinnipedii, *and "*M. canetti*" – were studied using* gyr*B-restriction fragment length polymorphism (*gyr*B-RFLP) analysis. A table was elaborated, based on observed restriction patterns and published* gyr*B sequences. To evaluate applicability of* gyr*B-RFLP at Instituto Adolfo Lutz, São Paulo, Mycobacterial Reference Laboratory, 311 MTBC clinical isolates, previously identified using traditional methods as* M. tuberculosis *(306),* M. bovis *(3), and* M. bovis *BCG (2), were analyzed by* gyr*B-RFLP. All isolates were correctly identified by the molecular method, but no distinction between* M. bovis *and* M. bovis *BCG was obtained. Differentiation of* M. tuberculosis *and* M. bovis *is of utmost importance, because they require different treatment schedules. In conclusion,* gyr*B-RFLP is accurate and easy-to-perform, with potential to reduce time needed for conventional differentiation methods. However, application for epidemiological studies remains limited, because it cannot differentiate* M. tuberculosis *from* M. africanum *subtype II, and "*M. canetti*",* M. africanum *subtype I from* M. pinnipedii, *and.* M. bovis *from* M. bovis *BCG.*

Key words: *Mycobacterium tuberculosis* complex - *gyr*B - restriction fragment length polymorphism

Tuberculosis is one of the leading causes of death due to infectious agents. Ninety-five per cent of cases occur in the developing world, where few resources are available for diagnostic and treatment (ATS 2000). Members of *Mycobacterium tuberculosis* complex (MTBC) are the causative agents of tuberculosis in humans and animals and, despite genetic close relationship, they differ in epidemiology, pathogenicity, geographic range, host preference, and in importance for tuberculosis disease in humans. MTBC includes *M. tuberculosis*, *M. bovis* subsp. *bovis*, *M. bovis* subsp. c*aprae*, *M. bovis* BCG vaccine strain, *M. africanum* (subtypes I and II), *M. microti*, and "*M. canetti*". The last member of this list has still not been officially recognized on the list of Bacterial Names with Standing in Nomenclature [http://www.bacterio.cict.fr]. A novel member, *M. pinnipedii*, proposed by Cousins et al. (2003), has been well-characterized as a specie of *M. tuberculosis* complex. Genetically, all members of this complex are extremely similar, having 99.9% similarity at the nucleotide level and identical 16SrRNA sequences (Brosch et al. 2002).

Early diagnosis is essential for tuberculosis control. The high degree of sequence conservation among members of MTBC makes differentiation of species in the clinical mycobacteriology laboratory a difficult task. Routine differentiation is still based on phenotypic characteristics, such as oxygen preference, niacin accumulation, nitrate reductase activity, colony morphology, and resistance to two compounds, 2-thiophen-carboxylic acid hydrazide (TCH) and pyrazinamide (PZA) (Collins et al. 1997). These tests need sufficient bacterial growth, are time-consuming and are not performed by many laboratories routinely. Species belonging to MTBC cannot be differentiated by small subunit rRNA (16SrRNA) or internal transcribed spacer (ITS) sequencing. Molecular techniques were designed to rapidly differentiate MTBC species, as Spoligotyping (Kamerbeek et al. 1997), methods to detect mutations in *pncA* and *oxyR* genes (Scorpio et al. 1996, Sreevatsan et al. 1996), *mtp40*-PCR (Del Portillo et al. 1991, Liébana et al. 1996), and PCR-amplification of regions of difference (RD) (Parsons et al. 2002, Huard et al. 2003), among others. There are some limitations for the use of these techniques. *pncA*, *oxyR*, and *mtp40* are useful for differentiation of *M. tuberculosis* and *M. bovis*, but not for other MTBC members. Spoligotyping requires analysis of several isolates in the same experiment and RD analysis requires several amplification steps.

The *gyr*B gene encodes the B subunit of DNA gyrase (topoisomerase II), an enzyme universally distributed and essential for bacterial replication. Kasai et al. (2000) obtained *gyr*B nucleotide sequences from different strains of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. mi-*

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croti. A two-step method for differentiation of these species was developed, based on *gyr*B. In the first step, a 1020 bp fragment of the gene is amplified with specific primers, which do not generate amplicons from other species of mycobacteria. In the second step, the amplicon is digested with restriction enzymes. Analysis of restriction fragment length polymorphisms (RFLP) allows distinction of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. Niemann et al. (2000) extended these observations and proposed a diagnostic algorithm of *gyr*B-RFLP patterns to differentiate *M. tuberculosis/M. africanum* II from *M. africanum* I, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae* and *M. microti*.

In order to implement *gyr*B-RFLP in the Reference Laboratory at Instituto Adolfo Lutz, in São Paulo, Brazil, we analyzed *gyr*B-RFLP patterns from reference strains, including *M. tuberculosis* H37Rv (ATCC 27294), *M. bovis* (AN5), and *M. bovis* BCG (Pasteur), "*M. canetti*" isolate 140010059 and *M. africanum* isolate 140010068 from the National Reference Laboratory for Mycobacteria at Institut Pasteur (Paris, France) culture collection, kindly provided by Dr Véronique Vincent, and *M. pinnipedii* isolate 1458, kindly provided by Amelia Bernardelli (Senasa, Buenos Aires, Argentina). Amplifications were performed using purified DNA or bacteria lysed by freezing and thawing, using the protocol described by Kasai et al. (2000), with modifications. Mycobacteria DNA was added to PCR mix containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1U of *Taq* DNA polymerase (Invitrogen), 0.2 mM of each deoxynucleoside triphosphate, and 100 pmol of each primer MTUBf (5'TCGGACGCGTATGCGATATC) and MTUBr (5'ACATACAGTTCGGACTTGCG). After initial denaturation for 10 min at 95ºC, 35 amplification cycles were completed, each consisting of 1 min at 94ºC, 1 min at 65ºC and 1,5 min at 72ºC. A final extension of 10 min at 72ºC was applied. The amplification product was analyzed by restriction with *Rsa*I, *Taq*I and *Sac*II. Restriction digests were separated in 2% agarose gels together with 50 bp DNA ladder (Invitrogen), applied in a separate lane. Gels were stained with ethidium bromide and photographed on UV transiluminator. RFLPs in the 1020-bp *gyrB* fragments were analyzed using GelCompar II v. 2.5 program (AppliedMaths, Sint-Martens-Latem, Belgium) (Fig. 1).

After analysis of the results we observed that some bands showed different sizes from those described by Niemann et al. (2000). Also, small bands, not considered by Kasai et al. (2000) and Niemann et al. (2000), were clearly visible and helped in species differentiation (Fig. 1). Thus, we developed a new table based on band sizes calculated using GelCompar and confirmed by analysis of sequences deposited by Kasai et al. (2000) in DNA DataBank of Japan (http://www.mbio.co.jp/icb/icb.html) (Fig. 2). This new table describes for the first time the *gyr*B-RFLP patterns of "*M. canetti*", indistinguishable from *M. tuberculosis*, and of *M. pinnipedii*, indistinguishable from *M. africanum* I. This last result confirms findings described by Cousins et al. (2003), demonstrating that seal isolates were genetically closer to *M. tuberculosis* and *M. africanum* than to *M. bovis*.

To evaluate the clinical applicability of *gyr*B-RFLP, we analyzed 311 MTBC isolates cultured from patients living in different cities of the state of São Paulo. A set of 307 clinical MTBC isolates, were consecutively received at the Mycobacteriology Laboratory at Instituto Adolfo Lutz (IAL) for susceptibility and identification tests from Jan 9 2003 to Mar 7 2003. From these isolates, 83 (27.1%) came from cities of the state of São Paulo and 224 (72.9%) from the Metropolitan Region of Great São Paulo. They were cultured from sputum (268 samples), bronchial washes (8), lymph nodes (9), pleural fluids (4), urine (4), blood (4), cerebrospinal fluids (4), gastric washes (3), abscesses from unknown sites (2), and skin biopsy (1). For differentiation of MTBC, isolates were examined using biochemical and drug susceptibility tests. Biochemical tests include nitrate reduction, niacin accumulation test (BBL Taxo TB niacin test, Becton Dickinson, US) and growth in the presence of TCH (5 µg/ml), p-nitrobenzoic acid (PNB) (0.5 mg/ml) and cycloserine (0.02 mg/ml). Drug susceptibility was determined by the resistance ratio method in Löwenstein

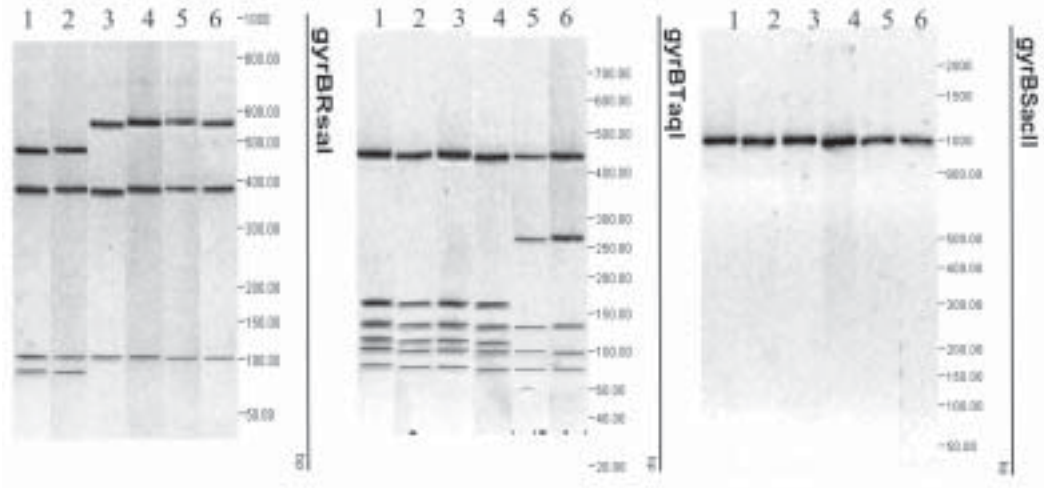

Fig. 1: *gyr*B patterns analyzed using GelCompar II. Lanes 1: *Mycobacterium bovis* BCG; 2: *M. bovis* subsp. *bovis*; 3: *M. pinnipedii*; 4: *M. africanum*; 5: "*M. canetti*"; 6: *M. tuberculosis*

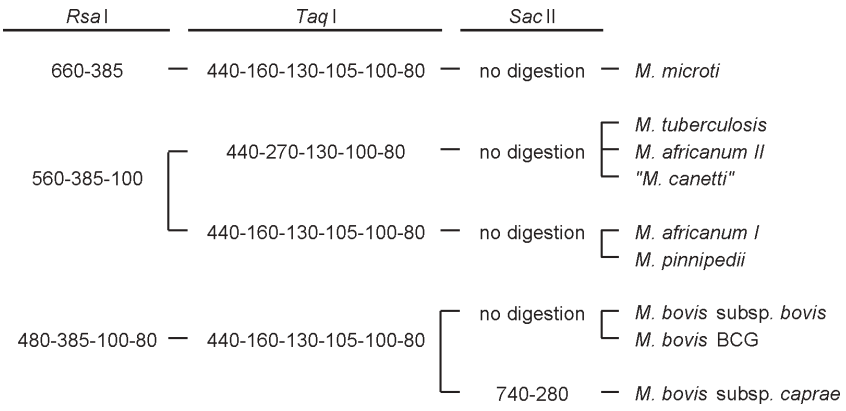

Fig. 2: table of *gyr*B patterns for differentiation of *Mycobacterium tuberculosis* complex species, based on gel images analyzed using GelCompar II and published sequence data.

Jensen (LJ) medium containing isoniazid (INH), rifampicin (RF), ethambutol (EMB) and streptomycin (SM) (Collins et al. 1997). Pyrazinamide (PZA) susceptibility was tested by detection of pyrazinamidase activity (Collins et al. 1997). At Instituto Adolfo Lutz, drug susceptibility test is performed under specific criteria. All isolates cultured from cases of HIV positive, multidrug resistance tuberculosis, relapses, default and treatment failure are tested to INH, RF, and PZA. If some resistance occurs, the isolates are further tested to EMB and SM. Isolates not matching any of these criteria were investigated exclusively by biochemical tests. According to the IAL criteria, 198 isolates were subjected to susceptibility tests and 109 isolates to biochemical tests for identification. Out of 307 isolates, 306 (99.7%) were identified as *M. tuberculosis* and one as *M. bovis* BCG. Two clinical isolates of *M. bovis* and two of *M. bovis* BCG belonging to IAL strain collection were also included for analysis of *gyr*B patterns.

*gyr*B-RFLP revealed 306 isolates with the profile common to *M. tuberculosis*, *M. africanum* II, and "*M. canetti*" and five with the pattern common to *M. bovis* subsp. *bovis* and *M. bovis* BCG. Lack of restriction with *Sac*II excluded the presence of *M. bovis* subsp. *caprae* (data not shown).

Despite evidences that the prevalence of *M. africanum* II and *"M. canetti"* in non-African populations is extremely low, data presented here cannot confirm that these species are not present among the analyzed isolates, first because *M. tuberculosis*, *"M. canetti"*, and *M. africanum* subtype II are indistinguishable by *gyr*B-RFLP, and second because this study was not specifically designed to verify this point. *M. bovis* and *M. bovis* BCG were also not differentiated by *gyr*B-RFLP. Both are sensitive to the usual antituberculosis drugs, isoniazid and rifampin, but resistant to pyrazinamide. Therefore, from the point of view of treatment, simple differentiation of *M. tuberculosis* from *M. bovis*, achieved by *gyrB*-RFLP, is satisfactory.

No association between *gyr*B genotype and drug resistance was found, as expected. Among 198 isolates subjected to susceptibility tests, 165 (83.3%) were sensitive to INH, RF, PZA, and 33 (16.7%) were resistant at least to one drug (data not shown). All 198 isolates presented indistinguishable *gyr*B-RFLP pattern common to *M. tuberculosis*, *"M. canetti"*, and *M. africanum* II.

In conclusion, *gyr*B-RFLP can differentiate MTBC species with easy-to perform PCR, enzymatic restriction and gel electrophoresis. Consequently, it holds many advantages for the clinical laboratory, in terms of improvement in accuracy and savings in time and cost. It has the potential to reduce time required for identification when compared to conventional methods. The test can be simplified by the use of only two enzymes, *Rsa*I and *Taq*I, which are sufficiently discriminatory. The third enzyme, *Sac*II could be reserved for situations in which differentiation of *M. bovis* subsp. *bovis* and *M. bovis* subsp. *caprae* may be required.

Application of *gyr*B-RFLP for epidemiological studies remains limited, because it cannot differentiate *M. tuberculosis* from *M. africanum* subtype II and "*M canetti*", *M. africanum* subtype I from *M. pinnipedii*, and *M. bovis* from *M. bovis* BCG. For this purpose, other strategies have to be pursued.

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