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The use of selected commercial molecular assays for the microbiological diagnosis of tuberculosis

Zastosowanie wybranych komercyjnych testów molekularnych w mikrobiologicznej diagnostyce gruźlicy

Abstract

Introduction: We performed a retrospective assessment of the AMPLICOR *Mycobacterium tuberculosis* (MTB) assay for the molecular diagnosis of tuberculosis based on our own determinations between 1999 and 2009 and a preliminary assessment of the Xpert MTB/RIF system, which we are currently using.

Materials and methods: The study groups comprised 1875 samples (including 104 inhibited samples) and 213 samples, respectively.

Results: The sensitivities of the AMPLICOR MTB and the Xpert MTB/RIF assays were 81.9% and 81.8%, respectively, and their specificities were 97.2% and 99.5%, respectively, versus culture on Loewenstein-Jensen medium. Both assays showed a considerable difference in sensitivity depending on whether the test samples were smear-positive (AFB+) or smear-negative (AFB–). The sensitivities of the AMPLICOR MTB and the Xpert MTB/RIF assays were 97.8% and 100.0%, respectively, for AFB+ samples and 58.1% and 50.0%, respectively, for AFB– samples.

Conclusions: Our results confirm full usefulness of the Xpert MTB/RIF assay for routine diagnosis in the case of smear-positive clinical samples.

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Introduction

Nucleic acid amplification (NAA) techniques are increasingly used for the direct examination of clinical samples in the laboratory diagnosis of tuberculosis, in addition to the standard methods, which involve microscopy and isolation of mycobacteria on a solid or liquid medium. For the past 12 years, NAA-based assays have been included in the diagnostic algorithm used by our Department.

Between 1999 and 2009 we used the AMPLICOR *Mycobacterium tuberculosis* (MTB) assay (Roche Diagnostics, Switzerland) approved by the European Centre for Disease Prevention and Control (ECDC, EU); the AMPLICOR MTB assay is based on polymerase chain reaction (PCR) [1]. For the past year and a half our Department has been using the Xpert MTB/RIF system (Cepheid, USA), which utilises real-time PCR [2]. The system was awarded the European Certificate in 2009 and only a few papers on its use in clinical diagnosis have been published so far.

A 584-bp DNA fragment within a larger region encoding for 16S rRNA, common for *Mycobacterium* spp., was chosen as the target sequence in the AMPLICOR MTB assay. Of the possible amplification products, thanks to the use of a plate coated with a specific probe, *Mycobacterium tuberculosis* complex (MTBC) comprising *M. africanum*, *M. bovis*, BCG, *M. microti*, *M. canetti* [3], and *M. pinnipedii* [4] can be detected. The AMPLICOR MTB assay has the advantage of internal control, which

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is a safeguard against false negative results arising from inhibition of the polymerase. The assay also has a built-in system (uracil-*N*-glycosylase) that secures against contamination by amplicons from previous determinations, which is the most common cause of false positive results in routine work.

The real-time PCR technique employed in the Xpert MTB/RIF system brings a new quality to the molecular diagnosis of tuberculosis. It allows the simultaneously use of 5 probes in the amplification reaction of the *rpoB* gene fragment, hence enabling the identification of MTBC and at the same time the detection of most strains resistant to rifampicin (RIF) [5]. The *rpoB* gene is a highly conservative gene encoding the beta subunit of RNA polymerase. The enzyme is deactivated by RIF in sensitive strains. Most mutations in the *rpoB* region do not result in a loss of polymerase activity, but they do make this enzyme insensitive to RIF.

The Xpert MTB/RIF system is fully automated and all the processes comprising the PCR technique, i.e. DNA release, the polymerase chain reaction proper, and detection of the amplicon, are integrated in a disposable element (a cartridge) containing several reaction chambers. Each reaction occurs in a separate chamber and freeze-dried reagents are released from the capsules as the process progresses. This technological solution minimises the risk of contamination (including contamination as a result of transfer of amplicons from previous determinations) and virtually rules out the possibility of false positive results. Another protection measure against false negative results involves the control of the degree of mycobacterial disintegration and polymerase activity, which has been indirectly solved in the Xpert MTB/RIF system as follows: *Bacillus globigii* spores have been included as internal control, and a sixth probe detecting *B. globigii* DNA has been added. Only clinical samples that give a negative signal for MTBC and positive signal for *B. globigii* are true negative.

The Xpert MTB/RIF system is easy to use and thanks to being fully automated the duration of determination does not exceed 2 hours. An additional positive effect of the automation is the minimisation of the risk of contamination and the maximum safety for the staff.

In 2009, the manufacture of the AMPLICOR MTB assay was discontinued. Of the commercially available certified NAA assays for the diagnosis of tuberculosis we selected the Xpert MTB/RIF system due to the possibility of simultaneous detection of resistance to RIF directly in the clinical sample. It was not, however, possible to compare both of these methods on the same clinical material. Our study is retrospective in nature: the diagnostic effectiveness of the AMPLICOR *Mycobacterium tuberculosis* (MTB) assay (Roche Diagnostics, Switzerland) was assessed on the basis of our own determinations performed for more than 10 years. We then assessed the effectiveness of the Xpert MTB/RIF system (Cepheid, USA) based on the results collected since November 2009 in order to gain a preliminary insight into whether the parameters of sensitivity and specificity of MTBC genome detection using the new system match or surpass the parameters of the previously used assay.

Material and methods

As part of the routine diagnostic procedure, a smear stained for acid-fast bacilli (AFB) using the Ziehl-Nielsen method and a culture on Loewenstein-Jensen (L/J) solid medium were performed after liquefaction and decontamination of the clinical sample using sodium lye with *N*-acetylcysteine and sodium citrate, followed by inspissation according to standard procedure [6]. The cultured mycobacterial strains were typed in accordance with the Centres for Disease Control and Prevention (CDC, USA) guidelines [7] employing mycolic acid analysis with the use of high-pressure liquid chromatography (HPLC), as described previously [8, 9]. In justified cases, genotyping was performed using the molecular assay GenoType Mycobacterium CM/AS (Hain Lifescience, Germany) [10]. The molecular assays AMPLICOR MTB or Xpert MTB/RIF were used to directly examine the clinical samples in cases ordered by the clinician.

The AMPLICOR MTB assay was performed in accordance with the instructions of the manufacturer, Roche Diagnostics, Switzerland. All the reagents, controls, and plates, coated with specific probes for the detection of MTBC DNA and for internal control (IC), were supplied in the kit. One run requires at least 6 samples and 2 controls (positive and negative). The assay procedure consists of 3 stages completed in separate rooms: (1) preparation of the samples, positive control and negative control, (2) the polymerase chain reaction proper, and (3) specific detection of the reaction product and control. In brief, the procedure is as follows:

1. 100 μl of inspissated clinical sample, after washing and centrifugation with 500 μl of Respiratory Specimen Wash Solution, was incubated with 100 μl of Respiratory Specimen Lysis Reagent at 60°C for 45 minutes. The negative control, MYCO(–)C, and the positive

control, MYCO(+)C, were prepared in parallel with the test sample. The sample and the controls were neutralised with 100 μl of Respiratory Specimen Neutralisation Reagent. For the purposes of PCR, 50 μl of the resulting DNA matrix were collected.

2. 50 μl/reaction of Working Master Mix were prepared by mixing the following components in the proportions indicated by the manufacturer: MYCO IC (Mycobacterium Internal Control), AmpErase LD (Low DNA Uracil-*N*-Glycosylase), and MYCO MMX (Mycobacterium Master Mix), a reagent containing Taq DNA polymerase, a mixture of the following nucleotides: dATP, dCTP, dGTP, and dUTP, and a mixture of the following biotinylated primers: KY18 and KY75. The final PCR volume was 100 μl. The reaction was conducted as follows: 1 cycle, 600 s 50°C/2 cycles, 20 s 98°C, 20 s 62°C, 45 s 72°C/41 cycles, 20 s 94°C, 20 s 62°C, 45 s 72°C/1 cycle, 300 s 72°C/final temperature 72°C.
3. The samples were denatured immediately after removing from the thermal cycler by adding 100 μl of Denaturation Solution to each of the samples. Detection was carried out in parallel on MTB MWPs (*M. tuberculosis* Microwell Plates) and IC MWPs (Internal Control Microwell Plates) by hybridisation with specific probes. The hybridisation product was detected using a colour enzymatic reaction with Avidin-Horseradish Peroxidase Conjugate. All the stages of the procedure are considered to have been completed correctly, if extinction values for both MTB MWP and IC MWP are: MYCO(–)C ≥ 0.25 and MYCO(+)C ≥ 2.0. Table 1 summarises interpretation of the results.

Determination takes 7–8 hours to complete, excluding pretreatment of the clinical samples.

The Xpert MTB/RIF assay was performed in accordance with the instructions of the manufacturer (Cepheid, USA), which enable determination of liquefied, decontaminated and inspissated clinical samples as well as samples without pretreatment. A rule was adopted to determine samples after pretreatment and a decision not to apply this rule was made only in isolated justified cases. 1.5 ml of the sample reagent (SR) was added to 0.5 ml of the sample, mixed and incubated for 15 minutes at room temperature, and mixed again between 5 and 10 minutes of incubation. The mixture was then transferred to the cartridge and the cartridge was placed in the GeneXpert system, controlled by the computer, and the reaction, which took 2 hours to complete, was initiated. The process was fully automated and the results were interpreted by integral software in accordance with the following algorithm:

1. The result is positive for MTBC if at least 2 out of 5 *rpoB* probes give a positive signal no later than in the two subsequent cycles.
2. The result is negative for MTBC if condition 1 is not met and the probe for *B. globigii* gives a positive signal.
3. Samples negative for both MTBC and *B. globigii* are classed as indeterminable.
4. Resistance to RIF is identified if condition 1 is met and at least 1 out of the remaining 3 *rpoB* probes is negative.

Results

From 12 January 1999 to 16 November 2009 a total of 1875 clinical samples were examined using the AMPLICOR MTB assay. In 1771 cases the polymerase chain reaction was not inhibited and the results were diagnostically relevant. Concordant results using the AMPLICOR MTB assay and the L/J culture were obtained for 90.5% (1697/1875) of the samples tested: in both tests, there were 127 positive and 1570 negative samples. Discordant results were obtained for 4.0% (74/1875) of the samples. For 46 samples, the AMPLICOR MTB assay results were positive but cultures were negative. In 28 cases, tuberculosis was confirmed by L/J culture only with the AMPLICOR MTB assay being negative. In most cases these were bacterioscopy-negative materials (24 samples), but in 2 cases the samples were positive on bacterioscopy. The results are summarised in Table 2.

Table 1. AMPLICOR MTB Test (Roche Diagnostics, Switzerland) — interpretation of results

Mtbc A_{450}	IC A_{450}	Interpretation
< 0,35	≥ 0,35	Negative result. MTB DNA not detected
< 0,35	< 0,35	Inhibitory Specimen. MTB DNA, if present, would not be detectable
≥ 0,35	Any	Positive result. Specimen is positive for the presence of MTB

Mtbc — Mycobacterium tuberculosis complex, IC — internal control

Table 2. Results of the AMPLICOR MTB Test (Roche Diagnostics, Switzerland)

Microscopy		AFB(+)	AFB(–)	Not performed	Total	
PCR(+)	L/J(+)	91	33	3	127	173
	L/J(–)	31	12	3	46	
PCR(–)	L/J(+)	2	24	2	28	1598
	L/J(–)	101	1430	39	1570	
Total	L/J(+)	93	57	5	155	1771
	L/J(–)	132	1442	42	1616	

AFB — acid fast bacilli, L/J — Loewenstein-Jensen medium, PCR — polimerase chain reaction

Table 3. Results of the Xpert MTB/RIF (Cepheid, USA)

Microscopy		AFB(+)	AFB(–)	Not performed	Total	
Xpert(+)	L/J(+)	14	4	0	18	19
	L/J(–)	1	0	0	1	
Xpert(–)	L/J(+)	0	4	0	4	194
	L/J(–)	7	181	2	190	
Total	L/J(+)	14	8	0	22	213
	L/J(–)	8	181	2	191	

AFB — acid fast bacilli, L/J — Loewenstein-Jensen medium

Based on the above results, we calculated the sensitivity and specificity of the AMPLICOR MTB assay obtaining the following values: 81.9% (127/155) and 97.2% (1570/1616), respectively. The sensitivity of the molecular assay was then analysed in groups. For the 225 samples for which bacterioscopy was positive (AFB(+) samples), the sensitivity of the AMPLICOR MTB assay was 97.8% (91/93). In the group of the remaining 1546 samples (1499 AFB(–) samples and 47 samples for which, for justified reasons, bacterioscopy was not performed), the sensitivity of the assay was 58.1% (36/62).

In one case, from an AFB(+) sample which was initially positive for MTBC in the AMPLICOR MTB assay an NTM strain was cultured, which was subsequently typed by HPLC as *M. chelonae*. The examination was repeated using another molecular test, GenoType Mycobacterium CM/AS, which yielded a result that was concordant with the AMPLICOR MTB assay, confirming the presence of MTBC DNA. It was eventually established that the *M. chelonae* strain was a contaminant.

In the tested group, the PCR was inhibited in 104 cases. Inhibition was most common in the bronchoalveolar lavage fluid (BALF) and in the urine. For 23% of the BALF samples and 12% of the urine samples tested no PCR result was obtained.

The Xpert MTB/RIF system is routinely used in the laboratory diagnosis of tuberculosis in our Department at present. Table 3 summarises results for 213 clinical samples. In 208 cases (97.6%), the results of the new molecular assay and those of cultures were concordant. In 18 cases, we obtained positive results both in the Xpert MTB/RIF system and in the L/J culture. In both tests, 190 clinical samples were negative. Of the remaining 5 samples for which the results were discordant, MTBC strains were cultured from 4 AFB(–) samples despite the negativity in the molecular assay. The sensitivity of the assay was estimated at 81.8% (18/22) and its specificity at 99.5% (190/191). Taking bacterioscopy results into account, sensitivity was 100% (14/14) for samples positive with Ziehl-Neelsen stain and 50% (4/8) for bacterioscopy-negative samples.

In the Xpert MTB/RIF system, no cases of inhibition reaction occurred: in a few cases (3 sputum samples, 1 bronchial lavage fluid sample, 1 bronchial discharge sample) the result was considered indeterminable by the system, but reliable results were obtained on retest.

In the study group of clinical samples, nontuberculous strains were cultured in 7 cases with no cross-reaction being observed in any instance. No resistance to RIF was identified in the study group.

Discussion

Our results indicate that both the sensitivity and the specificity of the AMPLICOR MTB assay were very high: 81.9% and 97.2%, respectively.

While assay specificity was not affected by whether the sample was rich or poor in mycobacteria, assay sensitivity depended on the type of clinical material and was high (97.8%) for AFB(+) samples and much lower (58.1%) for AFB(–) samples. With these parameters characterising the diagnostic effectiveness of the AMPLICOR MTB assay, a positive result confirms tuberculosis for a sample positive for Ziehl-Neelsen stain and is strongly suggestive of tuberculosis if the smear is negative. An isolated negative result of the assay does not rule out tuberculosis if the sample is negative in microscopy. If the sample is positive for Ziehl-Neelsen stain, a negative result of the AMPLICOR MTB assay strongly suggests the presence of non-tuberculous mycobacteria. Our results are consistent with bibliographical data [11, 12].

The Xpert MTB/RIF system was implemented by us in November 2009, half a year after it had been approved for routine diagnosis in the European Union by the ECDC. True results (true positive and true negative results) were obtained in 97.6% of the cases, which is slightly higher than the true result rate for the AMPLICOR MTB assay (90.5%). In our study, the specificity of the Xpert MTB/RIF system was slightly higher than that of the AMPLICOR MTB assay (99.5% vs. 97.2%), while the sensitivity was on a similar level (81.8% vs. 81.6%). After including microscopy results in the analysis, the sensitivity for both systems was very high (100% for Xpert MTB/RIF and 97.8% for AMPLICOR MTB) and much lower for samples poor in mycobacteria and negative on bacterioscopy (50.0% for Xpert MTB/RIF and 58.1% for AMPLICOR MTB). It seems, however, that due to the small number of 8 AFB(–) samples which were examined using the Xpert MTB/RIF system, drawing conclusions on the sensitivity of this system for samples poor in mycobacteria is not justified. In connection with the use of real-time PCR one should rather expect a much higher diagnostic sensitivity in the Xpert MTB/RIF system compared to the traditional NAA assays. Other team of investigators has obtained much higher sensitivity (72.5%) for AFB(–) clinical materials while examining isolated samples. Sensitivity was even higher (85.1%) if two samples were tested with the Xpert MTB/RIF system and even reached 90.2% when three examinations were performed [13].

Our study indicates that some of the diagnostic parameters of the Xpert MTB/RIF system are slightly higher than those of the AMPLICOR MTB. However, taking into account the World Health Organization recommendation [14] to use the Xpert MTB/RIF system in the first step of the laboratory procedure in the diagnosis of tuberculosis in adults suspected of multidrug resistant tuberculosis (MDR TB) or tuberculosis in the course of HIV infection and, possibly, for other patients, as a second test for AFB(+) samples in the smear, it seems necessary for us to conduct further research on the sensitivity of the method in AFB(–) samples poor in mycobacteria.

Based on our study we cannot address the specificity of the Xpert MTB/RIF system if mixed mycobacterial strains are present in the sample, as those did not occur. Helb et al. [15] conclude that the Xpert MTB/RIF system is characterised by a high specificity and the ability to detect MTBC strains even in samples contaminated with non-tuberculous mycobacteria. In the cited study, none of the 22 non-tuberculous mycobacterial strains tested, out of the 22 *Mycobacterium* species, cross-reacted. The test correctly detected MTBC in the presence of *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. malmoense*.

Our assessment of the Xpert MTB/RIF system in terms of detection of resistance to RIF remains another open question. In regions with high rates of MDR TB, defined as simultaneous resistance to RIF and isoniazid (INH), 70–95% of *M. tuberculosis* strains resistant to RIF had a mutation in one of the following three loci of the *rpoB* gene: 516, 526, or 531 [16, 17]. Prammananan et al. presented a discussion in their study on the prevalence of various mutations determining resistance to RIF depending on the geographical region [18]. Because the emergence of resistance to INH usually precedes resistance to RIF, the latter is often treated as a substitute marker of multidrug resistance [19]. In regions where MDR TB is rare, the use of resistance to RIF as a marker of multidrug resistance depends on the prevalence of monoresistance to RIF in a given population [20]. Augustynowicz-Kopeć showed that in the Polish population, in previously treated patients, genetically determined resistance to RIF may be a substitute marker of MDR TB [21]. It seems, therefore, that the Xpert MTB/RIF system may be useful for determining at least secondary resistance of the MDR type, although we could not address this issue in our study as no strains resistant to RIF were present in the study group.

Conclusions

1. Our study assessed the use of the AMPLICOR MTB assay and the new system, Xpert MTB/RIF, for the molecular diagnosis of tuberculosis.
2. Our results demonstrate complete usefulness of the Xpert MTB/RIF system for routine dia-

gnosis of clinical samples, positive in the microscopic examination of the smear (i.e. AFB(+) samples).

3. Determination of the sensitivity of the Xpert MTB/RIF system for smear-negative samples requires further research with participation of a larger number of patients.
4. It is necessary to determine the specificity of the Xpert MTB/RIF system for *M. tuberculosis* strains contaminated with environmental mycobacteria and to examine the effectiveness of the system for the detection of strains resistant to RIF.

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