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Whole genome sequencing to complement tuberculosis drug resistance surveys in Uganda

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ABSTRACT

Understanding the circulating *Mycobacterium tuberculosis* resistance mutations is vital for better TB control strategies, especially to inform a new MDR-TB treatment programme. We complemented the phenotypic drug susceptibility testing (DST) based drug resistance surveys (DRSs) conducted in Uganda between 2008 and 2011 with Whole Genome Sequencing (WGS) of 90 *Mycobacterium tuberculosis* isolates phenotypically resistant to rifampicin and/or isoniazid to better understand the extent of drug resistance.

A total of 31 (34.4 %) patients had MDR-TB, 5 (5.6 %) mono-rifampicin resistance and 54 (60.0 %) mono-isoniazid resistance by phenotypic DST. Pyrazinamide resistance mutations were identified in 32.3% of the MDR-TB patients. Resistance to injectable agents was detected in 4/90 (4.4%), and none to fluoroquinolones or novel drugs. Compensatory mutations in *rpoC* were identified in two patients. The sensitivity and specificity of drug resistance mutations compared to phenotypic DST were for *rpoB* 88.6% and 98.1%, *katG* 60.0% and 100%, *fabG1* 16.5% and 100%, *katG* and/or *fabG1* 71.8% and 100%, *embCAB* 63.0% and 82.5%, *rrs* 11.4% and 100%, *rpsL* 20.5% and 95.7% and *rrs* and/or *rpsL* 31.8% and 95.7%.

Phylogenetic analysis showed dispersed MDR-TB isolate, with only one cluster of three Beijing family from South West Uganda.

Among tuberculosis patients in Uganda, resistance beyond first-line drugs as well as compensatory mutations remain low, and MDR-TB isolates did not arise from a dominant clone. Our findings show the potential use of sequencing for complementing DRSs or surveillance in this setting, with good specificity compared to phenotypic DST. The reported high confidence mutations can be included in molecular assays, and population-based studies can track transmission of MDR-TB including the Beijing family strains in the South West of the country.

1. Introduction

Tuberculosis remains one of the major global health problems with more than 1.8 million deaths worldwide (WHO, 2014). The increasing prevalence of multi-drug resistant tuberculosis (MDR-TB), resistant to rifampicin (RIF) and isoniazid (INH), has continued to threaten public health efforts towards tuberculosis control. The emergence of extensively drug-resistant (XDR) tuberculosis, i.e. MDR-TB with additional resistance to any fluoroquinolone and at least one of the second-line injectable agents (kanamycin, amikacin or capreomycin), has further complicated control efforts (WHO, 2010). The prevalence of MDR-TB in Uganda in the year 2011 was reported as low among new cases (1.4%), and, as expected, higher among previously treated patients, 12.1% (Lukoye et al., 2013).

Previous studies have reported imperfect agreement between genotypic and phenotypic drug susceptibility testing (DST) methods (Ocheretina et al., 2014; Rigouts et al., 2013). The recent WHO, 2015 guidelines for surveillance of drug resistant tuberculosis recommends incorporation of molecular technologies into surveys, either alone or as a screening tool prior to culture-based methods (WHO, 2015). However, drug resistance conferring mutations that are normally missed in phenotypic DST (Jamieson et al., 2014; Ocheretina et al., 2014; Walker et al., 2015) or by the rapid molecular methods such as XpertMTB/RIF assay, for resistance to RIF(Sanchez-Padilla et al., 2015),

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have been reported. Most of the patients with these mutations, but with a susceptible phenotypic DST, have been found to have poor clinical outcomes (Ho et al., 2013; Van Deun et al., 2015). Genotypic assays on the other hand, are known to produce rapid results but they may also miss certain clinically important mutations that may be outside the target region or due to other mechanisms of resistance (Merker et al., 2013; Sun et al., 2012). RIF resistance conferring mutation *rpoB* S531L is associated with the acquisition of *rpoA* and *rpoC* compensatory mutations, a combination that is found to be strongly associated with improved transmissibility of strains in patient populations (Casali et al., 2014; de Vos et al., 2013; Lanzas et al., 2013). From a model-based analysis, fitness costs of resistance-conferring mutations have been reported as key determinants for the future burden of drug resistant tuberculosis (Knight et al., 2015). Moreover, use of WGS data has been found to be vital in prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance (Walker et al., 2015).

To-date, the contribution and agreement of phenotypic and genotypic testing for such resistance-conferring mutations to first and second-line tuberculosis drugs in Uganda, where MDR-TB treatment has been available since 2012, is unknown. Documenting the prevalent resistance-conferring mutations, especially before an MDR-TB treatment programme, can provide evolutionary lessons that are vital in the implementation of molecular diagnostic tools (Niemann and Supply, 2014), anti-TB drug resistance surveillance, and interruption of the transmission chain of these strains (Nardell and Dharmadhikari, 2010; Trauner et al., 2014; Wells et al., 2013).

In the current study, we aimed at complementing the conventional DST used in the anti-tuberculosis DSRs conducted in Uganda with WGS. This study contributes baseline estimates of the proportions of resistance-conferring mutations to first and second-line tuberculosis drugs in Uganda, which can be applied in the design and deployment of future drug regimens, as well as monitoring resistance to new drug classes.

2. Materials and methods

2.1. Study setting and population

The demographic and clinical information, as well as *Mycobacterium tuberculosis* isolates, were obtained from two tuberculosis DRSs done in Uganda: one done in the capital city of Kampala (August to December 2008), and a nationwide survey (December 2009–February 2011) (Lukoye et al., 2013, 2011), with no overlap between surveys. For the present study, we considered participants having isolates with phenotypic resistance to RIF and/or INH, the two most powerful first-line anti-tuberculosis drugs.

2.2. Culture and drug susceptibility testing

Standard Löwenstein-Jensen (LJ) proportional method was used for primary isolation and to test for susceptibility to RIF (40 mg/mL), INH (0.2 mg/mL) and streptomycin (STR) (10 mg/mL) for which results were interpreted at week six, and for ethambutol (EMB; 2 mg/mL) which was interpreted at week four (Lukoye et al., 2013, 2011). All MDR isolates were phenotypically tested for kanamycin and ofloxacin resistance and no phenotypic resistance to either drug was reported. For external quality control, random samples resistant to RIF and/or INH-15 from the Kampala survey and 73 from the national survey-were retested at the supra-national reference laboratory in Borstel (Germany). Accuracy was confirmed to exceed 95% for all first-line drugs tested (Lukoye et al., 2013, 2011).

2.3. Spoligotyping

From a portion of the frozen stock of *Mycobacterium tuberculosis* isolates received at the Institute of Tropical Medicine (ITM), Antwerp, Belgium, we performed spoligotyping. Primers (DRa and DRb) targeting the direct repeat (DR) region of the genome of *Mycobacterium tuberculosis* and an in-house membrane were used according to the standard spoligotyping protocol as described (Kamerbeek et al., 1997).

2.4. Genomic DNA extraction

A portion of each frozen stock was sub-cultured on LJ medium for WGS. Scraped colonies were transferred to 150 μl of a buffer containing 0.5 M Tris (PH 8.5), 0.5 M EDTA and boiled for 5 minutes in a biosafety level three laboratory. The boiled lysates underwent genomic DNA (gDNA) extraction as previously described (Kaser et al., 2009), followed by purification in the Maxwell® 16 DNA purification Kit AS1020 (Promega, 2014). The extracted gDNA was checked for integrity and purity using agarose gel electrophoresis and the yield was estimated by a Qubit 2.0 fluorometer with dsDNA BR assay kits. The purified gDNA was used for WGS, at either Genoscreen (Lille, France) or the Beijing Genomic Institute (BGI; Hong Kong, China), with resulting data analyzed at ITM.

2.5. DNA sequencing and sequence analysis

WGS of the *Mycobacterium tuberculosis* isolates was performed following Illumina TruSeq DNA sample preparation recommendations. The fastq read pairs were processed in an on-line program, PhyResSE,version 1.0 available at https://bioinf.fz-borstel.de/mchips/phyresse/ (Feuerriegel et al., 2015). This tool maps fastq reads to the *Mycobacterium tuberculosis* reference strain H37RV (NC_000962.3) to produce drug resistance and phylogenetic single nucleotide polymorphisms (SNPs). It allows fastq files of up to 2.1 GB and approximately 1,000× coverage. These SNPs are further used to assign lineage from the literature (Feuerriegel et al., 2015; Steiner et al., 2014), experiments and other public sources (Sandgren et al., 2009) and to identify both high confidence (well supported in the literature) and low confidence (some supporting evidence) drug-resistance related mutations. The low confidence mutations were verified for their references and classification in the Tuberculosis Drug Resistance Mutation Database (TBDreamDB)(Sandgren et al., 2009). The list of genes and positions analysed for each tuberculosis drug was based on the well-characterized SNPs collected in the PhyResSE, ResiList-Master.v27 (Feuerriegel et al., 2015) available at https://bioinf.fz-borstel.de/mchips/phyresse/.

2.6. Analysis of phenotypic and genotypic discordance for RIF

2.6.1. Determination of RIF MIC

We performed MIC testing for discordant RIF resistant isolates between survey results and WGS analysis. RIF MICs were performed using LJ with drug concentrations 10, 20, 40, 80, 160, and 320 μg/mL. Bacterial suspensions were prepared in sterile 0.01% Tween 80 and adjusted to McFarland 1. Both drug containing and plain (control) LJ slants were inoculated with 10^{-2} and a second control inoculated with 10^{-4} of the bacterial suspensions. The inoculated tubes were incubated at 35 to 38 °C and read after 4 and 6 weeks of incubation. The lowest concentration with growth less than the 1/100 diluted control (10^{-4}) tube at week six was interpreted as the MIC-99 value as per the proportion method (Canetti et al., 1963).

2.6.2. rpoB sequencing

From the isolates with RIF discordance between phenotypic DST and mutation analysis, the *rpoB* gene was sequenced and analysed as previously described (Rigouts et al., 2007).

Sequence graphs for the RIF discordant isolates were investigated for evidence of hetero-resistance in terms of resistant sub-populations.

2.6.3. Phylogenetic analysis by phenotypic resistance and district

For each set of fastq reads, we undertook quality control using the nesoni version 0.13 pipeline (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) with a minimum read quality of 10, read length of 45 and removal of sequence adaptors. Sequences were aligned to the most to the recent common ancestor of the *M. tuberculosis* complex (MTBc; H37rv_NC_018143.2) (Comas et al., 2010, 2013). The nesoni consensus tool was used for SNP calling with reads that mapped to more than 1 position removed, a minimum coverage of 10 reads, minimum mapping quality of 20 and minimum base call consensus of 66%. SNPs and indels per isolate were generated using nesoni nway and a SNP alignment was created using custom python scripts. We constructed a maximum likelihood tree using Randomized Axelerated Maximum Likelihood (RAxML) version 8.2, based upon the SNP alignment and employed a generalized time-reversible (GTR) CAT model with Stamatakis ascertainment bias correction (Stamatakis, 2014). The phylogenetic tree was visualized in FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/ accessed 22nd December 2015).

2.7. Data management and analysis

Data were exported to Stata, (Stata Corp LP, College Station TX, USA) for analysis of frequencies and proportions of lineages and drug resistance mutations in relation to phenotypic testing results. Sensitivities and specificities of the resistance-conferring mutations were calculated with phenotypic drug susceptibility results as the reference standard.

Data for the drug resistance-conferring mutations as well as the phylogenetically informative mutations identified are provided, supplementary file 1, computed on 26th October 2015 using PhyResSE v1.0 and H37RV reference strain (NC_000962.3).

2.8. Ethical considerations

For the surveys, ethical approvals were obtained from the Research and Ethics Committees of Makerere University College of Health Sciences, Kampala and the Uganda National Council of Science and Technology. The present sub-study obtained additional approval from the Institutional Review Board of the ITM (Antwerp, Belgium).

3. Results

3.1. Clinical and phenotypic characteristics of participants

Of the 136 eligible patients with INH and/or RIF resistance from both surveys, isolates 25/41 (61.0%) from the Kampala survey and 88/95 (92.6%) from the nationwide survey were available for the current

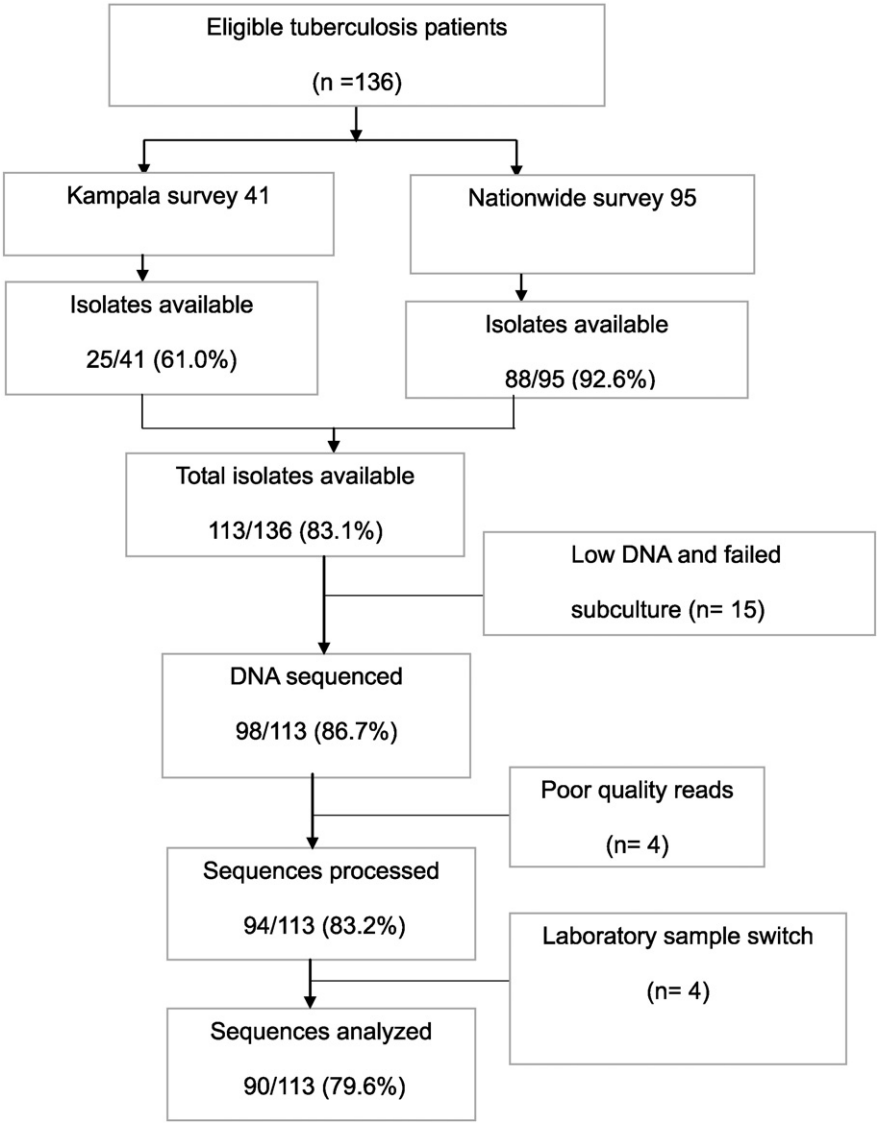

Fig. 1. Flow diagram showing the number of participants enrolled.

Table 1
Clinical and phenotypic characteristics of participants with *Mycobacterium tuberculosis* resistant to rifampicin and/or isoniazid (n = 90).

Parameter	Category	n (%)
Gender	Male	62 (68.9)
	Female	28 (31.1)
Age category (years)	18–24	18 (20.0)
	25–34	25 (27.8)
	35–44	22 (24.4)
	>44	25 (27.8)
	Median age (IQR)	35 (26–45)
HIV-status	Negative	63 (70.0)
	Positive	27 (30.0)
Treatment category	New	26 (28.9)
	Previously treated	64 (71.1)
Drug resistance	Mono-RIF	5 (5.6)
	Mono-INH	54 (60.0)
	MDR	31 (34.4)

MDR (multi-drug resistant) = Resistant to both rifampicin and isoniazid, IQR = interquartile range, RIF = rifampicin, INH = isoniazid.

study. Of the 113 total patient isolates, fifteen had low DNA quantity and failed subculture. Of the 98 (86.7%) sequenced, four samples had poor quality sequences and four, processed in one batch, were excluded from analysis due to laboratory sample switch, recognized through mismatches of spoligotype patterns between genomes and spoligotype films. The remaining 90/113 (79.6 %), isolates with WGS results were analysed, Fig. 1.

Of the 90 patients, 62 (68.9 %) were male, median age was 35 years (IQR; 26–45), 27(30.0%) were HIV-infected and 64 (71.1%) were previously treated for tuberculosis. Isolates with phenotypic mono-RIF resistance were 5 (5.6%), 54 (60.0%) were mono-INH resistant, and 31 (34.4%) were MDR-TB, Table 1. None of the MDR-TB patients' isolates had phenotypic resistance to kanamycin or ofloxacin.

From phylogenetic analysis, only two MDR-TB patients from Mbarara district and one from Rakai district were in a cluster of Beijing family isolates with identical drug resistance mutations, Fig. 2.

3.2. Frequency of *Mycobacterium tuberculosis* resistance-conferring mutations

Using sequencing results to predict drug resistance, resistance-conferring mutations to RIF were identified in 32/90 (35.6%) isolates. The most prevalent RIF resistance-conferring mutation was *rpoB*.S531L, in 16 (50.0%). Two of the MDR-TB isolates with *rpoB*.S531L also had a compensatory mutation in *rpo*C, N698S and V483A; both were from patients previously treated for tuberculosis, one of whom was HIV-infected. Mutations in the *rpoB* 526 position had the most variation with five amino acid exchanges, all classified as RIF resistant by phenotypic DST.

Resistance-conferring mutations to INH were found in 61/90 (67.8%) of the isolates. Mutations of the *katG*.S315T alone were found in 44/61 (72.1%) whereas mutations in the *fabG1* promotor region were present in 9/61 (14.8%) of the isolates and none had *inhA* mutations. One INH resistant patient had the *ahpC*-48 G/A mutation Table 2A).

Resistance-conferring mutations to EMB in the *embCAB* loci were found in 28/90 (31.1 %) isolates, with *embB*.M306V being the most

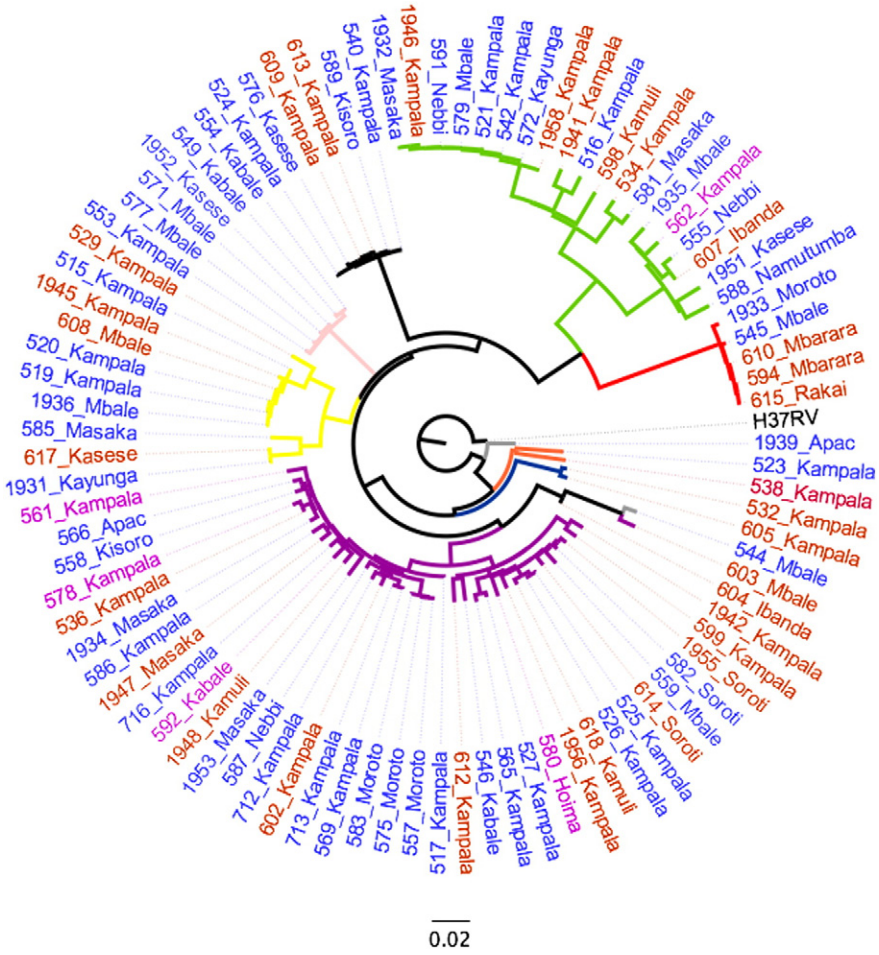

Fig. 2. Phylogenetic tree showing the distribution of RIF and/or INH resistance by districts in Uganda. Taxa colour; MDR-TB (red), INH-mono resistance (blue) and RIF-mono resistance (purple). Lineage colour; Beijing (red), CAS-Dehli (green), Harlem (blue), LAM (yellow), S-type (pink), T-unclassified (orange), T2-Uganda (purple), X1 (black), unknown (grey).

common at 7/28 (25.0%). Only one mutant, *embB*.M306V, was phenotypically sensitive to EMB.

Resistance to STR due to mutations in the *rpsL* and *rrs* loci was found in 16/90 (17.8%) isolates. Mutations in the *rpsL* were more frequent at K43R, 7/16 (43.8%). Resistant-conferring mutation *rpsL*.K43R was also found in two isolates which were phenotypically susceptible to STR. None had mutations in the *gidB* promoter.

Resistance to PZA at the *pncA* locus was identified in 11/90 (12.2 %) of the isolates and none with *rpsA*. A total of nine different resistance-conferring mutations were identified (Table 2B). Patients with MDR-TB (32.3%) also had *pncA* mutations.

Four (4.4%) MDR-TB patients had resistance-conferring mutations to injectable agents; one to kanamycin, amikacin and capreomycin in the *rrs* gene at positions G1484T and *C1402A*, one with *rrs*.A581C and two with *rrs*.C517T, the last two conferring resistance to both STR and amikacin. SNP analysis of the WGS results revealed no resistance-conferring mutations to fluoroquinolones (FQ), linezolid (*rrl*, *rplC*), para-aminosalisylic acid (*thyA*), ethionamide (*ethA*, *inhA*, *ndh*, *mshA*), STR, amikacin, capreomycin kanamycin (*gidB*, *tlyA*) or kanamycin (*eis*) (Table 2B).

Of the 90 *Mycobacterium tuberculosis* isolates, SNP analysis of the WGS for lineages revealed 5 (5.3 %) isolates of the Beijing family (lineage 2), 18 (20.0 %) CAS-Delhi (lineage 3), 2 without any specific lineage defining SNPs, but with spoligotype pattern of U and T3-ETH according to http://tbinsight.cs.rpi.edu/ (Shabbeer et al., 2012), and the remainder were sub-families within lineage 4 (11 (12.2 %) LAM, 7 (7.8 %) S-type, 36 (40.0%) T2-Uganda, and 11(12.2%) "ill-defined" (X1 = 6, T2 = 1, T = 2 and H1 = 2) (Shabbeer et al., 2012). The majority of the resistant variants in lineage 4 belonged to the T2-Uganda sub-lineage (Table 3).

3.3. Comparison of genotypic and phenotypic drug resistance testing

Analysis of sequence graph revealed mixed coverage for one discordant isolate with MIC >320, which was excluded from this comparison.

Table 2A
Frequency of *Mycobacterium tuberculosis* resistance-conferring mutations.

Drug	Locus	Mutation[ab]	Phenotype[d]	Frequency	Relative frequency (%)
RIF	*rpoB*[c]	L511P	Sensitive	1	3.1
		Q513L	Resistant	1	3.1
		D516V	Resistant	2	6.3
		D516Y + *I572F*	Resistant	1	3.1
		D516Y	Resistant	2	6.3
		H526D	Resistant	4	12.5
		H526L	Resistant	1	3.1
		H526R	Resistant	1	3.1
		H526T	Resistant	1	3.1
		H526Y	Resistant	2	6.3
		S531L	**Resistant**	**16**	**50.0**
		Total	N/A	32	N/A
	rpoC	N698S	Resistant	1	–
		V483A	Resistant	1	–
INH	*KatG* + *ahpC*	S315T + ahpC-48 G/A	Resistant	1	1.6
	katG + *fabG1*	S315T + fabG1.pro-15 C/T	Resistant	1	1.6
	KatG	**S315T**	**Resistant**	**44**	**72.1**
		S315N	Resistant	2	3.3
		S315R	Resistant	2	3.3
	fabG1	pro-8 T/C	Resistant	1	1.6
		pro-15C/T	Resistant	9	14.8
	ahpC	48G/A	Resistant	1	1.6
		Total	N/A	61	N/A
EMB	*embB* + *embA*	G406D + *embA.pro-12 C/T*	Resistant	1	3.6
		Q497R + *embA.pro 12 C/T*	Sensitive	1	3.6
		Q497R + M306I + *embA.pro 12 C/T*	Resistant	1	3.6
	embB	M306I	Resistant	1	3.6
		M306I	Sensitive	4	14.3
		M306V	**Resistant**	**6**	**21.4**
		M306V	Sensitive	1	3.6
		M306L	Resistant	3	10.7
		G406A	Resistant	2	7.1
		G406A	Sensitive	3	10.7
		G406S	Sensitive	1	3.6
		Q497R	Resistant	1	3.6
		Q497R	Sensitive	1	3.6
		M306V + G406D	Resistant	1	3.6
		G406A + *G406R* + M306I	Sensitive	1	3.6
STR	*rpsL*	Total	N/A	28	N/A
		K43R	**Resistant**	**7**	**43.8**
		K43R	Sensitive	2	12.5
		K88R	Resistant	2	12.5
	rrs	A514C	Resistant	1	6.3
		C517T	Resistant	2	12.5
		905 C-A	Resistant	1	6.3
		906 A-G	Resistant	1	6.3
		Total	N/A	16	N/A

Bold values indicate significance at most frequent resistance mutation relative to drug.
–Locus number very small to yield meaningful comparisons.
[a] Italicized mutation = Low confidence mutations.
[b] All mutations are reported in TBdreamDB (Sandgren et al., 2009).
[c] BASED on the Escherichia coli *rpoB* codon numbering system.
[d] Using LJ; RIF (40 mg/mL), INH (0.2 mg/mL) and STR (10 mg/mL) interpreted at week six and EMB (2 mg/mL) interpreted at week four.

Table 2B
Resistance conferring mutations to other drugs.

Drug	Locus	Mutation[a]	Frequency	Relative frequency (%)
Pyrazinamide		D12A	1	9.1
		P54Q	1	9.1
		D63G	1	9.1
		F94L	1	9.1
	pncA	K96R	1	9.1
		H137R	1	9.1
		R154G	1	9.1
		V180F + *T142R*	**1**	9.1
		V180F	**3**	**27.3**
	rpsA	None	0	N/A
		Total	11	N/A
Fluoroquinolone	*gyrA*[b]/*gyrB*	None	–	N/A
Kanamycin/ amikacin/capreomycin	*rrs*	G1484T + *C1402A*	1	–
STR, amikacin, capreomycin kanamycin	*gidB*, *tlyA*	None	0	N/A
STR and amikacin	*rrs*	C517T	2	–
		A514C	1	–
Linezolid	*rrl*/*rplC*	None	0	N/A
Para-Aminosalisylic_Acid	*thyA*	None	0	N/A
Ethionamide	*ethA*, *inhA*, *ndh*, *mshA*	None	0	N/A

Bold values indicate significance at most frequent resistance mutation relative to drug.
–Locus number very small to yield meaningful comparisons.
[a] Italicized mutation = Low confidence mutations.
[b] Excludes phylogenetically informative mutation gyrA.T80A.

Considering phenotypic DST as the gold standard, 31/35 patients with a resistant RIF phenotype had *rpoB* mutations (sensitivity 88.6 %). One previously treated patient of 54 patients with a susceptible RIF phenotype had the *rpoB*.L511P mutation (specificity 98.1)1 (Tables 2A and 4), as well as *katG*.S315T, *rrs*.A514C and *embB*.M306V resistance-conferring mutations and was with the isolate having a RIF MIC of 320 μg/ml. Of the four isolates having phenotypic resistance without detectable RIF resistance-conferring mutations and no evidence of mixed coverage/infection, three had MIC around the cut-off (MIC = 40 μg/ml) and one had MIC indicating low level RIF resistance (MIC = 80 μg/ml), Table 5.

Of the 85 patients with an INH-resistant phenotype, 51 had *katG* mutations (sensitivity 60.0%) whereas 14/85 had *fabG1* mutations, including one with *ahpC* mutation (sensitivity 16.5 %). A combination of *katG and fabG1* together with *ahpC* increased the sensitivity for INH-related mutations to 71.8%. All isolates with INH resistance-conferring mutations had INH-resistant phenotypes resulting in genotype specificity of 100%, (Tables 2A and 4).

Of the 27 patients with a resistant phenotype to EMB, 17 had *embAB* mutations (sensitivity 63.0 %). Eleven patients had a susceptible phenotype to EMB, but with *embAB* mutations (specificity 82.5 %).

Of the 44 patients with a STR- resistant phenotype, five had mutations in the *rrs* locus (sensitivity 11.4 %) and nine in the *rpsL* locus (sensitivity 20.5 %). A combination of *rrs* and *rpsL* increased the sensitivity for STR resistance-conferring mutations to 31.88% (Tables 2A and 4).

Table 3
Mycobacterium tuberculosis lineages stratified by resistance variants assigned by SNP analysis.

Sub-lineage	Overall n (%)	RIF	INH	EMB	STR
Beijing (lineage 2)	5 (5.3)	3	3	3	3
CAS-Delhi (lineage 3)[a]	18 (20.0)	7	13	10	4
Euro-American (Lineage 4)					
LAM	11 (12.2)	4	10	3	2
S-type	7 (7.8)	1	4	0	0
T2-Uganda	36 (40.0)	16	19	7	7
ill-defined (lineage 4)[*]ǂ	11 (12.2)	4	10	4	0
Unknown[*]	2 (2.1)	1	2	1	0
Total	90	32	61	28	16

ǂ = X1 = 6, T2 = 1, T = 2, and H1 = 2, Unknown = U and T3-ETH, RIF = rifampicin, INH = isoniazid, EMB = ethambutol, STR = streptomycin.
[*] Lineage assigned by http://tbinsight.cs.rpi.edu/.
[a] One with no resistance SNP detected had mixed coverages of lineage 3 and lineage 4.

4. Discussion

Molecular diagnostic methods are revolutionizing drug resistance testing in tuberculosis, especially in the high-burden countries where phenotypic methods are scarcely available. Ours is one of the first studies to use WGS on a population-representative sample of RIF and/or INH-resistant strains from sub-Saharan Africa, showing the potential use of sequencing for complementing DRSs or surveillance in this setting. Specifically, our results indicate which mutations may be important to include in assays to replace phenotypic DST where this reference standard for drug resistance surveillance cannot be feasibly applied.

However, these findings also raise questions about the value of phenotypic DST versus sequencing, since among survey patients who were reported as resistant to RIF and INH, WGS for RIF, compared to DST on LJ proportion (culture) method, had a sensitivity of only 88.6% and specificity of 98.1%. The low sensitivity of *rpoB* mutations for detecting phenotypic resistance is unusual, yet the incomplete specificity may indicate poor performance of the reference standard. The only patient reported as susceptible by phenotypic DST had *rpoB*.L511P (MIC 320), a mutation that has been previously found to be missed by culture-based DST (Rigouts et al., 2013; Van Deun et al., 2013). This study also documents a variety of mutations in *rpoB*, which have been found to have different MICs including reproducibility challenges in liquid culture systems (Jamieson et al., 2014; Ocheretina et al., 2014; Rigouts et al., 2013). Such discordances, with poor clinical outcome documented for these mutations, challenge the recommendation that preferably phenotypic DST should be undertaken using liquid culture, in countries where these 'disputed' mutations are likely to be more frequent. One patient had D516Y + I572F, and the *rpoB*.I572F mutation, which is outside the *rpoB* "hot-spot" region, and has been found in 30% of RIF resistant patients in Swaziland and at lower frequency among clinical isolates in Australia (Sanchez-Padilla et al., 2015; Yuen et al., 1999). The mutations outside the *rpoB* "hot spot" region are more likely to be missed in an Xpert MTB/RIF-based drug resistance survey/surveillance. Our findings further support a recommendation for combined phenotypic and molecular RIF DST.

Table 4
Sensitivity and specificity of genotypic compared with phenotypic drug susceptibility testing.

		Resistant		Susceptible		Accuracy values	
Drug	Locus[ab]	with mutation	without mutation	with mutation	without mutation	Sensitivity (%)	Specificity (%)
RIF	*rpoB*	31	4*	1	53	88.6	98.1
INH	*katG*	51	34	0	5	60.0	100.0
	fabG1 + *ahpC*	14	71	0	5	16.5	100.0
	katG and/or *fabG1* + *ahpC*	61	24	0	5	71.8	100.0
EMB	*embAB*	17	10	11	52	63.0	82.5
STR	*rrs*	5	39	0	46	11.4	100.0
	rpsL	9	35	2	44	20.5	95.7
	rrs and/or *rpsL*	14	3	2	44	31.8	95.7

RIF = rifampicin, INH = isoniazid, EMB = ethambutol, STR = streptomycin.
* Excludes one patient with no genotypic resistance but with mixed infection.
[a] Includes high and/or low confidence according to TBdreamDB and patients with more than one resistance conferring mutations.
[b] Excludes phylogenetically informative mutations, shown in supplementary file 1.

On investigation of the (4/35, or 11.4 %) patients' isolates that were phenotypically resistant to RIF yet with wild-type genotype, despite excellent results of QC and EQA in the surveys, mixed sequence coverage as well as MICs around the break-point were the most likely explanations of these discordances.

The two MDR-TB patients with *rpoB*.S531L concurrent with compensatory *rpoC* N698S and V483A mutations suggest that these strains have circulated longer and were able to select for compensatory mutations that may enhance transmission (de Vos et al., 2013), although both patients were previously treated for tuberculosis and these mutations may have developed *de novo* in these patients. Of the three MDR-TB patients involved in a cluster, all of Beijing lineage, one had *rpoC* N698S. While the DRS design does not permit inferences on transmission rates, the diversity of resistance mutations and low clustering suggest that MDR-TB in Uganda is not caused by a limited number of resistant clones that are actively transmitted. Follow-up surveillance of the one small Beijing MDR cluster would however be warranted.

Mutations at the *katG* gene were the most frequent resistance markers for INH, followed by *fabG1*. Mutations at *katG* codon 315 have been associated with unfavourable treatment outcome and high-level INH resistance (Ando et al., 2010; Huyen et al., 2013; Tolani et al., 2012). Although there is limited data on the role of *ahpC* mutations towards INH resistance, the only patient with an *ahpC* mutation also had an INH resistance phenotype. At global level the proportion of mutations in the *katG* is correlated with tuberculosis incidence, i.e. transmission rates (Cohen et al., 2004). However, the low-level INH resistance conferred by *fabG1* alone, or even the higher level resistance conferred by *katG*, may still be overcome by higher doses of INH (Katiyar et al., 2008). Furthermore, the combination of *katG* and *fabG1* promoter mutations tends to confer very high MICs (Vilcheze and Jacobs, 2014) that obviate the further use of INH. Twenty-four (44.4%) of the INH phenotypic resistant patients were found to have wild type *Mycobacterium tuberculosis* in *ahpC*, *inhA*, *fabG1*, *katG* or *ndh*, although we do not know the level of INH resistance, as the survey results were only interpreted at 0.2 μg/mL confirming the limited sensitivity of INH susceptibility testing using genotypic methods. Since the impact on phenotypic resistance of each variant is different, a sequencing approach could offer an optimally early and accurate diagnosis.

The high frequency of EMB discordance between genotypic and phenotypic testing reported in previous and the current study indicates the challenges of EMB resistance testing using conventional methods. The difference between the epidemiological cutoff (ECOFF) and MIC for EMB is small (Cui et al., 2014). This complicates EMB phenotypic DST, warranting larger studies to directly correlate different EMB mutations with patient outcome so as to clarify their clinical importance.

Mutations at *rpsL* codon 43 have been previously associated with MDR-TB (Jnawali et al., 2013; Spies et al., 2011) and found to carry no fitness cost (Spies et al., 2013). Indeed, we found 7/9 (77.8%) of patients with *rpsL* codon 43 to have MDR-TB, however, two patients with mutations at codon 43 had susceptible STR phenotype. Mutations at the *rrs* locus have been associated with resistance to aminoglycosides (Georghiou et al., 2012; Jugheli et al., 2009). We found 4/5 (80.0 %) patients with mutations at the *rrs* locus to have MDR-TB, which indicates pre-XDR TB. Mutations at *rpsL* and *rrs* occurred independently, indicating a possible unique mechanism of their occurrence, which can be exploited in new molecular diagnostic tools for STR resistance. Overall, looking at the four main TB drugs, our findings are in agreement with the previous studies (Dominguez et al., 2016).

Phenotypic DST for PZA was not performed in the current study. Similar to previous studies (Cuevas-Cordoba et al., 2013; Napiorkowska et al., 2014), among the eleven patients with *pncA* mutations, nine (81.8%) different mutations were identified. In Uganda PZA is included in both treatment regimens for susceptible and MDR-TB patients. The fact that 32.3% of the MDR-TB patients also had *pncA* mutations raises the question whether continued use of PZA in MDR-TB treatment regimens for these patients still adds benefit (Chang et al., 2012; Miotto et al., 2015), a question which has to date not been sufficiently addressed.

Only four (4.4%) MDR-TB patients were found to have resistance-conferring mutations to injectables, of which three were mutations

Table 5
Clinical, phenotypic and genotypic characteristics of *Mycobacterium tuberculosis* strains with rifampicin discordance between phenotypic DST, MIC and *rpoB* mutation analysis.

ID	Age/sex	HIV	Previous TB treatment	Phenotypic drug resistance†	Lineage	WGS	rpoB-PCR	MIC99 (μg/ml)	Final interpretation
561	25, F	Negative	Yes	RIF	T2-Uganda	WT	WT	40	S
581	24, M	Positive	Yes	INH, STR	CAS-Delhi	*rpoB*.L511P, *embB*.M306V, *katG*.S315T, *rrs*.A514C	Pro511(CCG)	320	R
592	28, M	Negative	Yes	RIF, STR	T2-Uganda	WT	WT	80	R
602	20, M	Negative	Yes	RIF, INH, EMB, STR	T2-Uganda	WT	WT	40	S
607*	42, M	Negative	Yes	RIF, INH, STR	CAS-Delhi	*katG*.S315T	WT	>320	R
618	35, M	Negative	Yes	RIF, INH, STR	T2-Uganda	WT	WT	40	S

RIF = rifampicin, INH = isoniazid, EMB = ethambutol, STR = streptomycin, MTB = *Mycobacterium tuberculosis*, MIC = minimum inhibition concentration, WGS = whole genome sequencing, † = drug resistance testing (DST) performed using Lowenstein Jensen (LJ); RIF (40 mg/mL), INH (0.2 mg/mL) and STR (10 mg/mL) interpreted at week six and EMB (2 mg/mL) interpreted at week four.
* Mixed coverage of resistance and lineage.

conferring resistance to both STR and amikacin. Lack of evidence for presence of resistance conferring mutations to fluoroquinolones, linezolid (*rrl*), para-aminosalisylic acid (*thyA*), ethionamide (*ethA*, *inhA*, *ndh*, *mshA*), cross-resistance to STR, amikacin, capreomycin kanamycin (*gidB*, *tlyA*) or kanamycin (*eis*) among these patients confirms that most MDR-TB in Uganda is not yet complicated by resistance to 2nd line drugs.

Our study has limitations. First, our selection criteria only considered patients with RIF and/or INH phenotypic resistance, precluding prevalence estimates across the entire DRSs. While this selection did allow us to look at gene mutations in a population-representative sample of isolates with phenotypic resistance to the two drugs that define MDR-TB, it could provide only limited information about resistance-conferring mutations in isolates that were phenotypically susceptible. As a consequence, specificity estimates for genotypic resistance for RIF related to isolates that were phenotypically resistant to INH and vice versa, which may have introduced bias.

Similarly, our results for EMB, STR and PZA may not be representative for all resistance to these drugs in Uganda. Secondly, we only managed to obtain isolates from 66.2% of the eligible patients, and had less power to correlate patient characteristic and drug resistance mutations. Nevertheless, the strength of this study, one of the first of its kind, consists of the genome sequence based screen for all resistance conferring mutations embedded in a nationwide drug resistance survey.

5. Conclusions

Among tuberculosis patients in Uganda, resistance beyond first-line drugs as well as compensatory mutations remain low, and MDR-TB isolates did not arise from a dominant clone. The reported important "high confidence" mutations, if included in molecular assays, can replace phenotypic DST where this reference standard for drug resistance surveillance cannot be feasibly applied. However, the imperfect correlation of drug resistance-conferring mutations with phenotypic testing raises concerns, resolution of which requires larger studies that include analysis of clinical outcomes. The one clone of MDR-TB of Beijing family isolates in the South Western Uganda that needs to be monitored. Together, these results are encouraging from a public health perspective, as effective MDR treatment regimens, combined with solid patient support, stand a good chance to prevent the emergence of XDR-TB in this setting.

Conflict of interests

None of the authors had a conflict of interest to declare.

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The raw sequence reads were deposited in the European Nucleotide Archive at (http://www.ebi.ac.uk/ena/data/view/PRJEB10533).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.02.019.

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