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LETTER

Systematic rifampicin resistance errors with Xpert® MTB/RIF Ultra: implications for regulation of genotypic assays

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Dear Editor,

With its improved ability to detect low concentrations of *Mycobacterium tuberculosis* complex DNA using trace results, Xpert® MTB/RIF Ultra (Ultra; Cepheid, Sunnyvale, CA, USA) is increasingly replacing Xpert® MTB/RIF (Xpert; Cepheid).[1,2] Although trace results do not provide genotypic drug susceptibility testing (gDST) results for rifampicin (RIF), Ultra was hypothesised to have improved performance to detect RIF resistance compared with Xpert because of the following four features.[3] First, Ultra has a better limit of detection for low-frequency mutations.[4] Second, it does not appear to have a higher rate of false resistance results with samples with very low bacillary loads.[4] Third, two synonymous mutations at the *rpo*B codons 432 and 433 no longer cause false resistance results.[4] Finally, Ultra melting temperatures (Tms) can be used to distinguish between some mutations (although it should be noted that this analysis is not integrated into the Cepheid software and must be performed separately using a script).[5–7] However, the study on which the WHO based their endorsement of Ultra only demonstrated that the specificity for RIF resistance (based on a the research use only version 2 [RUO2] of Ultra, which corresponds to Ultra version 1 that became available clinically) was non-inferior to version 5 (v5) of Xpert G4, whereas the sensitivity was similar but non-inferiority was not achieved.[2]

Because the instructions for Ultra do not reference the lack of false resistant results due to the two aforementioned synonymous *rpo*B mutations, we investigated whether this feature might also extend to other mutations.[6] Testing was carried out from positive cultures at three WHO Supranational Reference Laboratories in Borstel (Germany), Brisbane (Australia) and Johannesburg (South Africa) using eight different synonymous mutations in 10 strains that were susceptible to RIF by phenotypic DST (pDST). We also included 67 phenotypically resistant strains with 29 different non-synonymous mutations, insertions, deletions in *rpo*B, or combinations thereof to assess whether any of these are missed.[8] We used Xpert G4 v5, Ultra v2 and Ultra version 3 (v3), all of which are currently used clinically, to test 27, 59 and 18 strains of the 77 total strains (see Table). The Ultra Tms were used to predict the corresponding mutations according to Cao et al.[7] Nine susceptible and 10 resistant strains were also tested with the WHO-endorsed Hain Lifescience GenoType MTBDR*plus* v2.0 (FL-LPA). Ethics approval was not needed for this laboratory-based study using stored, anonymised strains.

With the exception of the Phe433Phe mutation, seven of the eight synonymous mutations resulted in false resistant results with either Ultra v2 or v3 (Table). By contrast, false

resistance for the FL-LPA and Xpert was only avoided for Gly453Gly as this mutation lies outside of the *rpo*B regions interrogated by these assays.[4,9]

Xpert G4 did not miss resistance in any of the strains, whenever tested (Table). This is in contrast to Ultra v2, which missed some RIF resistance for strains that only had mutations in codons 431–433. Specifically, only Gln432Lys and the deletion of codon 432 were reported as resistant, whereas four different mutations at these codons were missed completely. The Ser431Arg/Gln432Leu double mutant tested indeterminate. Ultra v3 results were available for two of these mutations (i.e. Gln432Leu and Gln432Pro, which tested indeterminate). Notably, the results for Gln432Leu and Gln432Pro were reproducible with both versions of the assay, indicating that these were systematic limitations (i.e., that repeat testing cannot be used to overcome them, as is the case with random errors).

Ultra v2 detected all other resistance mutations, with the exception of His445Arg and a combined synonymous mutation and deletion affecting codons 427–429, which were all found to be indeterminate (Table). This was in line with an earlier report that found that His445Arg was indeterminate in three of four strains.[5] Ultra v3, Xpert G4, and the FL-LPA all consistently reported His445Arg as causing RIF resistance.

The script by Cao et al. did not yield any matches for 31 Ultra Tms. Where a match was obtained, the predictions were consistent with the actual mutation in 36 of 46 cases, of which 21 were unambiguous matches (i.e., where only the exact change was predicted). Our study was limited by the fact that not all strains were tested with all assays because our primary focus was to evaluate Ultra (i.e., FL-LPA and Xpert results were only included where available). Specifically, each laboratory used the version of Ultra that it relied on for routine clinical care, with the exception of Johannesburg, which also tested some strains with v3. Each site tested locally available strains, which meant that we were unable to include the Gln432Gln mutation.

Despite these limitations, our data support three conclusions. First, even if the script by Cao et al. is refined, the prediction of the precise mutation(s) based on Ultra Tms is unlikely to be as accurate as sequencing given the large number of combinations of *rpo*B mutations. Nevertheless, we believe that it would be helpful to include it as a feature in the Cepheid software, provided this is accompanied by a disclaimer that this represents a best guess. This information could be used for a variety of purposes, such as to investigate the potential reasons for discordant DST results (e.g., when Gly453Gly is only inferred by Ultra but not the FL-LPA because this codon is only interrogated by the former assay[4,9]). Second, systematic false resistant results due to synonymous mutations are possible with Xpert G4 and Ultra v2/3. This

should be reported to clinicians as 'resistance to RIF inferred' instead of 'RIF resistance detected', as recommended by the European Laboratory Initiative based on guidance from the Global Laboratory Initiative for the FL-LPA.[10,11] In most settings, synonymous mutations are rare relative to the frequency of resistance mutations in *rpo*B. However, locally frequent strains with synonymous mutations are possible, resulting in a poor positive predictive value, as demonstrated for gDST for fluoroquinolones.[12] Countries that do not perform adequate confirmatory testing, therefore, run the risk of missing this phenomenon. Third, our observations that Ultra systematically missed some resistance mutations, which was not the case for Xpert, and that the performance of v2 appeared to differ from v3 have more profound implications for the evaluation and regulation of gDST assays.[13] In our view, any WHO-endorsement should be for a particular version of a gDST assay and should not be automatically extended to later versions as these cannot be assumed to be equivalent. Instead, manufacturers of diagnostic equipment should conduct a risk analysis (e.g., according to ISO 14971 guidelines) and provide appropriate data to justify changes, which would have to be reviewed independently and communicated to users. For example, the changes between Ultra v2 and v3 appear to have improved the ability to infer His445Arg, which is welcome, but might have adversely affected the performance of Ultra in other ways. Moreover, it is not sufficient to simply assess the performance of a gDST assay by analysing its overall sensitivity and specificity, as occurred during the Ultra validation, given that these figures are driven by the dominant resistance mutations in the study in question.[1,2] Instead, the performance for individual mutations has to be calculated.

To address this question systematically, we propose testing reference collections that are enriched for rare resistance mutations—for example, the mutations that are either completely missed or reported as indeterminate by Ultra v2. These account for 2.3% (95% CI 1.4–3.5) of RIF resistance based on WHO surveillance data from seven countries (Table).[13,14] In fact, testing such collections has already revealed that other gDST assays, including the FL-LPA, miss some *rpo*B resistance mutations.[9,15] Based on such studies, the instructions for use for each diagnostic test should clearly list the mutations that can be detected, inferred, or are missed. This data would need to be updated as new evidence becomes available. In addition, the instructions for use should disclose whether any mutations are intentionally masked or excluded by the analysis software or through the probe design. For instance, it is possible that the goal to avoid false resistance due to the synonymous mutations at codons 432 and 433 may have resulted in some resistance mutations at these codons not being reported at all by Ultra v2, or being reported as indeterminate by Ultra v3.

Clinicians have to weigh the risks and benefits of medication. In our view, the same approach should be applied to diagnostic tests. Clearly, Ultra has advantages compared with Xpert.[1–5,7] However, deficiencies in the current approach to evaluating and regulating all gDST assays have resulted in some of its limitations being unrecognised. In particular, any decision to improve the specificity of gDST assays at the expense of the sensitivity has to be disclosed for appropriate diagnostic guidelines to be developed.[10] This is especially important for Ultra because in many settings it is effectively the only assay available to diagnose RIF resistance (i.e., the diagnostic algorithms do not include routine secondary testing).

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Table FL-LPA, Xpert G4 and Ultra results and predicted mutations

Type of mutation(s) (RIF pDST result at 1 mg/L in MGIT™)	Mutation(s)	RIF resistance (R) result[†] FL-LPA (*n* = 19)	Xpert G4 v5 (*n* = 27)	Ultra v2 (*n* = 59)	Ultra v3 (*n* = 18)	Predicted mutation(s) based on Ultra Tms[‡]
Synonymous (S)	Gly426Gly (ggC/ggG)	R inferred	nt	R detected	nt	No match
	Gly426Gly (ggC/ggT)	R inferred	R detected	nt	R detected	No match
	Thr427Thr (acC/acT)	R inferred	R detected	nt	R detected	No match
	Leu430Leu (ctG/ctT)	R inferred x2	nt	R detected x2	nt	No match x2
	Phe433Phe (ttC/ttT)*	R inferred	R detected	nt	R not detected*	No match
	Pro439Pro (ccG/ccT)	R inferred x2	nt	R detected x2	nt	435Asp (GAC) deletion or 435Val (gTc) & 437Asp (Gac) x2*
	Leu452Leu (Ctg/Ttg)	nt	R detected	R detected	nt	No match
	Gly453Gly (ggG/ggC)[§]	R not detected*	R not detected*	nt	R detected*	No match
Non-synonymous (R)	Leu430Pro (ctg/cCg)	nt	nt	R detected	nt	430Pro (cCg)
	Leu430Arg (cTg/cGg) & Asp435Tyr (Gac/Tac)	nt	nt	R detected	nt	429Leu (cTg) & 435Tyr (Tac)*
	Ser431Arg (Agc/Cgc) & Gln432Leu (cAa/cTa)*	nt	R detected	R indeterminate*	nt	No match
	Gln432Leu (cAa/cTa)*[¶]	R inferred x2	R detected x3	R not detected x4*[#]	R indeterminate x3*	No match x7
	Gln432Lys (Caa/Aaa)	nt	R detected	R detected x2	nt	No match x2
	Gln432Pro (cAa/cCa)*[¶]	R inferred x2	R detected x4	R not detected x10*,**	R indeterminate x4*	No match x14
	Asp435Gly (gAc/gGc) & Leu452Pro (cTg/cCg)	nt	nt	R detected x4	nt	435Gly (gGc) & 452Pro (cCg) x4
	Asp435Tyr (Gac/Tac)	nt	nt	R detected	nt	434Ile (aTt) & 435Tyr (Tac) or 435Tyr (Tac)
	Asp435Val (gAc/gTc)	nt	nt	R detected x3	nt	435Val (gTc); 435Tyr (Tac) or 435Val (gTc) x2
	Ser441Leu (tCg/tTg)	nt	nt	R detected x2	nt	441Leu (tTg) x2
	Ser441Val (TCg/GTg)	nt	nt	R detected	nt	No match
	His445Arg (cAc/cGc)*[¶]	R inferred x3	R detected x5	R indeterminate x5*[††]	R detected x4*	No match x6; 445Arg (cGc) x3
	His445Asn (Cac/Aac)	nt	nt	R detected	nt	445Asn (Aac) or 445Leu (cTc)
	His445Asp (Cac/Gac)	nt	nt	R detected x3	nt	445Asp (Gac) or 450Phe (tTC); 445Asp (Gac) or 450Phe (tTC) or 445Ter (TaA) x2
	His445Cys (CAc/TGc)	nt	nt	R detected x2	nt	445Cys (TGc); 445Ter (TaA) or 445Cys (TGc)
	His445Leu (cAc/cTc)	nt	nt	R detected x2	nt	445Asn (Aac) or 445Leu (cTc) x2
	His445Tyr (Cac/Tac)	nt	nt	R detected x4	nt	445Asn (Aac) or 445Leu (cTc) or 445Tyr (Tac) x4
	Ser450Phe (tCG/tTT)	R inferred x2	R detected x2	nt	R detected x2	445Ser (AGc); 445Ser (AGc) or 445Cys (TGc)*
	Ser450Leu (tCg/tTg)	nt	nt	R detected x5	nt	450Leu (tTg) x5
	Ser450Trp (tCg/tGg)	nt	nt	R detected x2	nt	450Trp (tGg) x2
	Leu452Pro (cTg/cCg)	nt	nt	R detected x2	nt	452Pro (cCg) x2

Non-synonymous and synonymous (R)	Leu449Leu (ctG/ctC) & Ser450Phe (tCG/tTT)	R inferred	R detected	nt	R detected	449Met (Atg) & 450Phe (tTC)*
Insertion (R)	432Arg (CGC)*¶	nt	R detected	R not detected*	nt	No match
	433Phe (TTC)*¶	nt	R detected x3	R not detected x3*	nt	Wild type x3*
Deletion (R)	Gln432	nt	nt	R detected	nt	No match
	Asp435	nt	nt	R detected	nt	435Asp (GAC) deletion or 435Val (gTc) & 437Asp (Gac)
Deletion and synonymous (R)	Thr427Thr (acA/acG) & Ser428-Gln429*	nt	R detected	R indeterminate*	nt	No match
Deletion and non-synonymous (R)	Gln432His (caA/caC) & Phe433-Asp435 del	nt	nt	R detected x2	nt	No match x2
	Met434Asn (aTg/aAc) & Asp435 del	nt	nt	R detected	nt	434Ile (atT) & 435Tyr (Tac) or 435Tyr (Tac)*

* Notable gDST results or predicted mutations.

† Some strains were not tested. The reporting language recommended by European Laboratory Initiative <AQ>Author: Please confirm. Ed</AQ> and Global Laboratory Initiative was used for the Hain GenoType MTBDR*plus* v2.0.[10,11] Unless otherwise specified in a footnote, all gDST results were for a single strain and for different strains if more than one result are given (detailed results available on request).

‡ This is not a feature of the Cepheid software but was generated using the experimental script by Cao et al.[7]

§ Outside of *rpo*B regions interrogated by the FL-LPA and Xpert.[4,9]

¶ Account for 2.3% (95% CI 1.4–3.5) of resistant strains in a population-based surveillance study conducted by the WHO in Azerbaijan, Bangladesh, Belarus, Pakistan, the Philippines, South Africa, and Ukraine.[14]

Includes three repeats for one strain tested.

** Includes three repeats for one strain and four repeats for another. This mutation in a phenotypically resistant strain was also missed using Ultra RUO2 but detected using Xpert G4 v5 in the original validation study of Ultra.[2]

††Includes three repeats for one strain tested. This mutation tested 'indeterminate' three times and 'detected' once with Ultra v2 in an independent study.[5]

FL-LPA = first-line line-probe assay; RIF = rifampicin; pDST = phenotypic drug susceptibility testing; MGIT™ = Mycobacteria Growth Indicator Tube; Tm = melting temperature; nt = not tested; gDST = genotypic DST.