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Detection of *Mycobacterium tuberculosis* in paucibacillary sputum: performances of the Xpert MTB/RIF ultra compared to the Xpert MTB/RIF, and IS*6110* PCR

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ABSTRACT

The Xpert MTB/RIF Ultra assay has recently been launched to improve the detection of smear negative disease. This retrospective study compares the sensitivity of Xpert MTB/RIF Ultra with that of Xpert MTB/RIF tests and IS*6110* real-time PCR in sputum. Diagnostic performance of three molecular tests was evaluated using 48 culture-positive clinical respiratory specimens diluted to obtain paucibacillary sputum specimens. Xpert MTB/RIF Ultra had the highest sensitivity of 100% compared to 42% ($P < 0.001$) for Xpert MTB/RIF and 64.5% ($P = 0.02$) for IS*6110*-PCR. All "very low" or "low" positive specimens using Xpert MTB/RIF Ultra were tested positive using IS*6110*-PCR, but 35.4% were found negative using Xpert MTB/RIF. Xpert MTB/RIF Ultra is more sensitive than the two other tests for sputum with a low bacterial load. Adding detection of IS*6110* and IS*1081* to *rpoB*, is a key evolution of the assay and improves the detection of *Mycobacterium tuberculosis*' DNA in paucibacillary sputum.

1. Introduction

Tuberculosis (TB) remains one of the deadliest infectious diseases worldwide. Improvements in diagnosis are needed to end the global TB epidemic. World Health Organization (WHO) has set up a target of 80% for case detection, but this rate was estimated at 61% (10.4 million people) in 2017 (WHO, 2017). One of the major reasons for this gap is the lack of accessible and highly sensitive diagnostic assays (Uplekar et al., 2015) to detect *Mycobacterium tuberculosis* (MTB). Mycobacterial culture is still considered as the gold standard test for TB diagnosis. Although culture is of high analytical sensitivity, it requires expensive laboratory infrastructures while the obtainment of culture results often suffers from significant delay. Despite poor sensitivity, conventional smear microscopy remains the most widely used primary laboratory method for TB diagnosis in most but not only resource-limited countries (Espy et al., 2006). Over the years, significant improvement of molecular technologies has been achieved with the development of molecular assays dedicated to TB diagnosis. In 2010 and 2016 respectively, WHO recommended an automated cartridge-based PCR system Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) to enhance the detection of both TB cases and rifampicin resistance (Boehme et al., 2010; Steingart et al., 2014), and a method based on isothermal amplification (WHO, 2016).

Nucleic acid amplification tests (NAATs) provide highly sensitive detection of smear positive specimens. Sensitivities close to 100% were reported using the Xpert MTB/RIF (Xpert), and over 96.8% using LAMP (Loop – mediated – isothermal – amplification) assay (Habeenzu et al., 2017). Diagnosis of smear-negative pulmonary TB remains, however, a trickier challenge, and needs to be evaluated using paucibacillary samples (< 10,000 Acid Fast Bacilli). Sensitivities of 67% and 71% have been reported using Xpert when testing two specimens (Helb et al., 2010) and 49% using the LAMP assay (Mitarai et al., 2011). The sensitivity in smear-negative TB cases can be improved by up to 5% while testing three sputum specimens per patient (Mase et al., 2007). Hence, suboptimal performances in paucibacillary specimens remain a limitation of NAATs and a clinical issue because of the poor access to biosafety level 3 facilities (BSL-3). Cepheid has recently launched a new molecular assay, Xpert MTB/RIF Ultra (Xpert Ultra) with the objective to improve the detection rate of biological specimens with low bacilli concentration. Compared to the first version of Xpert, the sensitivity of Xpert Ultra is improved essentially due to the incorporation of two different multicopy amplification targets (IS*6110* and IS*1081*) (Chakravorty et al., 2017). The

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IS*6110* element is the most abundant multi-copy insertion sequence of the genome of *MTB* (Thierry et al., 1990), with a mean of 10 copies per bacilli (ranging from 1 to 25 copies per bacilli depending on the strain). IS*6110* target is specific to the *Mycobacterium tuberculosis* complex (MTBC) and is used in several commercial and laboratory PCR (Armand et al., 2011; El Khéchine et al., 2009). Studies have reported higher performances for TB detection using PCR assays based on IS*6110* detection when compared to the Xpert MTB/RIF assay based on *rpoB* amplification (Armand et al., 2011; Kim et al., 2015).

Here, we tested paucibacillary specimens for *MTB* DNA using Xpert Ultra assay and compared the results with those from the Xpert assay and IS*6110* PCR to evaluate the additional sensitivity gained by the Xpert Ultra assay.

2. Materials and methods

2.1. Specimen collection, storage, and preparation

Seventy-eight sputa samples (48 TB culture-positive and 30 control TB negative-culture) were collected from patients hospitalized at Montpellier University Hospital, included in the study. The study was approved by the Méditerranée III Ethical committee and Montpellier University Hospital (NCT number: NCT02898623). Digested and decontaminated sputum with MycoPrep® kit (Becton Dickinson, Baltimore, United States) were stained using auramine-O stain for acid-fast bacilli (AFB) microscopic examination, were inoculated for 2 to 8 weeks in both BACTEC Mycobacterial Growth Incubation Tube (MGIT) 960 (Becton Dickson Microbiology Systems, Sparks, United Sates) and Löwenstein-Jensen medium (Heipha Diagnostika Biotest, Germany). MTB culture was used as gold standard to compare the sensitivities of the three NAATs. Isolates were identified by the MPT64 antigen detection (Capilla TB, Tauns laboratory) and molecular tests (GenoType MTBC or CM, Hain Lifescience, Germany). Drug susceptibility testing (DST) for first line drugs was applied to all 48 isolates in MGIT 960 system.

The sample preparation is outlined in the flow diagram on the Fig. 1. Frozen decontaminated sputum samples were thawed and mixed vigorously by vortexing in 2-ml tube containing beads to disrupt bacterial clumps. Culture positive sputa were tested by IS*6110* PCR for quantification of MTB DNA level using H37Rv commercial standard (Advanced Biotechnologies Inc., Eldersburg, MD, United States). Excepted for six samples with MTB DNA below 160 IS*6110* copies/ml, serial dilutions in sterile phosphate buffer saline were prepared to obtain paucibacillary specimens, below, around and above the LOD (1712 IS*6110* copies/ml of equivalent of diluted sputum, 95% CI 1328–2080). Seventeen samples had MTB DNA below the LOD (< 1600 IS*6110* copies/ml, group A), 20 around the LOD (1712 IS*6110* copies/ml, group B), and 11 around 10X-LOD (16,000 IS*6110* copies/ml, group C). A volume of 500 µl was used for the Xpert assays and 300 µl for the IS*6110* PCR, respectively.

2.2. IS6110 PCR assay

The lysis of bacilli and the extraction of DNA in clinical samples were processed using Chelex® method adapted from Heginbothom et al. (Heginbothom et al., 2003). Briefly, 300 µl of samples were incubated with 900 µl of Chelex® 100 resin (Bio-Rad) suspension, in heater block at 100 °C for 15 minutes and placed in an ultrasonic bath for 15 min. After centrifugation, 400 µl of supernatant were recovered. Five µl of supernatant (1.25%) were added to 15 µl of master mix (Omunis, France, Clapiers) for the PCR assay. The primers and probe design were based on IS*6110* sequence homology in species of MTBC (Gene ID 12500611), as previously described (Kolia-Diafouka et al., 2018): IS*6110* forward 5′-CATGTCCGGAGACTCCAGTT-3′, IS*6110* reverse 5′-GGTACCTCCTCGATGAACCA-3′ and IS*6110* probe 5′-AAAGGATGGGGTCATGTCAG-3′.

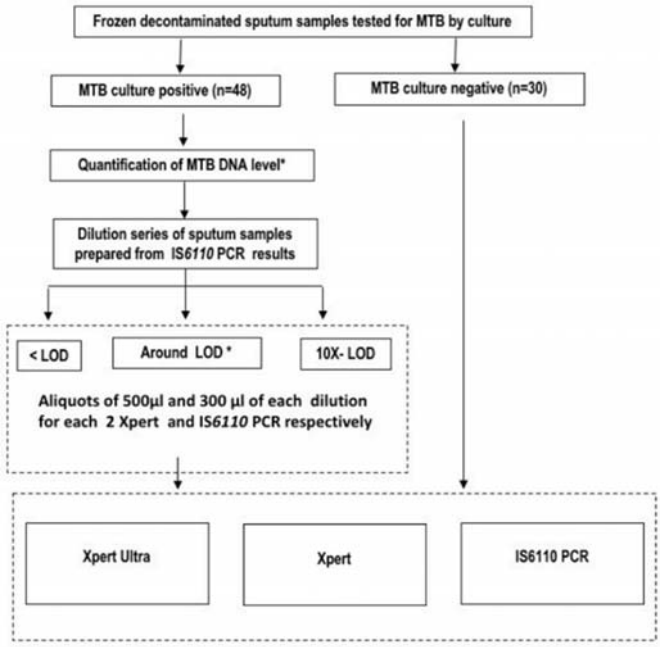


Fig. 1. Preparation of Mycobacterial dilutions for Xpert Ultra, Xpert and IS*6110* PCR assays. After IS*6110* PCR quantitation, sputum samples were serial diluted in 3 groups according to the limit of detection (LOD). From this dilution, 48 aliquots from each dilution for each group were prepared. Three aliquots of 500 µl and 300 µl for each 2 Xpert and IS*6110* PCR assays, respectively were compared. *LOD = 1712 IS*6110* copies/ml (95% CI: 1328–2080).

The PCR was performed using a LightCycler 480 Real-Time PCR System (Roche Applied Science, Germany), in 20 µl final reaction volume. The following thermal profile was used: polymerase activation at 95 °C for 15 min followed by 50 amplification cycles of 15 sec at 95 °C and 1 min at 60 °C each. The assay includes an internal control based on a heterologous amplification system to ensure that nucleic acids were successfully extracted and amplified. External standard MTB H37Rv (Advanced Biotechnologies Inc., Eldersburg, MD, USA) was used for calibration curve. Results were expressed as IS*6110* copies/ ml of equivalent of diluted sputum. The lower limit of detection of the IS*6110* PCR assay was estimated to 1712 IS*6110* copies/ml (95% CI: 1328–2080), using Probit analysis with 95% rate of detection (Kolia-Diafouka et al., 2018). To confirm the species specificity of the qPCR, genomic DNA was tested for the following non-tuberculosis mycobacteria: *Mycobacterium abscessus, Mycobacterium smegmatis, Mycobacterium marinum, Mycobacterium fortitium, Mycobacterium gordonae and Mycobacterium chelonae* (data not shown).

2.3. Xpert MTB/RIF and Xpert MTB/RIF ultra assays

Xpert Ultra and Xpert were performed according to the manufacturer's instructions. Briefly, 500 µl for each Xpert test, that is 1 ml of diluted samples were mixed with 1:3 ratio using sample reagent of the kit for 15 minutes at room temperature and transferred into 2 single-use disposable cartridge of Xpert and Xpert Ultra. Comparative characteristics of the two assays are presented in Table 1. The Xpert assays provide semi quantitative results, — "high", "medium", "low", and "very low" – based on the cycle threshold (CT) of the first positive *rpoB* probe. In addition, the Xpert Ultra assays include a "trace" category for samples tested positive for IS*6110* and/or IS*1081* detection in the absence of a signal for a least three out of five *rpoB* probes. The "trace" category was designed to identify samples with the lowest number of *MTB* targets.

Table 1
Comparison of operational characteristics of the three real-time PCR assays.

	IS6110-PCR (Kolia-Diafouka et al., 2018)	Xpert MTB/RIF (Dorman et al., 2018)	Xpert MTB/RIF Ultra (Bisognin et al., 2018)
Principle	Real-time PCR	Hemi-nested RT-PCR	Nested RT-PCR, HRMT
Target (s)	IS*6110*	*rpoB*	*rpoB*, IS*6110*, IS*1081*
Sample format	Microplate, 96 wells[b]	Individual cartridge[c]	Individual cartridge[c]
Volume/PCR	20 μ	25 μ	50 μ
End result	MTBC	MTBC, RIF	MTBC, RIF
Limit of detection	107 copies H37Rv/ml	131 CFU/ml	11.8 CFU/ml
Level of complexity	High	Low	Low
Turn-around-time[a]	2 h 35	2 h	1 h15 to 1 h30
Result of interpretation	Automated	Automated	Automated
Laboratory infrastructure	Open system requiring three separate work areas	Closed system	Closed system

HRMT= High resolution melt technology; CFU = colony forming unit.
[a] Including DNA extraction (manual for IS6110 real-time PCR).
[b] Depends on the microplate used (96, 364 wells).
[c] Depends on which module you have (e.g. 2,4, 16, or 48).

2.4. Statistical analysis

All the results were tabulated according to the TB culture status. Sensitivity was calculated as proportion of positives over the true positives. Statistical comparison for categorical variables was made using chi-square and Mc Nemar tests. A *P* value of ≤0.05 was considered significant.

3. Results

3.1. Sample characteristics

Among the MTB culture positive sputum, 42 out of 48 samples were sputum smear positive. MTB DNA concentrations ranged from 10^3 to 10^7 MTB copies/ml using the IS*6110* assay (mean: 3.3×10^5, 95% CI: 2×10^3 to 5×10^7).

Fig. 2 demonstrates the variation of IS*6110* PCR values according to smear grade and correlation between IS*6110*-PCR values with time to positivity culture (TTP). Smear negative samples had a median $\log_{10}$ MTB DNA copies of 2.093 (IQR 1.732–2.562) compared to 5.143 (4.976–5.502) for smear positive samples. Levels of MTB DNA copies values strongly correlated with smear with a Spearman's ρ of 0.91 (0.86–0.96) (Fig. 2A).

When data for sputum specimens were pooled (Fig. 2B), TTPs and MTB DNA copies/ml values were correlated (Spearman coefficient of 0.7395, $P < 0.0001$). The median TTP for the 48 sputa was 9 days (IQR 8–12 days), with a range of 1–35 days: 83.3% (40/48) were detected within 14 days, 14.6% (7/48) within 21 days, and 2.1% (1/48) within 28 days.

Serial dilutions were performed to obtain paucibacillary samples with a target concentration ranging from 340 to 5.8×10^4 IS*6110* copies/ml (mean: 1.3×10^4, 95% CI: 9760 to 1.3×10^5).

3.2. MTB DNA detection in paucibacillary specimens using the Xpert MTB/RIF ultra, Xpert MTB/RIF and IS6110 assay

All the specimens from the tuberculosis group were positive using Xpert Ultra test (sensitivity 100%, 95% CI: 98–100%). By comparison, 31 out of 48 samples were tested positive using the IS*6110* assay (sensitivity 64.5%, 95% CI: 57–71%) and 20 out of 48 samples were tested using the Xpert test (sensitivity 42.0%, 95% CI: 35–59%) (Fig. 3). Using Xpert, the detection rate in specimens with MTB DNA levels $<1.6 \times 10^3$ IS*6110* copies/ml and C (10 X-LoD: $>1.6 \times 10^4$ IS*6110* copies/ml) were 45% and 100%, respectively. Xpert Ultra test sensitivity was significantly better compared to the IS*6110* assay ($P < 0.001$) and Xpert assay ($P < 0.001$). The sensitivity of the IS*6110* assay was higher to the Xpert test ($P = 0.02$). All the samples of the control group were tested negative for MTB DNA with the three assays (specificity 100%, 95% CI: 93–100%) (Fig. 3).

3.3. Comparison of MTB DNA levels using the Xpert MTB/RIF ultra, Xpert MTB/RIF and IS6110 assay

Semi-quantitative assessment of MTB DNA using Xpert assays and results of MTB DNA quantitation using the IS*6110* PCR were compared (Fig. 4). Among the samples tested positive with Xpert Ultra, 17 (35.4%) had a result considered as a "trace". All had an estimated MTB DNA value below 1.6×10^3 IS*6110* copies/ml. None of these 17 samples were tested positive using IS*6110* PCR or Xpert (Fig. 4). All specimens detected as "very low" or "low" positive using Xpert Ultra were also tested positive using IS*6110* PCR, but 11 out of 31 (35.4%) were not detected using Xpert. Eight out of 11 specimens (72%) tested low positive using Xpert Ultra were also low positive with Xpert and three were considered as very low positive.

3.4. Testing of rifampicin resistance

Rifampicin resistance by Xpert Ultra testing was not detected in 28 cases (positive with "very low" or "low" quantification), and 17 cases were indeterminate (positive with "trace" quantification). Three isolates (6.26%) were detected Rifampicin resistance. Hence, molecular Rifampicin susceptibility was determined for 31 (64.5%) and 20 (42%) specimens, respectively for Xpert Ultra and Xpert assays. No discordance occurred with resistance testing by Xpert Ultra, Xpert and culture.

4. Discussion

Molecular diagnosis of TB in specimens with low MTB DNA level remains a challenge because of the suboptimal sensibility of the NAAT on smear negative samples (Vittor et al., 2014). Our results confirm previous findings that, adding repeated insertion sequence detection in the Xpert Ultra multiplex PCR assay, increases significantly the analytical sensitivity of the GeneXpert system. This study provides also comparative data about detection of low MTB DNA concentrations and biological significance of "trace" detection using the Xpert Ultra assay. The clinical performances of the Xpert assay and IS*6110* PCR have been evaluated on sputum smear negative samples in different independent studies (Laraque et al., 2009; Pai et al., 2003; Vittor et al., 2014) and thus can be considered as reference assays to assess the sensitivity gained by the Xpert Ultra assay. The better sensitivity of the Xpert Ultra assay is attributable to the detection of IS*6110* and IS*1081* MTB repeated sequences in addition to *rpoB* target, which justified also to assess *rpoB* and IS*6110* detection separately for comparison.

In our study, the multiplex format of the PCR in the Xpert Ultra assay based on amplification of two repeated insertion sequences and a nested PCR method appeared noticeably superior to the PCR based on IS*6110* amplification and *rpoB* alone since all TB positive samples were detected using the Xpert Ultra assay whereas the IS*6110* PCR and

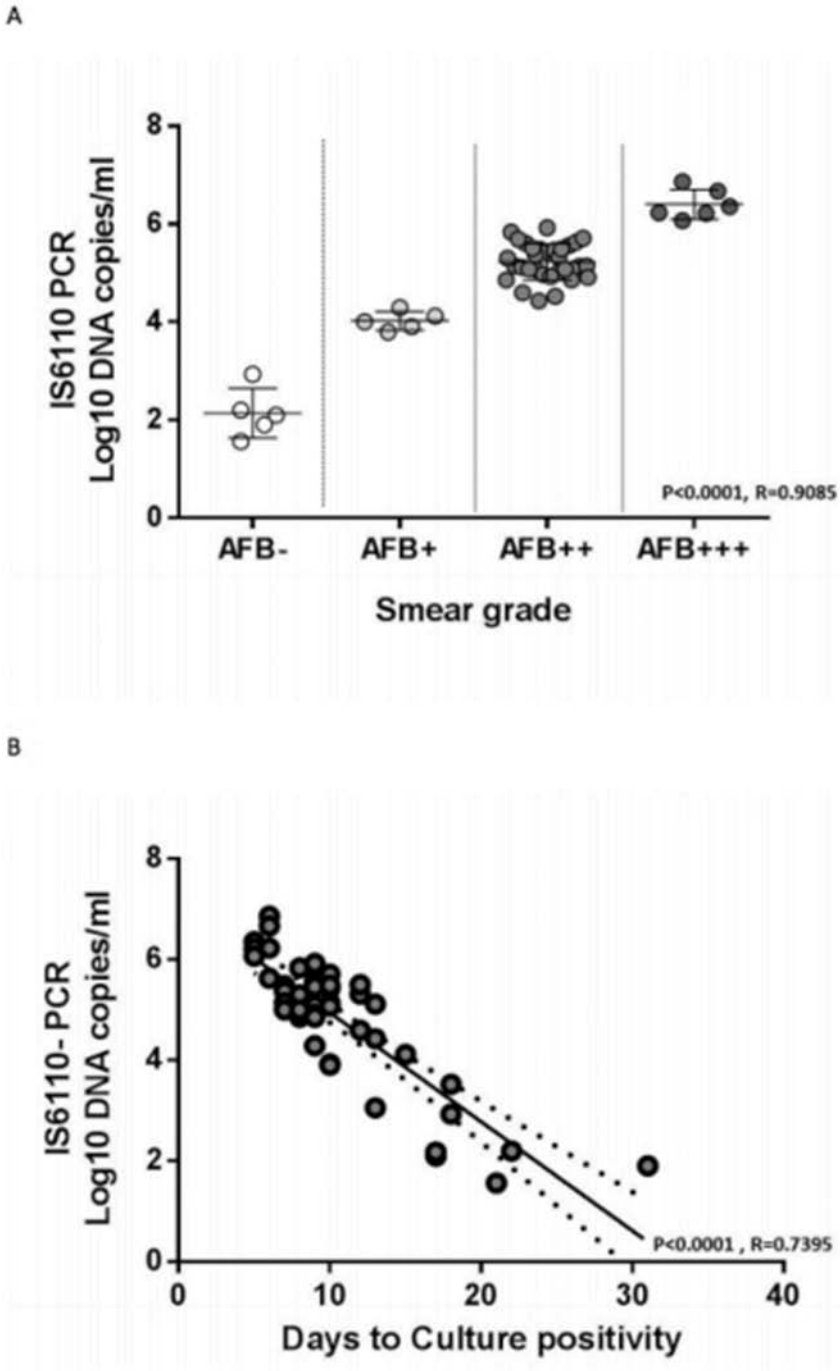
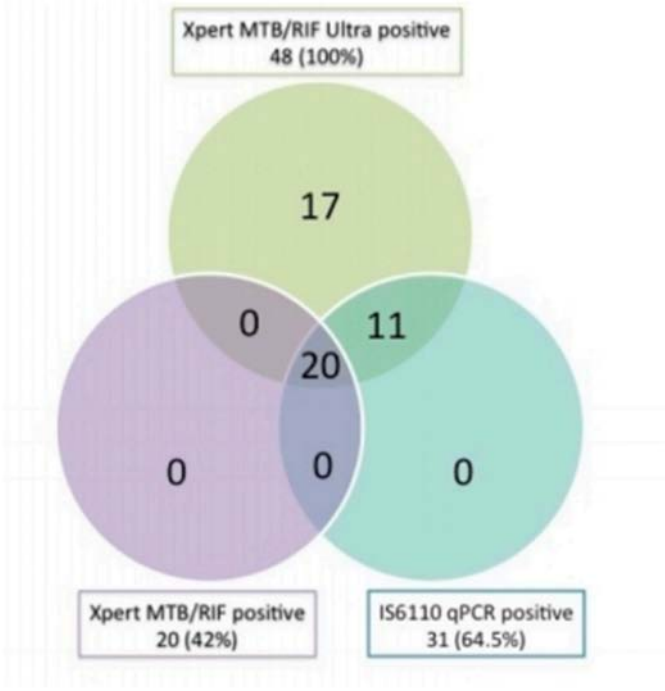


Fig. 2. IS*6110* PCR according to smear grade and time to culture positivity (TTP). (A) Specimens were tested by IS*6110* PCR and compared to results of smear microscopy and TTP into negative to 3- plus (1+, 2+, 3+) (Ait-Khaled and Enarson, 2003), and classified in four group: group 1, white circles AFB negative; group 2, light gray, from 10 to 99 AFB per 100 fields; medium gray circles, from 1 to 10 AFB per field; dark gray circles, more than 10 AFB >9 per field. Bars indicate the median DNA load copy numbers in each group. (B) Correlation between IS*6110* PCR values and TTP in days, in 48 TB culture-positive sputa.

Xpert assay failed to amplify MTB DNA in various samples. Chakravorty et al. have recently reported an overall sensitivity of 78.9% using Xpert Ultra versus 66.1% using the Xpert assay, in smear-negative/culture positive sputum (Chakravorty et al., 2017). Likewise, in another study, a sensitivity of 63% was observed in smear-negative/culture positive sputum using the Xpert Ultra versus 46% using the Xpert assay (Dorman et al., 2018).

MTB DNA was detected as "trace" in all the samples containing concentration below 1.6×10^3 IS*6110* copies/ml. Results responded in the "trace" category is IS*6110*/IS*1081* positive but *rpoB* negative, allowing to keep undetermined the resistance for rifampicin. Caution is required for interpretation of this results associated with detection of low concentration of MTB DNA. In our study conducted in a low prevalence setting (4741 active TB were notified in France in 2015, which corresponds to an incidence of 7.1 per 100,000 habitants), the result "trace" has a high specificity for TB. History of TB infection should be considered for interpretation of Xpert Ultra trace readout (Bahr et al., 2018), since subjects treated for TB may be tested "trace" positive. Although the specificity of



Positive specimens/Category	Group A (n=17)	Group B (n=11)	Group C (n=20)
Xpert MTB/RIF Ultra	17	11	20
Xpert MTB/RIF	0	0	20
IS*6110* PCR	0	11	20

Fig. 3. Venn diagram of overlap in MTB detection using Xpert Ultra, Xpert and IS*6110* PCR assays in sputum samples. The Venn diagram displays 48 specimens with IS*6110* DNA copies concentration ranged from 340 to 5.8×10^4 IS*6110* copies/ml. All samples positive by Xpert Ultra only had IS*6110* DNA copies concentration below 1600 IS*6110* copies/ml.

the assay has been questioned in previous studies (Dorman et al., 2018; Mitarai et al., 2011), we observed a full specificity in this study based on smear negative/culture negative sputum collected in a low TB incidence country. Our data confirm that results reported as "trace" and considered as positive for TB, contribute significantly to the performances of the assay in smear-negative/culture-positive sputum (Mitarai et al., 2011; Piersimoni and Scarparo, 2003).

All TB samples containing "trace" using the Xpert Ultra assay were undetected for MTB using the IS*6110* PCR, whereas all the samples with "very low" or "low" results using the Xpert Ultra assay were tested positive using the IS*6110* PCR. These results underline that, when *rpoB* target is detected using Xpert Ultra assay, the IS*6110* PCR was also able to detect MTB DNA. Another important message from this study relies on the better sensitivity observed when using the IS*6110* PCR compared to Xpert. These results are supported by previous findings from Armand et al., reporting a sensitivity of 48% versus 63% in smear-negative/culture positive specimens using the Xpert versus an IS*6110* PCR assay, respectively (Armand et al., 2011). This might be explained by a clear benefit of using multicopy target IS*6110* instead of single gene copy. IS*6110* multicopy target is present in 10 to 16 copies in most genome of MTBC (Flores et al., 2005). Previous studies showed the impact using multicopy elements to increase sensitivity of the real-time PCR (Hofmann et al., 2015; Sanosyan et al., 2017). Contrasting with these findings, Miller et al. observed a higher sensitivity for the Xpert assay than for IS*6110* PCR (Miller et al., 2011). Differences in lysis and/or extraction methods may explain this discrepancy. Indeed, laboratory PCR dedicated to MTB DNA detection are highly dependent on the DNA extraction methods used and elimination of PCR inhibitors from sputum (Nakatani et al., 2004). Here, boiling and sonication increase release of mycobacteria DNA and purification with Chelex® reduces PCR inhibitor activity. The Chelex® resin works by chelating magnesium ions in the cell wall, helps in effective removal of inhibitors and keeps the release DNA intact (Walsh et al., 1991).

The TB assays on the GeneXpert system are fully automated and random access molecular tests that integrate the different steps of molecular tests – bacterial lysis, DNA extraction and amplification – in an individual cartridge. The only manual step is the liquefaction and inactivation of the specimen with the sample treatment reagent. Consequently, the test is easy to perform and the turn-around-time is shortened: for Xpert Ultra 65 minutes if MTB is detected or 77 minutes if the result is negative. The IS*6110* PCR requires laboratory facilities but present also some interesting features. The lysis-extraction of MTB using the Chelex®, boiling and sonication method is performed on a single tube, thus limiting the risk of contamination. This simple and inexpensive method can be applied simultaneously on a large number of samples. That IS*6110* PCR

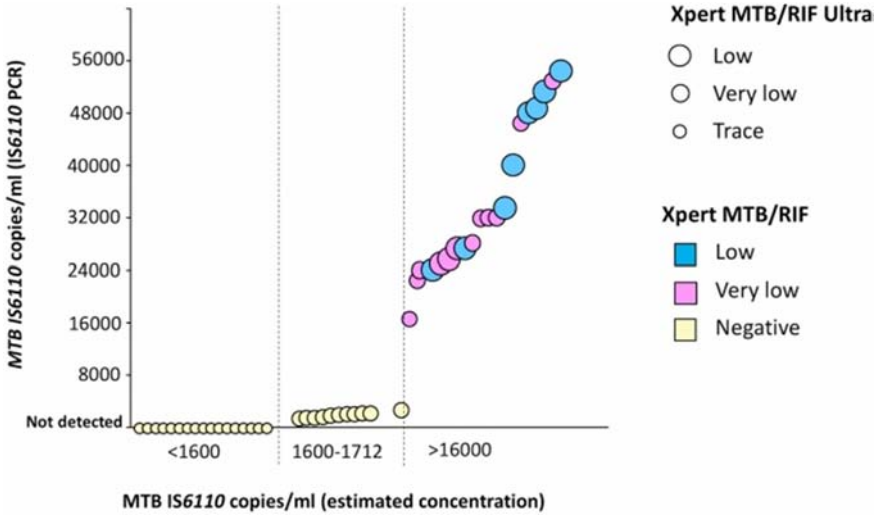


Fig. 4. Results of three assays compared to estimated IS*6110* copies/ml concentration. Sputum were diluted to obtain specimens with low MTB DNA. Samples were stratified in three groups indicated on X axis: <1600 IS*6110* copies/ml, around the LOD (1600–1712 IS*6110* copies/ml, and >16,000 IS*6110* copies/ml. Values on the Y axis indicate MTB DNA levels quantified using IS*6110* PCR. The colors indicate TB DNA levels using Xpert assay: blue, low positive; pink, very low positive and yellow, nt detected. The circle sizes indicate MTB DNA levels using Xpert Ultra assay: large, low positive; medium, very low and small, trace.

performed on the Lightcycler apparatus uses 96- or 364-well microplates makes it possible to test a large number of samples in a single run in only 90 minutes. TB testing on open polyvalent PCR platforms using IS*6110* detection should thus be considered for screening and mass diagnosis in central laboratories.

Adding detection of insertion sequenced to *rpoB* in the latest version of the Xpert assay is a key evolution of the assay and improves MTB DNA detection in paucibacillary sputum. IS*6110* amplification combined with efficient TB DNA extraction methods substantially contributes to improve the detection of MTB DNA in paucibacillary specimens.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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