HHS Public Access

Author manuscript

Diagn Microbiol Infect Dis. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Diagn Microbiol Infect Dis. 2016 May ; 85(1): 53–55. doi:10.1016/j.diagmicrobio.2016.01.009.

False-positive Rifampin Resistant Results with Xpert MTB/RIF Version 4 Assay in clinical samples with a low bacterial load

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Abstract

We report investigation of 22 TB cases with positive Xpert MTB/RIF result for resistance to Rifampin and “Very Low” MTB detection level. Twelve cases were false positive without *rpoB* mutations, 2 were false-positives with a silent mutation in *rpoB* codon T508 and only 10 were true positives.

Methods, Results and Discussion

Xpert MTB/RIF (Cepheid, CA, USA) is an automated, cartridge-based assay designed to simultaneously detect *Mycobacterium tuberculosis* (MTB) and resistance to rifampin (RIF) directly in clinical specimens using hemi-nested real-time PCR. The single use cartridge contains reagents for DNA extraction, PCR amplification, internal controls and five partially overlapping fluorescent probes A, B, C, D and E, targeting the 81 bp Rifampin Resistance Determining Region (RRDR) of MTB *rpoB* gene. The test provides semi-quantitative MTB detection based on the probes’ Cycle Threshold (Ct) – number of PCR cycles required to amplify MTB DNA to a detectable level. MTB detection result is reported as “High” (Ct<16), “Medium” (Ct 16–22), “Low” (Ct 22–28), or “Very Low” (Ct>28). In samples with non-mutated *rpoB* RRDR, all 5 probes exactly match to the PCR-amplified MTB DNA and their Ct values are similar. Presence of *rpoB* mutations changes dynamics of hybridization between the amplicon and the probe(s) corresponding to the mutated site, which causes difference between the Ct values of the probes.

While the Xpert MTB/RIF test didn’t change the output since its debut in 2009, by 2012 Cepheid had produced four generations of cartridges and software in an effort to improve sensitivity and specificity for detection of RIF resistance (1). False-sensitive results for RIF susceptibility were reported for clinical strains with *rpoB* mutations in codon L533 (2, 3)

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and in samples with mixed populations of RIF-sensitive and RIF-resistant bacilli (4). Some false-resistant Xpert results were associated with silent *rpoB* mutations (5, 6) and mutations causing sub-critical levels of resistance, which is not detectable by culture-based DST (6, 7, 8). In other cases differences between the Ct values of the fluorescent probes was caused by the unequal stability of the probe/wild-type target hybrids during the test and not by the presence of *rpoB* mutations (9, 10). The latest, 4th version of Xpert software interprets samples with >4 cycles difference in Ct values between any 2 probes as resistance to RIF. Because the assay terminates after 39 cycles, a sample might be reported indeterminate for RIF resistance if the first probe's Ct is >34.5 cycles and the last probe's CT is >38 cycles.

TB laboratory of the Groupe Haitien d'Etude du Sarcome de Kaposi et des Infections Opportunistes (GHESKIO) in Port-au-Prince, Haiti routinely tests one diagnostic specimen for every patient with suspicion for TB with Xpert MTB/RIF. Sputa and gastric aspirates (GA) are kept refrigerated after collection and tested with turn around time of 24 hours. Samples positive for MTB are cultured. Specimens resistant to RIF by Xpert are tested by an alternative molecular test - MTBDR*plus* (Hain Life Sciences, Nehren, Germany). Isolates from Xpert-resistant samples are subjected to DST to first and second line anti-tuberculosis drugs, *rpoB* sequencing and spoligotyping as described previously (6). As part of the QC procedures all MTB positive Xpert results are examined and signed off by the senior laboratory staff experienced in manual evaluation of real-time PCR results.

In 12 months from June 1st 2013 to May 30th 2014, 9,890 Xpert MTB/RIF tests from 9,629 sputa and 261 GA generated results, of them 2,000 and 24 respectively were positive for MTB (Table 1).

In 1614 samples (1606 sputa and 8 GA) Xpert MTB/RIF detected High, Medium or Low level of MTB. In all of those samples Xpert was able to determine RIF susceptibility status. 87 specimens (86 sputa and 1 GA) were classified as RIF-resistant. MTB was isolated from the gastric aspirate and from all but one sputum samples. 100% of isolates harbored *rpoB* mutations as demonstrated by Sanger sequencing and 93% of them tested RIF-resistant in culture-based DST. The discrepancies between the molecular and conventional susceptibility tests were explained by the presence of silent and "low level" *rpoB* mutations as described in our previous report (6).

In 410 MTB-positive by Xpert samples (394 sputa and 16 GA) detection level was "Very Low". For 113 out of those 410 samples (110 sputa and 3 GA) Xpert MTB/RIF was unable to determine RIF susceptibility status. 35 samples (31 sputa and 4 GA) tested RIF-resistant, the remaining 262 samples tested RIF-sensitive. Only 22/35 Xpert RIF-resistant samples with "Very Low" MTB detection level produced positive cultures, which were subjected to confirmatory testing. Sequencing demonstrated *rpoB* mutations in 10/22 isolates, of them 8 tested RIF-resistant by DST and 2 had silent *rpoB* mutation T508T and tested RIF-sensitive by DST. However 12/22 isolates from 9 sputa and 3 GA (Table 2) did not harbor *rpoB* mutations and tested sensitive to RIF and other anti-tuberculosis drugs by DST. Sanger sequencing trace files did not indicate mixed populations of mutated and non-mutated genotypes. When tested directly with MTBDR*plus*, 10 samples were sensitive to RIF and INH and 1 test failed. Finally for 2 out of the 12 patients a second sputum specimen

collected within 2 days tested RIF-sensitive by Xpert MTB/RIF. Discrepant results could not be explained by circulation of a particular artifact-producing MTB strain since we found 9 distinct spoligotypes among them.

The observed false-resistant results were obtained with 3 different reagent lots of Xpert MTB/RIF cartridges. In all 12 false-resistant tests probes D and E hybridized with delay (higher Ct values) of more than 4 cycles compared to the probes B and C, which suggests unequal dynamics of probe/wild-type target hybridization for different probes after extended number of PCR cycles. Snapshots of the 12 false-resistant Xpert MTB/RIF tests are provided in the Online Supplement. A snapshot of a test for one confirmed RIF-resistant sample is also provided for comparison.

Rollout of Xpert MTB/RIF revolutionized TB diagnostics in high burden countries where resources to routinely use culture-based methods are inadequate and smear microscopy is often the only available diagnostic tool. Current WHO guidelines recommend using Xpert MTB/RIF to diagnose TB in children and adults suspected of having MDR-TB or HIV co-infection (11) because bacterial load in their samples is often below the detection level of AFB smear. Since in 86% of cases AFB smear-negative samples test “Very Low” with Xpert (12), it is of concern that systematic diagnostic testing of children and HIV-co-infected individuals will lead to increased rates of false resistant RIF results and may result in inappropriate treatment with toxic second-line anti-tuberculosis drugs of those two vulnerable groups of TB patients. RIF-resistant diagnoses in tests with “Very Low” MTB detection grade should be confirmed with a “gold standard” culture-based DST. It is also important to determine specificity for detection of RIF resistance depending on the bacterial load of samples in clinical evaluation of future Xpert MTB/RIF versions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by the Training Grant 1D43TW010062 from the Fogarty International Center, grants 5K24AI098627 and 1U19AI111143 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health and PEPFAR grants 541, 545. We are very grateful to the Expand TB WHO Program, and the Global Fund for supplying GHESKIO laboratory with materials and reagents and to Foundation Merieux for helping to build and maintain the Biosafety Level 3 facility.

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Table 1

MTB-positive Xpert MTB/RIF results stratified by MTB detection level and RIF susceptibility status.

A
MTB-positive tests for sputum specimens

MTB Detection Level	N	Susceptibility to RIF "indeterminate"	RIF-sensitive	RIF-resistant N (%*)
High	359	0	334	25 (6.9%)
Medium	753	0	708	45 (6.0%)
Low	494	0	478	16 (3.2%)
Very Low	394	110	253	31 (10.9%)
TOTAL	**2000**	**110**	**1773**	**117 (6.2%)**

B
MTB-positive tests for gastric aspirates

MTB Detection Level	N	Susceptibility to RIF "indeterminate"	RIF-sensitive	RIF-resistant N (%*)
High	0	0	0	0
Medium	6	0	5	1 (16.7%)
Low	2	0	2	0
Very Low	16	3	9	4 (30.8%)
TOTAL	**24**	**3**	**16**	**5 (23.8%)**

• - % of RIF-resistant Xpert results in tests with determined RIF susceptibility status.

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Table 2

Clinical and laboratory characteristics of 12 cases with false-positive Xpert MTB/RIF result for susceptibility to RIF

Patient	Sample type	Spoligo-type (SIT)	Ct of *rpoB* probes					max ΔCt	MTBDR*plus* assay	
			A	B	C	D	E		RIF	INH
1	GA	91	32.1	↓31.5	31.6	36.2↑	36.1	5.1	n.t.	n.t.
2	GA	2	32.5	30.6	↓29.9	33.3	34.1↑	4.2	sens	sens
3	sputum	50	32.9	0	32.3	0	0	>30	n.d.	n.d.
4	sputum	4	32.7	↓31.4	31.8	39.0↑	37.8	7.6	sens	sens
5*	sputum	2	30.2	↓29.7	30.2	33.9↑	33.3	4.2	sens	sens
6	sputum	5	31.0	30.0	↓29.3	34.4↑	33.8	5.5	sens	sens
7	sputum	52	32.7	↓31.4	↓31.4	36.2↑	35.2	4.8	sens	sens
8	sputum	2	31.8	31.0	↓30.7	35.7↑	34.5	7.1	sens	sens
9*	GA	633	32.8	33.3	↓30.5	37.6↑	36.9	7.1	sens	sens
10	sputum	n.d.	31.5	↓30.9	31.5	36.5↑	34.8	6.6	sens	sens
11	sputum	2054	31.9	↓30.9	31.1	36.2↑	35.0	6.8	sens	sens
12	sputum	34	33.1	↓31.5	31.8	35.6	35.8↑	7.8	sens	sens

*second sample collected within 2 days tested RIF-sensitive by Xpert MTB/RIF

Lowest and highest Ct values are marked with arrows

n.t. - not tested
n.d. - test failed
sens - sensitive