Prevalence and drivers of false-positive rifampicin-resistant Xpert MTB/RIF results: a prospective observational study in Rwanda

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Summary

Background The Xpert MTB/RIF (Xpert) assay is used globally to rapidly diagnose tuberculosis and resistance to rifampicin. We investigated the frequency and predictors of false-positive findings of rifampicin resistance with Xpert.

Methods We did a prospective, observational study of individuals who were enrolled in a Rwandan nationwide diagnostic cohort study (DIAMA trial; NCT03303963). We included patients identified to have rifampicin resistance on initial Xpert testing. We did a repeat Xpert assay and used *rpoB* Sanger and deep sequencing alongside phenotypic drug susceptibility testing (pDST) to ascertain final rifampicin susceptibility status, with any (hetero)resistant result overriding. We used multivariable logistic regression to assess predictors of false rifampicin resistance on initial Xpert testing, adjusted for HIV status, tuberculosis treatment history, initial Xpert semi-quantitative bacillary load, and initial Xpert probe.

Findings Between May 4, 2017, and April 30, 2019, 175 people were identified with rifampicin resistance at initial Xpert testing, of whom 154 (88%) underwent repeat Xpert assay. 54 (35%) patients were confirmed as rifampicin resistant on repeat testing and 100 (65%) were not confirmed with resistance. After further testing and sequencing, 121 (79%) of 154 patients had a final confirmed status for rifampicin susceptibility. 57 (47%) of 121 patients were confirmed to have a false rifampicin resistance result and 64 (53%) had true rifampicin resistance. A high pretest probability of rifampicin resistance did not decrease the odds of false rifampicin resistance (adjusted odds ratio [aOR] 6·0, 95% CI 1·0–35·0, for new tuberculosis patients *vs* patients who needed retreatment). Ten (16%) of the 64 patients with true rifampicin resistance did not have confirmed rifampicin resistance on repeat Xpert testing, of whom four had heteroresistance. Of 63 patients with a very low bacillary load on Xpert testing, 54 (86%) were falsely diagnosed with rifampicin-resistant tuberculosis. Having a very low bacillary load on Xpert testing was strongly associated with false rifampicin resistance at the initial Xpert assay (aOR 63·6, 95% CI 9·9–410·4).

Interpretation The Xpert testing algorithm should include an assessment of bacillary load and retesting in case rifampicin resistance is detected on a paucibacillary sputum sample. Only when rifampicin resistance has been confirmed on repeat testing should multidrug-resistant tuberculosis treatment be started. When rifampicin resistance has not been confirmed on repeat testing, we propose that patients should be given first-line anti-tuberculosis drugs and monitored closely during treatment, including by baseline culture, pDST, and further Xpert testing.

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Introduction

Timely detection of rifampicin-resistant tuberculosis as a proxy for multidrug-resistant (MDR) tuberculosis has been achieved widely through the roll-out of rapid molecular diagnostic methods, such as the Xpert MTB/RIF (Xpert) assay.[1] These procedures allow swift initiation of appropriate MDR-tuberculosis treatment, which improves outcomes and interrupts transmission of resistance.[2,3]

Detection of rifampicin resistance by the Xpert assay is based on absence or delay of binding of five probes (labelled A–E) that cover the 81 bp rifampicin-resistance determining region, as measured by a significant difference in the PCR threshold cycle (Ct) value (ie, the number of PCR cycles needed for initial detection of amplification) between the different probes (ΔCt>4). The Ct value gives a semi-quantitative measure of the tuberculosis bacillary load in the sample; for example, values can range from less than 16 (Ct<16, classified as high *Mycobacterium tuberculosis* complex detected) to greater than 28 (Ct>28, categorised as very low *M tuberculosis* complex detected).[4]

The Xpert assay was initially reported with an imperfect specificity that was actually attributable to missed

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Research in context

Evidence before this study

We searched PubMed for all studies published before February, 2020, in which false rifampicin resistance results on Xpert MTB/RIF (Xpert) testing were reported, using combinations of keywords: ("false" OR "discordance" OR "discordant"), AND ("rifampicin-resistant" OR "rifampicin resistance"), AND ("Xpert MTB/RIF" OR "GeneXpert"). Only a few studies, mainly case reports or case series, reported false rifampicin resistance on Xpert testing, particularly in samples with a very low bacillary load. Findings of previous studies suggested an association between false rifampicin resistance and probe B or delayed binding of probes, but no estimates have been made of the frequency and predictors of false rifampicin resistance results on Xpert testing in the setting of a national tuberculosis programme.

Added value of this study

Our analysis includes patients enrolled into a nationwide cohort study. Our findings show at the population level that false rifampicin resistance on Xpert can be very frequent. In our setting of low prevalence of rifampicin resistance, close to half (47%) of rifampicin resistance diagnosed on Xpert was false. Our study confirmed previous findings that false rifampicin resistance results on Xpert testing are driven by a very low bacillary load in the sample. Moreover, a high pretest probability of rifampicin resistance (ie, in patients with previous tuberculosis) did not decrease the odds of false rifampicin resistance results on Xpert testing. Further, repeat Xpert testing might not rule out true rifampicin resistance, particularly in individuals with heteroresistance.

Implications of all the available evidence

The findings of this study warrant a modification of the recommended Xpert testing flowchart, in which patients who test rifampicin resistant on the initial Xpert assay currently receive treatment for multidrug-resistant (MDR) disease, regardless of having true or false rifampicin resistance. In addition to a low pretest probability as an indication to repeat the Xpert assay, repeat testing on a better sample (ie, obtained after clearly instructing patients or using an early-morning sample) should be done for samples with rifampicin-resistant results but a very low bacillary load. Moreover, if rifampicin-resistant tuberculosis is not confirmed at repeat Xpert testing, further testing during MDR-tuberculosis treatment and follow-up should be considered to overcome false rifampicin susceptibility because of heteroresistance.

rifampicin resistance as detected by the gold-standard test (mostly, phenotypic drug-susceptibility testing [pDST]).[5] However, in a few case reports, false rifampicin resistance was also noted on Xpert testing.[6,7] Findings of two studies identified false rifampicin resistance in small sets of paucibacillary sputum samples.[4,8] In another report, false rifampicin resistance was associated with Xpert probe B and probe binding delay.[9] Although the 2017 Global Laboratory Initiative (GLI) guideline recommends repeat Xpert testing in patients with a low pretest probability for rifampicin-resistant tuberculosis (eg, new patients who have not been in contact with a patient with rifampicin-resistant tuberculosis),[10,11] population-based data on the frequency of false rifampicin resistance on Xpert testing, and predictors for a false result, are not available.

The classic Xpert assay was introduced in six public hospitals in Rwanda in 2012, increasing to 68 centres in 2019. Currently, Xpert testing is easily accessible as a first-line tuberculosis diagnostic method for high-risk groups (eg, people aged ≥55 years, individuals with HIV co-infection, children aged ≤15 years, contacts of tuberculosis patients, and prisoners). The assay is also used as a diagnostic method for rifampicin-resistant tuberculosis for all patients with a positive smear.[12] Moreover, since 2017, Xpert has been used as a first-line diagnostic method for all patients with presumptive tuberculosis in Kigali, where more than 55% of patients with notified rifampicin-resistant tuberculosis in Rwanda reside.

The DIAgnostics for Multidrug resistant tuberculosis in Africa (DIAMA) trial is an ongoing trial of molecular diagnostic tests for MDR-tuberculosis in Rwanda (NCT03303963). During this trial, frequent discordance was seen between initial and repeat Xpert test results. Thus, we decided post hoc to do a population-wide, prospective observational study to investigate factors associated with false rifampicin resistance on Xpert testing.

Methods

Design and study population

We did a population-wide, prospective observational study at peripheral health facilities in Rwanda. We included in our analysis all people enrolled in the DIAMA trial who were diagnosed with rifampicin-resistant tuberculosis on initial Xpert testing. We excluded patients enrolled in the DIAMA trial who initiated MDR-tuberculosis treatment on the basis of clinical decision; who had a line probe assay resistance diagnosis; who had a GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) resistance diagnosis; who were judged to have extra-pulmonary rifampicin-resistant tuberculosis; or for whom an initial Xpert test result was not available.

The DIAMA study protocol was approved by the Rwandan national ethics committee (Institutional Review Board 00001497 of IORG0001100; ref no 0069/RNEC/2017). All participants provided informed consent or assent.

Procedures

For the initial Xpert assay (Cepheid, Sunnyvale, CA, USA), all individuals received a prelabelled container and

were instructed at their respective peripheral health facility how to provide a sputum sample. The specimen was stored at 2–8°C before shipment in a cool box (at approximately 4°C) to one of 68 peripheral Xpert testing laboratories in Rwanda. Samples were tested within 2 days after the initial collection date under a quality assurance programme (Xpert testing sites had implemented a continuous quality improvement scheme and successfully participated in annual US Centers for Disease Control and Prevention proficiency panel testing since 2018).

Per routine in Rwanda, all patients identified with rifampicin-resistant tuberculosis on the initial Xpert assay were referred to one of two MDR-tuberculosis clinics (located in Kigali and Huye). Before starting treatment for MDR-tuberculosis, three additional sputum samples were obtained, the first on the day of arrival (spot sample 1), the second overnight (sample 2; ie, sputum continuously produced in the same container during the night), and the third the next day (spot sample 3). All three samples and a data sheet were sent to the National Reference Laboratory (NRL) in Kigali on the same day the third sample was obtained in a cool box (at approximately 4°C). All patients started a short MDR-tuberculosis treatment regimen (9 months' duration) and were treated regardless of subsequent test results.

The repeat Xpert test was done at the NRL within 2 days of sample collection; the overnight sample was used for testing because sample 2 typically had a higher volume than did spot samples 1 and 3, allowing different assays to be done on the same sample. Sample 3 and the remainder of sample 2 were processed separately for mycobacterial culture at the NRL.[13] Sample 1 was stored for future testing. Positive cultures were confirmed for presence of acid-fast bacilli by Ziehl-Neelsen microscopy. Identification of *M tuberculosis* complex was confirmed using an immunochromatographic test (SD MPT64TB Ag kit; SD Bioline, Seoul, South Korea). Positive cultures underwent pDST for rifampicin and other anti-tuberculosis drugs (including isoniazid, ethambutol,

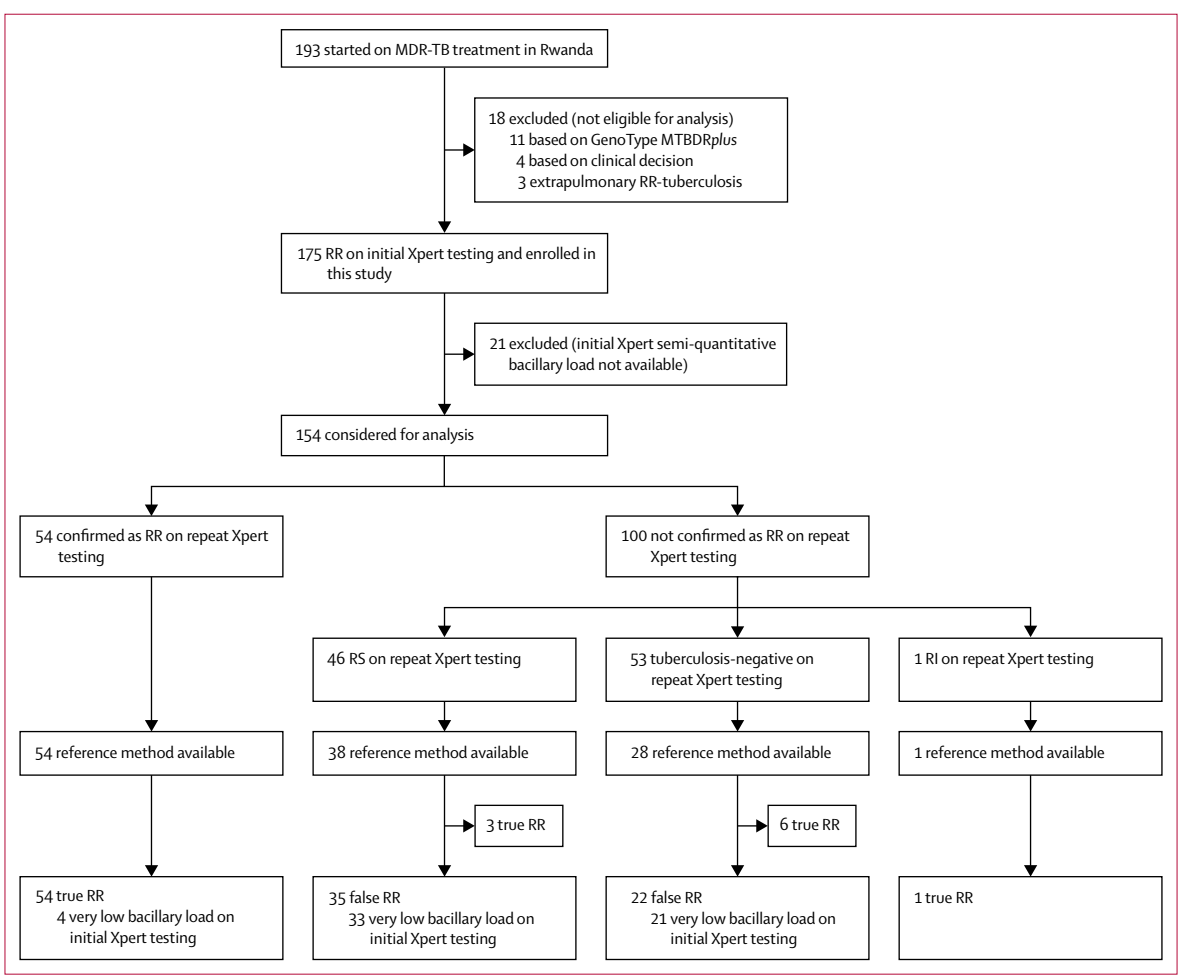

Figure 1: **Rifampicin susceptibility testing flowchart**
Repeat Xpert testing was done on an overnight sample obtained at specialised MDR-TB treatment clinics. GenoType MTBDR*plus* is an MDR-TB diagnostic test. Xpert=classic Xpert MTB/RIF assay. MDR-TB=multidrug-resistant tuberculosis. RR=rifampicin resistant. RS=rifampicin susceptible. RI=rifampicin indeterminate.

	Total (n=154)	Repeat Xpert result			
		Rifampicin resistant (n=54)	Rifampicin susceptible (n=46)	Indeterminate rifampicin resistance (n=1)	Tuberculosis-negative (n=53)
Sex					
Male	119	39	41	0	39
Female	35	15	5	1	14
Age, years					
<30	49	16	12	0	21
30–44	52	24	16	0	12
45–54	28	8	7	1	12
>54	25	6	11	0	8
Tuberculosis treatment history					
New	118	41	37	1	39
Retreatment	36	13	9	0	14
HIV status					
Positive	56	24	14	0	18
Negative	93	28	30	1	34
Unknown	5	2	2	0	1
Bacillary load of sputum sample*					
High (1+ to 3+)	44	38	5	0	1
Low (scanty)	41	12	19	1	9
Negative	69	4	22	0	43
Initial Xpert result, semi-quantitative bacillary load (Ct value)					
High (Ct<16)	26	23	1	0	2
Medium (16≤Ct<22)	21	19	1	1	0
Low (22≤Ct≤28)	13	8	2	0	3
Very low (Ct>28)	94	4	42	0	48
Initial Xpert result, absent or delayed probe binding					
Probe A	10	0	5	0	5
Probe B	19	10	3	0	6
Probe C	6	0	3	0	3
Probe D	19	2	10	0	7
Probe E	90	42	20	1	27
Probe X†	10	0	5	0	5

Data are number of patients. Xpert=classic Xpert MTB/RIF assay. Ct=PCR threshold cycle. *Ascertained by direct fluorescence microscopy. †More than one probe.

Table 1: **Characteristics of patients with rifampicin-resistant tuberculosis on initial Xpert testing, stratified by findings of repeat Xpert testing**

streptomycin, kanamycin, capreomycin, and ofloxacin) using the proportion method on Löwenstein-Jensen medium, with the final reading at 4 weeks for ethambutol and 6 weeks for the other drugs.[14]

If participants were not confirmed with rifampicin resistance on repeat Xpert testing, Sanger *rpoB* gene sequencing was done on remnants of sputum sample 2, using a nested PCR system covering both the rifampicin-resistance determining region and the non-rifampicin-resistance determining region, as previously described.[15] If tests on the sputum sample did not produce a result, the tests were repeated on the corresponding culture isolate, if available. Furthermore, some participants underwent next-generation sequencing using sample 2 or 3. Deeplex-MycTB (Deeplex; GenoScreen, Lille, France) is a targeted deep-sequencing assay that was done on sputum DNA extracts.[16] Moreover, whole-genome sequencing (WGS) was done on isolate DNA[17] on an Illumina HiSeq platform using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Single nucleotide polymorphisms (SNPs) were detected from WGS reads by first filtering out non-*M tuberculosis* DNA using Centrifuge version 1.0.3[18] and then calling SNPs against the reconstructed ancestor genome[19] using MTBseq.[20]

We obtained data for Xpert bacillary load for all tuberculosis patients with rifampicin resistance on the initial Xpert assay and smear positivity for all notified tuberculosis cases from routine programmatic records.

Final rifampicin resistance status was ascertained using a composite reference standard. If any documented rifampicin resistance-conferring mutations were identified by any sequencing technique, including mutations coexisting with wild-type bacilli (heteroresistance; with minimum filtering set at 3% minority reads in Deeplex and 5% in WGS analysis), the participant was judged to have true rifampicin-resistant tuberculosis.[21] Total absence of a resistance mutation (ie, wild-type) meant that the individual was regarded as susceptible to rifampicin and, thus, a false rifampicin-resistant result on the initial Xpert assay. When no sequencing result was available, the pDST result was regarded as the reference standard, particularly to confirm rifampicin resistance. If no sequencing and pDST results were available, rifampicin resistance was deemed unknown.

Statistical analysis

We used Pearson's χ^2 test and Fisher's exact test to investigate associations between false rifampicin resistance and potential predictors, comprising bacillary load on initial Xpert testing (high [Ct<16], medium [16≤Ct<22], low [22≤Ct≤28], and very low [Ct>28]), previous history of tuberculosis treatment (tuberculosis retreatment *vs* new tuberculosis), HIV co-infection status (HIV-positive *vs* HIV-negative), and specific probe reactions (probe E or probe B *vs* other probes combined, a binary variable grouping together probes with a higher frequency of false rifampicin resistance). Pearson's χ^2 test was used to investigate associations between bacillary load on initial Xpert testing and HIV co-infection, as well as between the year of diagnosis and the proportion of patients with a microscopy-positive smear. We judged a p value less than 0·05 statistically significant. Multivariable Firth logistic regression was used to assess predictors of discordance between the initial and the repeat Xpert and predictors of false rifampicin resistance, adjusted for HIV status, tuberculosis treatment history, initial Xpert semi-quantitative bacillary load, and initial Xpert probe. We judged an odds ratio (OR) significant if the 95% CI excluded 1·0. We used Stata version 14.2 for data analysis.

The DIAMA trial is registered with ClinicalTrials.gov, NCT03303963.

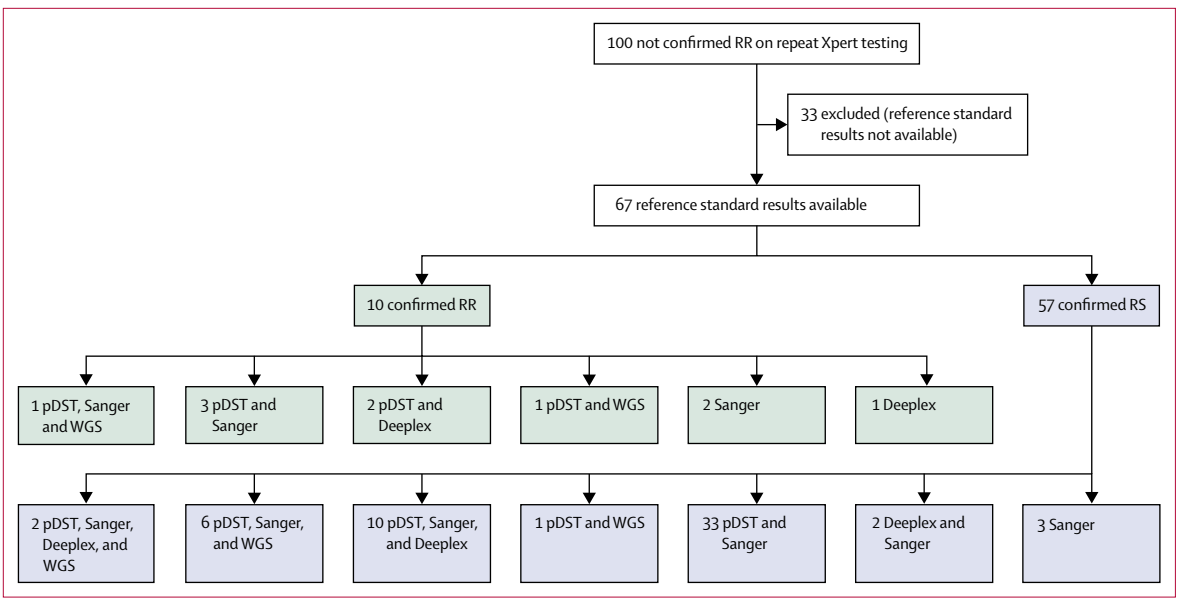

Figure 2: **Reference standard testing for patients not confirmed as RR on repeat Xpert testing**
All confirmatory tests were concordant. RR=rifampicin resistant. Xpert=classic Xpert MTB/RIF assay. RS=rifampicin susceptible. pDST=phenotypic drug susceptibility testing. Sanger=*rpoB* gene target sequencing by Sanger method. WGS=whole-genome sequencing. Deeplex=Deeplex-MycTB deep sequencing assay.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report. JCSN, BCdJ, and DA had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

From May 4, 2017, to April 30, 2019, 193 individuals in Rwanda started MDR-tuberculosis treatment. Among this population, 175 (91%) people were identified with rifampicin-resistant tuberculosis on initial Xpert testing and were eligible for our analysis, of whom 154 (88%) had both initial and repeat Xpert results available and were included in our analysis (figure 1; table 1). 21 patients excluded did not have data available on initial Xpert semi-quantitative bacillary load.

Of 154 patients with rifampicin resistance on initial Xpert testing, 100 (65%) did not have rifampicin resistance on repeat Xpert testing (figure 1; figure 2; table 1). 46 (30%) patients were deemed susceptible to rifampicin, one (1%) had indeterminate rifampicin resistance, and 53 (34%) were tuberculosis-negative (figure 1; table 1). Of 54 (35%) patients with rifampicin resistance confirmed on repeat Xpert testing, all had the same rifampicin resistance-determining probe reaction as on the corresponding initial Xpert assay; four (8%) patients had a very low bacillary load on the initial Xpert test (figure 1; appendix 2 p 5).

94 (61%) of 154 patients had a very low bacillary load on initial Xpert testing (table 2). These patients had a much higher probability of having a discordant repeat Xpert result (90 of 94 [96%]) compared with those with a high

	Patients (n=154)	Rifampicin resistance not confirmed on repeat Xpert testing, n (%)	Univariate analyses, odds ratio (95% CI)	Multivariable analyses, adjusted odds ratio (95% CI)*
HIV status				
Negative	93	65 (70%)	1 (ref)	1 (ref)
Positive	56	32 (57%)	0·6 (0·3–1·1)	0·6 (0·2–1·8)
Tuberculosis treatment history				
Retreatment	36	23 (64%)	1 (ref)	1 (ref)
New	118	77 (65%)	1·1 (0·5–2·3)	4·7 (0·9–24·3)
Bacillary load of sputum sample†				
High (1+ to 3+)	44	6 (14%)	1 (ref)	..
Low (scanty)	41	29 (71%)	15·3 (5·1–45·6)	..
Negative	69	65 (94%)	102·9 (27·3–388·0)	..
Initial Xpert result, semi-quantitative bacillary load (Ct value)				
High (Ct<16)	26	3 (12%)	1 (ref)	1 (ref)
Medium (16≤Ct<22)	21	2 (10%)	0·8 (0·1–5·3)	1·0 (0·2–5·6)
Low (22≤Ct≤28)	13	5 (38%)	4·8 (0·9–24·8)	4·6 (0·9–22·9)
Very low (Ct>28)	94	90 (96%)	172·5 (36·1–825·4)	118·4 (22·2–631·4)
Initial Xpert result, absent or delayed probe binding				
Probe E or B	109	57 (52%)	1 (ref)	1 (ref)
Other probes‡	45	43 (96%)	19·6 (4·5–85·0)	3·0 (0·5–17·0)

Rifampicin resistance was not confirmed on repeat Xpert testing in 100 patients. Xpert=classic Xpert MTB/RIF assay. Ct=PCR threshold cycle. *Adjusted for HIV status, tuberculosis treatment history, initial Xpert semi-quantitative bacillary load, and initial Xpert probe. †Ascertained by direct fluorescence microscopy; not included in multivariable analysis because measures of bacillary load are not independent. ‡Probes A, C, D, and more than one probe together.

Table 2: **Factors associated with failure to confirm initial rifampicin-resistant Xpert result on repeat Xpert testing**

	Patients (n=121)	False rifampicin resistance, n (%)	Univariate analyses, odds ratio (95% CI)	Multivariable analyses, adjusted odds ratio (95% CI)*
HIV status				
Negative	71	37 (52%)	1 (ref)	1 (ref)
Positive	46	18 (39%)	0·6 (0·3, 1·3)	0·6 (0·2–2·2)
Tuberculosis treatment history				
Retreatment	21	7 (33%	1 (ref)	1 (ref)
New	100	50 (50%)	2 (0·74–5·4)	6·0 (1·0–35·0)
Bacillary load of sputum sample†				
High (1+ to 3+)	44	5 (11%)	1 (ref)	..
Low (scanty)	35	18 (51%)	8·3 (2·63–25·9)	..
Negative	42	34 (81%)	33·2 (9·9–111·0)	..
Initial Xpert result, semi-quantitative bacillary load (Ct value)				
High (Ct<16)	24	1 (4%)	1 (ref)	1 (ref)
Medium (16≤Ct<22)	21	0	0·4 (0·0–9·4)	0·5 (0·0–12·5)
Low (22≤Ct≤28)	13	2 (15%)	4·2 (0·3–51·2)	3·3 (0·4–30·0)
Very low (Ct>28)	63	54 (86%)	138 (16·5–1153·0)	63·6 (9·9–410·4)
Initial Xpert result, absent or delayed probe binding				
Probe E or B	92	30 (33%)	1 (ref)	1 (ref)
Other probes‡	29	27 (93%)	27·9 (6·2–125·2)	8·6 (1·5–49·1)

121 patients had documented rifampicin results on reference standard, of whom 57 (47%) had false rifampicin resistance. Xpert=classic Xpert MTB/RIF assay. Ct=PCR threshold cycle. *Adjusted for HIV status, tuberculosis treatment history, initial Xpert semi-quantitative bacillary load, and initial Xpert probe. †Ascertained by direct fluorescence microscopy; not included in multivariable analysis because measures of bacillary load are not independent. ‡Probes A, C, and D together.

***Table 3*: Predictors of false rifampicin resistance on initial Xpert assay, in patients with rifampicin results ascertained by reference standard**

See Online for appendix 2

bacillary load on initial Xpert testing (three of 26 [11%]; adjusted OR 118·4, 95% CI 22·2–631·4; p<0·0001; table 2; appendix 2 p 5). HIV co-infection did not affect bacillary load on initial Xpert testing, with 30 (54%) of 56 patients with HIV co-infection and 61 (66%) of 93 HIV-negative patients having a very low bacillary load (p=0·40; appendix 2 p 5).

121 (79%) of 154 patients had a final known status for rifampicin susceptibility (figure 1; appendix 2 pp 1–4). Samples from the remaining 33 patients (of whom 25 were tuberculosis negative and eight were rifampicin susceptible on repeat Xpert testing) remained culture-negative and without a reference test result, despite repeated culture attempts. 57 (47%) of 121 patients with a final status were identified as false rifampicin resistant on initial Xpert testing (not questioning the specificity for detection of tuberculosis) whereas 64 (53%) had true rifampicin resistance (figure 1).

Of the 57 patients with false rifampicin resistance (figure 2), *rpoB* sequencing showed wild-type DNA for 54 (95%), and three had mutations outside the rifampicin-resistance determining region that were not associated with rifampicin resistance (Gly386Asp and Thr585Ile). Of the 54 patients with a wild-type *rpoB* sequence, 50 had a pDST result available, and all were rifampicin susceptible. Of the 57 patients with false rifampicin resistance, 50 were new tuberculosis patients and seven needed retreatment (five had initial treatment failure and two had relapse; adjusted OR 6·0, 95% CI 1·0–35·0; table 3).

Of the 64 patients with true rifampicin resistance on initial Xpert testing, 56 had MDR-tuberculosis with resistance also to isoniazid, whereas none had tuberculosis resistant to fluoroquinolones or second-line injectable drugs. Of the 54 patients with concordant rifampicin resistance on both initial and repeat Xpert testing, 21 (39%) were confirmed as rifampicin resistant by both pDST and deep sequencing, 31 (57%) by pDST alone, and two (4%) by deep sequencing alone. Among the remaining ten (16%) patients who did not have rifampicin resistance on repeat Xpert testing, but in whom rifampicin resistance was confirmed by reference methods (figure 2), four had heteroresistance (three on deep sequencing and one on Sanger sequencing) reported as rifampicin susceptible (n=3) and tuberculosis negative (n=1) by the repeat Xpert assay and six had fixed high-confidence resistance-conferring mutations in the *rpoB* gene (five on Sanger sequencing and one on WGS). Of these six patients, five had a tuberculosis-negative result on repeat Xpert testing and one was not interpretable (indeterminate rifampicin resistance) by the repeat Xpert assay. Among these ten patients who were not rifampicin resistant on repeat Xpert, five were detected as rifampicin resistant during MDR-tuberculosis treatment by follow-up Xpert assay (three tested rifampicin resistant at month 1 and another two tested rifampicin resistant at month 2), four remained tuberculosis negative, and one patient died before the first follow-up Xpert assay was done. Of these same ten patients not confirmed rifampicin resistant on repeat Xpert assay, nine were new tuberculosis patients whereas one had had treatment failure.

Among the 121 patients with a final known status for rifampicin susceptibility, the likelihood of false rifampicin resistance on initial Xpert assay was highest in patients with very low bacillary load (54 of 63 [86%]) compared with those with low (two of 13 [15%]), medium (none of 21 [0%]), or high (one of 24 [4%]) bacillary loads. By multivariable analyses, a very low bacillary load (adjusted OR 63·6, 95% CI 9·9–410·4) and an absent or delayed probe other than B or E on initial Xpert testing (8·6, 1·5–49·1) were associated with false rifampicin resistance (table 3).

Discussion

The findings of our population-based study in Rwanda show that the Xpert assay has a low positive predictive value (53%) for rifampicin resistance in sputum samples with a very low bacillary load. To be identified many years after global roll-out of the Xpert assay, this finding is alarming.

The low positive predictive value of the Xpert assay is the outcome of imperfect specificity in the context of low prevalence of rifampicin resistance and an increase in patients with paucibacillary presentation because of expanded testing indications, resulting in patients being

tested earlier during tuberculosis disease. Absence of probe binding seems to be caused by insufficient mycobacterial DNA in the sample, rather than being related to absence or delayed binding of a specific probe, such as B or E, as previously suggested.[4,9] Indeed, our findings showed that an absent or delayed probe other than B or E was associated with false rifampicin resistance. However, probe reactions might reflect distinct mutations, which can vary between settings, making the implications of this finding difficult to elucidate. Moreover, coexistence of multiple mutations, which together affect more than one probe, has been reported.[22] False rifampicin resistance on Xpert testing of paucibacillary sputum samples might be attributable to unequal dynamics of target hybridisation for different probes, which could have a greater effect after extended PCR cycles.[4,23]

In our setting, false rifampicin resistance was not associated with HIV co-infection, probably because the frequency of paucibacillary samples was similar among patients with HIV co-infection and those who were HIV-negative. In Rwanda, the diagnostic strategy for tuberculosis now favours earlier access to Xpert testing. Therefore, patients who are HIV-negative are also diagnosed at the paucibacillary stage of disease.

The classic Xpert assay continues to be used in most endemic areas and countries, although the novel Xpert Ultra assay has started to be deployed since 2017.[24] This newer assay has shown higher sensitivity to detect tuberculosis and greater specificity for prediction of rifampicin resistance compared with the classic Xpert assay. Moreover, the Xpert Ultra assay is apparently unaffected by very low bacillary load, although a rifampicin result is withheld in case of scanty results.[1,24] Unlike the classic Xpert assay tested in our study, with which resistance is ascertained on the basis of an absent signal (risking non-specific causes of error such as insufficient DNA), detection of rifampicin resistance with the Xpert Ultra assay is based on melting temperature shifts caused by rifampicin resistance-conferring mutations in the rifampicin-resistance determining region.[24] Rwanda plans to gradually implement the Xpert Ultra assay, beginning in July, 2020. The price of the Ultra and classic assays is the same and procedures are similar. Staff at peripheral health centres will be trained on interpretation of results. A postimplementation field study will need to confirm the higher specificity of the Xpert Ultra assay versus the classic Xpert test for detection of rifampicin resistance, which was previously assessed on spiked samples.[24]

One repeat Xpert assay proved insufficient to rule out rifampicin resistance, particularly in patients with heteroresistance, for which the classic Xpert assay limit of detection requires a minority population of more than 50% and is only slightly better with the Xpert Ultra assay, depending on the *rpoB* mutation.[24] Other retesting samples were tuberculosis negative, probably as a result of bacillary loads close to the limit of detection of the assay. In our setting, if the decision to start MDR-tuberculosis treatment had relied on one repeat Xpert assay, ten (16%) of 64 patients with true rifampicin resistance would have been missed. During MDR-tuberculosis treatment follow-up, half of these patients were identified as rifampicin resistant on Xpert after 1 or 2 months of treatment. Indeed, repeat testing on samples with a higher bacillary load (higher than very low) would increase the probability of detecting true rifampicin resistance.[25]

The GLI guideline recommends to accept the result of a repeat Xpert assay in patients with a low pretest probability of rifampicin resistance (eg, in new tuberculosis cases) and to consider the initial Xpert result of rifampicin resistance as definitive if the patient is at high risk of rifampicin resistance (eg, in patients needing retreatment).[10,11] However, our findings showed that a high pretest probability of rifampicin resistance (ie, in people previously treated for tuberculosis) did not lower the

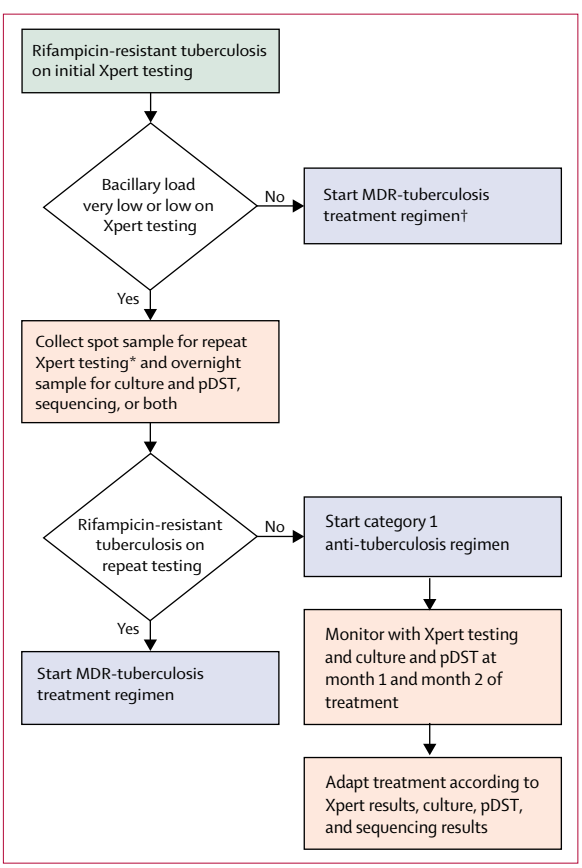

***Figure* 3: Proposed and currently implemented algorithm for initial rifampicin-resistant tuberculosis Xpert investigation implemented in Rwanda**

Category 1 anti-tuberculosis regimen consists of 6 months of rifampicin and isoniazid, complemented with ethambutol and pyrazindamid during the first 2 months of treatment. MDR=multidrug resistant. pDST=phenotypic drug susceptibility testing. Xpert=classic Xpert MTB/RIF assay. *Repeat Xpert testing should be done at the same place as the initial test, to limit delay. †Repeat Xpert testing should be done at the treatment centre, to rule out any clerical error that might happen at peripheral testing sites.

likelihood of false rifampicin resistance on Xpert testing, driven by a very low tuberculosis bacillary load rather than pretest risk. Thus, although the GLI guideline might prevent unnecessary MDR-tuberculosis treatment in new cases of tuberculosis, it would not prevent unnecessary MDR-tuberculosis treatment in many patients needing retreatment with false rifampicin resistance on one Xpert assay because of a very low bacillary load. The proposed algorithm being implemented in Rwanda since Jan 3, 2020 (figure 3) initiates an investigation of false rifampicin resistance in patients with very low or low bacillary load on Xpert testing, regardless of treatment history, whereas patients with a medium or high bacillary load continue to be swiftly transferred for MDR-tuberculosis treatment initiation at a specialised MDR-tuberculosis clinic, where a confirmatory repeat test will be done to exclude clerical error. Considering the prevalence of rifampicin-resistant tuberculosis in Rwanda, this modified flowchart could lead to approximately 60 repeat samples per year, divided over 68 testing facilities, implying a negligible increase of cost and workload.

In Rwanda, unpublished data from the National Tuberculosis control programme show the proportion of patients with a positive smear has fallen from 89% (95% CI 88–90) in 2016 to 68% (67–70) in 2017 and 41% (39–42) in 2018 ($p<0·0001$). The proportion of patients with a microscopy-negative smear, reflecting low bacillary loads, has steadily increased from 11% (95% CI 10–12) in 2016 to 59% (58–61) in 2018, as has the proportion with a very low bacillary load among the rifampicin-resistant population on Xpert—from 10% (95% CI 7–14) between 2012 and 2016 to 61% (53–68) between 2017 and 2019 ($p<0·0001$). This trend is probably attributable to the policy of using Xpert testing as the initial diagnostic method for all patients with presumptive tuberculosis in Kigali.[26] The number of patients with false rifampicin resistance is expected to increase when Xpert is used as the initial diagnostic test in all patients with presumptive tuberculosis in Rwanda. The Xpert assay is more sensitive than is smear microscopy, particularly for paucibacillary tuberculosis.[27] Moreover, patients are investigated at an early stage, typically with paucibacillary tuberculosis, because of active case-finding and the integration of tuberculosis services in the community health package.[12] Our findings and their implications in terms of a modified testing algorithm probably apply to many other settings in which the classic Xpert assay continues to be used for early diagnosis and active case-finding strategies, as per WHO recommendations.[28] Moreover, the higher risk of false rifampicin resistance with Xpert testing on samples with a low bacillary load is also important for children[29] and people with extrapulmonary tuberculosis,[30] who generally present with paucibacillary tuberculosis.

Compared with use of the Xpert assay as an initial diagnostic method (eg, in Kigali), the problem of false rifampicin resistance has not been seen when Xpert testing is used after a positive smear, including in drug-resistance surveys targeting smear-positive patients who are subsequently tested with the Xpert assay.[31] Our findings indicate that using Xpert for determination of rifampicin resistance in tuberculosis prevalence surveys, with testing irrespective of clinical symptoms or microscopy results, risks resulting in an unacceptably high error rate for false rifampicin resistance.

Our analysis had several important strengths. First, we used a large population-based sample to investigate the frequency of and factors associated with false rifampicin resistance results on Xpert assay. Since we used a national sample, our findings are representative of the general population of Rwanda, and probably of many other countries similarly characterised by a high tuberculosis notification rate, early tuberculosis diagnosis, low prevalence of rifampicin resistance, and universal Xpert testing. Second, we used a comprehensive set of reference standards, including classic Sanger sequencing, deep sequencing, and pDST. Our findings informed a revision of the Xpert testing algorithm in Rwanda and might trigger similar evaluations in other countries.

Our study also has some limitations. First, despite training, close supervision, and a quality improvement scheme initiated at all peripheral centres doing Xpert testing, the handling of samples at these sites might differ from how samples are obtained at the central MDR-tuberculosis clinic and tested at the NRL. However, our findings still represent how Xpert is used by the Rwandan tuberculosis programme. Second, the final rifampicin resistance status was not available for 54 (31%) of 175 patients (21 patients did not have data for the initial Xpert semi-quantitative bacillary load and 33 did not have a reference standard result). 40 (74%) of 54 patients had a very low bacillary load (appendix 2 p 5). As a result, we might have underestimated the proportion with false rifampicin resistance. Moreover, not all patients had results for all the different tests used in this investigation (figure 2)—in particular, deep sequencing for hetero-resistance, because of challenges related to studying paucibacillary tuberculosis. However, only one of four results found heteroresistance on deep sequencing that had been missed by the other tests. Finally, our study did not consider chronic comorbidities other than HIV. Moreover, data for CD4 cell counts, antiretroviral therapy, and viral load were not available.

In summary, a very low tuberculosis bacillary load in sputum is the main driver of false rifampicin resistance results in the Xpert assay, leading to an unacceptably low positive predictive value in settings with a low prevalence of rifampicin resistance. For as long as the classic Xpert assay is used in Rwanda, we will assess the effect of a modified Xpert testing algorithm. Among patients with paucibacillary tuberculosis, those with at least one repeat confirmed rifampicin resistance test will be treated with an MDR-tuberculosis regimen. However,

patients with no repeat Xpert test yielding rifampicin resistance at the time of diagnosis will be treated with first-line anti-tuberculosis treatment and monitored closely, including baseline culture, pDST, and Xpert testing during treatment. If the higher specificity of the Xpert Ultra assay is confirmed in field settings, and on specimens with a very low bacillary load, countries should plan to implement Xpert Ultra as soon as possible to overcome limitations associated with the classic Xpert assay, while it would be prudent to continue monitoring false rifampicin resistance in paucibacillary samples.

Contributors

BCdJ, JCSN, LR, PS, CSM, and DA designed the study. JCSN, PM, EBN, YMH, BU, EI, JMS, WBdR, FM, and JBM participated in patients' enrolment and data collection. JCSN, TD, CJM, and BCdJ analysed data. AVD, PS, TD, CSM, and GT critically revised the report. All authors contributed to writing of the report and approved the final version.

Declaration of interests

PS was a consultant for Genoscreen. All other authors declare no competing interests.

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