Detection of *Mycobacterium tuberculosis* complex organisms in the stools of patients with pulmonary tuberculosis

Amel El Khéchine, Mireille Henry, Didier Raoult and Michel Drancourt

URMITE CNRS-IRD, UMR 6236, Faculté de Médecine, IFR48, Université de la Méditerranée et Pôle de Maladies Infectieuses, Assistance Publique-Hôpitaux de Marseille, Marseille, France

The laboratory diagnosis of pulmonary tuberculosis mainly relies on the detection of *Mycobacterium tuberculosis* complex (MTC) organisms in the sputum. In patients who do not give sputum, alternative respiratory tract specimens can be obtained only by invasive procedures. Based on the known survival of MTC organisms in the gastric fluid, we hypothesized that swallowed MTC organisms would be detectable in stool samples. We compared the presence of MTC organisms in respiratory tract specimens and stool specimens collected in parallel from the same patients. MTC was detected in cultures grown on egg-based medium after appropriate decontamination, by microscopic examination after Ziehl–Neelsen staining and by real-time PCR detection of IS\textsubscript{6110} using internal controls. A case of pulmonary tuberculosis was defined by the presence of (i) clinical and radiological signs and symptoms suggestive of pulmonary tuberculosis, and (ii) culture of MTC organisms from at least one respiratory tract specimen or (iii) the presence of acid-fast bacilli in the sputum that were subsequently identified as MTC organisms by real-time PCR. The observation of 134 patients suspected to be suffering pulmonary tuberculosis led to the identification of 24 cases and 110 non-infected control patients. Cases and controls did not significantly differ with respect to sex but cases were significantly younger than controls. The sensitivity/specificity was 37.5 %/100 % for the microscopic examination of stools, 54.2 %/100 % for culturing and 100 %/97.3 % for real-time PCR. The positive predicted value was 100 %, 100 % and 88.9 %, respectively, and the negative predicted value was 88 %, 90.9 % and 100 %, respectively. In four patients, a stool specimen initially yielded the correct diagnosis of pulmonary tuberculosis before evaluation of the respiratory tract specimen confirmed the diagnosis. These data indicate that stools could be used in conjunction with sputum testing or as an alternative specimen upon which to base the diagnosis of pulmonary tuberculosis by molecular identification of acid-fast bacilli and culture. This non-invasive alternative procedure is of particular interest for patients who cannot expectorate.

INTRODUCTION

Pulmonary infection is the most prevalent form of tuberculosis (Saranchuk et al., 2007). Exposure to contact persons can cause secondary infection, and in some areas sustained high-level prevalence of this deadly infection (Johansen et al., 2002). The diagnosis of pulmonary tuberculosis currently relies upon the detection of *Mycobacterium tuberculosis* complex (MTC) organisms in sputum (Pfyffer, 2007). Some patients, however, are unable to produce sputum, including children (Oberhelman et al., 2006; Starke & Correa, 1995), immunocompromised patients and patients with neurological impairment (Andresen, 2007). In these situations, alternative specimens obtained by invasive procedures include nasopharyngeal aspirates (Owens et al., 2007), gastric aspirates (Chierakul et al., 2003), respiratory tract secretions obtained by bronchoscopy (Ding et al., 2008) and intestinal fluid obtained by the string test (Vargas et al., 2005). The invasive procedures required to obtain the above-mentioned specimens are not comfortable for the patient and are potentially harmful.

The facts that swallowed MTC organisms can be detected in the gastric fluid and have been recently demonstrated to resist acid (Vandal et al., 2008) suggest that MTC organisms have the potential to survive the enteric tract and be eliminated in stools. However, few systematic evaluations of the utility of stool specimens for the diagnosis of pulmonary tuberculosis have been reported (Donald et al., 1996). We therefore examined stool
specimens collected from patients in parallel with respiratory tract specimens for the presence of MTC organisms using a standardized protocol.

**METHODS**

**Clinical specimens.** Sputum specimens collected from patients suspected to be suffering pulmonary tuberculosis were routinely submitted to our laboratory for microbiological and molecular detection of MTC organisms. In addition, a stool specimen was collected into a sterile receptacle within 24 h of sputum collection. For cases in which several specimens were collected from a single patient, only the first sputum specimen and the first stool specimen were included in this study. The study was approved by a local Ethics Committee.

**Microbiological procedures.** Direct microscopic (×100) examination was performed after Ziehl–Neelsen staining (Quick-TB kit, Réactifs RAL) of one drop of sputum or filtered stool specimen (see below). Respiratory tract specimens were digested and decontaminated by the NALC-NaOH method (Kent & Kubica, 1985). A sample (200 μl) of this suspension was inoculated into a BACTEC bottle (Mycof-sputa; Becton Dickinson), supplemented with 5% oleic acid-albumin-dextrose-catalase and PANTA (Becton Dickinson) and incubated in an automated BACTEC 9000 MB system for two months.

Stool specimens were filtered using a faecal specimen filtration vial kit (Orion-Diagnostica-Fumouze-Division Diagnostics). The filtration vial contained the IS6110 gene copy number (Carcopino et al., 2006), vortexed for 15 min at room temperature, washed in PBS (pH 7.2). Its screw-on cap contained a spoon for sampling and a filter. We adapted this system by adding a macroporous compress, which has precise mesh openings and uniformly oriented fibres (Laboratoire Hydrex), to the vial cap so that the mycobacterial cells could not be trapped within the filter matrix. The attached spoon was used to place two spoonfuls of the faecal sample into the vial. The preparation was then shaken thoroughly in order to mix the sample with the buffer solution and the suspension was further filtered into a 50 ml conical centrifuge tube. About 5 ml of the stool filtrate was mixed with 3 vols 1% chlorhexidine digluconate (Sigma) (Best et al., 1999; Ferroni et al., 2006), vortexed for 15 min at room temperature, washed in PBS and centrifuged at 3000 g for 20 min at room temperature. The pellet was suspended in 1 ml PBS, and 200 μl of this specimen was inoculated onto a Löwenstein–Jensen slant (bioMérieux) and incubated at 37 °C for 2 months. The culture was examined daily for up to 1 week to check for any contamination, then twice weekly for 2 months to check for any growth of colonies. Colonies were identified as MTC organisms by Ziehl–Neelsen staining and molecular identification as described below.

**DNA isolation.** A filtered stool specimen or sputum sample (250 μl) was inactivated by heating at 95 °C for 1 h, then transferred into a sterile screw-cap Eppendorf tube containing 0.3 g acid-washed glass beads (Sigma) and shaken in a Bio 101 FastPrep instrument (Qiogene) at level 6.5 (full speed) for 45 s. The supernatant was incubated overnight at 56 °C with 25 μl proteinase K (20 mg ml⁻¹) and 180 μl T1 buffer from the Nucleospin Tissue Mini kit, according to the manufacturer’s recommendations (Macherey-Nagel). After a second mechanical lysis, performed as described above, and a 15 min incubation at 100 °C, total DNA was extracted using the Nucleospin Tissue Mini kit, according to the manufacturer’s recommendations. Extracted DNA was eluted into 100 μl elution buffer and stored at −20 °C until use. Sterile buffer (250 μl) was run in parallel for each batch of DNA extractions as a negative control.

**Molecular diagnosis.** Real-time PCR amplification and detection of IS6110 was done using PCR primers and the detection probe reported in Table 1. The IS6110 system was tested with seven MTC species and the non-tuberculous species Mycobacterium avium, Mycobacterium fortuitum and Mycobacterium chelonae in order to ensure specificity. We compared the sensitivity of the real-time PCR detection in sputum and stool specimens by seeding five sputum and five stool specimens with M. tuberculosis H37Rv CIP 64.31. Real-time PCR was performed in an MX3000 system (Stratagene Europe) using the QuantiTect PCR mix (Qiagen), plus 5 pmol each primer, 5 pmol probe and 5 μl DNA in a final volume of 25 μl. PCR products for IS6110 and the internal control (Fig. 1) were detected in the same assay by use of the IS6110 probe labelled with FAM and an internal control probe labelled with VIC (FAM and VIC labels were from Applied Biosystems). The internal control plasmid was included in every PCR in order to check for the presence of PCR inhibitors. The concentration of the internal control was adjusted in order to obtain a Cq value of 27. Its composition (chimeric preparation of HPV18 and human albumin targets) was designed such that it would not interfere with any of the organisms found in stool specimens. A chimeric nucleotide fragment was constructed as previously described for quantification of the IS6110 gene copy number (Carcopino et al., 2006; Menard et al., 2008) (Fig. 2). A dilution series from 10⁷ to 10 copies of the quantification plasmid was run in parallel. The chimeric fragment contained the IS6110, ITS 1 (the internal transcribed spacer 1) and the internal standard target sequences reported in Table 1. The ITS sequence was not evaluated in this study. M. tuberculosis H37Rv DNA was used as a positive control. Serial 10-fold dilutions of M. tuberculosis H37Rv DNA were used as a positive control. Serial 10-fold dilutions of M.

**Table 1.** Internal standard and IS6110 probe and primer sequences used for real-time, standard PCRs and sequencing

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard PCR</td>
<td>IS6110</td>
<td>5'-TTCAAGTCTCAGTGCCCTTC-3'</td>
<td>438</td>
</tr>
<tr>
<td>PCR_IS6110_dir primer</td>
<td>IS6110</td>
<td>5'-CGAATACTCAAGGAGCCACATCA-3'</td>
<td></td>
</tr>
<tr>
<td>PCR_IS6110_rev primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>IS6110</td>
<td>5'-GTAAAGGCTCGTGCAAGAG-3'</td>
<td>77</td>
</tr>
<tr>
<td>Q_IS6110_dir primer</td>
<td>IS6110</td>
<td>5'-GGCTGTGGGATGCACACCT-3'</td>
<td></td>
</tr>
<tr>
<td>Q_IS6110_rev primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS6110_FAM probe</td>
<td>IS6110</td>
<td>6FAM-ACTGATGCGAACCCTGCC</td>
<td>240</td>
</tr>
<tr>
<td>HPV18_dir primer</td>
<td>Internal control</td>
<td>5'-AAAAAGCGACATTCTACAATAGC-3'</td>
<td></td>
</tr>
<tr>
<td>Alb_rev primer</td>
<td></td>
<td>5'-AAACTCATGGGAGCTGTTGTC-3'</td>
<td></td>
</tr>
<tr>
<td>Albu_VIC probe</td>
<td></td>
<td>VIC-CCTGTCATGCCACACAAAATCTCTCC</td>
<td></td>
</tr>
</tbody>
</table>
potential contamination.

Xho shaded grey. The boxed sequences in normal font correspond to internal control are boxed and in bold, and the probe is in bold and shaded in grey, while the probe is shaded in dark grey. The nucleotide sequence of the target on the ITS gene of pan-mycobacteria and grey, with white text; probes for M. tuberculosis are in bold and shaded in light grey, while the probe is shaded in dark grey. The nucleotide sequence of the target on the ITS gene of pan-mycobacteria and M. tuberculosis is italicized and the primers are highlighted in black and white boxes. Probes for pan-mycobacteria are shaded in grey, with white text; probes for M. tuberculosis are shaded in grey, with black text. Primer sequences for the nucleotide sequence of the target DNA extracted from about 1 g of faeces or 250 μl of sputum.

dna sequencing. Standard PCR and sequencing reactions were performed for positive cases in order to confirm the specificity of the real-time PCR results. The PCR mixture contained 30.8 μl H2O, 2 μl 25 × MgCl2 (Qiagen), 5 μl 10 × dNTPs, 0.2 μmol l−1 of each primer (Table 1), 5 μl template DNA and 0.2 μl Hotstart Taq (Qiagen). Negative controls consisted of PCR mix without target DNA. The positive control consisted of M. tuberculosis H37Rv DNA. PCR was performed using the following programme: 15 min enzyme activation at 95 °C, followed by 49 cycles consisting of 95 °C for 30 s, annealing at 60 °C for 45 s and elongation at 72 °C for 1 min. A final elongation step was performed at 72 °C for 5 min. Sequencing was done as previously described (Djelouadji et al., 2008).

Case definition. A case of pulmonary tuberculosis was defined by observation of (i) clinical and radiological signs and symptoms suggestive of pulmonary tuberculosis, and (ii) culture of MTC organisms from at least one respiratory tract specimen or (iii) the presence of acid-fast bacilli in the sputum, identified as MTC organisms by real-time PCR.

RESULTS AND DISCUSSION

A total of 134 first sputum and stool specimens were analysed for the presence of MTC organisms in 134 patients. Pulmonary tuberculosis was diagnosed in 24 patients, including 22 culture-positive patients and 2 patients with microscopic detection of acid-fast bacilli (AFB) identified as MTC by real-time PCR and sequencing (case group; mean age: 37 ± 15 years; sex-ratio male/ female: 15/9). Tuberculosis was excluded in 110 patients (control group; mean age: 52 ± 18 years; sex-ratio male/ female: 87/23). Cases were younger than controls (chi-squared = 0.064) but did not differ with respect to sex. AFB were found in 11.2 % sputum and in 6.7 % stool specimens (Fig. 3). Molecular identification of AFB in stools was critical because Mycobacterium avium complex (Colebunders et al., 1990; Liesenfeld et al., 1995; Mavennyengwa & Nziramasanga, 2003; Mazurek et al., 1997; Poropatic et al., 1987; Yajko et al., 1993), Mycobacterium floridum (Tortoli et al., 2005) and Mycobacterium gordonae (Liesenfeld et al., 1995) have been previously detected in patients’ stools. Mycobacterium genavense DNA has been detected in colon biopsy samples of a few patients with colonic polyps and colonic cancer (Dumonceau et al., 1995) and in a few non-HIV, immunocompromised patients (de Lastours et al., 2008; Dumonceau et al., 1995). Also, MTC organisms can be detected in the stools of patients with digestive tuberculosis (Farid et al., 1999; Namisaki et al., 2004), a situation that was not addressed in this study. With respect to the

Fig. 1. Internal standard sequence. Primer sequences for the internal control are boxed and in bold, and the probe is in bold and shaded grey. The boxed sequences in normal font correspond to XhoI sites that were introduced into the DNA for the detection of potential contamination.

Fig. 2. Sequence of the chimeric nucleotide fragment (480 bp) inserted into the pCR II plasmid. The primers specific to the IS6110 gene of M. tuberculosis are in bold and shaded in light grey, while the probe is shaded in dark grey. The nucleotide sequence of the target on the ITS gene of pan-mycobacteria and M. tuberculosis is italicized and the primers are highlighted in black and white boxes. Probes for pan-mycobacteria are shaded in grey, with white text; probes for M. tuberculosis are shaded in grey, with black text. Primer sequences for the nucleotide sequence of the internal control are in normal font, underlined and in bold; the probe is boxed and in bold. The sequences in boxes and in normal font correspond to an XhoI site that was introduced for the detection of potential contamination.

Fig. 3. Distribution of 24/134 patients with a final diagnosis of pulmonary tuberculosis by three diagnostic procedures: microscopic detection of AFB, culture and PCR-based detection of IS6110 of sputum and stool specimens.
diagnosis of pulmonary tuberculosis, microscopic analysis of stools had a sensitivity of 37.5%, which is slightly higher than the 34% reported for non-tuberculous mycobacteria (Morris et al., 1993). Specificity was 100%, the negative predictive value (NPV) was 88% and the positive predictive value (PPV) was 100%. In one patient, stool AFB permitted the diagnosis of pulmonary tuberculosis a few days before additional clinical specimens were shown to be positive. In three additional patients, including one HIV-infected patient and one kidney-transplant patient, diagnosis of pulmonary tuberculosis was provided exclusively by stool specimens.

MTC organisms grew in 14.9% sputum and 9.7% stool specimens (Fig. 3). Culture of stools had a sensitivity of 54.2%, a specificity of 100%, a PPV of 100% and an NPV of 90.9%. Among protocols proposed for the decontamination of stools for the recovery of mycobacteria (Yajko et al., 1993), alkali is most widely used (Mavennyengwa & Nziramasanga, 2003; Oberhelman et al., 2006). A recent study of paediatric patients in Peru found that culture of a gastric aspirate (15/15 cases) was superior to stool culture (3/15) for the diagnosis of pulmonary tuberculosis (Oberhelman et al., 2006). The authors noted that culture of a larger volume of stool and improved decontamination/concentration techniques could improve the sensitivity of stool culture (Oberhelman et al., 2006). We set up a new protocol for this purpose after a preliminary study in our laboratory indicated that chlorhexidine was more efficient than alkali (data not shown). This approach was previously reported for sputum decontamination in cystic fibrosis patients (Ferroni et al., 2006).

Real-time PCR detected MTC DNA in 28/134 (20.9%) sputum and 27/134 (20.2%) stool specimens (Fig. 3). Its sensitivity and specificity for stools were 100% and 97.3%. The PPV was 88.9% and the NPV 100%. The real-time PCR results were regarded as authentic because negative controls remained negative, the molecular technique used sealed capillary tubes known to limit the risk of molecular contamination, and the results of various techniques were in agreement. We further confirmed the specificity of the IS6110 target, as this PCR did not amplify the tested nontuberculous mycobacterial species. Sequencing performed for specimens found to be positive by real-time PCR confirmed the specificity of PCR for 24 patients in the presence of negative controls. Artificially spiked sputum and stool specimens gave a limit of detection of one organism per 25 μl of PCR, with no significant difference in the C_value between sputum and stool specimens. Incorporation of the internal control indicated that 2/134 (1.5%) sputum specimens and 3/134 (2.2%) stool specimens were partially inhibited, a low value when compared with a reported 78% rate of inhibition using stool specimens (Reischl et al., 1998). This result indicated that the DNA extraction protocol we adopted in this study was highly efficient in removing PCR inhibitors from stools. We hypothesized that initial filtration and heat-inactivation steps contributed to this result. Moreover, we designed a DNA extraction protocol to optimize lysis of organisms with a thick and strong cell wall, including mycobacteria. This protocol uniquely comprises two mechanical lysis steps combined with strong enzymic lysis. C_t values varied from 21.7 to 38 (7.7–9.12 × 10^2 M. tuberculosis copies) for positive sputum specimens and from 22.8 to 37.5 (11.5–3.95 × 10^3 copies) for stool specimens. The number of IS6110 copies was higher in stools than in sputa in 11 cases; the copy number was higher in sputa than in stools in 6 cases and the other cases gave almost the same copy number in stools as in sputa. We further observed that the C_t values for 9 patients with positive AFB in stools were significantly lower than those for 15 patients without AFB in stools (P=0.0008). Likewise, the C_t value for 15 patients with positive AFB in sputum was significantly lower than that for 9 patients without AFB in sputum (P=0.0057).

There was a significant correlation between the delay of MTC growth and the C_t value for stool specimens (P=0.0007) and a marginally significant correlation for sputum (P=0.055). The fact that RT-PCR detected the presence of MTC DNA in otherwise culture-negative specimens has been previously reported (Soo et al., 2006). It may be due to the effectiveness of antituberculous drugs, which kill MTC organisms still excreting DNA, as documented in one patient in this series. Therefore, the detection of specific MTC DNA is not by itself a diagnosis of active pulmonary tuberculosis and it was not included in the case-definition in this report. Two patients were 7- and 14-year-old children, confirming the usefulness of stool analysis in paediatric patients. Another recent study in children also concluded the usefulness of stool PCR-based detection of IS6110 in stools but inhibition was not quantified and the sensitivity remained low, at 31–38% (Wolf et al., 2008).

**Conclusion**

The data presented herein indicate that laboratory investigation of stools is favourable compared to that of respiratory tract specimens for the diagnosis of pulmonary tuberculosis, including in children, who often lack productive sputum, thus rendering the diagnosis of pulmonary tuberculosis more difficult. Children have been estimated to constitute up to 25% of all persons with tuberculosis in areas of high transmission (Nelson & Wells, 2004), with an overall incidence of 9.6/100 000 person-years for patients <20 years of age in Taiwan (Chan et al., 2007). Patients with impaired mental status and immunocompromised patients, as herein reported, could also benefit from stool analyses for the diagnosis of pulmonary tuberculosis. Sensitivity, specificity and predictive values were not statistically different between respiratory tract specimens and stool specimens. Because these data were obtained in a prospective study performed during routine diagnosis in our laboratory, including more than 130 specimens, similar data should be obtained in other laboratories.
ACKNOWLEDGEMENTS

This study was supported by l’Oeuvre anti-tuberculeuse des Bouches du Rhône, 2007 and URMITE. The authors acknowledge Professor Hervé Richet for his help with the statistical analyses. Authors’ contributions: A. E. K. and M. H. performed the experiments, wrote the manuscript and contributed to drafting of the manuscript. D. R. and M. D. conceived the experimental design, provided support for the study and contributed to drafting of the manuscript. Conflict of interest: none.

REFERENCES


**Edited by:** M. Daffé