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Short Communication

Multiplex-PCR for differentiation of *Mycobacterium bovis* from *Mycobacterium tuberculosis* complex

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Abstract

We evaluated a multiplex-PCR to differentiate *Mycobacterium bovis* from *M. tuberculosis* Complex (MTC) by one step amplification based on simultaneous detection of *pncA* 169C > G change in *M. bovis* and the *IS6110* present in MTC species. Our findings showed the proposed multiplex-PCR is a very useful tool for complementation in differentiating *M. bovis* from other cultured MTC species.

Key words: bovine tuberculosis, tuberculosis, PCR-multiplex, diagnosis.

Mycobacterium tuberculosis, which belongs to *Mycobacterium tuberculosis* Complex (MTC) together with *M. bovis*, *M. bovis* BCG, *M. africanun*, *M. microti*, *M.canettii* and *M. pinnipedii*, is the most common etiological agent of human tuberculosis (TB). However *M. bovis* may also be a relevant human pathogen in countries with high prevalence of bovine TB (OIE, 2009). Immuno-suppressing diseases, especially caused by Human Immunodeficiency Virus (HIV), is a risk factor for the development of human TB by *M. bovis* (Thoen *et al.*, 2006; Cícero *et al.*, 2009). Since *M. bovis*-caused human TB is clinically, pathologically and radiologically undistinguishable from *M. tuberculosis*-caused TB (De La Rua-Domenech, 2006), the fast differentiation of the two species has a high epidemiologic importance (Leite *et al.*, 2003). Among molecular methods for detecting species belonging to the MTC, several markers have been employed. The *IS6110* is highly used since this marker is found in several copies in the MTC genome (Eisenach *et al.*, 1990; Ogusku *et al.*, 2004). The *pncA* gene, which encodes the pyrazinamidase enzyme (PZAase) that converts the prodrug pyrazinamide (PZA) to pyrazinoic acid (POA), has a single point mutation at nucleotide 169C > G (appears to be unique to *M. bovis*) is one of the markers that is commonly used to distinguish *M. bovis* from others MTC species (De Los Monteros *et al.*, 1998; Kidane *et al.*, 2002). In the current study we evaluated a rapid test to differentiate *M. bovis* from other MTC species by one step amplification based on simultaneous detection of *pncA* 169C > G change in *M. bovis* and the *IS6110* present in MTC species. DNA from *M. tuberculosis* H_{37}Rv (ATCC 27294), *M. bovis* AN5, *M. avium* (Central Laboratory - LACEN/PR), *M. smegmatis* (LACEN/PR), *M. kansasii* (LACEN/PR), *M. fortuitum* (LACEN/PR), *M. szulgai* (LACEN/PR), *M. massilienses* (LACEN/PR), *M. abcessus* (LACEN/PR), *M. chelonae* (LACEN/PR) reference strains, one *M. bovis* BCG, 17 *M. bovis*, isolated from bovine lymph nodes and indentified by biochemical and molecular methods in a previous work (Cardoso *et al.*, 2009), 30 *M. tuberculosis* clinical isolates indentified by biochemical and molecular methods in a previous work (Noguti *et al.*, 2010) were used. All *M. bovis* clinical isolates were previously characterized by spoligotyping (data not shown) and all *M. tuberculosis* by spoligotyping and MIRU (Noguti *et al.*, 2010). Amplification was carried out with specific primers for *M. tuberculosis* complex TB1 (5'-CCTGCGAGCGTAGGCGTCGG-3')

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and TB2 (5'-CTCGTCCAGCGCCGCTTCGG-3') (Eisenach *et al.*, 1990) which amplify a fragment of 123 base pairs (bp) of the *IS6110* sequence and with specific primers for *M. bovis pncA*TB-1.2 (5'-ATGCGGGCGT TGATCATCGTC-3') and *pncA*MB-2 (5'-CGGTGTGC CGGAGAAGCCG-3') (De Los Monteros *et al.*, 1998; Bannalikar *et al.*, 2006) which amplify a fragment of 186 bp of *pncA* gene. DNA extracts (1 µL) from all mycobacterial reference strains and clinical isolates, were amplified by multiplex-PCR using 24 µL reaction mixture containing 0.2 pmol/µL TB1 and TB2 primers (Invitrogen - Integrated DNA Technologies, Inc. Coralville, USA), 0.4 pmol/µL *pncA*TB-1.2 and *pncA*MB-2 primers (Invitrogen - Integrated DNA Technologies, Inc. Coralville, USA) and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA), according to manufacturer's instructions. Amplification was carried out in a Perkin-Elmer Gene Amp PCR System 2400 thermalcycler (Waltham, Massachusetts, USA) with an initial cycle of 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 68 °C, 1 min at 72 °C and a final extension at 72 °C for 10 min. *M. tuberculosis* H_{37}Rv (1 ng) and *M. bovis* (1 ng) as positive and water as negative controls were included in all assays. PCR products were separated by electrophoresis on 2% agarose gel (Biotools/M&B Laboratories, S. A., Uniscience do Brasil, São Paulo, Brazil) at 100 V for 1 h in TBE. Gel were 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). A 100 bp DNA standard (Invitrogen Life Technologies, São Paulo, Brazil) was used as a DNA size marker.

The multiplex-PCR showed to be specific to differentiate *M. bovis* from other MTC species by amplification of two DNA fragments sized 123 and 186 bp and a 123 bp DNA fragment respectively. No PCR product of specific sizes to *M. tuberculosis* and *M. bovis* were observed in amplification of DNA from nontuberculous mycobacteria reference strains except for *M. avium* that showed a DNA fragment sized more than 300 bp (Figure 1). Also, all *M. tuberculosis* and *M. bovis* clinical isolates (confirmed by spoligotyping) showed a single (123 bp) and two (123 and 186 pb) DNA fragments respectively, by multiplex-PCR.

The multiplex-PCR combining TB1/TB2 (Eisenach *et al.*, 1990) and *pncA*TB-1.2/*pncA*MB-2 (De Los Monteros *et al.*, 1998) primers, proposed in this study, could not differentiate among the six species of MTC, but it showed to be specific in differentiating *M. bovis* from the others. Exception was for *M. bovis* BCG, which could not be differentiated from wild *M. bovis* (clinical isolates), once the first one tested has the 169C > G mutation too.

The correct differentiation of *M. bovis* triggers to an appropriate therapy, knowledge on TB epidemiology and control of bovine infection (De Los Monteros *et al.*, 1998; Bannalikar *et al.*, 2006; De La Rua-Domenech *et al.*, 2006).

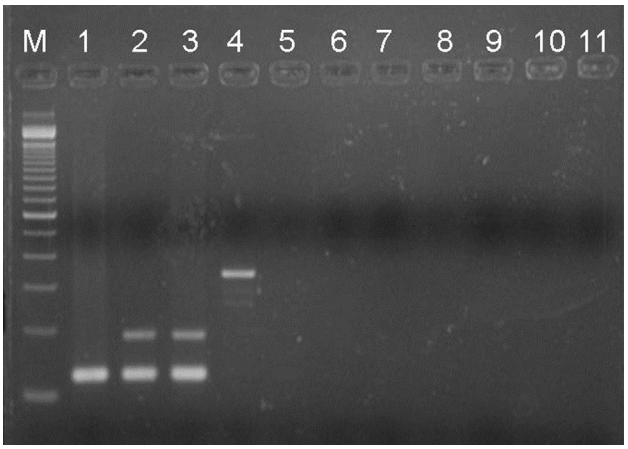

Figure 1 - Specificity of multiplex-PCR for differentiate *Mycobacterium bovis* from *M. tuberculosis* Complex (MTC). Electrophoresis on 2% agarose gel. Line M: DNA molecular weight marker (100 bp); Line 1: *M. tuberculosis* H37Rv; Line 2: *M. bovis* AN5; Line 3: *M. bovis* BCG*;* Line 4: *M. avium;* Line 5: *M. kansasii*; Line 6: *M. abcessus;* Line 7: *M. chelonae;* Line 8: *M. massiliense;* Line 9: *M. szulgai;* Line 10: *M. fortuitum;* Line 11: negative control.

In fact, the proposed multiplex-PCR seems to be a very useful tool for complementation in differentiating *M. bovis* from others MTC species, which are slow-growing mycobacteria, where a small number of bacilli in cultures hinder identification by biochemical tests. Additionally, biochemical identification methods are not able to provide satisfactory results and the multiplex-PCR also has the advantage of being a fast method with results provided in one day. As consequence, a specific, fast and low cost diagnosis method is highly relevant to differentiate *M. bovis* isolates from the *M. tuberculosis* complex (Bannalikar *et al.*, 2006).

The multiplex-PCR showed to be an important tool for differentiation of *M. bovis* from *M. tuberculosis* cultured isolates tested. Hopefully, the proposed multiplex-PCR should be tested with the other species from the MTC to set up its potential in differentiating them as the multiplex-PCR proposed by Yeboah-Manu *et al.* (2001). The multiplex-PCR added of a internal control should also be studied in clinical specimens as additional diagnostic tool mainly in regions where bovine and human TB coexist for the monitoring infection by *M. bovis* in human beings.

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