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Genotypic Drug Resistance using Whole-genome Sequencing of *Mycobacterium tuberculosis* Clinical Isolates from North-western Tanzania

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Abstract

Background—Drug resistant Tuberculosis (TB) is considered a global public health threat. Whole-genome sequencing (WGS) is a new technology for tuberculosis (TB) diagnostics and is capable of providing rapid drug resistance profiles and genotypes for epidemiologic surveillance. Therefore, we used WGS to determine genotypic drug resistance profiles and genetic diversity of drug resistant *Mycobacterium tuberculosis* isolates from Mwanza, North-western Tanzania.

Methods—A cross-sectional study was conducted at the Bugando Medical Centre (BMC) from September 2014 to June 2015. Consecutively, smear-positive newly diagnosed TB patients aged ≥18 years were enrolled. Sputum samples were cultured on Löwenstein-Jensen (LJ) slants. Mycobacterial genomic DNA was extracted for WGS to determine drug resistant mutations for first and second line drugs as well as the spoligotypes.

Results—A total of 78 newly diagnosed patients with pulmonary TB with a median age of 37 [IQR: 30 – 46] years were enrolled. Of these, 57.8% (45/74) were males and 34.6% (27/78) were HIV infected. *Mycobacterium tuberculosis* genomic DNA for WGS was obtained from isolates in 74 (94.9%) patients. Of the 74 isolates, six (8.1%) isolates harbored mutations for resistance to at least one drug. The resistance to the drugs was isoniazid 3/74 (4.1%), rifampicin mono-resistant 2/74 (2.7%), ethambutol 2/74 (2.7%) and streptomycin 1/74 (1.4%). None was isoniazid mono-resistant. Of the 74 only one (1.4%) patient had MDR-TB. The resistance to ethionamide, the

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AUTHORS CONTRIBUTIONS

Edited and reviewed critically the manuscript: BRK SEM OO DWF. Conceived and designed the experiment: BRK SEM OO DWF. Performed the experiment: BRK OO. Analyzed the data: BRK OO DWF Contributed reagents/materials/analysis tools: BRK SEM OO DWF.

second line drug, was detected in one patient (1.4%). None was resistant to pyrazinamide, fluoroquinolones, kanamycin, amikacin, or capreomycin. The mutations detected were *mabA-inhA* promoter region C(-15)T and *katG* Ser513Thr for isoniazid; *rpoB* His526Leu and *rpoB* Ser531Leu for rifampicin; *embB* Met306Val and *embB* Met306Ile for ethambutol; *rpsL* Lys43Arg for streptomycin; and *mabA-inhA* promoter region C(-15)T for ethionamide. The spoligotypes of the drug resistant *Mycobacterium tuberculosis* were distinct to all six isolates and belonged to T1, T2, T3-ETH, CAS1-DELHI, EAI5 and LAM11-ZWE lineages.

Conclusion—The genetic drug resistance profile of *Mycobacterium tuberculosis* isolates from North-western Tanzania comprises of the common previously reported mutations. The prevalence of resistance to first and second line drugs including MDR-TB is low. Six drug resistant strains exhibited different spoligotypes, suggesting limited transmission of drug resistant strains in the region.

INTRODUCTION

Drug resistant Tuberculosis (DR-TB) is considered a global public health threat [1]. Globally in 2015, WHO estimated that 3.9% of new cases and 21% of previously treated cases had multi drug resistant TB (MDR-TB), defined as resistant to both isoniazid and rifampicin [1]. In 2015 there were 480 000 incident cases of MDR-TB with 250 000 deaths [1]. Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB with additional resistance to any fluoroquinolone and one of the second-line injectable anti-TB drugs, amikacin, capreomycin or kanamycin. By the end of 2015, XDR-TB had been reported by 117 countries with 9.5% of MDR-TB cases being XDR-TB [1]. Both MDR-TB and XDR-TB require extensive treatment with multiple, potentially toxic drugs, and treatment outcomes remain unacceptably poor. Estimates show that 52% of patients with MDR-TB and less than 30% of patients with XDR-TB survive [1], [2].

Tanzania is not among the 30 countries with the severe burden of MDR-TB in the world and in 2015 WHO estimated that 1.3% of new cases and 4.7% of previously treated cases are MDR-TB [1]. In Tanzania, since 2000 to date eight studies have reported low prevalence of phenotypic TB drug resistance with the prevalence MDR-TB ranged from 0.4 – 5.7% among new cases and from 3.9 – 11.1% among previously treated cases [3 - 10]. Among new cases resistance to isoniazid is high at a prevalence range from 3.0 – 6.3%, whereas for rifampicin it ranges from 0.5 – 5.7% [3 - 10]. Estimates by WHO in 2015 show that in Tanzania less than 1% were notified tested for rifampicin resistance among new cases and 3.0% among previously treated cases. These studies in Tanzania have reported the resistance to any of the first line TB drugs among new patients to range from 5 – 11.4% [3 - 10] with the low occurrence of resistance to rifampicin, streptomycin and ethambutol and pyrazinamide [3 - 10]. Data are very limited on reported prevalence of resistance to the second line TB drugs.

Understanding the circulating *Mycobacterium tuberculosis* resistance mutations is vital for better TB control strategies, especially to inform a new MDR-TB treatment programme. However, in Tanzania only two studies, with a total number of 35 isolates analyzed, have reported the mutations conferring resistance to TB drugs by sequencing the *M. tuberculosis* genes; *rpoB* (rifampin), *inhA* (isoniazid, ethionamide), *katG* (isoniazid), *embB* (ethambutol),

gyrA (fluoroquinolones), *rrs* (streptomycin, amikacin, kanamycin, capreomycin), *eis* (kanamycin) and *pncA* (pyrazinamide) [5, 11]. In these studies the mutations reported for isoniazid were all at 531 *katG*, for rifampicin were all at the 81-bp rifampicin resistance determine region (RRDR). Mutations conferring resistance to ethambutol has been reported at *embB* gene position 378, 306, 406 and 319, and for pyrazinamide were at *pncA* gene position 128; 111, 49, 179, and 169. No mutation were detected at *gyrA*, *rrs* or *eis* [5, 11]. One previous study has shown that the common clades circulating in this region are T, LAM, CAS, EAI and Beijing [12].

HIV infection, increased urbanization and international migration, and refugees movements; coupled with poor control of infection easily allow the spread DR-TB. There are no recent data on drug resistance and the causative mutations from North-western Tanzania. This region is surrounded by Democratic Republic of Congo, Burundi, Rwanda, Uganda and Kenya where there has been instability and reports of increased drug resistance [1]. Therefore monitoring for drug resistance is essential, though continuous surveillance system for DR-TB is resource demanding, requires finance and personnel with high expertise. The last DR-TB surveillance in Tanzania was conducted during 2005 – 2009 [1].

WHO proposed expanding rapid testing and detection of cases to tackle the global DR-TB crisis [13]. Phenotypic methods for detection of TB drug resistance are expensive, technically complex and time consuming [14], with the possibility of sample contamination or failure to grow in specific testing media. Targeted molecular tests, such as Xpert MTB/RIF and other PCR-based methods, although more rapid, only examine a limited number of target regions [15]. WGS is a newer alternative for TB diagnostics, is capable of providing rapid and comprehensive view of drug resistance profiles, species identification and genotype of *Mycobacterium tuberculosis* within a clinically relevant timeframe [16, 17]. In addition, it provides the highest resolution when investigating outbreaks [18].

Therefore, we used WGS to determine genotypic drug resistance profiles and genetic diversity of drug resistant M*ycobacterium tuberculosis* isolates from North-western Tanzania. This insight is necessary for the epidemiology of TB as it may identify mutations and genetic diversity of drug resistant *Mycobacterium tuberculosis* isolates available in the region with the ultimate goal of improving management and control of tuberculosis infection. Furthermore, it provides more information about the mutations and their differences in transmission fitness.

PATIENTS, MATERIALS AND METHODS

Study setting and duration

This study was conducted at the Bugando Medical Centre (BMC), Mwanza Tanzania from September 2014 to June 2015. BMC is a 900-bed tertiary hospital serving a population of over 10 million people from six regions and has the Zonal TB Laboratory in North-western Tanzania (www.bugandomedicalcentre.go.tz).

Study design

This was a cross sectional study in which we enrolled, consecutively all consenting smear-positive newly diagnosed TB patients aged ≥ 18 years. Three consecutive sputum samples (spot, early morning and spot) were taken from each patient according the Tanzania National TB and Leprosy guidelines. Only the sample with the highest ZN smear grade for each patient was further processed and cultured.

Sputum sample processing and culture

Sputum samples (2.5 - 10 ml) were processed by the standard N-acetyl L-cystein (NALC)-NaOH method [19]. The sediment was reconstituted to 2.5 ml with phosphate buffer pH 6.8 [20] and inoculated on two Löwenstein-Jensen (LJ) slants, one containing 0.75% glycerol and the other containing 0.6% pyruvate. LJ slants were incubated at 37°C and examined weekly for growth. Cultures were considered negative when no colonies were seen after 8 weeks.

Genomic DNA Extraction

Colonies were harvested from LJ slants for genomic DNA extraction, transferred to Tris-HCl–EDTA buffer and heated for 2 hours at 90°C. Genomic DNA (gDNA) was extracted using the cetyltrimethylammonium bromide (CTAB)-phenol chloroform method [21], 2 ul of isolated gDNA was quantified using NanoDrop 2000 spectrtophotometer (Thermo Scientific, USA). 260/280 ratio of all gDNA preps was between 1.7 and 2.0. The gDNA was diluted to 30 ng/ul and 50 ul was shipped to Wadsworth Center, New York State Department of Health, Albany, New York, USA for WGS analysis.

Whole genome sequencing

The average DNA yields were measured by Qubit fluorometry, and the success rate for WGS was evaluated. The whole genome sequencing (WGS) was performed as described previously [17]. Briefly, the paired-end 250-bp DNA sequencing was carried out using the Illumina MiSeq platform following Nextera XT library prep with a 15-cycle PCR indexing step. Sequencing runs were composed fully of MTBC (15 to 17) samples. A negative control was included through each library preparation and on each sequencing run. The Wadsworth Center TB WGS bioinformatics pipeline was followed as described previously [17]. Briefly, the raw reads were mapped on the *M. tuberculosis* H37Rv reference genome using BWA-MEM version 0.7.12 [22]and sorted using SAMtools version 0.1.19 [23]. Read duplicates were marked using Picard tools version 1.129 (http://broadinstitute.github.io/picard/). Indels were realigned with GATK IndelRealigner, and SNPs and indels were called separately using GATK UnifiedGenotyper version 3.3 [24], allowing for a ploidy of 2 and a minimum mapping quality of Phred 20. A ploidy of 2 for SNP detection is required to detect emerging resistant subpopulations. Low-quality positions were automatically rejected and assigned “N” for an unknown state. Each genomic position was assessed and filtered with a minimum depth (DP) of 10, mapping quality score (MQ) of 40, minimum quality per depth (QD) of 2, maximum Fisher’s exact test to detect strand bias (FS) of 200, and a minimum read positive rank sum value of −20. All positions failing these requirements were also designated unknown (N) when creating the consensus sequence and identifying resistance-associated

mutations. Positions showing heterogeneity were also designated unknown (N) in the consensus sequence. Lumpy-SV version 0.2.9 [25] was used to screen for the presence of larger deletions in the sequenced genomes that could account for antibiotic resistance. Large deletions detected by Lumpy-SV must be confirmed by the absence of mapped reads over the deleted region to be valid. Reports generated by the Wadsworth Center TB WGS bioinformatics pipeline include species identification, spoligotype, and resistance-associated mutations for 10 drugs and drug classes (RIF, INH, PZA, EMB, SM, ETH, KAN, AMK, CPR and FLQ). The genes and non-coding regions studied were *rpoB* (rifampin), *mabA-inhA* promoter (isoniazid, ethionamide), *katG* (isoniazid), *mabA* (isoniazid, ethionamide) *oxyR-ahpC* (isoniazid) *embB* (ethambutol), *gyrA* and *gyrB* (fluoroquinolones), *rrs* (streptomycin, amikacin, kanamycin, capreomycin), *rpsL* (streptomycin) *eis* promoter (kanamycin) and *pncA* and *pncA* promoter (pyrazinamide). Any mutations present in 13 resistance-associated genes or noncoding regions were identified. However, only a select list of 64 “high-confidence” and selected “low-confidence” SNPs, insertions, and deletions across these 13 genes and noncoding regions were used to predict resistance as described previously [17]. The same negative control used for library preparation and sequencing was also used as a control for bioinformatic analysis. The control was considered passing if the DP of mapped reads to the H37Rv reference sequence was less than 5×. Samples exceeding a 40× genome-wide average DP, 20× DP for each SNP locus used to predict resistance, and reference genome coverage of at least 95% were considered acceptable and included in the analysis. Species identifications were made using Kraken version 0.10.5-beta [26] on the raw reads, utilizing a local database created from available fully sequenced and draft genomes of *Mycobacterium* species from NCBI. To improve runtime without affecting the accuracy of the SNP calling, samples exceeding 80× genome-wide average depth were downsampled using SAMtools to achieve an average depth of ~80× prior to the SNP calling step.

The resulting spoligotypes were compared to existing patterns in an international genotyping database SITVIT2, available at: http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/, and assigned spoligotype international type (SIT) if they shared identical spoligotype patterns with patterns present in the existing database. Spoligotypes that had a match with a single database isolate, not yet assigned a number, were considered as orphans. The *in-silico* spoligotypes derived from WGS were confirmed using the Luminex MagPlex magnetic microspheres spoligotyping method as described previously [27].

HIV testing

Blood samples were collected for determining HIV status as per Tanzania National AIDS Control Programme Guidelines [28].

Data analysis

Data were analyzed with STATA version 12 software. Summarization of categorical variables utilized percentages while medians with interquartile range (IQR) was calculated for continuous variables. The main outcome was genetic drug resistance. Descriptive statistics were done to describe the distribution of the main outcome across relevant strata (age groups, sex and HIV status).

Ethical considerations

The study protocol and consent forms were reviewed and approved by the joint CUHAS/BMC ethics review committee and the Weill Cornell Medical College Institutional Review Board (IRB). Patients were identified and managed according to the Tanzania NTLP guidelines. Written informed consent to participate in the study was obtained from all enrolled participants.

RESULTS

A total of 78 newly diagnosed patients with pulmonary TB with a median age of 37 [IQR: 30 – 46] years were enrolled. Of these, 57.8% (45/74) were males and 34.6% (27/78) were HIV positive. *Mycobacterium tuberculosis* genomic DNA suitable for WGS analysis was obtained in isolates from 74 (94.9%) participants.

Drug Resistance Mutations

There were 6/74 (8.1%) patients harboring isolates resistant to at least one drug. The resistance to the drugs was isoniazid 3/74 (4.1%), rifampicin mono-resistant 2/74 (2.7%), ethambutol 2/74 (2.7%) and streptomycin 1/74 (1.4%). None was isoniazid mono-resistant. MDR-TB was detected in one (1.4%) of 74 patients. The resistance to ethionamide, the second line drug, was detected in one patient (1.4%). None was resistant to pyrazinamide, fluoroquinolones, kanamycin, amikacin or capreomycin. Table 1 summarizes the genotypic drug resistance of the 74 *Mycobacterium tuberculosis* isolates.

The mutations detected were *mabA-inhA* promoter region C(-15)T and *katG* S513T for isoniazid; *rpoB* His526Leu and *rpoB* Ser531Leu for rifampicin; *embB* Met306Val and *embB* Met306Ile for ethambutol; *rpsL* Lys43Arg for streptomycin; and *mabA-inhA* promoter region C(-15)T for ethionamide. One of the isolates with *rpoB* mutation at Ser531Leu had a compensatory *rpoC* mutation Glyc332Arg. This was the MDR-TB isolate. Table 2 summarizes the descriptions of patients, genotypic resistance, mutations for resistance and spoligotypes.

Genetic Diversity of Drug Resistant *Mycobacterium tuberculosis*

The six drug resistant isolates were from six distinct spoligotype lineage: T1 (Ill defined), T2 (Ill defined), T3-ETH (Ill defined), CAS1-DELHI (Central Asian Strain), EAI5 (East-African_Indian) and LAM11-ZWE (Latin-American-Mediterranean). Of note, the MDR-TB aroused from T3-ETH. The *in-silico* spoligotypes derived from WGS had a perfect match with those obtained from Luminex MagPlex magnetic microspheres spoligotyping method. Table 2 summarizes the descriptions of patients, genotypic resistance, genetic mutations for resistance and spoligotypes.

Patients Treatment Outcome

All patients were newly diagnosed and were treated according to the National standard regimen for treating new smear positive pulmonary tuberculosis which includes four anti-tuberculous drugs, namely isoniazid, rifampicin ethambutol and pyrazinamide for two months followed by isoniazid and rifampicin for four months. Of the 74 patients, 66 (89.2%)

were cured, 6 (8.1%) died, one (1.4%) was transferred out and one (1.4%) defaulted. Table 2 summarizes the descriptions of patients and treatment outcome, genotypic resistance, mutations for resistance and spoligotypes.

DISCUSSION

WGS is a new tool in TB diagnostics and is capable of providing rapid drug resistance profiles while performing species identification and genotyping. Understanding the circulating *Mycobacterium tuberculosis* resistance mutations is vital for better TB control strategies, especially to inform a new MDR-TB treatment programme. We found low prevalence of resistance to first and second line TB drugs as well as for the MDR. Likewise, no resistance to pyrazinamide fluoroquinolones and kanamycin was detected. Our result is similar to the previous studies, except for the prevalence of resistance to isoniazid, which is low compared to most of the previous studies [3 – 10, 29]. Isoniazid had high rate of resistance as it is commonly used as a prophylactic drug. This low prevalence of resistance to primary TB drugs as well as for the MDR could be attributed to the effort of the TB control programme in Tanzania as well as to the control for transmission of HIV infection. Our prevalence of MDR-TB is low despite the fact MDR-TB is an emerging problem in many parts of the world, and levels of MDR-TB among new TB patients are increasing in sub-Saharan Africa. A Systematic review by Kidenya *et al.*, reports the prevalence of MDR-TB among new cases for the past 15 years in Tanzania ranges from 0.4 – 2.1% and within East Africa 0.4 – 4.4% [29].

We found common mutations conferring resistance to TB drugs as our findings are consistent with prior reports [29, 30]. The mutations detected were *mabA-inhA* promoter region C(-15)T and *katG* Ser513Thr for isoniazid. Over 90% of isoniazid resistant strains can be predicted using the high-confidence mutations on the *katG, inhA*(-15) and *oxy-ahpC.* The *katG* encodes for catalase-peroxidase and its mutation confers high level of isoniazid resistance. The *mabA-inhA* intergenic region encodes for enoyl-ACP reductase promoter and once mutated it causes low level of isoniazid resistance as well a resistance to ethionamide. The *oxy-ahpC* gene encodes for alky hydro-reductase and mutations thereof causes low level of resistance to isoniazid [31]. Of the three isoniazid resistant strains in our study, two had high level resistance with a *katG* mutation and one low level resistance with a *mabA-inhA* promoter region mutation. We found His526Leu and Ser531Leu *rpoB* mutations for rifampicin. Over 95% of rifampicin resistant strains can be predicted using the high-confidence mutations on the *rpoB* gene which encodes β subunit of the RNA polymerase [31, 32]. A review of all MDR-TB for the past 15 years in East Africa has shown that 88% had mutations in the short, 81 base pair core region of the *rpoB* gene [29]. We found the mutations on *embB* Met306Val and Met306Ile for ethambutol. About 70% of resistance to ethambutol can be predicted using the high-confidence mutations on the *embB* gene, particularly in codon 306. The *embB* gene is part of the *embCAB* operon which codes for arabinosyl transferases, involved in the biosynthesis of arabinogalactan and lipoarabinomannan, the key structural components of the mycobacterial cell wall [32]. The streptomycin resistant strain contained *rpsL* mutation Lys43Arg. Around 70% of strains resistant to streptomycin can be predicted using the high-confidence mutations on the *rpsL* and *rrs* genes coding for ribosomal protein S12 and 16S RNA respectively [32]. The MDR-

TB patient with *rpoB* Ser531Leu mutation had also a compensatory *rpoC* Gly332Arg mutation suggesting that this strain was able to select for compensatory mutations that may enhance transmission [33].

In our study, 6 *Mycobacterium tuberculosis* isolates with resistance to at least one drug were very diverse, each belonging to a different spoligotype-defined MTB lineage. All six spoligotypes were previously reported in East Africa [12, 34 – 42]. Five of them had an assigned SIT number and one was listed as an “orphan” with a single isolate from Serengeti, Northern part of Tanzania registered in SITVIT database [12]. Previous studies in East Africa have reported that there is no significant associations between MDR-TB and lineages [41 – 43]. The important finding that drug-resistant isolates were not related, underpins low possibility of local community spread of drug resistant tuberculosis.

Our study strength is the information emanating from the WGS sequencing as to the best of our knowledge this is the first study in our setting to detect the *Mycobacterium tuberculosis* spoligotypes and mutations conferring resistance. However our limitation is the small number of clinical isolates we have investigated.

Conclusion

The genetic drug resistance profile of *Mycobacterium tuberculosis* isolates from North-western Tanzania comprises of the common previously reported mutations. The prevalence of first and second line drug resistant TB as well as MDR-TB is low. Drug resistant strains were of difference clades, suggesting limited transmission of drug resistant tuberculosis in the region. Further studies using WGS will allow monitoring the spread of existing drug-resistant strains and the appearance of new ones.

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HIGHLIGHTS

- The genetic drug resistance profile of *Mycobacterium tuberculosis* isolates from Northwestern Tanzania comprises of the common previously reported mutations.
- The prevalence of first and second line drug resistant TB as well as MDR-TB is low.
- Drug resistant strains were of difference clades, suggesting limited transmission of drug resistant tuberculosis in the region.
- Further studies using WGS will allow monitoring the spread of existing drug-resistant strains and the appearance of new ones.

Table 1

Distribution of genotypic drug resistance among 74 *Mycobacterium tuberculosis* isolates

Drug	Mutation	Number (n)	Percent (%)
Any resistance		**6**	**8.1**
INH	*MabA-inhA* C(-15)T *katG* Ser315Thr	1 2	33.3 66.1
RIF	*rpoB* His526Leu *rpoB* Ser531Leu	1 2	33.3 66.7
EMB	*embB* Met306Ile *embB* Met306Val	1 1	50.0 50.0
SM	*rpsL* Lys43Arg	1	100.0
PZA	Wild	0	0.0
ETH	*MabA-inhA* C(-15)T	1	100.0
KAN	Wild	0	0.0
FLQ	Wild	0	0.0
AMK	Wild	0	0.0
CPR	Wild	0	0.0
Mono-resistance		**3**	**4.1**
RIF	*rpoB* Ser531Leu	2	66.7
SM	*rpsL* Lys43Arg	1	33.3
Poly-resistance not MDR		**2**	**2.7**
INH + EMB	*katG* Ser315Thr + *embB* Met306Val	1	50.0
INH + ETH	*MabA-inhA* C(-15)T	1	50.0
MDR		**1**	**1.4**
INH + RIF + EMB	*katG* Ser315Thr + *rpoB* Ser531Leu + *embB* Met306Ile	1	100

Table 2

Patients' characteristics, genotypic drug resistance, mutations and spoligotypes of the drug resistant *Mycobacterium tuberculosis* isolates

Sex	Age (years)	HIV Status	Genotypic Resistance	Mutations	SIT	Clade	Outcome
Female	26	Negative	INH + ETH	*MabA-inhA* C(-15)T	26	CAS1-DELHI	Cured
Male	52	Positive	SM	*rpsL* Lys43Arg	8	EAI5	Cured
Male	33	Negative	RIF	*rpoB* His526Leu	2487	LAM11-ZWE	Defaulted
Female	19	Negative	INH + RIF + EMB	*katG* Ser315Thr; *rpoB* Ser531Leu; *embB* Met306Ile	345	T3-ETH	Died
Female	53	Positive	INH + EMB	*katG* Ser315Thr; *embB* Met306Val	52	T2	Cured
Female	46	Negative	RIF	*rpoB* Ser531Leu	Orphan	T1	Transferred out