

Rapid detection and identification of *Mycobacterium tuberculosis* by Real Time PCR and Bactec 960 MIGT

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SUMMARY

We have developed a Real-Time PCR assay to detect *M. tuberculosis* using the iCycler iQ detection system by TaqMan assay directly on the clinical specimen. A total of 513 clinical samples were taken from patients with suspected tuberculosis and other patients that had an active mycobacterial infection, as well as patients with diagnosed tuberculosis who were receiving antitubercular therapy. The sensitivity and specificity of this assay, 10% and 100%, respectively, were compared to those of conventional microbiological methods.

KEY WORDS: Real Time quantitative PCR; *Mycobacterium tuberculosis*; Non-tuberculous mycobacteria; Molecular diagnosis of tuberculosis; Insertion sequence *IS6110*.

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Mycobacterium tuberculosis (MTB) remains a serious public health issue due to its risk of person-to-person transmission, and high level of morbidity and mortality. Currently, there are approximately 8 million new infections and 3 million deaths attributed to *M. tuberculosis* each year (Maher, 1999; Kraus *et al.*, 2001; Miller *et al.*, 2002).

The resurgence of TB in industrialized countries since the mid-1980s, primarily due to both the increased incidence of immunocompromised patients with AIDS, and the emergence of MDR-strains of *M. tuberculosis* has increased the

need for rapid diagnosis of this disease. Rapid detection of active TB infection is critical for the prompt detection of new cases, effective patient management and implementation of infection control measures, and to institute appropriate antimycobacterial therapy. The AFB (Acid Fast Bacilli) smear tests and cultures lack specificity, so there is need for a laboratory test for specific detection of the *M. tuberculosis complex* that can be performed within a short period of time. PCR-based assays for the detection of *M. tuberculosis* approach the sensitivity of conventional cultures yet have the advantage of greater specificity and rapidity (Sechi L.A. *et al.*, 1997; Gail L. Woods, 2001; Hanna Soini and James M. Musser, 2001; Miller N. *et al.*, 2002).

Nucleic acid amplification methods have been applied in the clinical laboratory with great success. However, these procedures are often labor-intensive and the FDA-approved nucleic acid amplification-based assays for MTB display high

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specificity but variable sensitivity, several stages are required in the amplification and detection steps involving user manipulations at each point of the assays that have the potential for error and sample contamination. The Real time PCR technique is considerably simpler and faster with respect to the standard PCR technique. This system, involving fluorogenic probes, has been successfully used for the rapid detection and identification of a variety of microorganisms, Real time assay has also been shown to be useful for the detection of mycobacteria including *M. tuberculosis* (Eishi Y. *et al.* 2002; Bruijnestein van Coppenraet E.S., 2004; Isik S.J., 2004; Lemaitre N. *et al.*, 2004). In this report we describe the development of a TaqMan assay (Biorad) for the quantification of *M. tuberculosis* DNA by monitoring the real time amplification of a sequence within the insertion sequence IS6110 present in the MTB genome in multiple copies (Thierry D. *et al.*, 1990a; Thierry D. *et al.*, 1990b).

The clinical samples were obtained from different clinics of the University of Sassari and from Nuoro Hospital and were analysed in the clinical diagnostic laboratory of Mycobacteriology of the Department of Biomedical Science, University of Sassari. The samples were taken from 505 patients with suspected tuberculosis and other patients with an active mycobacterium infection, as well as patients with diagnosed tuberculosis who were receiving antitubercular therapy. All the samples, with the exception of those obtained from sterile sites, were decontaminated, centrifuged to concentrate mycobacteria by standard procedures (Robert GD *et al.*, 1991), and used for direct microscopy, culture and DNA extraction. The positive AFB was confirmed by Ziehl-Neelsen staining and the cultures were incubated in a Bactec System MGIT 960 (Becton Dikson) in BBL MGIT 7 ml tubes (Mycobacteria Growth Indicator Tube) and observed for 60 days before being considered negative. DNA extraction was carried out directly from 500 µl of sample or culture, with the DNeasy Tissue Kit (QIAGEN), which is designed for the rapid purification of total DNA. *M. tuberculosis* DNA was eluted in 100 µl of TE and subjected to amplification by Real Time PCR. The reaction was optimized to obtain the best amplification kinetics, the cycle condition was performed for 1 cycle, 3 min at 95°, 30 s at 95°C and 50 s at

60°C for 50 cycles. The samples were tested in triplicate and the reaction mixture was performed in a final volume of 150 µl (30 µl for each well). Moreover, a negative control was included in each experiment. The Real time PCR mixtures containing a final concentration of 1X Buffer, 2.5 mM MgCl₂, 0.2 mM of each nucleotide, 1 U/µl Taq pol (QIAGEN), and the target specific primers and probes were used at a final concentration of 0.5 uM and 0.5 µM respectively, finally 18 µl of template. The primers and the probe sequence were selected from a region of the IS6110: Primers IS6110 D-1 (5'-acctgaaagacgttatccacat-3') and IS6110 D-2 (5'-cggctagtgcattgtcatagga-3') which amplify a 100 bp fragment; the probe: (5'-[6 FAM]tccgaccgcgctccgaccgacg[TAMRA-Q]3') was synthesized and conjugated with the reporter dye FAM and TAMRA quencer dye, which were covalently linked to 5' and 3' ends oligonucleotide respectively. The primers and the probe did not show homology with other known nucleotide sequences. The control DNA was extracted from the *Mycobacterium tuberculosis* H37Rv strain and measured with a spectrophotometer. Considering that the H37Rv genome weighs 4 picograms and that the number of IS6110 multicopy insertion elements in the genome of H37Rv is 16, the concentration of DNA was expressed in terms of the number of bacterial genomes/µl, since the genome of strains of *M. tuberculosis* isolates from clinical samples is expected to contain approximately the same number of copies as IS6110 (Brosce R. *et al.*, 1998). The standard curve obtained with a serially diluted *M. tuberculosis* H37Rv DNA preparation, was linear over 6 orders of magnitude with a coefficient of correlation of 0.999 and a slope of 3.514, corresponding to a PCR efficiency of 92.6%. In accordance with the standard curve generated by the analysis of known amounts of genomic *M. tuberculosis* H37Rv DNA with the IS6110 TaqMan assay it was possible to detect 10 bacterial genomes/µl. Three serial dilutions of the *Mycobacterium tuberculosis* strain H37Rv DNA (1/10, 1/100, 1/1000) were used for all assays as standard for quantification, based on a standard curve, to quantify the unknown bacterial load in the clinical specimens. We evaluated the sensitivity of Real Time PCR to detect the bacterial loads as number of bacterial genomes/µl in different clinical samples. We selected negative spec-

imens obtained from different sites, urine, sputum, broncho-aspirate, gastric aspirate, spinal fluid, lymph node, and others and made them positive with a known amount of the *M. tuberculosis* H37 Rv strain. PCR restriction fragment length polymorphism analysis of the *hsp65* gene (*hsp65* PRA) was used for the identification of mycobacteria other than tuberculosis (MOTT). The PRA, based on the amplification of a 439-bp fragment of the *hsp65* gene, was performed with primers Tb11 (5'-ACCAACGATGGTGT-GTCCAT) and Tb12 (5'-CTTGTCGAACCGCAT-ACCCT) and the PCR product digested by *Bst*EII and by *Hae*III, then the digestion products were visualized using 3% metaphore agarose gel and finally the patterns obtained were analysed (Devallois Anne *et al.*, 1997).

A total of 505 clinical samples were collected: 159 urine sample, 122 sputum samples, 51 gastric aspirates, 49 broncho aspirates, 45 spinal fluid samples, 23 pleural fluid samples, 18 lymph node samples, 17 cutaneous biopsies, 6 medullar aspirates, 5 pus and 18 other samples. Concerning the sensitivity of the diagnostic methods utilised in this study, 14 (2,7%) of the 513 samples

analysed tested positive by microscopy, 53 (10.5%) of the samples were positive by culture method and 51 (10%) tested positive by Real Time PCR (Table 1).

Forty-six (9%) of the isolates were identified as *M. tuberculosis* by Real Time PCR assay while seven (1.5%) of the samples analysed tested positive only with real time, this result has been explained because they were collected from patients under antitubercular therapy. On the other hand, 5 samples that yielded negative results with the TaqMan assay, testing positive in the culture method, were Mycobacteria Other Than Tuberculosis (MOTT) and were identified by the *hsp65* PRA as: *M. abscessus* isolated from a gastric aspirate, *M. xenopi* isolated from a broncho aspirate, 2 isolates as *M. chelonae* from two cutaneous biopsies, and another that generated a new pattern, isolated from asitic fluid, was identified as *M. austroafricanum*. In this study different diagnostic methods used to diagnose tuberculosis infection were compared. The most rapid and cost-efficient method is Bacilli Acid Alcohol Resistant (BAAR) searching, but this method has the disadvantage of

TABLE 1 - Sensitivity and specificity of Real Time PCR assay compared to those of conventional microbiological methods, direct microscopy and culture: a total of 513 samples were collected, 53 strains were isolated, 47 of these were identified as *M. tuberculosis* by Real Time assay, 5 as MOTT by *hsp65* PRA; 7 samples positive only with TaqMan assay were collected from patients under antitubercular therapy

N° Samples	AAR 960	Bactec MGIT PCR	Real Time PRA	<i>hsp 65</i>	Identification
159 Urine	1	5	8		8 <i>M. tuberculosis</i>
122 Sputum	9	16	16		16 <i>M. tuberculosis</i>
51 Gastric Aspirate	1	10	9	1	9 <i>M. tuberculosis</i> 1 <i>M. abscessus</i>
49 Broncho Aspirate	2	5	4	1	4 <i>M. tuberculosis</i> 1 <i>M. xenopi</i>
45 Spinal Fluid		2			2 <i>M. tuberculosis</i>
18 Lymphonodes		2	2		2 <i>M. tuberculosis</i>
17 Cutaneous Biopsie	1	3	1	2	1 <i>M. tuberculosis</i> 2 <i>M. chelonae</i>
52 Other		10	11	1	11 <i>M. tuberculosis</i> 1 <i>M. austro-africanum</i>
505 Total	14 (2.7%)	53 (10.5%)	51 (10%)	5 (0.9%)	

being neither particularly sensitive nor specific. The culture method is more sensitive, and it is the only one able to show the viability of mycobacteria and bacterial isolates are necessary to perform the *in vitro* susceptibility of antitubercular drugs and the identification of MOTT. However the method lacks specificity and sensitivity as it does not detect dead bacteria. The data obtained suggested that the Real Time PCR assay showed a high degree of sensitivity, similar to the culture method. Furthermore, none of the genomic DNA from the 5 mycobacteria species tested and identified as MOTT produced an amplification product, demonstrating the high specificity (100%) of this IS6110 TaqMan assay. The most common type of the samples taken from clinicians was urine (31%), yet only 5 (10%) of these were positive, while the highest number of positive samples were detected in sputum, 16 (34%) and in gastric aspirates 10 (21%), these samples were 24% and 10% of the total of the samples analysed, respectively. Five broncho-aspirates samples, 10% of the total, also tested positive. The number of bacterial genomes/ μl was detected in different samples to emphasize the type of clinical samples where it is possible to detect a higher load of mycobacteria. Analysing the bacterial load can be useful for clinicians that often do not take the samples correctly, and it is very important to consider the site of TB infection carefully. A higher number of bacterial genomes was detected in sputum (150.000 genomes/ μl) and in broncho-aspirates (150.000 genomes/ μl) with respect to gastric aspirates (139.000 genomes/ μl), urine (32.000 genomes/ μl) and spinal fluids (416 genomes/ μl). In the light of these results, considering that most common site of infection of *M. tuberculosis* is the lung, sputum may be the best sample type for the isolation of this mycobacteria, however, taking this type of sample, may be problematic, especially if the patients are children when gastric aspirate is recommended. Two spinal fluids tested positive by Real Time PCR, but not by culture, this outcome has been explained because the patient were undergoing antitubercular therapy. However, the Taqman assay was useful for rapid and accurate diagnosis in cases highly suspected of meningitis TB and also for the assessment of antitubercular treatment response in spite of negative

results obtained on conventional methods. The Real Time technique has also been used to evaluate the MTB DNA in the samples obtained from two tuberculosis patients during treatment with antitubercular drugs. The specimens were taken at different times for throughout a one year follow-up period and were all tested by microscopy, culture method and TaqMan assay. Our results based on TaqMan assay showed the reduction of the bacterial loads in the different specimens taken at different times from a renal tuberculosis patient and in a patient with tuberculous meningitis during antitubercular therapy, hence monitoring the success of the therapy, demonstrating the importance of obtaining multiple samples; chemotherapy gave satisfactory results for both (Fig. 1).

Ziehl-Neelsen staining of the first samples of the renal tuberculosis patient gave a positive result, the culture became positive after 15 days, the Taqman assays gave positive result after 24 h (3800 bacterial genomes/ μl). Follow-up microscopy and culture rapidly yielded negative results during the 4th month of therapy, while the Taqman assay was still positive, but the number of bacteria had decreased (304 bacterial genomes/ μl). We observed that the number of bacterial loads was still decreasing after 7 months (50 bacterial genomes/ μl) and after 9 months all the samples analysed, the last three, were negative with all the methods. A case of tuberculous meningitis in a 32-year-old man with AIDS was also reported. We obtained 5 samples, the aspect of Cerebrospinal fluid (CSF) was clear and the patient had a 12 month follow-up. The first and the second samples tested positive only with Real Time PCR, with bacterial loads of 416 and 275 bacterial genomes/ μl , respectively, on the contrary, the last three samples tested negative with Real Time PCR as well as with microscopic and culture methods. Real Time PCR results gradually converted from positive to negative, correlating with the improvement in clinical conditions during the course of treatment.

Real Time PCR is necessary for rapid diagnosis of TB in the clinical laboratory however some problems of sensitivity when the samples contain small amounts of *M. tuberculosis* DNA may arise (detection limit was 10 bacterial genomes/ μl). Moreover, it could be a useful method for assessing treatment response in

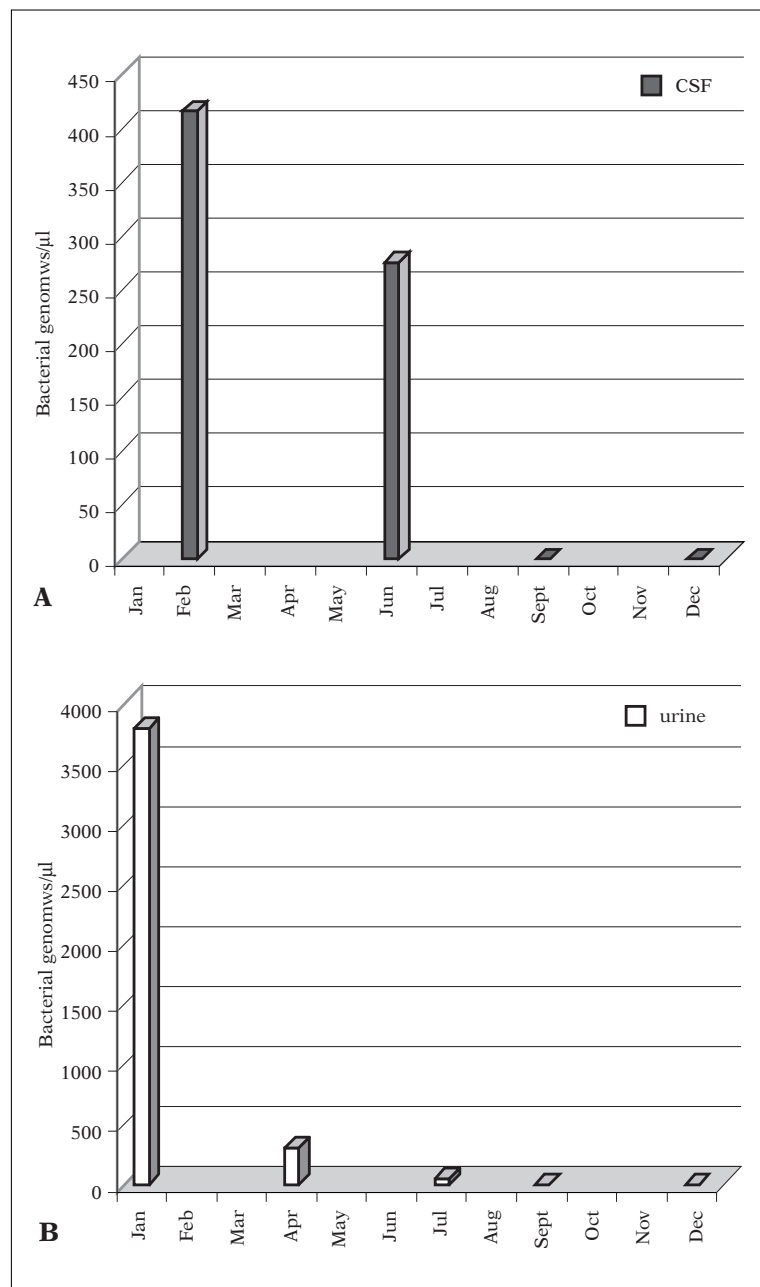


FIGURE 1 - MTB load quantified by Real Time PCR in A) Cerebrospinal Fluid (CSF) samples collected from a patient with Tuberculous meningitis and in B) urine samples collected from a patient with Renal tuberculosis at different times in the follow-up therapy period: Real Time PCR assays gave a positive result in the first two samples of CFS, bacterial loads decreased from 416 bacteria/μl to 275 bacteria/μl after 5 months of therapy, while the last three samples tested negative with all the methods; in the first sample of urine, Taqman assays gave also a positive result (3800 bacterial genomes/μl), during a 4 months follow-up therapy period the Taqman assay was still positive, but the number of bacteria was decreased (304 bacterial genomes/μl), after 7 months (50 bacterial genomes/μl), after 9 months the last three analysed were negative with all the methods.

patients with TB. Real Time PCR assay showed a high degree of specificity, sensitivity and especially rapidity of detection of tuberculosis, the latter being a very important factor in patient management in terms of initiating appropriate antitubercular therapy. In conclusion, the Real Time PCR approach represents a fully controlled, diagnostic tool for the rapid identification of MTB infection directly on clinical specimens which

might be useful also for the clinical monitoring of antitubercular treatment.

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