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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, Resi-List-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.