Evaluation of INNO-LiPA assay for direct detection of mycobacteria in pulmonary and extrapulmonary specimens

Francesca Perandin, Gabriele Pinsi, Costantino Signorini, Nino Manca
Department of Experimental and Applied Medicine, Microbiology and Virology, University of Brescia, Italy

SUMMARY

The INNO-LiPA Mycobacteria kit has been developed for detecting mycobacteria in liquid and solid cultures through amplification of the 16S-23S rRNA mycobacterial spacer region and the use of species-specific probes. The aim of this study was to verify the possible direct use of the kit on clinical samples. The study was performed retrospectively on a total of 129 specimens (104 pulmonary and 25 extrapulmonary) and the results were compared to those obtained from culture. For pulmonary specimens, the overall clinical sensitivity of INNO-LiPA Mycobacteria kit was 79.5% and its specificity 84.6%. For extrapulmonary samples, the kit had an overall clinical sensitivity of 71.4%. In both cases no false positive results were found.

KEY WORDS: INNO-LiPA, mycobacteria, direct identification.

INTRODUCTION

There is an increasing interest in mycobacteria - whether Mycobacterium tuberculosis or non-tuberculous mycobacteria (NTM) - in the industrialized and developing countries. Tuberculosis remains the bacterial disease with the highest worldwide mortality rate, with some 8 million new infections and 2 million deaths reported each year (World Health Organization, 2003). Prompt and accurate identification of mycobacteria is necessary for patient management, appropriate therapeutic decisions and to prevent the spread of infection (Wright et al., 1994; Kremer et al., 2002). Though not infallible, cultivation of mycobacteria is considered the gold standard for diagnosis of mycobacterial infection. However, this gold standard has some limitations, in particular in terms of speed. Many mycobacteria grow extremely slowly and may require up to eight weeks to develop, though they can be collected far sooner in liquid media - especially from smear positive specimens (Vestal, 1975). Growth rates, colony pigmentation and biochemical tests are suitable for the identification of most clinically-relevant mycobacteria. On the other hand, they could be inconclusive for certain isolates with variable features. Chromatographic analysis of mycobacterial lipids (mycolic acids or cellular fatty acids) has been suggested as an alternative approach, but the method is unable to identify all species of mycobacteria (Pinsi et al., 2001). For these reasons, bolstered by recent evidence on mycobacterial gene sequences, various molecular approaches have been developed for identification of isolates and amplification of specific gene fragments: e.g. the use of gene probes, gene amplification (PCR), DNA fingerprinting (RFLP), pulsed field gel electrophoresis, and genomic sequence analysis.
DNA strip technology - based on reverse hybridization of PCR products - has been employed for simultaneous detection and identification of mycobacteria. This technique is available through commercial kits containing gene probes, primers and buffer solution for the detection and identification of mycobacterial species after culture. The assay is easy to perform, interpretation of positive lines is clear-cut and the tool is useful for rapid species differentiation. The INNO-LiPA Mycobacteria kit (Innogenetics N.V., Ghent, Belgium), developed for direct use on positive liquid and solid cultures, amplifies the 16S-23S rRNA mycobacterial spacer region (Miller et al., 2000; Suffys et al., 2001; Makinem et al., 2002; Tortoli et al., 2003). Its protocol is quick (5-6 hours), reliable, specific, straightforward and suitable for routine laboratory testing. More recently, some researchers have looked into molecular laboratory methods for direct use on biological samples (Piersimoni et al., 2002; Tansuphasiri et al., 2004; Wang et al., 2004). This approach is clinically interesting and could lead to significantly shorter diagnostic times.

The purpose of the present study was to verify the possible direct use, with slight modifications, of the INNO-LiPA Mycobacteria kit on clinical samples. In particular, we investigated respiratory and extrapulmonary specimens previously assayed by conventional routine procedures.

**PATIENTS AND METHODS**

This study was performed retrospectively on a total of 129 specimens (104 pulmonary and 25 extrapulmonary) sent to the microbiology laboratory of Brescia hospital (Spedali Civili) for investigation of suspect mycobacterial infection. In particular we consider the following samples: 104 pulmonary samples, including 82 sputa and 22 bronchoalveolar lavages (BAL), and 25 extrapulmonary samples including 9 lymphnodes, 8 stools, 6 pus, 1 urine and 1 gastric liquid (Table 1).

The samples were liquefied and decontaminated following the standard N-acetyl-L-cysteine-sodium-hydroxide method. Each specimen was split into two parts: one for the culture and the other for the INNO-LiPA test. Each decontaminated sample was inoculated on Löwenstein-Jensen solid and MGIT liquid media (Becton Dickinson, NJ) and the media were incubated at 37 °C for 8 weeks. The presence of acid-fastness bacilli in positive cultures was investigated through smears stained with auramine-rhodamine fluorescent stain. The identification of isolated mycobacteria on culture was performed by standard biochemical tests.

The DNA template was obtained using the High Pure Template Preparation kit (Roche Applied Science, Penzberg, Germany), with some adjustments. Briefly, 500 µl of each decontaminated sample, stored at -20 °C until molecular analysis, were centrifuged at 13,000 g for 10 min and the supernatant was poured off. The pellet was resuspended in 200 µl of tissue lysis buffer, added with 40 µl of proteinase K and incubated at 55 °C for one hour; after incubation, 200 µl of binding buffer were added and the solution was incubated at 70 °C for 30 min. The subsequent steps of DNA preparation were conducted according to the manufacturer’s instructions. All the 129 DNA template samples were tested.

<table>
<thead>
<tr>
<th>Pulmonary specimens (104)</th>
<th>Sputum</th>
<th>82</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bronchoalveolar lavage</td>
<td>22</td>
</tr>
<tr>
<td>Extrapulmonary specimens (25)</td>
<td>Stool</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Pus</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Lymphnode</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Gastric liquid</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>129</strong></td>
</tr>
</tbody>
</table>

*Two samples were unsuitable due to the presence of Taq polymerase enzyme inhibitors.
by β-actin gene amplification for the presence of DNA polymerase inhibitory factors using a TaqMan β-actin Control Reagents kit (Applied Biosystems, Foster City, CA). Any PCR-inhibitory substances present in the specimen would be detected by failure to amplify the β-actin gene. The template was amplified by real-time ABI Prism 7700 Analytical PCR (Applied Biosystems) with a species-specific primer set and its corresponding probe, according to the manufacturer's instructions. A reaction mixture without DNA was included in each run as negative control. During amplification, the instrument sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The reporter dye signal was measured against the internal reference dye signal to normalize for non-PCR related well-to-well fluorescence fluctuations.

In order to amplify the mycobacterial DNA, we used the INNO-LiPA Mycobacteria v2 kit (Innogenetics) based on PCR amplification and the DNA strip technology. The PCR amplifies the 16S-23S rRNA spacer region, and the DNA probes hybridize the species-specific region to identify the following mycobacteria: M. tuberculosis complex, M. kansasii (three subgroups), M. xenopi, M. gordonae, M. genavense, M. simiae, M. marinum plus M. ulcerans, M. celatum, M. avium complex, M. intracellulare, M. scrofulaceum, M. malmoense, M. haemophilum, M. chelonae (three subgroups), M. fortuitum - M. peregrinum complex and M. smegmatis. The strip also has an additional probe for mycobacterial identification at genus level and a line for enzymatic reaction control. PCR-based amplification was carried out according to the manufacturer's instructions, with slight adjustments. Briefly, 10 µl of extracted DNA solution were transferred to a 0.2 ml amplification tube containing 10 µl of biotinilated MYC primer solution, 10 µl of amplification buffer solution, 19.7 µl of distilled water and 0.3 µl of 5 U/µl Taq polymerase (Taq DNA polymerase, Applied Biosystems). All the reagents except thermostable Taq DNA polymerase, were supplied with the INNO-LiPA Mycobacteria v2 kit. In each run, a positive control (DNA extracted from M. tuberculosis culture, strain ATCC 27294) and a negative control (sample without DNA) were included. To prevent cross-contamination, different sets of pipettes and work areas were used for template and PCR preparation. Amplification was carried out using GenAmp PCR System 2700 (Applied Biosystems) with initial denaturation at 95 °C for 1 min, followed by 45 cycles at 95 °C for 30 s, at 62 °C for 30 s and at 72 °C for 30 s. The success of the amplification step was checked by loading 10 µl of amplified product on ethidium bromide-stained 2% agarose gel. The amplified target appeared as a single band of 400-550 base pairs. After PCR, the hybridization procedure was conducted according to the manufacturer's instructions. Sample identification was performed by evaluating the strip against an interpretation chart supplied with the kit.

RESULTS

Pulmonary specimens
The cultural and biochemical tests performed on the 104 considered samples revealed the presence of 78 positive specimens for the presence of mycobacteria, whereas the remaining 26 samples were negative. In sputa (n = 82) we found M. tuberculosis (n = 63) and M. avium (n = 5) and in BAL samples (n = 22) M. tuberculosis (n = 7) and M. avium (n = 3). When tested by β-actin gene amplification - as described in the Patients and Methods section - these 104 samples did not reveal the presence of polymerase enzyme inhibitors. Therefore the samples were analyzed using the INNO-LiPA Mycobacteria v2 kit, based on mycobacterial DNA extraction and amplification followed by hybridization. Mycobacterial DNA was found in 71 samples. The method revealed mycobacteria at species level (n = 66) or only at genus level (n = 5), whereas the remaining samples (n = 33) were negative. Among culture positive samples (n = 78), the INNO-LiPA kit revealed the same type of mycobacteria in 62 cases, with an overall clinical sensitivity of 79.5%. In cultural negative samples (n = 26) the kit found M. tuberculosis in 4 BAL cases, with an overall specificity of 84.6% (Table 2).

Extrapulmonary specimens
Out of the 25 samples considered, 23 were culture positive for the presence of mycobacteria, whereas the remaining 2 were negative. More specifically, we identified M. tuberculosis (n = 15),
M. genavense (n = 3), M. malmoense (n = 2) and M. avium (n = 1). Among these, the inhibitory of polymerase enzyme (β-actin gene amplification test) were found in 2 stool samples, which were not considered in this study. The 23 specimens were therefore analyzed using the direct method under evaluation. Mycobacterial DNA was found in 19 samples at species level (n = 15) or only at genus level (n = 2), whereas the remaining samples (n = 4) resulted negative. Among culture positive samples (n = 21), the INNO-LiPA kit revealed the same type of mycobacteria in 15 cases, with an overall clinical sensitivity of 71.4%. In the culture negative stool samples (n = 2) INNO-LiPA identified the presence of M. genavense (Table 3).

**DISCUSSION**

In 104 pulmonary samples the INNO-LiPA kit found a clinical sensitivity of 79.5% (62/78) and a specificity of 84.6% (22/26); in the 23 extrapolummary samples, it found 19 DNA positive samples, with an overall clinical sensitivity of 71.4% (15/21). Turning to discordant results, out of 6 INNO-LiPA positive but culture negative specimens (4 pulmonary and 2 extrapolummary), the microscopy examination of the 4 pulmonary samples revealed the presence of mycobacteria; moreover the samples were collected during drug treatment from patients with a clinical diagnosis of tuberculosis. These discordant results might account for the ability of
INNO-LiPA to detect also nonviable microorganisms. In the remaining 2 extrapulmonary stool samples, INNO-LiPA identified the presence of *M. genavense*. This mycobacterial strain required specific growth media, which are used only if there is clinical evidence advising the laboratory to test for this type of microorganism. However in these two patients, the presence of *M. genavense* was confirmed by hemocultural results obtained on blood samples collected at the same time of stool samples. Finally, 15 INNO-LiPA negative specimens (11 pulmonary and 4 extrapulmonary) were culture positive and these false-negative results might be attributed to an unsuitable sensitivity of the method. In addition, extrapulmonary samples represent a major diagnostic problem for amplification techniques, as they could contain polymerase enzyme inhibitors.

The INNO-LiPA assay performed well in the detection and identification of mycobacteria. In terms of workload, the system requires around 6 hours and the diagnostic time is considerably reduced by using DNA taken directly from biological samples instead of cultured bacteria. Although we did not evaluate the INNO-LiPA assay’s sensitivity, PCR amplification showed increased sensitivity compared to other probe-based analytical systems. Furthermore, the INNO-LiPA assay has the advantages of targeting stable DNA and requiring small amounts of bacteria including nonviable microorganisms (Tortoli et al., 2003). One of the disadvantages of molecular DNA amplification is the potential presence of inhibitory factors which could lead to false negative results. For this reason, before using the direct method we checked for the presence of inhibitory factors in DNA extracted from clinical samples by real-time PCR β-actin gene amplification.

In the light of such evidence, it is reasonable to conclude that this direct molecular biology method may prove useful when a rapid response is required. Of course, the method cannot replace conventional microscopy examination, cultures and biochemical tests because - as shown above - molecular biology produced some false negative results. Though licensed only for use with cultured specimens, the INNO-LiPA assay should be evaluated also for direct application to biological specimens. The results reported here need further studies on a larger number of samples, especially in the case of extrapulmonary specimens.

ACKNOWLEDGMENTS

The authors would like to thank Paola Piscioli for her technical assistance in this study.

This work was partially supported by the Ministry of University and Scientific Research, FIL (40%).

REFERENCES


