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Need for caution when interpreting Xpert® MTB/RIF results for rifampin resistance among children

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SUMMARY

BACKGROUND: Recommended by the World Health Organization as an initial diagnostic test for TB in children, Xpert® MTB/RIF is widely implemented in many countries, including Kenya.

METHODS: Three hundred HIV-positive and negative children (<5 years) were enrolled in Kisumu County, Kenya, from October 2013 to August 2015. Multiple specimen types were collected from each child and tested using Xpert, liquid culture, and phenotypic drug susceptibility testing (DST). Samples positive for rifampin (RIF) resistance on Xpert were tested using line-probe assay and sequencing.

RESULTS: Of 32 children with bacteriologically confirmed TB, 27 had positive Xpert results. Of these, 3/27 (11%, 95% CI 4–28) had RIF resistance detected on Xpert, but not by phenotypic DST, line-probe assay, or sequencing. For these three children, five Xpert tests showed RIF resistance; all five tests had semi-quantitative "very low" results and delay or absence of probe D signal, whereas no Xpert results with higher semi-quantitative results showed RIF resistance. All three children responded well to standard TB treatment.

CONCLUSIONS: False RIF resistance may be detected in pediatric specimens. Further study is needed to determine if false RIF resistance is associated with low bacterial load.

RÉSUMÉ

Recommandé par l'Organisation mondiale de la santé en tant que test diagnostique initial de la TB chez l'enfant, le test Xpert® MTB/RIF est largement utilisé dans de nombreux pays, dont le Kenya.

Trois cents enfants (<5 ans) infectés ou non par le VIH ont été inclus dans le comté de Kisumu, Kenya, d'octobre 2013 à août 2015. Divers types d'échantillons ont été prélevés chez chaque

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enfant, pour être analysés par test Xpert, culture en milieu liquide et test phénotypique de sensibilité aux médicaments (DST). Les échantillons positifs pour résistance à la rifampicine (RIF) par test Xpert ont été analysés par hybridation inverse sur bandelette (LPA) et séquençage.

Sur 32 enfants atteints de TB bactériologiquement confirmée, 27 avaient des résultats positifs au test Xpert. Une résistance à la RIF a été détectée par test Xpert chez trois de ces 27 enfants (11%, IC 95% 4–28), mais pas par DST phénotypique, test LPA ou séquençage. Pour ces trois enfants, cinq tests Xpert ont montré une résistance à la RIF ; les cinq tests ayant été associés à des résultats semi-quantitatifs « très faibles » et à un retard ou une absence de signal de sonde D, alors qu'aucun résultat semi-quantitatif plus élevé par test Xpert n'a montré de résistance à la RIF. Les trois enfants ont tous bien répondu au traitement antituberculeux standard.

Une fausse résistance à la RIF peut être détectée dans les échantillons pédiatriques. D'autres études sont nécessaires pour déterminer si la détection erronée de résistance à la RIF est associée à une faible charge virale.

Keywords

tuberculosis; Xpert MTB/RIF; children; rifampin resistance; cycle threshold; false-positive

TB is a leading cause of death by a single infectious agent worldwide. In 2019, 10 million (range 8.9–11.0 million) people had TB disease, and 1.2 million people died from the disease.[1] Over 95% of new TB diagnoses and deaths occur in developing countries.[2] Detecting acid-fast bacilli using sputum smear microscopy is the main diagnostic test for TB used in much of the world, but this method has poor sensitivity in certain populations, including children.[3] Compared to sputum smear microscopy, Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA) is a more sensitive molecular assay for detecting *Mycobacterium tuberculosis* (MTB) and resistance to rifampin (RIF) without the need for culture.[4] The WHO recommends Xpert as the initial diagnostic test in all patients if resources allow, and especially, in children and adults who may have multidrug-resistant TB (MDR-TB) or HIV-associated TB.[5] For RIF resistance detection in adults, Xpert has a sensitivity of 93% (95% confidence interval [CI] 90–95) and a specificity of 98% (95% CI 96–98).[6–8]

In 2019, Kenya had a TB incidence of 267 (range: 163–396) /100,000 and a TB-HIV coinfection rate of 26%. The estimated percentage of new MDR- or RIF-resistant TB (MDR/RR-TB) in new patients is respectively 1.3% and is 4.6% among patients previously treated for TB.[1] Although Xpert has been implemented in most sub-county hospitals throughout Kenya, the occurrence of possible false-positive RIF resistance results due to Xpert has not been studied in our setting. We prospectively investigated RIF resistance among 300 children enrolled in a TB diagnostic study in Kisumu County, Kenya; we identified three children with bacteriologically confirmed TB and apparent false-positive RIF resistance results on Xpert.

METHODS

Enrollment, sample collection, and treatment

Three hundred HIV-positive and HIV-negative children aged <5 years who had symptoms of TB disease were enrolled in Kisumu County, Kenya (October 2013–August 2015).[9] Up to two samples each of the following specimen types were collected from each participant at the time of enrollment: gastric aspirate, induced sputum, nasopharyngeal aspirate, string test, urine, and stool. Additionally, cervical lymph node aspirate biopsy (LNAB) was collected if indicated. Each collected sample was tested using fluorescence microscopy, Xpert and MGIT™ (Mycobacterial Growth Indicator Tube) liquid culture (BD, Sparks, MD, USA). MGIT drug susceptibility testing (DST) was performed on at least one culture isolate per participant. Molecular DST using line-probe assay and sequencing was performed using any remaining frozen specimen pellets to confirm RIF resistance detected by Xpert. Treatment decisions were made by the Kenya National TB Program (NTP).

Culture and drug susceptibility testing

Non-sterile specimens were decontaminated using an equal volume of freshly prepared mixture of *N*-acetyl-L-cysteine-sodium hydroxide-sodium citrate at a final sodium hydroxide concentration of 1%.[10] Pelleted material was re-suspended in phosphate-buffered saline (pH 6.8) and split for testing by Xpert (0.5 mL), fluorescence microscopy, and MGIT culture (0.5 mL). LNAB samples were considered sterile specimens and were inoculated directly into liquid culture media. Phenotypic DST (pDST) using the streptomycin, isoniazid, RIF, and ethambutol (SIRE) kit (BD, Sparks, MD USA) was performed on at least one positive culture isolate from each participant according to the manufacturer's instructions.[11]

Xpert MTB/RIF

Specimens were processed according to the manufacturer's instructions for sputum using 0.5 mL of processed sediment from non-sterile specimens or 1.0 mL of raw sample from sterile samples. Xpert Assay G4 v5 was used. Delta cycle threshold (ΔC_T max) was calculated by subtracting the largest from the smallest cycle threshold (C_T) value for probes A–E. The Xpert assay G4 assigns a result of RIF resistance detected if any of the five probes (A–E) fails to amplify or if the ΔC_T Max is >4 (probe delay).[12] An indeterminate RIF resistance result indicates that the first probe's C_T is >34.5 cycles, and the last probe's C_T is >38 cycles.[12]

Line-probe assay testing

DNA was prepared from 1.0-mL frozen specimen pellets using a Genolyse kit (Hain Lifescience, Nehren, Germany) per the manufacturer's instructions. The GenoType MTBDR*plus* v2.0 (Hain Life-science) assay was used to detect gene mutations in *rpo*B, *kat*G, and *inh*A gene regions according to the manufacturer's instructions.[13]

Sequencing

Sample template DNA was amplified using polymerase chain reaction (PCR) with primers F: 5′-CTTGCACGAGGGTCAGACCA-3′ and R: 5′-ATCTCGTCGCTAACCACGCC-3′ (Integrated DNA Technologies Skokie, IL, USA) using the following amplification cycle: 95°C for 15 min, then 50 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, then 72°C for 7 min.[14] Amplified products were purified using the QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Cycle sequencing was performed on the purified products using the automated geneAmp 9700 PCR system (Applied Biosystems Inc, Foster City, CA, USA) set at 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min.[14] Sequencing products were purified using Big Dye XTerminator Purification Kit (Applied Biosystem Inc). Sequence data were generated using the automated ABI 3130XL Genetic Analyzer (Applied Biosystem Inc), and sequence variations were identified by comparing the sample sequence to a reference *rpo*B gene using Sequencher software v5.1 (Gene Corporation, Suite, MI, USA). The target RIF resistance determining region of the *rpo*B gene was analyzed for any mutations, and these were compared to the Xpert results.

Statistical analysis

A semiquantitative grade is categorized according to the C_T of the first probe that detects *M. tuberculosis* as "high" ($C_T \leq 16$), "medium" ($16 < C_T \leq 22$), "low" ($22 < C_T \leq 28$), and "very low" ($C_T > 28$).[12] Proportions of Xpert tests with RIF resistance were compared for tests with semi-quantitative result "very low" vs. "low," "medium," and "high" results using Fisher's exact test, whereby *P* values <0.05 were considered statistically significant. Binomial confidence intervals using Wilson Score bounds were calculated for proportions.

Ethics

This study was approved by the Institutional Review Boards of the U.S. Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA), the Kenya Medical Research Institute (Kisumu, Kenya), and the Jaramogi Oginga Odinga Teaching and Referral Hospital (Kisumu, Kenya). Children's Hospital Boston/Harvard Medical School (Boston, MA, USA) relied on the review and oversight of the CDC Institutional Review Board. Parents or guardians of all participants provided written informed consent for participation in the study.

RESULTS

Among 300 children enrolled in the study, 32 (11%) had bacteriologically confirmed TB disease on MGIT or Xpert or both from any specimen. Of these, 27 had a positive Xpert result from at least one specimen. Among these, 3/27 (11%, 95% CI 4–28) had a positive result for RIF resistance for at least one specimen. For these three participants, all pDST results showed susceptibility to RIF, isoniazid, streptomycin, and ethambutol. Line-probe assay and sequencing results showed susceptibility to RIF; no mutations associated with RIF resistance were found on sequencing (Table 1).

Among the three participants with RIF resistance detected using Xpert, two had a combination of positive, negative, and indeterminate Xpert results from different specimens;

the third participant had only one single positive Xpert result from one sample, which tested positive for RIF resistance (Table 2). For all three participants with RIF resistance, the semi-quantitative result was “very low” for all Xpert results regardless of RIF resistance result.

Among the 27 participants with a positive Xpert result on at least one specimen, 273 Xpert tests were performed among the multiple samples collected; of the 108 (40%) that were positive for *M. tuberculosis* complex (MTBC), five (5%) showed RIF resistance. Among 108 positive Xpert results, 24 (22%) were from gastric aspirates, 14 (13%) were from the string test, 19 (18%) were from induced sputum, 23 (21%) were from nasal pharyngeal aspirates, 19 (18%) were from stool, 6 (6%) were from urine, and 3/108 (3%) were from fine-needle aspirates. Xpert results showing RIF resistance were from induced sputum, gastric aspirate, string test, and stool specimens. Of the 108 positive Xpert test results, 5/55 with semi-quantitative result “very low” detected RIF resistance, whereas none of 53 tests with other semi-quantitative results (“low,” 27; “medium,” 21; and “high,” 5) detected RIF resistance ($P = 0.06$).

For the five Xpert results in this study with RIF resistance detected, probe D failed to amplify for one specimen, and ΔC_T max was >4 for the remaining specimens. In all instances where ΔC_T max was >4, probe D had delayed amplification relative to the other probes (Table 2). For the participant who had a RIF-resistant result for multiple specimens, probe D failed for one specimen result and was delayed for the other two specimen results. In all instances of probe delay, the $\Delta C_T$was ≥5.

The three children with RIF resistance on Xpert were aged 12, 16, and 18 months, and all were HIV-negative. None had received TB treatment previously. The child aged 12 months presented to outpatient care with fever despite antimalarial and antibiotic treatment, as well as severe malnutrition (weight for height Z-score <−3) despite therapeutic feeding. The other two children were identified as household contacts of adults (a mother and a father) with pulmonary TB; both children presented with prolonged cough, fever, and cervical lymphadenopathy despite antibiotic treatment. For both parent contacts, the result of sputum smear microscopy was unknown or not done, and neither had drug resistance testing results. One parent died in the first month of treatment, before the child was enrolled in this study; the other parent was in the fourth month of standard TB treatment at the time the child was enrolled in the study. All three children initiated standard anti-TB therapy pending confirmatory drug resistance testing results and maintained this regimen for 6 months because of the negative confirmatory testing results for drug resistance. All three children improved clinically with standard treatment. The child with fever and severe malnutrition reported no fever at any of the follow-up visits and reached normal weight for height by 6 months after starting TB treatment. The other two children with cough, fever, and cervical lymphadenopathy did not report any symptoms at any of the follow-up visits.

DISCUSSION

We identified RIF resistance using Xpert in 11% (95% CI 4–28) of children diagnosed with TB on Xpert, a much higher rate than the national rate in Kenya of 1.3% among patients

with newly diagnosed active TB disease.[1] However, susceptible results for other tests of drug resistance and good clinical response to standard first-line TB treatment suggest that these Xpert RIF resistant results were false-positive.

Xpert showed qualitative results of “very low” grade for all tests performed for the three participants with RIF resistance detected. Xpert has high sensitivity and specificity for detecting RIF resistance.[6,7,15] However, false-positive RIF resistance has been reported for Xpert G4 when MTB detected result was “very low”. [16–22] In our study, for most (four of five) instances of RIF resistance detected, the resistance determination was based on probe delay. In all cases, RIF resistance determination was based on performance of probe D. Discordant RIF results on Xpert have been associated with probe delay, although the specific probe with delay differs between reports.[23,24] Our findings differ from these reports in that the ΔC_T for probes with delay was not near the threshold, but well above.

Mechanisms for false-positive RIF resistance on Xpert may include heterogeneous infection with drug-resistant and drug-susceptible MTB populations,[25] or silent mutations that affect probe binding.[16] Conversely, certain mutations are known to generate false-susceptible results on pDST, particularly in the MGIT system.[26,27] However, these mechanisms for RIF resistance are not likely in our case because clinical outcome was good after standard treatment and because sequencing results, where available, are not consistent with these explanations.

Our study had several limitations. First, we were able to obtain molecular DST results using line-probe assay or sequencing for only one of the three children in our analysis. Second, as RIF pDST was performed using a critical concentration of 1 mg/L, rather than the recently revised concentration of 0.5 mg/L, we may have missed resistance that would have been identified at the lower breakpoint.[28] Third, pDST was not performed on all culture isolates from each patient, and some samples that were RIF-resistant on Xpert did not grow in culture. Fourth, genotyping was not performed on the multiple isolates to rule out mixed infection with multiple strains of MTB or cross-contamination during sample processing.[29]

Although our results were not statistically significant, our findings are consistent with other reports of false-positive RIF resistance on Xpert in case of “very low” MTB results. It should be noted that our study extends this observation to diagnostic testing in young children and merits particular consideration because most diseases in this population is paucibacillary. Our findings in children aged <5 years underscore the importance of considering the possibility of false-positive RIF results from pediatric samples with Xpert MTB results “very low”.

In clinical practice, it is difficult to differentiate between false-positive and true RIF resistance in real time, especially in settings with a low prevalence of MDR-TB. Considering the possibility of false-positive results when interpreting RIF resistance results could improve patient care. In areas with low MDR-TB prevalence, and particularly, in young children with paucibacillary disease (i.e., Xpert MTB result “very low”), unless other clinical factors suggest differently, our results underscore the importance of additional drug resistance testing to confirm RIF resistance. Because false RIF-resistant results have been

observed with the Xpert G4 assay when the semi-quantitative result is “very low”, additional testing may include molecular diagnostic testing of the culture isolate. The diagnostic sensitivity of the Xpert® MTB/RIF Ultra (Cepheid) assay for RIF resistance is similar to Xpert, but specificity is slightly higher; this may be due to improved differentiation of certain silent mutations associated with RIF resistance and the identification of hetero-resistance.[30,31] However, false RIF resistance results may occur on Ultra due to synonymous mutations; also, Ultra misses some true resistance mutations identified using Xpert G4.[32] Ultra has recently been recommended as a first-line test in children.[33]

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Table 1

Summary of investigational tests to confirm RIF-resistant results from Xpert, stratified by patient (n=3) in a TB diagnostic study of 300 HIV-positive and HIV-negative children aged <5 years who had symptoms of TB disease in Kisumu County, Kenya, October 2013–August 2015*

Sample type	No.	Xpert MTB grading	Xpert RIF resistance result	Culture result	Rifampin DST result	MTBDR*plus* RIF result	*rpo*B sequencing result
Patient 1							
Gastric aspirate	1	Very low	Indeterminate	MTBC	—	—	—
	2	Very low	Not detected	MTBC	Susceptible	—	—
String test	1	No MTB	N/A	Negative	—	—	—
	2	No MTB	N/A	Negative	—	—	—
Induced sputum	1	Very low	Detected	MTBC	Susceptible	Susceptible	Wild-type
	2	Very low	Not detected	MTBC	Susceptible	—	—
NPA	1	No MTB	N/A	MTBC	Susceptible	—	—
	2	No MTB	N/A	MTBC	Susceptible	—	—
Stool	1	Very low	Detected	Negative	—	No MTB	No amplification
	2	Very low	Detected	Contaminated	—	No MTB	No amplification
Urine	1	No MTB	N/A	Negative	—	—	—
LNAB	1	—	—	—	—	—	—
Patient 2							
Gastric aspirate	1	No MTB	N/A	MTBC	Susceptible	—	—
	2	No MTB	N/A	Negative	—	—	—
String test	1	Very low	Detected	Negative	—	No MTB	No amplification
	2	No MTB	N/A	Negative	—	—	—
Induced sputum	1	No MTB	N/A	Negative	—	—	—
	2	No MTB	N/A	Negative	—	—	—
NPA	1	No MTB	N/A	Negative	—	—	—
	2	No MTB	N/A	Negative	—	—	—
Stool	1	No MTB	N/A	Negative	—	—	—
	2	No MTB	N/A	Negative	—	—	—
Urine	1	No MTB	N/A	Negative	—	—	—
LNAB	1	No MTB	N/A	Negative	—	—	—
Patient 3							

Sample type	No.	Xpert MTB grading	Xpert RIF resistance result	Culture result	Rifampin DST result	MTBDR*plus* RIF result	*rpo*B sequencing result
Gastric aspirate	1	Very low	Detected	MTBC	—	—	—
	2	No MTB	N/A	MTBC	—	—	—
String test	1	No MTB	N/A	Negative	—	—	—
	2	No MTB	N/A	MTBC	—	—	—
Induced sputum	1	Very low	Not detected	MTBC	—	—	—
	2	No MTB	N/A	MTBC	—	—	—
NPA	1	Very low	Indeterminate	MTBC	Susceptible	—	—
	2	No MTB	N/A	MTBC	—	—	—
Stool	1	No MTB	N/A	Negative	—	—	—
	2	—	—	—	—	—	—
Urine	1	No MTB	N/A	Negative	—	—	—
LNAB	1	Very low	Indeterminate	MTBC	—	—	—

*Results are shown as composites over all samples for each participant.

RIF=rifampin; MTB=*Mycobacterium tuberculosis*; DST=drug susceptibility testing; MTBC=*Mycobacterium tuberculosis* complex; N/A = not applicable; NPA = nasopharyngeal aspirate; LNAB = lymph node aspirate biopsy.

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Table 2

Xpert MTB/RIF Ct values for participants with RIF resistance results in a TB diagnostic study of 300 HIV-positive and HIV-negative children aged <5 years who had symptoms of TB disease in Kisumu County, Kenya, October 2013–August 2015

				Probe						
Patient	**Sample**	**Xpert MTB grading**	**Xpert RIF-R result**	**A**	**B**	**C**	**D**	**E**	**ΔCt max***	**RIF-R criterion†**
1	GA 1	Very low	Indeterminate	34.6	33.0	33.4	35.3	36.1	3.1	N/A
	GA 2	Very low	Not detected	30.9	29.9	30.7	31.7	32.2	2.3	N/A
	IS 1	Very low	Detected	32.9	31.5	31.9	37.7≠	35.8	6.2≠	Delay
	IS 2	Very low	Not detected	29.6	29.1	29.2	31.1	31.0	1.9	N/A
	Stool 1	Very low	Detected	31.8	31.0	30.7	36.2≠	34.7	5.5≠	Delay
	Stool 2	Very low	Detected	32.5	31.2	31.6	0≠	37.4	37.4≠	Failure
2	String	Very low	Detected	33.7	31.8	32.9	37.4≠	36.8	5.6≠	Delay
3	GA	Very low	Detected	30.7	29.2	30.2	40.7≠	38.1	11.5≠	Delay
	IS	Very low	Not detected	28.8	29.2	29.5	30.5	30.9	1.7	N/A
	NPA	Very low	Indeterminate	33.2	32.8	33.1	34.7	36.3	3.5	N/A
	FNA	Very low	Indeterminate	34.9	34.0	34.0	35.3	36.6	2.6	N/A

*ΔCt max is >4 = probe delay.

†Criterion for RIF-R is probe delay or amplification failure.

≠Probe D Ct and ΔCt max values for samples with RIF-R detected.

Ct=cycle threshold; RIF=rifampin; MTB=*Mycobacterium tuberculosis*; RIF-R=RIF-resistant; ΔCt=delta Ct; GA=gastric aspirate; IS=induced sputum; N/A=not applicable; NPA=nasal pharyngeal aspirate; FNA=fine-needle aspirate.