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The 500-Base-Pair Fragment of the Putative Gene RvD1-Rv2031c is also Present in the Genome of *Mycobacterium tuberculosis*

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Abstract. *Background: It has been proposed that differentiation between M. bovis and M. tuberculosis is possible by using a PCR assay for the 500bp fragment present only in the M. bovis genome. Materials and Methods: Forty clinical samples and 16 clinical isolates from the Department of Microbiology, as well as 4 clinical isolates obtained from another laboratory, were tested for the purpose of this study. As controls we tested 2 M. bovis (M. bovis BCG Pasteur TMC1011 and M. bovis BCG Copenhagen), 1 H37Rv M. tuberculosis strain, 2 M. avium (ATCC15765 and ATCC1975, respectively) and 1 M. paratuberculosis (ATCC19698) strains. Results: None of the mtp40- negative clinical isolates amplified the 500bp fragment, whereas 4 out of 17 mtp40-positive clinical isolates scored positive for the 500bp fragment. All clinical isolates scored positive for IS6110, mtp40, the pncA and oxyR PCR's. All but one of the clinical isolates amplified the 500bp fragment. Sequence analysis of the pncA and oxyR PCR products revealed the presence of nucleotide C at position 169 and G at position 285 respectively, suggesting M. tuberculosis as the causative agent. Conclusion: Our data suggest that the 500bp PCR fragment is present not only in M. bovis but also in M. tuberculosis.*

Although *Mycobacterium tuberculosis (M. tuberculosis)* is the most common cause of tuberculosis in humans, an unknown proportion of human tuberculosis is caused by *Mycobacterium bovis (M. bovis)* (1, 2). Since *M. bovis* and *M. tuberculosis* have almost identical genomes (3), it is difficult to make a distinction between them. Due to the intrinsic resistance of *M. bovis* to pyrazinamide, a rapid method is urgently needed for diagnosis and proper treatment.

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First Rodriguez and colleagues (4) reported the presence of a 500 bp fragment in *M. bovis* and its absence in *M. tuberculosis*. Also Sechi and colleagues (2) concluded, based on a study of 30 strains, that the 500bp region was *M. bovis* specific and suggested this PCR for the differentiation between *M. tuberculosis* and *M. bovis*. These data prompted us to use the primers and PCR conditions for exclusion of *M. bovis* infection in clinical samples already found positive for the *M. tuberculosis* complex (IS*6110*-positive and *mtp40*-negative). According to the literature, IS*6110* proved to be specific for the *M. tuberculosis complex* (5) and *mtp40* was identified as species specific for *M. tuberculosis* (6).

Furthermore, it has been reported (1, 7-9) that polymorphic regions in the *pncA* and *oxyR* genes can differentiate *M. tuberculosis* from *M. bovis*. Scorpio and colleagues (9) reported that 87/89 *M. bovis* strains could be distinguished from *M. tuberculosis* strains on the basis of the mutation at position 169 of the *pncA* gene. In another study, Espinosa and colleagues (7) reported that DNAs from 121 *M. tuberculosis* isolates had the expected base (guanine) at position 285.

This study aimed to differentiate between the genomes of *M. tuberculosis* and *M. bovis*.

Materials and Methods

In the present study we tested 40 clinical specimens such as urine, bronchoalveolar lavage, cerebrospinal fluid, gastric fluid, tissue, bone marrow and faeces. We also examined 16 clinical isolates, which were obtained after cultivation of clinical samples in the Department of Microbiology and 4 clinical isolates obtained from another laboratory. As controls we tested 2 *M. bovis* (*M. bovis* BCG Pasteur TMC1011 and *M. bovis* BCG Copenhagen), 1 H37Rv *M. tuberculosis* strain, 2 M. *avium* (ATCC15765 and ATCC1975, respectively) and 1 *M. paratuberculosis* (ATCC19698) strains. Specimens were processed as previously described (4, 5, 10). DNA isolation was carried out using Talent Seek-Viral DNA kit (TA50SKVD), according to the manufacturer's instructions (Talent srl). PCR detection of *M. tuberculosis* was conducted by two modified in-house PCR's (IS*6110* and *mtp40*) (8, 10). The *M. bovis* 500bp PCR was carried out as previously reported (2). In order to increase the sensitivity of the PCR proposed by Sechi and colleagues (2), we designed a semi-nested PCR

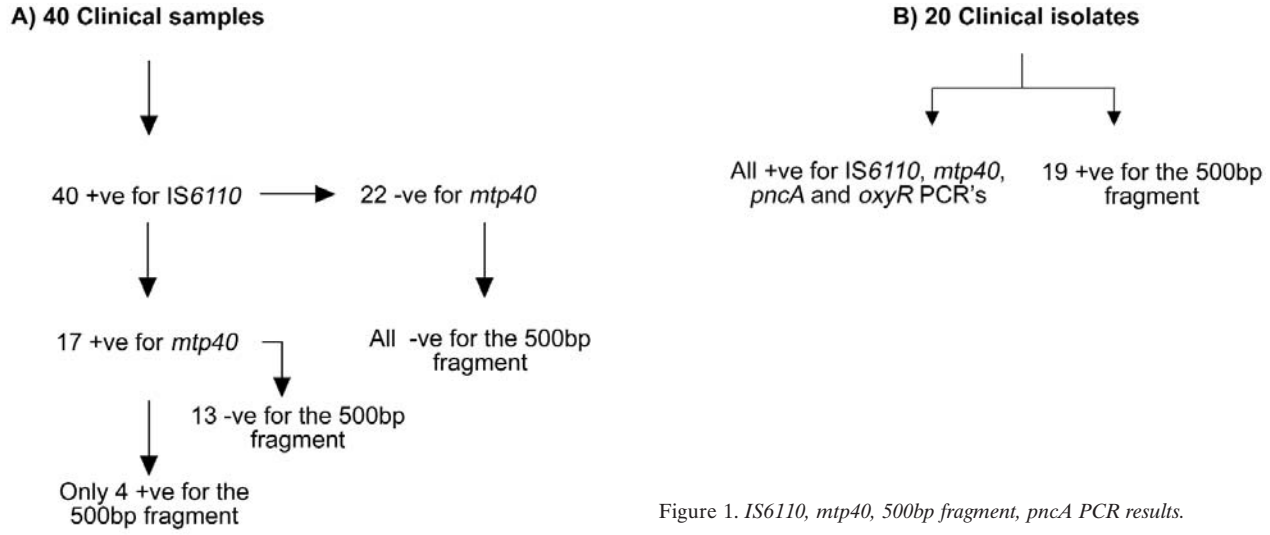

Figure 1. *IS6110, mtp40, 500bp fragment, pncA PCR results.*

for detection of the 500bp PCR fragment. By using the new antisense oligonucleotide JB23 (5'-GTCACCATCGCCGGCATTC-3') with JB21 in a second round PCR, the obtained fragment was 210bp. The PCR's for the *pncA* and *oxyR* genes were carried out as previously reported (7). Control experiments to exclude toxicity and inhibitory factors in the clinical samples were carried out. All PCR products were analysed in a 2% agarose gel and, when necessary, in 8% non-denaturing PAGE. Automated cycle sequencing was performed with the ABI Prism® 310 Genetic Analyzer using the Big-Dye DyeDeoxy terminator cycle sequencing kit. Sequences obtained were aligned, using Sequencher® PC software, with sequences from Genbank (Accession number Z83860 AL123456).

Results

Figure 1 summarizes the results from several experiments for 40 clinical samples. The general conclusion from Figure 1A is that the 500bp fragment is present in the *M. tuberculosis* genome, since 4 out 17 *mtp40*-positive clinical samples amplified the 500bp fragment.

Figure 1B summarizes the results from several experiments carried out in clinical isolates. All 20 clinical isolates scored positive for the IS*6110* and *mtp40* PCR's, whereas 19 (Figure 1B and Figure 2) scored positive for the 500bp fragment PCR. In order to further verify the amplified fragments of 500bp and 210bp we used *bcl-1* restriction enzyme for RFLP analysis and sequencing. Furthermore we did not observe any difference between the one round and our semi-nested PCR.

The 20 clinical isolates (Figure 1B) scored positive for both *pncA* and *oxyR* genes. Sequencing and alignment of the obtained sequences revealed the presence of nucleotide C in position 169 and G at position 285 of the *pncA* and *oxyR* genes, respectively. The *M. bovis* strains (*M. bovis* BCG Pasteur TMC1011 and *M. bovis* BCG-Copenhagen) contain the 500bp fragment and the sequenced *pncA* and *oxyR* PCR products revealed the presence of *M. bovis*. The H37Rv *M. tuberculosis* strain and one of our clinical isolates, as well as the *M. avium* strains and the *M. paratuberculosis strain* did not amplify the 500bp fragment.

Discussion

As far as the clinical samples are concerned, it is evident that differentiation between *M. tuberculosis* and *M. bovis* was not possible, since 4 *mtp40*-positive samples also amplified the 500bp fragment. For the remaining 13 clinical samples which scored negative for the 500bp fragment, one could argue that the causative agent was the human pathogen of tuberculosis since all 13 were found positive with the *mtp40* PCR.

Because these results disagree with the published data (2), we decided to elucidate the presence of the 500bp fragment in the *M. tuberculosis* genome by studying clinical isolates, where there is enough material for several experiments.

In total, 20 clinical isolates were examined and found positive for the IS*6110* and *mtp40* PCR's, whereas the 500bp fragment was amplified in all samples. Our data differ from the published data of Sechi and colleagues (2) who were not able to amplify the 500bp PCR fragment in 20 *M. tuberculosis* clinical isolates. Also Rodriguez (4) reported that, in 20 *M. tuberculosis* clinical isolates, the 500bp fragment was absent. However the same group mentioned in a later study (8), without presenting data, that some *M. tuberculosis* isolates render the 500bp amplification band with the 500bp PCR fragment primers.

Since *mtp40* is species-specific for *M. tuberculosis* and the 500bp fragment suggests the presence of *M. bovis*, we decided to use the *pncA* and *oxyR* PCR's for discrimination between *M. tuberculosis* and *M. bovis*. Sequence analysis of the *pncA* and *oxyR* PCR products of all 20 clinical isolates revealed the presence of nucleotide C in position 169 and

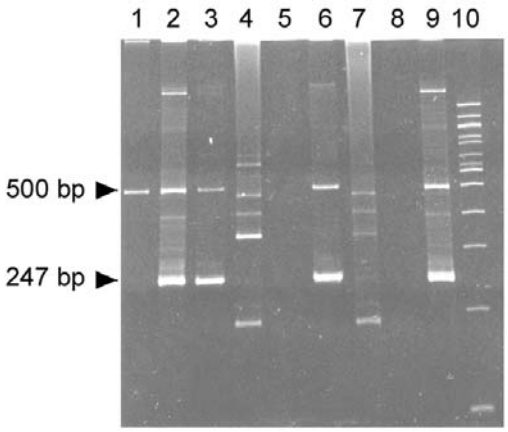

Figure 2. *Polyacrylamide gel electrophoresis of the PCR products in clinical samples and clinical isolates. Lanes 1, 2: M. tuberculosis clinical isolate (first and second round PCR, respectively). Lane 3: Positive control. M. bovis strain (M. bovis BCG Pasteur TMC1011) (second round PCR). Lane 4: Negative clinical sample (second round PCR). Lane 5: Negative control. Lane 6: Sputum clinical sample (second round PCR). Lane 7: Negative clinical sample (second round PCR). Lane 8: H37Rv M. tuberculosis strain (second round PCR). Lane 9: M. tuberculosis clinical isolate (second round PCR). Lane 10: 100bp DNA ladder.*

G at position 285 of the *pncA* and *oxyR* genes, respectively suggesting *M. tuberculosis* as the causative agent. Hence differentiation between the genome of *M. tuberculosis* and *M. bovis* was successful.

It seems that, although the 500bp fragment PCR can identify *M. bovis* specimens, it cannot differentiate between *M. bovis* and *M. tuberculosis*. One reason for the discrepancies between our and the published data could be the low bacterial load in the published examined clinical isolates that scored negative for the 500bp PCR fragment. Taking into account that the H37Rv *M. tuberculosis* strain and one of our clinical isolates did not harbour this sequence, the clinical isolates tested negative (4, 5, 11) could belong to this category.

In conclusion, our data suggest that the 500bp PCR fragment is not *M. bovis* specific, which was also verified by BLAST search where it is evident that the primer sequences are also present in the CDC1551 *M. tuberculosis* strain. Only the polymorphisms found at position 169 and 285 of the *pncA* and *oxyR* genes, respectively, allow differentiation between *M. bovis* and *M. tuberculosis*. Additional studies are needed in order to clarify, from an epidemiological point of view, the presence of the 500bp fragment in the *M. tuberculosis* genome in different countries.

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