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Evaluation of two tuberculosis PCR assays.

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Running head: Evaluation of tuberculosis PCR assays.

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Evaluation of two high-throughput tuberculosis PCR assays for routine use in a clinical setting of low population and low tuberculosis prevalence.

Today there are numerous different molecular diagnostic assays for detection of tuberculosis (TB) allowing optimization of rapid detection of TB according to the clinical need. In this study, two high throughput TB PCR assays with combined antimicrobial resistance detection, Anyplex™ II MTB/MDR (Seegene) and RealTime MTB + RealTime MTB RIF/INH Resistance (Abbott), were evaluated for routine use in a clinical setting of low population and low TB-prevalence in Finland.

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The RealTime MTB assay was 100% concordant (22/22 positive, n=169) with the reference methods (culture and Xpert MTB/RIF PCR assay, Cepheid). However, with a limitation of four separate PCR-cycles per kit, the routine use in a low TB-prevalence setting would easily lead in wasting most of the RIF/INH Resistance reagents. The Anyplex™ II MTB/MDR assay usability was more adaptive to suit the clinical setting but the assay sensitivity was considerably lower (86%, 19/22 positive, n=76) being closer to the sensitivity of smear microscopy.

The findings of this study suggest that the evaluated high-throughput MTB/MDR assays are evidently suboptimal for routine use in a low population, low TB-prevalence setting. In addition, neither of the two assays covers non-tuberculous mycobacteria and could therefore not fully replace acid-fast staining as the initial screening method.

Keywords: Anyplex; PCR; RealTime; Tuberculosis

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1. Introduction

Tuberculosis (TB) is a well-known infectious disease causing over a million deaths annually and affecting every country over the globe (1). It has been recently re-estimated that up to one fourth of the world's population may carry the infectious agent, *Mycobacterium tuberculosis* (Mtb) (2). TB is an excellent example of an infectious disease with an extremely widespread but uneven distribution. For instance, the incidence of TB rises over 1000 cases per 100 000 population in some high TB burden countries (1) whereas corresponding incidences in low burden Nordic countries are below 10 cases per 100 000 population (3). At a local level, this may mean less than a single case per month. On the other hand, low incidence of active TB may leave more space for recognizing latent TB and non-tuberculous mycobacteria (NTM) (4,5). Together with the gross differences in available

resources it is obvious that laboratory diagnosis of TB needs to be optimized to best fit a local setting.

For decades, culture and smear microscopy have been the cornerstones of TB laboratory diagnosis (6). Culture still represents the gold standard as the most sensitive method. However, it takes several weeks before definitive results are obtained. Smear microscopy provides preliminary results the same day but does not separate between tuberculous and non-tuberculous mycobacteria. In addition, the method is relatively subjective and requires high level of expertise. The era of PCR brought definitive laboratory diagnostics of TB down to hours instead of weeks (7). Today there are a number of nucleic acid amplification assays allowing different strategies for laboratory diagnostics of TB such as simultaneous analysis of antibiotic resistance or non-tuberculous mycobacteria (NTM) in addition to Mtb detection, point-of-care testing and analysis of virtually any kind of clinical specimen type (7-12).

The purpose of this study was to evaluate the suitability of two different TB-PCR systems, Anyplex™ II MTB/MDR (Seegene, Seoul, Korea) and RealTime MTB + RealTime MTB RIF/INH Resistance assay (Abbott Laboratories, Texas United States), in a clinical setting of low population and low TB-burden. Both of the assays are high-throughput tests detecting isoniazid (INH) and rifampicin (RIF) resistance in addition to Mtb complex organisms.

2. Materials and methods

2.1. Clinical material

169 clinical specimens were available for this retrospective study, collected in 2016 at Pirkanmaa Hospital District (Fimlab Laboratories), and Hospital district of Southwest Finland (Public Utility Tyks-Sapa). Most of the samples (n=163) were NALC-sedimented respiratory specimens (e.g. sputum, bronchial washing). Other than respiratory specimens, three urine, two tissue biopsy and one ascites fluid specimens were analyzed. Clinical specimens were stored frozen (-20 °C) and thawed upon analysis. Due to low prevalence of Mtb or NTM positive samples, to increase the number of mycobacterial isolates to be tested an additional 18 artificial specimens (AS) were prepared: 200 µl of positive mycobacterial cultures from the MGIT™ (Mycobacteria Growth Indicator Tubes, Becton Dickinson, New Jersey, United States) was added in sputum samples tested negative for Mtb, giving a total of 187 samples.

2.2. Methods

The study was performed at the Clinical Microbiology laboratory of Fimlab laboratories Ltd., Tampere, Finland. Two different Mtb PCR assays, Anyplex™ II MTB/MDR Detection (Seegene, Seoul, Korea) and RealTime MTB (Abbott Laboratories, Illinois, United States), were evaluated in this study. The RealTime system was further separated in two different PCR assays: one for MTB detection and the other for RIF/INH resistance detection. For analysis, samples were first inactivated to kill any live mycobacteria with a method previously described by Qi (13). This was done to ensure safe working outside a biosafety cabinet. Briefly, samples were mixed with inactivation reagent (0.6% sodium hydroxide [wt/vol], 60% isopropanol [vol/vol], and 1.8% Tween 20 [vol/vol]) at a ratio of 1:3 and vortexed vigorously twice during a one to 24-hour incubation at room temperature. DNA was extracted using the automated Abbott m2000sp system. RealTime MTB PCR was set up, amplified and analyzed automatically with the m2000sp and m2000rt system, respectively. Mtb-positive samples were assayed for antibiotic resistance with a semi-automated reflex-feature including a second mastermix protocol and RealTime MTB RIF/INH Resistance PCR assay. Anyplex™ II MTB/MDR PCR was set up manually according to manufacturer's instructions. PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad, California, United States) and results analyzed with Seegene viewer software. PCR kits were kindly provided for evaluation by Abbott Laboratories and Triolab Ltd. This set the limit for the study: all the 187 samples (18 AS) were analyzed with RealTime MTB assay but only 94 (18 AS) with Anyplex™ II MTB/MDR assay.

Results of the two PCR assays were evaluated against mycobacterial culture (Löwenstein-Jensen agar and BD BACTEC™ MGIT™ growth indicator tubes). In addition to culture results, smear microscopy (auramine staining) results of the clinical samples and mycobacterial species identification (GenoType® Mycobacterium CM VER 2.0 assay, Hain Lifescience GmbH, Nehren, Germany) and antimicrobial susceptibility results (determined by National Institute for Health and Welfare, Finland) were available for the study. Samples with controversial results between PCR and culture were analyzed with another PCR method, the Xpert® MTB/RIF assay (Cepheid, California, United States) using the GeneXpert system (Cepheid). The added and main value of the Xpert assay was to serve as a reference method to compare different PCR processes in terms of hands-on-time, time to result, general assay convenience in the clinical setting in question. To evaluate this, the duration of each step of each PCR method was measured with minimal and maximal assay capacity per run.

2.3. Clinical setting

To see how the capacities of the Anyplex and RealTime Mtb PCR assays suit the clinical setting of this study, numbers of mycobacterial laboratory tests and positive findings in Fimlab area were listed from four previous years (2014–2017).

3. Results

3.1. Analytical performance of Mtb and antibiotic resistance detection

Of the 187 samples (including 18 artificial samples, AS) included in this study, 60 (18 AS) were positive for mycobacteria with reference methods (Table 1). 25 samples (3 AS) were confirmed positive for Mtb and the rest 35 (15 AS) for non-tuberculous mycobacteria. 18 of the 22 Mtb positive clinical samples (81%) were smear positive.

Analytical performance data of the evaluated PCR tests are summarized in Table 2. The RealTime MTB assay identified all of the 22 Mtb-positive clinical samples whereas the sensitivity of the Anyplex™ II MTB/MDR assay was lower, 86% (19/22). Both tests identified the additional three Mtb-positive artificial samples. Of the three samples that gave a false-negative result with the Anyplex assay, one was culture and smear positive, one was culture positive but smear negative, and one was smear and culture negative and only positive with the other PCR assays (Xpert and RealTime). Specificity for both Anyplex and RealTime assays was 100%.

Two of the 25 (3 AS) Mtb-positive samples contained isolates resistant against first-line TB drugs, one for INH only and one (AS) for both INH and RIF. The antibiotic resistance profiles of these isolates were correctly identified by both Anyplex and RealTime assays. Neither of the assays reported any false positive antibiotic resistance results. However, four of the 25 RealTime MTB positive samples had Mtb concentrations below the RealTime MTB RIF/INH resistance assay's limit of detection.

3.2. Clinical setting

Local data was examined to scale the performance capacity of the different TB-PCR assays in a clinical setting. From 2014 to 2017, 6357–9152 clinical samples per year were cultured for mycobacteria. 56–59% of these were sputum samples. Blood samples were excluded from analysis. Of all samples, 332–401 (5.2–4.4%) were culture positive for acid fast bacilli (afb) confirmed by microscopy. Most of the afb findings involved non-tuberculous mycobacteria as there were only 27–45 new cases of Mtb. In summary, an average of 17–25 samples was cultured per day, one afb was found per day and one new Mtb case per 8–14 days.

3.3. Assay workflow

The workflows of the two PCR assays are shown in Figure 1 in relation to Xpert MTB/RIF (Cepheid). The advantages of these high-throughput PCR assays were clearly apparent with high sample counts. Both Anyplex and RealTime assays allow up to 93 tests per run (96-well format with three assay control). Time to first result was similar for Anyplex and RealTime assays, from 5 h 30 min to 9 h 15 min depending on the number of samples per run. That is, both PCR systems mostly allow detecting Mtb within clinical laboratory opening hours. In the case of the Anyplex assay, manual sample labeling before PCR and separate analysis of PCR data were additional steps compared to RealTime, with no significant effect to the analysis time whatsoever. With the RealTime assay, having the detection of Mtb and antibiotic resistance separated in two subsequent PCR cycles, final results were available from 7 h 45 min to 11 h 45 min. The protocols included the shortest possible inactivation step (1–1.75 h), automated extraction step (2–5 h), manual (Anyplex) or automated (RealTime) PCR preparation step (0.25–0.75 h) and amplification step (2 h) with minimal and maximal sample capacity, respectively. In terms of hands-on-time, the most laborious step was the initial inactivation step (up to 1.25 h). Test abortion occurred twice in sample preparation and twice in amplification step for both assays. No cross-contamination was detected in any of the nucleic acid method used (extraction or amplification). Considering routine use, the manufacturers allowed five separate PCR cycles (thaw and freeze) for 50 reaction Anyplex kit reagents and four cycles for 96 reaction RealTime MTB and 24 reaction RealTime RIF/INH Resistance kit reagents.

4. Discussion

Unlike Xpert MTB/RIF assay which suits even the needs of smallest clinical laboratories, Anyplex™ II MTB/MDR and RealTime MTB assays, in contrast, are more demanding in terms of equipment and working facilities. However, both assays allow excellent control over high sample frequencies and may therefore better suit the needs of central laboratories with high sample counts. As a bonus, the evaluated systems also allow better possibilities in subsequent molecular testing as having separate extraction and amplification steps. The RealTime MTB RIF/INH Resistance assay serves an example of such 'downstream' molecular testing. The fluency of the high-throughput workflow could be greatly enhanced by having the time-consuming inactivation step automated. This would also increase test sensitivity, as the inactivation step dilutes the original specimen. However, automatic handling of clinical samples may prove to be difficult with as mucous sample material as sputum (14). Despite the challenging sample material, the study showed low PCR error rates which reflect the efficiency of the Abbott m2000sp extraction system, but also of the inactivation protocol in use.

Considering analytical performance, the RealTime MTB assay performed excellently with 100% concordance with reference methods. The Anyplex™ II MTB/MDR assay missed three out of 22 Mtb-positive clinical samples (14%). The difference is clinically significant as any false negative Mtb result could exhibit a significant clinical impact considering early administration of antimicrobials and disease transmission (15). It is noteworthy that this study was performed using NALC sedimented, frozen and thawed samples instead of fresh untreated samples which may have affected the quality of this study. This was inevitable due to low TB incidence and retrospective nature of the study.

The Abbott's reflex system of antibiotic resistance detection would conceptually be ideal in a low burden setting where PCR was the primary screening method for TB. With only occasional Mtb-positive hits per samples series, a strategy where only Mtb-positive samples are analyzed for antibiotic resistance seems cost-efficient. However, the RealTime MTB RIF/INH assay is relatively expensive and the number of allowed runs is very limited (four separate times per reagent kit). With an average of one new TB case per one to two weeks, this easily leads in wasting expensive unused tests. In fact – and perhaps paradoxically – it would have been more affordable not to use the RealTime MTB RIF/INH Resistance assay but the more adaptable and less expensive Anyplex™ II MTB/MDR assay for the 'reflex testing' of RealTime MTB analyzed Mtb-positive samples. In addition,

in RealTime MTB RIF/INH Resistance assay the extracted DNA is dispensed in three separate wells which occasionally lead to dilution of Mtb DNA below assay sensitivity.

The valid detection of INH monoresistance by both assays first suggests that rapid detection of both INH and RIF resistance is necessary. However, it is under controversy whether this INH monoresistance affects treatment results with drug-sensitive TB regimen (16,17). In fact, WHO no more recommends drug-resistant TB treatment for INH-monoresistant TB whereas RIF monoresistant TB has similar recommendations with MDR-TB (18). In this sense, rapid detection of INH+RIF resistance – although advantageous – would not seem to be a necessity in the way that rapid detection of RIF resistance is.

In a low-burden clinical setting where smear microscopy still is the primary rapid TB-testing method, the capacities of the evaluated RealTime and Anyplex systems were too high to meet the actual need as a confirmatory test. The high throughput systems would thus suit more appropriately for rapid screening purposes. As a technology, PCR would be ideal for replacing the subjective, experience-demanding and laborious acid-fast staining as the preliminary diagnostic method. Reproducible PCR methods have been shown to defeat smear microscopy methods in analytic sensitivity (19,20). However, PCR loses its advantage as a rapid diagnostic method if batches of samples must be collected for several days due to low sample frequency. In the case of RealTime, an average of at least 22 samples per run and subsequently at least 4 Mtb-positive samples are required for not wasting any identification or resistance PCR reagents, respectively. The local data for daily number of samples shows that this is right in the lower limit of the assay capacity. In this sense, the Anyplex™ II MTB/MDR assay was much more adaptable with less restriction for separate cycles per PCR kit.

Finally, if a PCR method was to replace acid-fast staining as the preliminary TB screening method it should ideally cover not only Mtb but also other mycobacteria, furthermore discriminating between Mtb and non-tuberculous mycobacteria (NTM). Although not as critical as rapid detection of Mtb and other Mtb complex organisms, leaving laboratory diagnosis of NTM to rest only on mycobacterial cultures would be a major drawback for laboratory diagnosis of NTM. After all, NTM account for the majority of mycobacterial findings in some low TB-prevalence countries (21). As a conclusion, neither of the evaluated assays was optimal in the clinical setting in question. Therefore

low-throughput, random access strategies for molecular diagnosis of TB remain more useful for most laboratories in low population, low TB prevalence areas.

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Ethical statement

The authors have no conflict of interest to declare.

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Table 1. Mycobacterial species present in the study.

Mycobacterial species	Clinical Samples	Artificial Samples	Total
<i>M. tuberculosis</i> complex			
<i>M. tuberculosis</i>	22	3	25
Non-tuberculous mycobacteria			
<i>M. avium</i>	10	1	11
<i>M. lentiflavum</i>	2	2	4
<i>M. abscessus</i>	2	1	3
<i>M. chelonae</i>	1	2	3
<i>M. goodii</i>	1	2	3
<i>M. fortuitum</i>		2	2
<i>M. intracellulare</i>		2	2
<i>M. xenopi</i>	1		1
<i>M. bohemicum</i>		1	1
<i>M. marinum</i>		1	1
<i>M. neoaurum</i>		1	1
<i>Mycobacterium</i> spp.	3		3
Total	42	18	60

Table 2. Analytical performance data of Anyplex II MTB/MDR and RealTime MTB assays.

	Reference methods	Anyplex II MTB/MDR	RealTime MTB
n, total	169 (+18 AS)	76 (+18 AS)	169 (+18 AS)
n, Mtb positive	22 (+3 AS)	19 (+3 AS)	22 (+3 AS)
n, Mtb negative	147 (+15 AS)	57 (+15 AS)	147 (+15 AS)
Sensitivity, %		86	100
Specificity, %		100	100

AS: artificial sample, excluded from sensitivity and specificity analysis

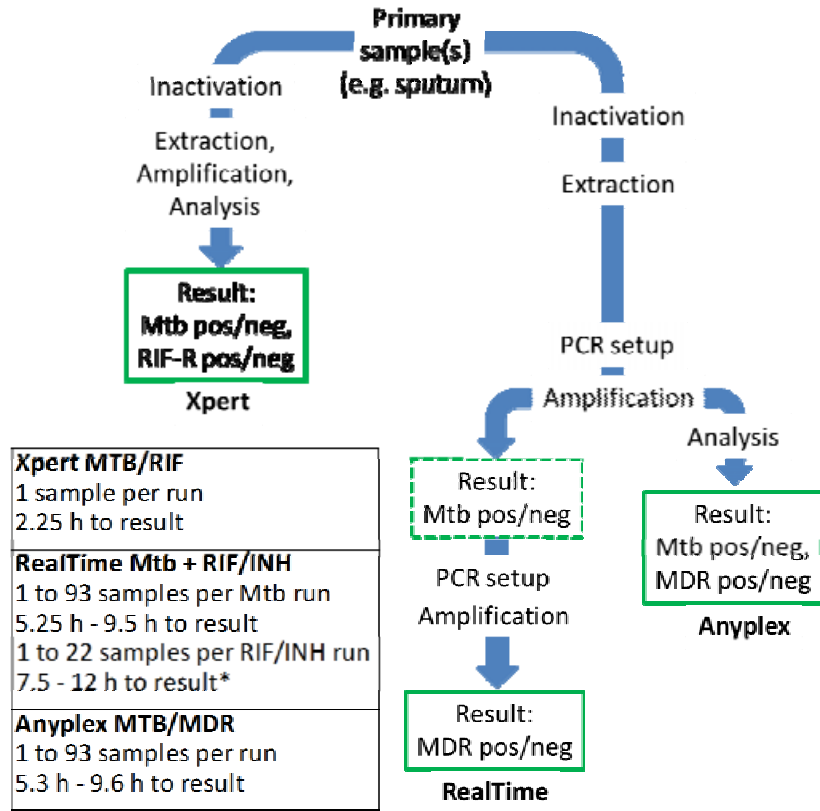


Figure 1. Diagrammatic workflows of reference PCR assay Xpert MTB/RIF and evaluated high-throughput PCR assays RealTime MTB + RealTime MTB RIF/INH and Anyplex™ II MTB/MDR.

*Final result of with Mtb detection and resistance data.