

Evaluation of the insertion-sequence-6110-based polymerase chain reaction for detection of pathogenic mycobacteria

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Abstract

The insertion sequence IS6110 is a mobile genetic element typical for *Mycobacterium tuberculosis* complex (MTC) bacteria. Due to its narrow host range, this insertion element can be used for rapid identification of pathogenic mycobacteria. The aim of this study was to evaluate the diagnostic value of the IS6110 element in comparison with common molecular and microbiological methods. A total of 170 clinical specimens from tuberculosis (TB) patients were tested for the presence of IS6110 by polymerase chain reaction (PCR). Detection of MTC by IS6110-PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to commercial LCx assay (27.6 %) and to the commonly used methods for estimating bacteria growth: BACTEC system (20.6 %) and on Lowenstein-Jensen medium (15.3 %). IS6110-PCR was shown to be a rapid and specific method for identification of the *Mycobacterium tuberculosis* complex. It was found to be especially useful for confirming diagnosis in cases of smear- and culture-negative results, when the clinical expansion of tuberculosis was obvious.

Key words: Identification, insertion sequence 6110, *Mycobacterium tuberculosis* complex, polymerase chain reaction.

Introduction

Mobile genetic elements like insertion sequences (IS) and transposons are found in bacteria, archaeobacteria and eukaryotes. Insertion sequences are DNA fragments encoding their own ability to transpose within the genome. The function of most mobile genetic elements is unknown and they are usually considered to be genome parasites. In the genus *Mycobacterium*, there are three groups of IS, which are usually host specific. IS are between 880 bp and 2260 bp long and are found in one to more than 20 copies in the chromosome (Guilhot et. al. 1999). One of those, a 1361 bp long insertion sequence named IS6110, first described by Thierry et al (1990), is present in the *Mycobacterium tuberculosis* complex (MTC) only. MTC contains four genetically and serologically related pathogens causing disease in human and in cattle: *M. tuberculosis*, *M. bovis*, *M.*

africanum and *M. microti*. The majority of investigated MTC strains contained between 5 and 20 copies of IS6110 located in various places of the genome (van Soolingen et al. 1993).

Recent achievements in molecular microbiology such as complete genome sequencing of the *M. tuberculosis* H37Rv strain opened new perspectives in the study of mycobacteria (Cole et al. 1998). At the same time, a dramatic increase of tuberculosis (TB) incidence worldwide in the last decades induced an urgent need for new molecular markers enabling rapid detection of MTC, a main causative agent for TB. It is known that smear examination may not be effective in non-pulmonary, pediatric or paucibacillary disease, or where HIV is prevalent (Rigouts, Portaels 2001). Recently developed molecular methods based on detection of mycobacterial proteins (protein antigen B), 16S rRNA or DNA fragments, are dissolving this problem. Of these methods, the amplification of insertion sequences is most commonly used. IS6110-based PCR enables detection of nucleic acids from MTC directly in clinical specimens: sputum, bronchial washings, urine etc. (Eisenach et al. 1988; Hance et al. 1989). IS6110-PCR is a more rapid, sensitive and specific in comparison to other methods routinely used in clinical laboratories; such as smear microscopy, and culturing on solid and on liquid media (American Thoracic Society Workshop 1997). Detection of MTC in cerebrospinal fluid (CSF) from patients suspected to have tuberculous meningitis is of great value since cultivation and microscopy may often fail to reveal a low amount of bacteria.

In this study, IS6110 was chosen due to several reasons. Firstly, a narrow host range of the IS6110 allows use of this element for precise identification of MTC. Secondly, it is usually present in more than five copies and can be used as a molecular marker for strain differentiation in epidemiological studies (van Soolingen et al. 1993). The aim of our study was to evaluate the sensitivity and specificity of the IS6110 amplification method on smear-negative samples, in comparison with smear microscopy and culturing on solid Lowenstein-Jensen medium. We expected a higher sensitivity of IS6110-PCR in comparison to commercially available systems for TB detection and MTC culturing, such as the Abbott LCx assay and BACTEC. LCx assay is a DNA-related TB diagnostic system based on protein antigen b gene fragment amplification (Andresen, Hansen 1989), but perhaps, is not as sensitive as IS6110-PCR since only one copy of the antigen b gene is present in the MTC genome. BACTEC is a precise radiometric system for mycobacteria growth detection, but it usually requires several weeks to grow a sufficient amount of bacteria.

Materials and methods

In total more than 250 specimens of bronchial washings (76 %), sputum (11 %), cerebrospinal fluid (4.3 %), urine (3.5 %), smear (2.6 %) and stomach washings (2.6 %) were obtained from Latvian patients admitted in 1998-1999 to the State Centre of Tuberculosis and Lung Diseases with suspected and confirmed TB. The patients had clinical symptoms of lung diseases or had suspicion of pulmonary/extrapulmonary tuberculosis shown by clinical and/or X-ray examination. Each patient was represented by one specimen or, in several cases, by various specimens from different locations. The specimens were directed for TB detection by the Abbott LCx (Ligase Chain Reaction) commercial kit (Abbott laboratories, Illinois, USA), mostly in cases when bacteria growth

was negative on the BACTEC system (Becton-Dickinson, Sparks, USA) or with repeated smear-negative bacterioscopy results. In our study, 157 smear-negative specimens were selected, allowing to test whether IS6110-PCR is more sensitive in comparison to the commonly used methods. A small control group of 13 specimens with smear-positive bacterioscopy results was included. The results of our study were interpreted in the context of the clinical findings, TB history and the efficiency of chemotherapy.

All specimens were collected and processed by the standard *N*-acetyl-L-cysteine-NaOH procedure (Kubica et al. 1963). Smears were analyzed by fluorescent microscopy and the corresponding samples were inoculated on solid (Lewenstein-Jensen) and liquid (BACTEC) mediums. The LCx assay was performed at the Centre of Tuberculosis in accordance with the manufacturer's recommendations (Ausina et al. 1997). DNA isolates for LCx reaction contained 0.5 ml LCx buffer, 27 mM MgCl₂, NaN₃ and glass beads. After the LCx analysis IS6110-PCR was directly applied to samples. The DNA concentration method (Boom et al. 1990) was used for extraction of DNA from several smear-negative specimens of CSF and bronchial washings received in year 2001.

The 245 bp fragment (from 633 to 877 nucleotide positions of the IS6110 sequence) was amplified in house using primers 5'-CGTGAGGGCATCGAGGTGGC-3' and 5'-GCGTAGGGCGTCGGTCACAAA-3' (Hermans et al. 1990). Initially, the nested PCR method was used. At the first step, a 1224 bp fragment (from 47 to 1270 nucleotide positions) of IS6110 was amplified and was used as a reaction template at the second step, where a 245 bp fragment was produced. Later, the first step of nested PCR was omitted since it did not show any advantages and the 245 bp fragment was directly amplified from specimens. Five μ l of DNA isolate in LCx buffer was added to the PCR mixture [containing PCR buffer with (NH₄)₂SO₄, 200 μ M of each dNTPs, 10 pmoles of each primer and 1 U of Taq DNA Polymerase 9MBI Fermentas, Vilnius, Lithuania] to a final volume of 25 μ l. The optimized cycling protocol was used a *Progene* cycler (Techne, Cambridge, England) with initial denaturation at 95 °C for 2 min, followed by 40 cycles (95 °C for 15 s, 65 °C for 20 s, 72 °C for 1 min) and a final extension (72 °C for 10 min). PCR products were analyzed by electrophoresis in 2 % agarose gel (BioRAD, Hercules, USA) and were visualized with ethidium bromide. A positive PCR signal indicated the presence of the MTC genome. In each set of reactions, one negative control and one positive control (DNA from the *M. bovis* BCG vaccine strain) were included. The reaction specificity was confirmed by digestion with PvuII (MBI Fermentas, Vilnius, Lithuania) and by hybridization with an appropriate ³²S-labeled fragment. Testing was repeated on specimens with negative amplification results, with an external addition of control DNA from the *M. bovis* BCG strain in order to evaluate the presence of a PCR inhibitor.

Results

The detection limit of IS6110-PCR, estimated by amplification of serial dilutions of DNA from the *M. bovis* BCG strain containing only one copy of IS6110, was 5 fg per probe. This amount of DNA can be isolated from two bacterial cells, meaning that our PCR method is able to detect at least two bacteria, each containing one copy of IS6110. The fact that MTC usually contains more than one IS6110 copy increases the detection level of our in house method.

The comparative results of MTC detection in 170 specimens are shown in Table 1.

Table 1. Comparison of the in house IS6110-PCR method and TC clinical laboratory results obtained for 170 specimens. *, number of PCR false positive after adjustment with LCx results and samples suspected in cross contamination

Method used	MTC positive (total n=170)	Discrepancy (PCR-positive and 1-4 method-negative)
Bacterioscopy	13 (7.6 %)	56 (32.5 %)
Growth on Lowenstein-Jensen medium	26 (15.3 %)	39 (22.9 %)
Growth in BACTEC system	35 (20.6 %)	44 / 25* (25.8 % / 14.7 %*)
LCx assay	47 (27.6 %)	32 (18.8 %)
IS6110-PCR	69 (40.6 %)	-

Detection of MTC by IS6110-PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to LCx assay (27.6 %) and to the commonly used methods for estimation of bacteria growth: on BACTEC (20.6 %) and on Lowenstein-Jensen (15.3 %) mediums. Smear microscopy (bacterioscopy), which is reliable in detecting MTC bacteria only when present in high amounts, was the least sensitive with 13 (7.6 %) MTC positive specimens. This group of 13 specimens was MTC positive by all of the remaining methods (100 % specificity for smear-positive specimens).

IS6110-PCR was the most sensitive method in comparison to LCx assay and to other clinical laboratory methods. However, the lack of a “golden standard” for amplification methods does not exclude false positive results. Therefore, cultivation on BACTEC radiometric liquid system is still considered to be the most reliable tool for detection of MTC (Nordhoek et al. 1994). Sensitivity and specificity of PCR was estimated on the basis of BACTEC results (Elder et al. 1997). Thus, 91 PCR negative results and 26 PCR positive results (together 68.8 %) were in accordance with BACTEC system, and were considered as true negative and true positive, respectively. PCR gave positive results in 44 (25.8 %) cases. Of 44 PCR-positive results 12 were correlated with LCx assay results, but not with BACTEC results, a method internationally considered as a standard method for detection of *M. tuberculosis*. Therefore, these PCR results should be considered as false positive, and the overall specificity for IS6110-PCR estimated as 67.0 %. After charging of 32 PCR and LCx discrepant results plus seven suspected cross-contaminated samples, the total number of false positive results was 25 and the PCR specificity was 79.0 %. Nine (5.2 %) discrepant PCR-negative and BACTEC-positive results were observed, thus the estimated overall PCR sensitivity was 72 %. There were nine (5.2 %) discrepant PCR-negative and LCx-positive cases and four (2.3 %) discrepant PCR negative and Lowenstein-Jensen culture-positive cases. False positive LCx or BACTEC results may have been caused by laboratory contamination. False negative results may be explained also by the absence of IS6110 fragment in the *M. tuberculosis* pathogen also. Inhibition of amplification reaction by remains of clumps, blood and urine present in a specimen is another probable cause for false negative PCR (Forbes 1997).

Clinical material isolated from CSF was studied as in this case there was an obvious requirement for rapid and precise diagnostics. In our study, seven smear and culture-negative CSF specimens showed negative results by both amplification methods (IS6110-

Table 2. Evaluation of PCR-based methods applied on clinical material isolated by Boom method. SCF, cerebrospinal fluid; L, bronchial washing; -, negative PCR signal; +, positive PCR signal, indicating the presence of MTC genome; ++, strong positive signal; +w, weak positive signal; WT, wild type (no resistance encoding mutation found). H37Rv strain was used as a PCR positive control. B and C types are different groups of genetic patterns, revealed by molecular typing of MTC bacteria

Clinical number	IS6110-PCR	Rifampin suscept. by <i>Inno-LiPA</i> kit	Rifampin suscept. by <i>rpoB</i> gene sequencing	Isoniazid suscept. by <i>katG</i> gene sequencing	Mixed linker PCR typing pattern	Evaluation based on PCR results	Clinical diagnosis
CSF 3024	+	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
CSF 3025	++	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
L-202	+	WT	WT	WT	C type	Drug suscept. TB	Lung TB
L-204	+w	WT	WT	-	Atypical	False positive	Atypical pneumonia
H37Rv strain	++	WT	WT	WT	Typical	-	-

PCR and LCx). The results were correlated with the clinical findings. Later, several specimens were received from patients with suspicion of TB or tuberculous meningitis, although they had smear-negative results. The specimens were analyzed using the Boom (Boom et. al. 1990) method, by increasing the DNA concentration. All of these samples showed positive IS6110-PCR results, confirming the presence of MTC bacteria. In addition to IS6110-PCR, drug susceptibility molecular tests and PCR-based molecular typing were performed in the Biomedical Research and Study Centre. The results of PCR-based methods for MTC detection, molecular drug susceptibility testing and molecular typing of several specimens are summarized in Table 2. In three cases (CSF-3024, CSF-3025 and L-202), our results were in good agreement with the clinical findings and were used for elaboration of therapeutic strategy. In one case (L-204), the PCR methods showed a weak amplification signal and an atypical genetic pattern in Mixed Linker PCR. A suspicion of a false positive result was confirmed by the diagnosis of atypical pneumonia. Unfortunately, Abbott LCx assay was not applied to these specimens, and laboratory investigations, except as smear negative bacterioscopy, were not available. Anti-TB chemotherapy was begun for these drug-susceptible persons.

Interestingly, only two of these methods, IS6110-PCR and Mixed-Linker PCR, were initially designed for a low amount of DNA present in clinical material. The described molecular methods for drug susceptibility testing (*Inno-LiPA* kit, *rpoB* and

katG gene fragment sequencing) are usually applied for larger amounts of DNA isolated from bacterial culture. In most cases, a low content of DNA is an obstacle for further application of molecular methods in clinical material. In our study, these methods were shown to be sensitive enough to give correct results for clinical material when DNA was concentrated using the Boom method.

Discussion

Nucleic acid amplification gives the best chance of detecting MTC in smear negative samples. IS6110-PCR is rapid and relatively simple method; PCR results are available in 1 or 2 days instead of several weeks needed to grow mycobacteria in the BACTEC system. IS6110-PCR was shown to be able to detect a mycobacterial genome in variable clinical material – sputum, bronchial lavage, CSF etc. The results were comparable with other studies in which PCR was used for diagnosis of (mainly pulmonary) TB, where the sensitivity and specificity ranged between 70-100 % when a culture of MTC was taken as golden standard (Forbes 1997). However, in practice, many problems occur due to inhibition or due to cross-contamination with the products from previous PCRs (Nordhoek et al. 1994). We separated rooms and instruments used at each PCR step: PCR mixture preparation, sample addition and PCR product analysis. Also, specimens with negative PCR results were controlled for the presence of inhibitors. For this aim, PCR-negative specimens were repeatedly tested with a parallel addition of *M. bovis* DNA. Absence or weak amplification of *M. bovis* BCG DNA indicated the presence of inhibiting factor.

As PCR is able to detect both dead and live MTC bacteria, some patients may remain PCR-positive for mycobacterial DNA several months after treatment. Therefore, 44 discrepant PCR-positive and BACTEC-negative samples were evaluated in the context of the clinical findings. Clinical data was available for 38 patients. Fifteen (39.5 %) of them had active TB, confirmed clinically and by X-ray examination. Eight patients had already received a drug therapy course for a period from three days to several months and seven patients have not started treatment. Interestingly, only four of those patients were detected as MTC-positive by LCx assay. Eight (21 %) patients had inactive TB with after-effects, thus confirming that PCR can detect dead or dormant mycobacteria. In general, the clinical findings were in agreement with the shown presence of the MTC genome among 66 % of the false positive samples. Seven (18 %) specimens were, possibly, contaminated during the bronchoscopy procedure. Extrapolation of the PCR results for the remaining six (16 %) patients was problematic.

This study showed the advantages of PCR-based TB detection prior to mycobacteria culturing and admitting of chemotherapy. It is likely that, in the cases of smear positive results, there is no additional need in IS6110-PCR, and it is useful only to distinguish MTC from the other acid-fast bacteria. Therefore, IS6110-PCR can be recommended in cases of smear- or BACTEC-negative results. Invention of PCR-based detection methods may increase a number of recovered TB cases and may dramatically shorten the time required for TB diagnostics.

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Uz inserciju secības IS6110 balstīta polimerāzes ķēdes reakcija: lietojums patogēno mikobaktēriju noteikšanai

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Kopsavilkums

Insercijas sekvenca IS6110 ir *Mycobacterium tuberculosis* kompleksam (MTC) raksturīgs mobilais ģenētiskais elements. Saimnieku loks šim insercijas elementam ir šaurs, un tādēļ to var lietot mikobaktēriju ātrai noteikšanai. Darba mērķis bija salīdzināt šā ģenētiskā elementa diagnostisko vērtību ar pazīstamām molekulārām un mikrobioloģiskām metodēm. Dažādus klīniskos paraugus no 170 tuberkulozes slimniekiem pārbaudīja uz IS6110 klātbūtni ar polimerāzes ķēdes reakciju (PCR). MTC noteikšana ar IS6110-PCR deva visaugstāko pozitīvo atbildi (40,6 %), salīdzinot ar LCx testu (27,6 %) un tādām noteikšanas metodēm kā baktēriju kultivēšana BACTEC sistēmā (20,6 %) vai uz Levenšteina-Jensena barotnes (15,3 %). Parādīts, ka IS6110-PCR ir ātra un specifiska MTC identifikācijas metode. Tai ir īpaša vērtība tajos gadījumos, kad ir nepieciešams apstiprināt mikobaktēriju klātbūtni, ja mikroskopiskā izmeklēšana un kultivēšana bijušas nesekmīgas.