Low Cycle Threshold Value in Xpert MTB/RIF Assay May Herald False Detection of Tuberculosis and Rifampicin Resistance: A Study of Two Cases

Jean Claude S. Ngabonziza,1,2,3 Tom Decroo,4,5 Rodrigue Maniliho,6 Yves M. Habimana,7 Armand Van Deun,8 and Bouke C. de Jong2

1National Reference Laboratory Division, Department of Biomedical Services, Rwanda Biomedical Centre, Kigali, Rwanda, 2Mycobacteriology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium, 3Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium, 4Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium, 5Research Foundation Flanders, Brussels, Belgium, 6Tuberculosis Laboratory, Kibagabaga Hospital, Kigali, Rwanda, 7Tuberculosis and Other Respiratory Diseases Division, Institute of HIV/AIDS Disease Prevention and Control, Rwanda Biomedical Center, Kigali, Rwanda, 8Independent TB Consultant, Leuven, Belgium

We report 2 cases for whom Xpert MTB/RIF falsely signaled rifampicin-resistant tuberculosis, based on unusually low cycle threshold and 3 of 5 probes missing. Other mycobacterial tests were negative. Further optimization of the Xpert MTB/RIF algorithm is warranted.

Keywords: active case finding; false detection of TB; rifampicin resistance; Xpert MTB/RIF assay.

CASE REPORT

The Xpert MTB/RIF G4 assay ([Cepheid] hereafter referred to as “Xpert”) has greatly improved diagnosis of tuberculosis (TB) and its resistance to rifampicin [1, 2]. Many TB control programs rely on its results for rapid diagnosis of rifampicin-resistant (RR) and initiation of appropriate treatment [3].

Xpert amplifies an 81-base pair region of the rpoB gene [4], using 5 probes (labeled A–E). At least 2 of 5 probes need to become positive within a certain cycle threshold (Ct) window to signal “MTB detected.” In case of complete probe dropout(s) (Ct zero) or delayed Ct (ΔCt max is >4.0) of 1 to 3 probes, Xpert also signals RR [1, 5].

Despite its excellent specificity for the detection of TB and RR [6], false signalization for the presence of TB has been reported. One study revealed that a higher bacterial load (10⁶ CFU/mL) led to false TB detection for 5 (Mycobacterium abscessus, Mycobacterium marinum, Mycobacterium smegmatis, Mycobacterium phlei, and Mycobacterium aurum) of 12 non-TB mycobacteria (NTM) tested [7]. Another study did not confirm this cross-reactivity [8]. Moreover, because the assay detects deoxyribonucleic acid (DNA), and does not distinguish between dead and active bacilli, patients who were previously successfully treated for TB may continue to test positive, occasionally years later [9–11]. This limitation applies to all molecular methods, which target TB DNA that may persist in the patients’ lungs after cure [10, 11].

In this study, we report a full investigation of Xpert falsely signaling the presence of TB with RR in one patient with no prior history of TB and in a second who was previously treated for TB but was disease-free for more than 4 years after cure. Both were spared unnecessary treatment.

In Rwanda, the current guideline states that TB symptoms screening and/or chest x-ray should be positive to justify Xpert testing.

Case 1

During active case finding activities among people living with human immunodeficiency virus (HV), a 38-year-old woman, infected with HV with no history of TB, without TB symptoms, and with a negative chest x-ray, was erroneously deemed eligible for Xpert testing, which was done the same day.

Xpert yielded a high load of Mycobacterium tuberculosis and also showed RR. The patient was admitted to start multidrug-resistant (MDR)-TB treatment at the Kibagabaga hospital. At admission, a second Xpert test was negative for TB. This conflicting result prompted further investigation. Reanalysis of the initial Xpert result showed that TB detection was based on hybridization of only 2 of 5 probes with an unusually low Ct (Ct = 9) and jagged rather than smooth amplification curves (Figure 1A). Rifampicin resistance was reported, because 3 of 5 probes (A, B, and D) were missing (Figure 1A).

The result was submitted for in-depth analysis to Cepheid, with rapid feedback, mentioning that the curves were completely abnormal, therefore requiring repeat testing, but without a clear answer on the potential cause.

A third Xpert was also negative for TB. Both samples used for repeat Xpert testing were tested a posteriori on smear microscopy and were negative. Moreover, there was no growth from these 2 samples inoculated in mycobacterial growth indicator tube and on Löwenstein Jensen media at the National Reference Laboratory (NRL). The initial sample was not available for additional testing.

Because 2 repeat Xpert tests were negative, and because the patient did not present clinical signs of TB, the patient was not...
started on MDR-TB treatment and was discharged the next day. She remains well to date without receipt of TB-specific treatment.

**Case 2**

The second case involves a 34-year-old male, infected with HIV, on antiretroviral therapy, and an inmate for 7 months. The patient was previously treated for TB but was disease-free for more than 4 years after cure. He was deemed eligible for Xpert testing due to sizable weight loss (from 75 to 62 kg) accompanied by a minor cough. As in Case 1, Xpert showed a high bacterial load for *M. tuberculosis* and showed RR. The patient was admitted to the MDR-TB treatment clinic at Kabutare hospital. The initial Xpert result was sent to the NRL for further interpretation. Tuberculosis detection was also based on the hybridization of...
only 2 of 5 probes, with an unusually low Ct (Ct = 9) and straight lines rather than amplification curves (Figure 1B). Xpert reported RR, because 3 of 5 probes (A, B, and D) were missing (Figure 1B). Two Xpert tests plus smear microscopy performed on separate samples at the NRL were all negative for TB. Moreover, this patient was not started on MDR-TB treatment. The patient remains well to date without receipt of TB-specific treatment.

Patient Consent Statement
Written consent was obtained from all patients included in this study. The study design has been approved by the Rwanda National Ethical Committee (Institutional Review Board 00001497 of IORG0001100; Reference Number 0069/RNEC/2017), as part of the DIAgnostics for Multidrug Resistant Tuberculosis in Africa trial (https://clinicaltrials.gov/ct2/show/NCT03303963).

RESULTS
Based on the findings described in these cases, we reviewed 175 nationwide Xpert results from 2017 to 2019 for unusually low Ct values (Ct < 10) reported as RR on Xpert, and we uncovered...
3, of which 2 had dropout or delay of at least 2 probes (Figure 1C and D). These 2 patients did have active TB disease, confirmed by culture, but rpoB gene sequencing—used as reference—showed wild type, thus revealing false RR. Unfortunately, these patients were inappropriately treated with the MDR-TB regimen [5]. The remaining patient with Ct <10 had dropout of probe E only and was confirmed as RR-TB.

DISCUSSION

To the best of our knowledge, the first case is the first report of Xpert falsely signaling the presence of TB and RR in a patient with no prior history of TB. Fortunately, discordant Xpert results triggered further investigations that identified this result to be false, and the patients were not unnecessarily exposed to 9 or more months of potentially toxic MDR-TB treatment. However, the root cause and frequency of these false results remains unknown. For Case 1, the polymerase chain reaction (PCR) curves of the initial Xpert test did not show clear amplification for any of the probes and showed an unusually low Ct for the sample processing control (Figure 1A). For Case 2, instead of standard PCR amplification curves, 2 straight lines corresponding to probes C and E were displayed, whereas the other 3 probes were missing (Figure 1B). In an ideal setting, these results would have been reported as invalid. Manual interpretation of PCR curves or Ct values is rarely done at Xpert testing sites, due to time constraints or insufficient knowledge on quantitative PCR-curve interpretation. Xpert information for users does not indicate any substance interference that would trigger a false-positive signal [12, 13]. Moreover, poor quality sample would rather lead to a negative or invalid result [12, 13].

The specificity of Xpert for the detection of TB is not 100%, but the true specificity remains unknown because most discrepancies with the reference standard—culture isolation—were explained by the imperfect performance of culture [1, 6]. In 2017, Cepheid released the upgraded Xpert MTB/RIF Ultra (Xpert Ultra) with improved sensitivity for the detection of TB and a higher specificity for RR detection [14, 15], which awaits confirmation after wider implementation in different settings [16]. Whether Xpert Ultra will also suffer from the same unknown mechanism and falsely signal the presence of TB needs to be monitored. Xpert Ultra detects RR based on a shift in melting curves. A false RR on Xpert Ultra due to a different reason (ie, distorted melt curves interpreted as double peaks) has already been reported [17].

Active case finding relying on molecular diagnostic tools, such as Xpert and Xpert Ultra, for the early diagnosis of TB as well as universal drug-susceptibility testing, is a key component of the End-TB strategy [18]. However, the positive predictive value of any test is lower when the pretest probability is low, for instance, when asymptomatic patients are referred for TB screening during active case finding.

The value of smear microscopy has been neglected in TB diagnosis. Indeed, smear microscopy had not been done on the initial sample from our patients. The low Ct values reported in the initial Xpert tests correspond to the highest grade of acid-fast bacilli on smear microscopy [19]. If sputum smear microscopy had been performed in the peripheral testing center, the negative smear results from the same samples would have raised suspicion of false Xpert results. The opposite situation, a positive smear but TB negative Xpert, is highly suggestive of NTM, another useful application of microscopy in the Xpert era. This highlights risks associated with the complete omission of smear microscopy in the TB diagnostic cascade. The use of smear microscopy for all Xpert positives, or at least those with high bacterial load, seems justified, especially when Ct values are <10 and/or the pretest probability is low (such as in active case finding or whenever TB symptoms screening criteria are loose, eg, presence of any cough in HIV-coinfected patients).

The World Health Organization recommends repeat Xpert testing when RR is detected in new TB patients not in contact with RR-TB [20]. However, we recently showed that a low bacterial load was strongly correlated with having false RR, rather than the patient’s treatment history [5]. Moreover, all RR results with dropout of more than 1 probe were false. Even though coexistence of multiple mutations/dropout of more than 1 probe can occur [21], especially the “elusive” mutations that cause failure of rifampin-based treatment yet are difficult to confirm in phenotypic drug-susceptibility testing [22, 23], dropout of multiple probes should be a flag for caution and additional testing.

CONCLUSIONS

Our findings highlight critical gaps in the Xpert algorithm. All results with Ct <10 for the lowest probe and missing at least 2 probes turned out to be false. Because Xpert will continue to be used for a while, we encourage Cepheid to review the assay’s analytical window and to optimize the algorithm towards higher specificity, even if this will predictably yield more “invalid” results. Meanwhile, we encourage healthcare workers at all diagnostic levels to suspect a false-positive result on Xpert when the Ct is low, prompting additional testing such as microscopy and repeat Xpert, in addition to in-depth analysis of the Xpert report by a skilled biologist. Increasing collective knowledge on the strengths and weaknesses of rapid molecular tools for the diagnosis of TB and its resistance will provide optimal care for future patients with presumed TB.

Acknowledgments

We acknowledge the clinical staff at Kibagabaga MDR-TB clinic and Innocent Habiyambere for patient management, Cepheid for in-depth analysis of Xpert result, and Leen Rigouts, Gabriela Torrea, and Dissou Affolabi for important inputs and critical review of this manuscript.

Disclaimer. The views and opinions of authors expressed herein do not necessarily state or reflect those of EDCTP.
Financial support. This publication is based on findings produced in the context of the DIAMA study, which is part of the EDCTP2 programme supported by the European Union (Grant Number DRIA2014–326 [DIAMA]). InnoR3TB study with financial support from ITM’s SOFI programme supported by the Flemish Government, Science & Innovation, and Belgian Directorate General for Development Cooperation (PhD Fellowship to J. C. S. N.).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

1. Boehme CC, Nabeta P, Hilleman D, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 2010; 363: 1005–15.
2. Albert H, Nathavitharana RR, Isaacs C, et al. Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better? Eur Respir J 2016; 48:516–25.
3. Ngabonziza JS, Habimana YM, Decroo T, et al. Reduction of diagnostic and treatment delays reduces rifampicin-resistant tuberculosis mortality in Rwanda. Int J Tuberc Lung Dis 2020; 24:329–39.
4. Teleni A, Imboden P, Marchesi F, et al. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet 1993; 341:647–50.
5. Ngabonziza JCS, Decroo T, Migambi P, et al. Prevalence and drivers of false-positive rifampicin resistant Xpert MTB/RIF results: a prospective observational study in Rwanda. The Lancet Microbe 2020; 1:74–83.
6. Steingart KR, Schiller I, Horne DJ, et al. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev 2014. doi: 10.1002/14651858.CD009593.pub3.
7. Pang Y, Lu J, Su B, et al. Misdiagnosis of tuberculosis associated with some species of nontuberculous mycobacteria by GeneXpert MTB/RIF assay. Infection 2017; 45:677–81.
8. Huh HJ, Song DJ, Ki CS, Lee NY. Is Cross-reactivity with nontuberculous mycobacteria a systematic problem in the Xpert MTB/RIF assay? Tuberc Respir Dis (Seoul) 2019; 82:88–9.
9. Miotto P, Bigoni S, Migliori GB, Matteelli A, Cirillo DM. Early tuberculosis treatment monitoring by Xpert® MTB/RIF. Eur Respir J 2012; 39:1269–71.
10. Theron G, Venter R, Calligaro G, et al. Xpert MTB/RIF results in patients with previous tuberculosis: can we distinguish true from false positive results? Clin Infect Dis 2016; 62:995–1001.
11. Boyle TH, Hughes J, Cox V, et al. False-positive Xpert® MTB/RIF assays in previously treated patients: need for caution in interpreting results. Int J Tuberc Lung Dis 2014; 18:876–8.
12. Cepheid. Xpert® MTB/RIF_GXMTB/RIF-10. 2019; (Information For Users, Cepheid, Sunnyvale, CA, USA). Available at: https://www.cephied.com/Package%20Insert%20Files/Xpert-MTB-RIF-ENGLISH-Package-Insert-301-1404-Rev-E.pdf. Accessed 12 June 2020.
13. Cepheid. Xpert® MTB/RIF _GXMTB/RIF-US-10. 2019; (Information For User, Cepheid, Sunnyvale, CA, USA). Available at: https://www.cephied.com/Package%20Insert%20Files/Xpert-MTB-RIF-ENGLISH-Package-Insert-301-1404-Rev-E.pdf. Accessed 12 June 2020.
14. Chakravorty S, Simmons AM, Rowneki M, et al. The new Xpert MTB/RIF ultra: improving detection of Mycobacterium tuberculosis and resistance to rifampin in an assay suitable for point-of-care testing. mBio 2017; 8:e00812–17.
15. Horne DJ, Kohli M, Zizfodya JS, et al. Xpert MTB/RIF and Xpert MTB/RIF Ultra for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev 2019; 6:CD009593.
16. Schumacher SG, Wells WA, Nicol MP, et al. Guidance for studies evaluating the accuracy of sputum-based tests to diagnose tuberculosis. J Infect Dis 2019; 220:599–107.
17. Ng KCS, Rigouts L, de Jong BC, Lynen L. False rifampicin resistance in Xpert ultra applied to lymph node aspirate: a case report. Open Forum Infect Dis 2020; 7:oofa204.
18. World Health Organization. The End TB Strategy. Available at: http://www.who.int/tb/strategy/en/. Accessed 5 October 2020.
19. Beynon F, Theron G, Respeito D, et al. Correlation of Xpert MTB/RIF with measures to assess Mycobacterium tuberculosis bacillary burden in high HIV burden areas of Southern Africa. Sci Rep 2018; 8:5201.
20. World Health Organization. GLI practical guide to TB laboratory strengthening. Available at: http://stoptb.org/wg/gli/assets/documents/GLI_practical_guide.pdf. Accessed 15 July 2019.
21. Zaw MT, Emran NA, Lin Z. Mutations inside rifampicin-resistance determining region of rpoB gene associated with rifampicin-resistance in Mycobacterium tuberculosis. J Infect Public Health 2018; 11:605–10.
22. Van Deun A, Aung KJ, Bossain A, et al. Disputed rpoB mutations can frequently cause important rifampicin resistance among new tuberculosis patients. Int J Tuberc Lung Dis 2015; 19:185–90.
23. Van Deun A, Aung KJ, Bola V, et al. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol 2013; 51:2633–40.