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BRIEF REPORT

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

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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 ($10^6$ 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 HIV 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 resistantce 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

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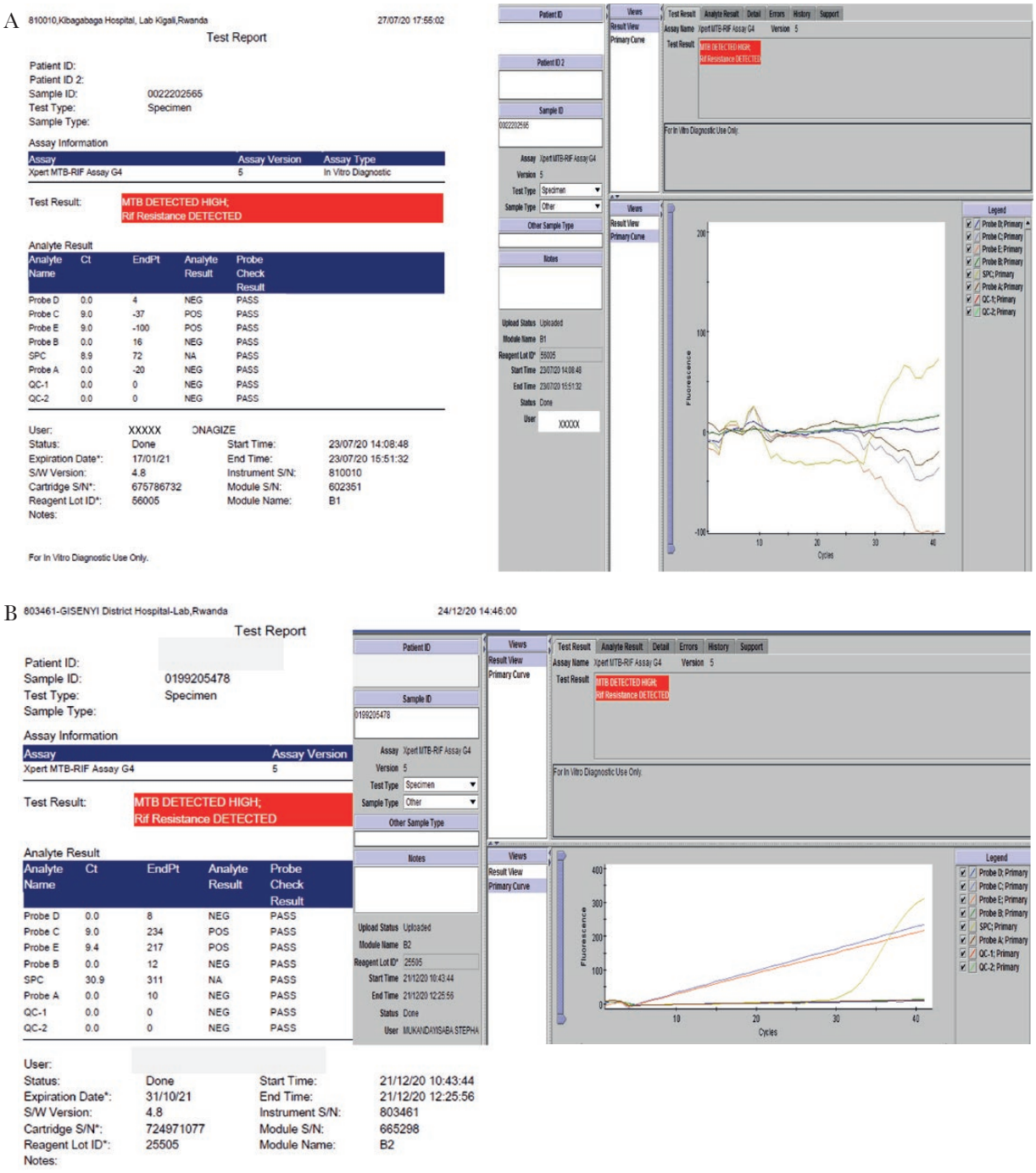


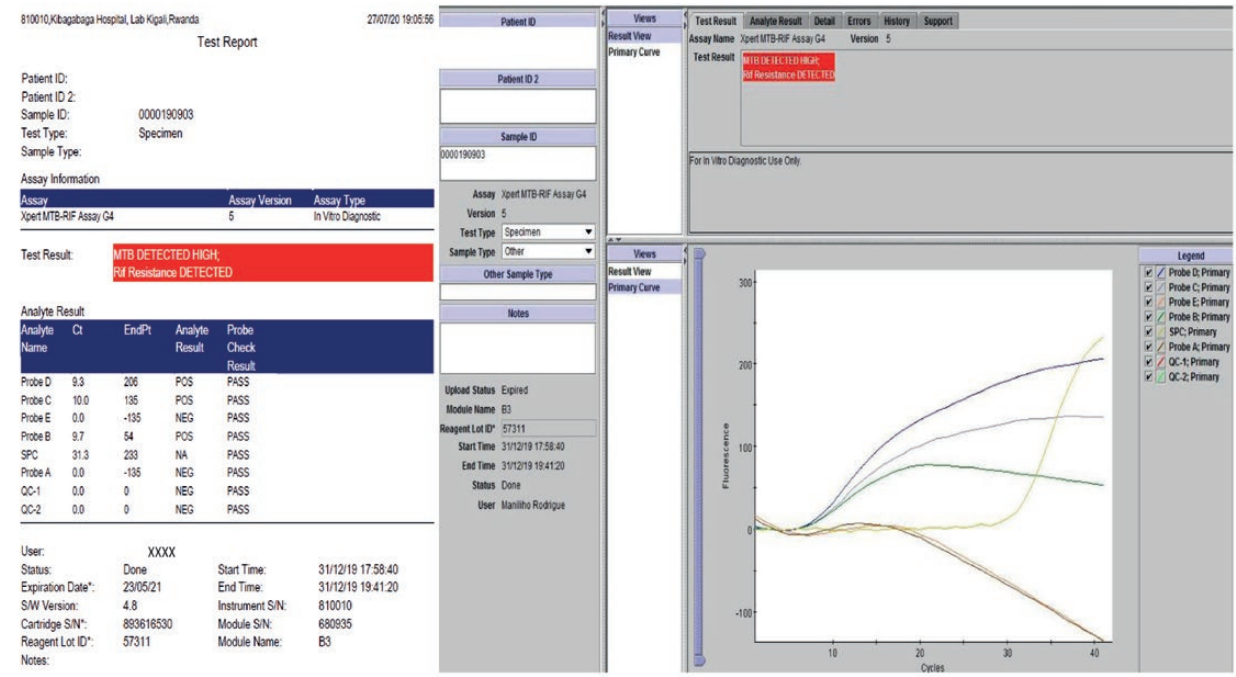

**Figure 1.** False signals for detection of tuberculosis (TB) and/or rifampicin resistance results. (A) Test report of false TB detection showing low probe cycle threshold (Ct) values and jagged amplification curves. (B) Test report of false TB detection showing low probe Ct values and straight lines instead of standard polymerase chain reaction amplification curves. (C) Test report of false rifampicin resistance showing low probe Ct values and amplification curves. (D) Test report of false rifampicin resistance showing a low Ct value for probe E.

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

C



810010,Kibagabaga Hospital, Lab Kigali,Rwanda 27/07/20 19:05:56

Test Report

Patient ID:
Patient ID 2:
Sample ID: 0000190903
Test Type: Specimen
Sample Type:

Assay Information

| Assay | Assay Version | Assay Type |
|---|---|---|
| Xpert MTB-RIF Assay G4 | 5 | In Vitro Diagnostic |

Test Result: MTB DETECTED HIGH; Rif Resistance DETECTED

Analyte Result

| Analyte Name | Ct | EndPt | Analyte Result | Probe Check Result |
|---|---|---|---|---|
| Probe D | 9.3 | 206 | POS | PASS |
| Probe C | 10.0 | 135 | POS | PASS |
| Probe E | 0.0 | -135 | NEG | PASS |
| Probe B | 9.7 | 54 | POS | PASS |
| SPC | 31.3 | 233 | NA | PASS |
| Probe A | 0.0 | -135 | NEG | PASS |
| QC-1 | 0.0 | 0 | NEG | PASS |
| QC-2 | 0.0 | 0 | NEG | PASS |

User: XXXX
Status: Done
Expiration Date*: 23/05/21
S/W Version: 4.8
Cartridge S/N*: 883616530
Reagent Lot ID*: 57311
Notes:
Start Time: 31/12/19 17:58:40
End Time: 31/12/19 19:41:20
Instrument S/N: 810010
Module S/N: 680935
Module Name: B3

Patient ID
Patient ID 2
Sample ID 0000190903
Assay Xpert MTB-RIF Assay G4
Version 5
Test Type Specimen
Sample Type Other
Other Sample Type
Notes
Upload Status Expired
Module Name B3
Reagent Lot ID* 57311
Start Time 31/12/19 17:58:40
End Time 31/12/19 19:41:20
Status Done
User Manlitho Rodrigue

Views
Result View
Primary Curve

Test Result | Analyte Result | Detail | Errors | History | Support
Assay Name Xpert MTB-RIF Assay G4 Version 5
Test Result MTB DETECTED HIGH; Rif Resistance DETECTED
For In Vitro Diagnostic Use Only

Legend
Probe D; Primary
Probe C; Primary
Probe E; Primary
Probe B; Primary
SPC; Primary
Probe A; Primary
QC-1; Primary
QC-2; Primary

Fluorescence
Cycles

D

801740,Biryongo Health Centre, Kigali Rwanda 27/02/19 07:41:49

Test Report

Patient ID:
Sample ID: Xpert M 270918074436
Test Type: Specimen
Sample Type:

Assay Information

| Assay | Assay Version | Assay Type |
|---|---|---|
| Xpert MTB-RIF Assay G4 | 5 | In Vitro Diagnostic |

Test Result: MTB DETECTED HIGH; Rif Resistance DETECTED

Analyte Result

| Analyte Name | Ct | EndPt | Analyte Result | Probe Check Result |
|---|---|---|---|---|
| Probe D | 32.4 | 70 | POS | PASS |
| Probe C | 31.0 | 102 | POS | PASS |
| Probe E | 9.9 | 269 | POS | PASS |
| Probe B | 31.3 | 73 | POS | PASS |
| SPC | 25.0 | 286 | NA | PASS |
| Probe A | 31.3 | 90 | POS | PASS |
| QC-1 | 0.0 | 0 | NEG | PASS |
| QC-2 | 0.0 | 0 | NEG | PASS |

User:
Status: Done
Expiration Date*: 10/11/19
S/W Version: 4.7b
Cartridge S/N*: 624055832
Reagent Lot ID*: 51701
Notes: Muhanga Prison
Start Time: 27/09/18 07:45:28
End Time: 27/09/18 09:28:40
Instrument S/N: 801740
Module S/N: 615706
Module Name: A3

**Figure 1.** Continued.

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 rifampicin-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.

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