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Genetic Determinants of Drug-Resistant Tuberculosis among HIV-Infected Patients in Nigeria

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Tuberculosis (TB) is the most common opportunistic infection in human immunodeficiency virus (HIV)-infected patients and the emergence of drug-resistant tuberculosis (DR-TB) is a growing problem in resource-limited settings. Adequate infrastructure for testing drug sensitivity and sufficient evidence of first-line resistance are currently unavailable in Nigeria. We collected sputum samples from HIV-infected patients enrolled in the Harvard PEPFAR/APIN Plus program over 12 months at two PEPFAR antiretroviral therapy (ART) clinics in the southwest and north central regions in Nigeria. Smear-positive sputum samples were submitted for GenoType MTBDRplus testing ($n = 415$); mutations were confirmed through sequencing. Our results show high rates of DR-TB in Nigerian HIV-infected individuals (7.0% for rifampin [RIF] and 9.3% for RIF or isoniazid [INH]). Total RIF resistance indicative of MDR-TB in treatment-naive patients was 5.52%, far exceeding the World Health Organization predictions (0 to 4.3%). RIF resistance was found in 6/213 (2.8%) cases, INH resistance was found in 3/215 (1.4%) cases, and MDR-TB was found in 8/223 (3.6%) cases. We found significantly different amounts of DR-TB by location (18.18% in the south of the country versus 3.91% in the north central region [$P < 0.01$]). Furthermore, RIF resistance was genetically distinct, suggesting possible location-specific strains are responsible for the transmission of drug resistance ($P < 0.04$). Finally, GenoType MTBDRplus correctly identified the drug-resistant samples compared to sequencing in 96.8% of cases. We found that total DR-TB in HIV-infection is high and that transmission of drug-resistant TB in HIV-infected patients in Nigeria is higher than predicted.

Human immunodeficiency virus (HIV) greatly increases the risk for tuberculosis (TB), and the two epidemics continue to fuel one another (31). HIV-infected patients are significantly more likely to develop active TB diseases than non-HIV-infected people and are more likely to die from TB (13, 27, 28). In sub-Saharan Africa, 30% of HIV-infected patients who are diagnosed with TB die 12 months after the initiation of treatment (12, 33). With an estimated national prevalence of HIV in Nigeria of 3.6% (7), the number of people living with HIV (3.3 million) represents the second largest burden of disease on the continent (32). Nigeria has the world's third largest TB burden, with the prevalence of 830,000 cases. The World Health Organization (WHO) estimates that 26% of patients with TB infection in Nigeria are HIV infected (37).

Multidrug-resistant TB (MDR-TB), defined by resistance to isoniazid (INH) and rifampin (RIF), is a growing global health problem (5, 19, 22). While MDR-TB emerges as a consequence of poor adherence to anti-TB treatment (34, 35), it can also be transmitted. MDR-TB results in significantly higher mortality rates in HIV-infected patients than drug-susceptible TB (18). The estimates based on modeling predict MDR-TB prevalence in Nigeria to range from 1.8% (0.0 to 4.3%) for new cases up to 7.7% (0.0 to 18.0%) for previously treated patients (36). Currently in Nigeria, streptomycin is the only treatment available for patients previously treated for TB or suspected of infection with MDR-TB. Furthermore, MDR-TB in HIV-infected individuals leads to higher mortality compared to mortality in non-HIV-infected patients or HIV-infected individuals with susceptible TB (18, 24). These findings, combined with alarming evidence that MDR-TB can be transmitted, calls for close monitoring of the incidence of drug resistance, especially in HIV-infected populations (6).

The conventional methods of drug resistance testing involve growth of *Mycobacterium tuberculosis* on liquid or solid culture medium (35). Culture methods are costly and time-consuming, thus limiting both utility for patient care and likelihood of timely treatment. Recently, several new commercial tests have been described that identify MDR-TB based on the genetic sequence; one is a line probe assay named GenoType MTBDRplus, which diagnoses TB and identifies drug resistance. It specifically examines samples for classic genetic mutations that confer resistance to both INH and RIF. Due to the reported efficiency and low cost of this test, it represents an alternative to conventional drug sensitivity testing through culture. The test has been successfully used in several locations worldwide with high sensitivity rates for RIF (>95.5%) and INH (>81.8%) resistance (2, 4, 15, 17, 21, 23) and 100% specificity (2, 4, 15, 17, 21, 23).

The GenoType MTBDRplus test is also reliable as a method for surveillance of drug resistance (26). The surveillance and monitoring of both INH- and RIF-resistant *M. tuberculosis* is not only beneficial for an individual patient but also for the HIV-infected population as a whole, since they are more susceptible to *M. tuberculosis* infection (10). Implementation of the GenoType

TABLE 1 Patient characteristics at study entry by site location[a]

Characteristic	Patients ($n = 224$)		P
	NIMR ($n = 90$)	JUTH ($n = 134$)	
Previous TB treatment	17	37	0.13
Median age in yrs (IQR)	35.62 (29.4–43.3)	35.99 (30.8–42.6)	0.72
Gender			
Female	58	72	0.11
Male	32	62	
Median no. of wks (IQR) between symptom onset and clinic presentation, no. of observations[b]	3 (2–3), 86	4 (3–8), 123	<0.01

[a] NIMR, Lagos state, South-west region; JUTH, Plateau state, North-central region. Data refer to numbers of patients except as noted in column 1. IQR, interquartile range.
[b] Data available for a subset of patients.

MTBDRplus test as a routine test can have a significant impact by improving the lives of HIV-infected patients with TB. It is therefore imperative to identify the individuals at highest risk of acquiring the drug-resistant *M. tuberculosis* strains in Nigeria in order to develop a programmatic policy to prevent further transmission.

MATERIALS AND METHODS

Two geographically distinct locations in Nigeria were chosen, the Nigerian Institute for Medical Research (NIMR) in Lagos and the Jos University Teaching Hospital (JUTH), located in the southwest and north central zones of the country, respectively. NIMR data were collected from June 2009 to June 2010, and JUTH data were collected between August 2009 and November 2010. The ethical approval was obtained from the institutional review boards at NIMR, JUTH, and Harvard School of Public Health (approval 16430-103).

At regular clinic visits, HIV-infected patients were screened for symptoms of pulmonary TB, including chest pain, cough lasting more than 2 weeks, fever, night sweats, and weight loss. Upon identification, patients were asked to participate in the study and provided written informed consent. Consented patients were queried about their TB history to assess their treatment exposure prior to the study and asked to provide three sputum samples. All samples were decontaminated using the modified Petroff method and stained directly for acid-fast bacilli (AFB) using the Ziehl-Neelsen method (20). Patients with AFB-positive samples were enrolled in the study for a 12-month period.

Samples identified as sputum smear positive (SS$^+$) for AFB were included in the study. Crude DNA extraction was performed on site, followed by PCR and hybridization on test strips, according to the GenoType MTBDRplus (Hain Lifesciences, Nehren, Germany) protocol (11). Isolated DNA was stored at −20°C until the genotypic resistance testing was performed. GenoType MTBDRplus test instructions were followed for *M. tuberculosis* DNA amplification and hybridization (11). The tests strips were scored for resistance based on the presence of a mutant strain or the absence of wild-type DNA.

Multiplex-nested PCR and DNA sequencing of resistance genes. Samples diagnosed as resistant with the GenoType MTBDRplus test and 16 susceptible samples from the same cohort were sequenced. Portions (5 to 10 μl) of crude lysate were used to amplify the four resistance-conferring genes (*rpoB*, *katG*, the *inhA* promoter, and the *oxyR-ahpC* intergenic regulatory region) using a multiplex PCR. The PCR was established using 1× KOD polymerase buffer, 0.2 mM deoxynucleoside triphosphate (each), 1.5 mM MgSO$_4$, 5% dimethyl sulfoxide, 100 nM concentrations of each primer, and 1% KOD Hot Start polymerase (Toyobo, Osaka, Japan). Cycling consisted of 1 cycle of 3 min at 95°C, followed by 25 cycles of 20 s at 95°C, 10 s at 63°C, and 15 s at 72°C, and then 1 cycle of 2 min at 72°C. A nested PCR was performed individually on each gene using 5 to 10 μl of the multiplex sample. The reagent concentrations were identical to the multiplex PCR, except that 200 nM inner primer was used. The nested

PCR protocol consisted of 1 cycle of 3 min at 95°C, followed by 30 cycles of 20 s at 95°C and 10 to 15 s of annealing/extension at various temperatures (*rpoB* and *inhA*,10 s for 60°C and 5 s for 72°C; *katG*, 10 s for 65°C; *oxyR-ahpC*, 10 s for 62°C and 5 s for 72°C).

The nested PCR products were then separated through electrophoresis on a 2% NuSieve (Lonza, Rockland, ME) agarose gel. The bands of the appropriate sizes were excised and purified from the gel using a MinElute kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After the PCR fragment concentrations were evaluated using a Quant-iT PicoGreen (Invitrogen, Carlsbad, CA), 10 ng of the DNA was used for sequencing with a BigDye Terminator (v1.1; Applied Biosystems, Carlsbad, CA) cycle sequencing kit on an Applied Biosystems 3100 sequencing instrument. Primers used for sequencing were the inner primers of the multiplex-nested PCR, except for the *inhA* promoter region that used separate primers (see Table S1 in the supplemental material).

Data analysis. Experimentally obtained sequences were aligned with the known genetic sequence of a susceptible *M. tuberculosis* strain H37Rv using Lasergene (DNAStar, Madison, WI) and examined for previously reported resistance associated mutations. The sequence consensus of each sample was compared to the GenoType MTBDRplus results to assess the two genotypic evaluations of resistance. Groups of resistant samples were compared using the Fisher exact test, while patient characteristics at study entry were compared using the chi-square or Wilcoxon rank-sum test as appropriate. Significance threshold was set to $P < 0.05$.

RESULTS

A total of 940 patients presented with signs and symptoms of TB, while 535 had at least one AFB$^+$ sputum. Of 415 available patient samples that were tested with GenoType MTBDRplus, genes representative of RIF susceptibility could be evaluated for 213 cases, INH susceptibility for 215 cases, and MDR-TB for 223 cases. RIF resistance with the GenoType MTBDRplus test was evaluated with the hybridized band profile of the *rpoB* gene, while INH was evaluated by hybridization to the *katG* open reading frame and the *inhA* promoter sequence. When we compared patients by site, there were no statistically significant differences in age (median, 36 years; $P = 0.72$), evidence of previous TB treatment (JUTH, $n = 37$; NIMR, $n = 17$ [$P = 0.17$]), or the percentages of female patients with 53.7% at JUTH and 64.4% at NIMR ($P = 0.11$). There were differences in time from symptom onset to clinic presentation (4 weeks at JUTH versus 3 weeks at NIMR; $P < 0.01$) (Table 1).

A greater number of resistant samples were observed at NIMR ($n = 14$) than at JUTH ($n = 5$) ($P = 0.001$). The percentage of resistant samples for each drug, but not for both (MDR-TB), demonstrated significantly higher rates of resistance at NIMR com-

TABLE 2 *M. tuberculosis* drug resistance in Nigerian HIV-infected patients by location as determined by GenoType MTBDRplus[a]

Resistance type	No. of patients/total no. of patients (%)		P
	NIMR	JUTH	
INH resistant	9/85 (10.59)	3/130 (2.31)	<0.02
RIF resistant	11/81 (13.58)	4/132 (3.03)	<0.01
MDR-TB	6/89 (6.74)	2/134 (1.49)	<0.07
Any resistance	14/77 (18.18)	5/128 (3.91)	<0.01

[a] NIMR, Lagos state southwest region; JUTH, Plateau state north central region.

TABLE 3 *M. tuberculosis* drug resistance in Nigerian HIV-infected patients by TB treatment history as determined by GenoType MTBDRplus

Resistance type	No. of patients/total no. of patients (%)		P
	Previously treated	Treatment naive	
INH resistant	4/50 (8.00)	8/165 (4.85)	0.48
RIF resistant	6/50 (12.00)	9/163 (5.52)	0.12
MDR-TB	3/54 (5.56)	5/169 (2.96)	0.40
Any resistance	7/46 (15.22)	12/159 (7.55)	0.15

pared to JUTH (Table 2). Fifty-four patients had previous exposure to TB treatment, while 170 were treatment naive. The stratification based on patient's prior treatment status showed no significant differences between the prevalence of drug resistance in naive versus pretreated patients (Table 3).

All of the polymorphisms found with the GenoType MTBDR-plus test, the codons they identify, and the numbers of samples with each profile by location are listed in Table 4. The GenoType MTBDRplus resistance profile for RIF differed by study site, where *rpoB* mutations from JUTH samples were predominantly found in codons 526 to 529 (wt[7]), while the NIMR samples showed mutations in codons 530 to 533 (wt[8]) (LG7 excluded, $P = 0.04$). Genotype resistance profiles also indicate that mutations conferring INH resistance in Nigeria occur at a similar rate in the *inhA* promoter and the *katG* S315T region, in contrast to previous reports (3, 14, 16). Furthermore, all of the mutations found in *katG* were the same point mutation, S315T1, and the *inhA* promoter was mutated at the C−15T position.

Using the multiplex/nested PCR technique, we amplified and sequenced most of the resistant samples (exceptions included LG12, LG13, and LG14), as well as 16 susceptible samples directly from sputum, to examine exact mutations found in resistance conferring genes. The GenoType MTBDRplus susceptible samples showed no polymorphisms previously reported to be associated with resistance (Table 4). Interestingly, each of the three samples from JUTH with a mutation in codon 526 of the *rpoB* gene had a different point substitution responsible for resistance. The most common mutation spanning the *rpoB*[wt8] codon in NIMR samples was S531L.

Only 2 of the 16 resistant samples (LG7 and LG4) and none of 16 susceptible samples had discordant results obtained from the GenoType MTBDRplus test and sequencing. Sample LG7 was still resistant to RIF, and one of the three mutations indicated by GenoType MTBDRplus was concordant (*rpoB*[wt4] D516A). Instead of the mutations in the *rpoB*[mut1] or *rpoB*[wt8] region, a mutation was observed in the *rpoB*[wt2] region L511P.

In addition, although sample LG7 exhibited no mutations conferring INH resistance in the *katG* or *inhA* promoter regions, it was the only sample that had a C→T substitution at location −25 (in relation to the *ahpC* transcription start) of the *oxyR-ahpC* regulatory region, previously described only in *inhA* resistant strains (14, 16, 25). Therefore, LG7 was not misdiagnosed for RIF resistance, but potential *inhA* resistance could have been missed by not including the *oxyR-ahpC* regulatory region in the assay.

Sample LG4 was designated as INH resistant by the GenoType MTBDRplus test but was not confirmed by sequencing; instead, the sample appeared to be susceptible according to its sequence

analysis. With LG4 identified as susceptible, INH resistance still differed significantly by site (NIMR INH resistance = 9.4% [$P < 0.03$] versus JUTH; NIMR any resistance = 16.9% [$P < 0.01$] versus JUTH). There was not a significant difference in any resistance, including INH between treatment-naive and experienced individuals.

In total, 1 of 31 sequenced samples (3.2%) did not confirm the resistance diagnosis obtained with the GenoType MTBDRplus; therefore, the concordance rate for DR-TB diagnosis was 96.8% (*oxyR-ahpC* promoter region excluded). We sequenced a total of 93 regions (*rpoB*, *katG*, and *inhA*) that are analyzed by the GenoType MTBDRplus test. GenoType MTBDRplus misidentified only four mutations and was therefore 95.7% specific.

DISCUSSION

In Nigeria, HIV/TB coinfection rates are as high as 30% in antiretroviral therapy (ART) clinic settings, and the national prevalence of MDR-TB is unknown. Our results show high rates of transmitted drug-resistant TB (5.5%), inferred by rates of rifampin resistance in treatment-naive patients. This rate exceeds the upper limit of the WHO MDR-TB models (4.3%). Furthermore, AFB sputum smears, using the Ziehl-Neelsen stain, lack sensitivity in identifying TB cases, and some cases of *M. tuberculosis* infection could have been missed. Since resistant bacteria are more likely to be less fit than sensitive bacteria (1, 8, 9, 30) and therefore cause paucibacillary disease, our results may represent an underestimate of drug resistance. This indicates that transmission of drug-resistant TB is a more serious problem than previously anticipated.

The GenoType MTBDRplus test correctly identified mutations with a high concordance rate. In recent literature, the gene-based identification of MDR-TB has gained prominence. The Gene-Xpert MTB/RIF is considered an appropriate new technology for diagnosing both TB and rifampin drug resistance. Although both GeneXpert and GenoType MTBDRplus work on a similar principle—gene amplification and subsequent hybridization—Gene-Xpert MTB/RIF does not examine INH resistance. Mutational analysis of INH resistance is more complex than RIF because it requires evaluating more genes. Furthermore, the genotypic analysis of *rpoB* for RIF resistance is thought to be sufficient for evaluating the public health threat of drug-resistant TB. However, recent reports indicate that this remains controversial (29). In our study, we observed 1.4% INH monoresistance and 2.8% RIF monoresistance, highlighting the importance of evaluating both drug susceptibilities. Although one case of INH resistance was incorrectly identified with GenoType MTBDRplus, the inclusion of *inhA* and *katG* mutation analysis in this test correctly identified three INH-resistant, RIF-susceptible strains. This is particularly important in HIV-prevalent settings where INH prophylaxis is

TABLE 4 Resistance profiles of samples from NIMR (LG) and JUTH (JS) in Nigeria

	GenoType MTBDRplus-diagnosed resistance[a]		Polymorphism determined by sequencing[b]			
Sample	RIF	INH	rpoB	katG	inhA	oxyR
JS1	wt[7], mut[2A]	sens.	H526Y CAC→CAA	wt	wt	wt
JS2	mut[3]	wt[katG], mut[katG1]	S531L TCG→TTG	S315T AGC→ACC	wt	wt
JS3	wt[7]	sens.	H526N CAC→AAC	wt	wt	wt
JS4	sens.	wt[katG], mut[katG1]	wt	S315T AGC→ACC	wt	wt
JS5	wt[7]	wt[inhA], mut[inhA1]	H526R CAC→CGC	wt	C−15T	wt
LG1	wt[8]	sens.	L533P CTG→CCG	wt	wt	wt
LG2	wt[7], mut[2B]	wt[katG], mut[katG1]	H526D CAC→GAC	S315T AGC→ACC	wt	wt
LG3	sens.	wt[katG], mut[katG1]	wt	S315T AGC→ACC	wt	wt
LG4	sens.	mut[inhA2c]	N/A	wt	wt	wt
LG5	wt[8], mut[3]	sens.	S531L TCG→TTG	wt	wt	wt
LG6	wt[8], mut[3]	sens.	S531L TCG→TTG	wt	wt	wt
LG7	wt[4,8,d] mut[1]	sens.	L511P CTG→CCG D516A GAC→GCC	wt	wt	C−12T[e]
LG8	sens.	wt[katG], mut[katG1]	wt	S315T AGC→ACC	wt	wt
LG9	mut[3]	mut[inhA1]	S531L TCG→TTG	wt	C/T−15T (mixed population)	wt
LG10	wt[8], mut[3]	wt[katG], mut[katG1]	S531L TCG→TTG	S315T AGC→ACC	wt	wt
LG11	wt[8], mut[3]	wt[inhA], mut[inhA1]	S531L TCG→TTG	wt	C−15T	wt
LG12	wt[8], mut[3]	wt[inhA], mut[inhA1]	NA	NA	NA	NA
LG13	wt[8], mut[3]	wt[inhA], mut[inhA1]	NA	NA	NA	NA
LG14	wt[8], mut[3]	N/A	NA	NA	NA	NA
Controls	sens.	sens.	wt ($n = 16$)	wt ($n = 15$)	wt ($n = 15$)	wt ($n = 15$)

[a] GenoType MTBDRplus-diagnosed resistance (wt, hybridization to wild-type band missing; mut, hybridization to mutant band present); N/A, not available. sens., sensitive.

[b] "wt" refers to the H37Rv sequence. NA, not amplified in multiplex/nested PCR.

[c] Mutation not observed by sequencing.

[d] Mutations on codon 530–533(wt[8]) and mut[1] D516V were not confirmed by sequencing; however, the lack of a wt[4] band was confirmed (D516A), and an additional mutation L511P (wt[2]) was found.

[e] Mutation previously reported in resistant sample.

being considered. Such preventative measures might not be effective and could increase the rates of INH resistance, exacerbating the diagnostic challenges for MDR-TB. Furthermore, misdiagnosing patients as MDR-TB when they are only RIF monoresistant would lead to inappropriate second-line treatment, when such treatment in resource-limited settings is already limited.

The baseline characteristics of age, gender, and prior treatment status of patients did not show major site differences, indicating that the sites were comparable. Although the duration of symptoms prior to clinic visit differed, this difference might be due to the fact that patients visiting the clinic in Jos traveled longer distances and therefore were at a disadvantage to be adherent to clinic visits. In these two geographically distinct study populations, a significantly higher number of drug-resistant TB was found in Lagos compared to Jos. This phenomenon could be explained by societal differences in the two cities, which might facilitate transmission, Lagos being more densely populated with crowded living arrangements and a congested public transportation system compared to Jos. Transmission of drug-resistant TB in Lagos is further suggested by the fact that 9 of 13 patients with drug-resistant TB were treatment naive (versus 2/5 in Jos). An alternative explanation is that the *M. tuberculosis* strains differed by location and therefore so did their transmission and/or mutation rates. Although we were not able to evaluate the *M. tuberculosis* strains or their characteristics in the present study, the mutations in the *rpoB* gene are suggestive of such differences. All of the Jos mutations in the *rpoB* gene were distinct, implying that they were acquired through separate mutagenesis events, whereas most of the Lagos

strains had the same mutation (S531L) belonging to a potentially more transmissible strain.

In summary, our study demonstrated high rates of drug-resistant TB in HIV-infected patients. We also showed that the cost-effective GenoType MTBDRplus test correctly identified resistance-conferring mutations in the majority of samples examined (95.7%). Although our sample size was small, this is the first multisite study of MDR-TB in HIV-infected population in Nigeria. Consistent with WHO recommendations, our results support the urgent need for systematic drug resistance testing in all HIV-infected patients with symptoms suggestive of TB.

ACKNOWLEDGMENTS

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