Abstract

Background: Drug-resistant tuberculosis (DR-TB) continues to be a challenge in developing countries with poor resources. Despite the high prevalence of primary DR-TB, its routine screening prior to the treatment is not performed in public hospitals in Nigeria. Data regarding drug resistance and its genetic determinant among follow-up patients with TB are lacking in Nigeria. Hence, the aim of this study was to determine the prevalence and genetic determinant of drug resistance among the follow-up patients with TB in a tertiary hospital in Nigeria. Materials and Methods: This was a cross-sectional, laboratory-based study conducted on 384 sputum samples collected from consented follow-up patients with TB. Standard microbiology methods (Ziehl–Neelsen staining and microscopy) and polymerase chain reaction (PCR; line probe assay [LIPA]) were used to analyze the collected samples. Pearson’s Chi-square test was used to analyze the generated data. Results: Out of 384 sputum samples analyzed for Mycobacterium tuberculosis and DR-TB, 25 (6.5%) tested positive for acid-fast bacilli. These samples were subjected to PCR (LIPA), of which 18 (72%) tested positive for DR-TB. Of these 18 samples, mutations conferring resistance to rifampicin (rpoB) and isoniazid (katG and/or inhA) were detected in 12 (66.7%) and 6 (33.3%) samples, respectively. Transmission dynamics of DR-TB was not significantly (P > 0.05) dependent on demographic characteristics. Conclusion: There is a need to strengthen the laboratory capacity for the diagnosis of TB and drug resistance testing and make these services available, affordable, and accessible to the patients who need them.

Keywords: Drug resistance, genetic determinant, intensive phase, Nigeria, tuberculosis

Introduction

Tuberculosis (TB) continues to be a major public health concern globally despite the discovery of an effective cure and preventive measures decades ago. With the incidence of 133/100,000 population, Nigeria ranks ninth among the 22 countries that account for 80% of TB burden in the world. Mortality also remains high with an estimated mortality rate of 81/100,000 population. The most serious problem hindering TB treatment and control is nonadherence of patients to the treatment, which is believed to fuel the increasing incidence of TB and the rising prevalence of drug-resistant-TB (DR-TB). However, the introduction of the Directly Observed Treatment, Short-course (DOTS) strategy is aimed at revolutionizing the prognosis of patients with TB and impedes the transmission and emergence of DR-TB. The DOTS strategy in Nigeria requires that patients swallow their drugs under the direct observation of the health-care workers at least in the first 2–3 months of treatment with free anti-TB drugs and medical laboratory test. However, since its implementation in 1993, it has achieved a case detection rate of only 30% and treatment success rate of 79%, which yet remains below the global target of 70% and 85% case detection and success rates, respectively.

The key to preventing the rapid rise and spread of DR-TB is its early detection and appropriate case management. The detection of “rifampicin (RIF) resistance,” the surrogate marker of multidrug-resistant (MDR)-TB, using a rapid molecular technique like real-time polymerase chain reaction (PCR) could save precious time and lives. Line probe assay (LiPA),...
which has been identified as a reliable method to detect isoniazid (INH) and RIF resistance, has only recently been introduced in Nigeria and available in only a few selected public hospitals. However, pretreatment resistance testing is not routinely performed and is offered only to patients with suspected treatment failure. Therefore, drug resistance testing using LiPA is only performed if patients’ sputum samples do not convert to smear negative after the intensive phase. Using this protocol, patients with primary drug resistance will only be detected after the intensive phase of treatment, thereby posing the risk of emergence of resistance to more drugs. This was primarily a laboratory-based study focusing on the prevalence of DR-TB, patterns of DR-TB distribution, and determination of the genes associated with resistance among the follow-up patients with TB after the intensive phase of first-line anti-TB treatment.

**Materials and Methods**

At regular clinic visits, patients with pulmonary TB who have completed the 2-month intensive phase of the first-line anti-TB treatment were identified. On identification, a written informed consent was provided and those who consented were enrolled in the study. A close-ended questionnaire was administered seeking information on demographic variables and history of previous exposure to anti-TB treatment. Those who were previously treated for TB were excluded from the study. Patients were then asked to provide three sputum samples in a sterile, wide-mouth, screw-capped, labeled, and transparent container, which were immediately transported in a cold box to the laboratory for analysis. All samples were decontaminated using the modified PetrOFF’s method and stained directly for acid-fast bacilli (AFB) using the Ziehl–Neelsen method. Samples identified as sputum smear positive 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 assay (Hain Lifescience, Nehren, Germany).[7] Isolated DNA was stored at −20°C until the genotypic resistance testing was performed. GenoType MTBDRplus assay (Hain Lifescience, Nehren, Germany) instructions were followed for Mycobacterium tuberculosis DNA amplification and hybridization. The test strips were scored for resistance based on the presence of a mutant strain or the absence of wild-type (WT) DNA.

**Line probe assay**

The prevalence of DR-TB was determined using LiPA on AFB-positive sputum samples by DNA extraction, amplification, hybridization, and detection methods.

**DNA extraction**

The specimens were sonicated after partial cell lysis through a heat-killing step. A 500-μL decontaminated sputum sample was transferred into a 1.5-mL screw-capped tube and centrifuged for 15 min at 10,000 x g at 2–4°C in a refrigerated centrifuge. The pellets were resuspended (after decanting supernatant) in 100 μL of an alkaline lysis buffer (Hain Lifescience, Nehren, Germany) and incubated for 5 min at 95°C in a water bath. Subsequently, 100 μL of neutralization buffer was added to the lysate. The mixture was vortexed and centrifuged for 5 min at 14,000 x g. The extracted DNA was kept in the freezer at −20 or −80°C. For LiPA, 5 μL of the supernatant (extracted DNA) was used.

**Polymerase chain reaction (line probe assay) analysis**

**MasterMix preparation**

Primer nucleotide mix (35 μL) (Hain Lifescience, Nehren, Germany) was pipetted and dispensed into sterile tubes. Next, 5 μL of ×10 PCR buffer, 2 μL of MgCl₂, 3 μL of molecular grade water, and 0.2 μL of Taq polymerase were added. The solution was mixed well.

**Procedure for preparation of DNA amplification mixture**

The numbers of amplified specimens were determined. Negative and positive controls were included. Prepared MasterMix (45 μL) was pipetted and dispensed into each sterile PCR tube. Next, 5 μL of extracted DNA was added into each of the tubes, except in the controls. Finally, 5 μL of the controls (positive and negative each) were added to the respective control tubes.

**DNA amplification**

The Hot 40 program was selected (for sputum sample) in a thermocycler. PCR was run for 2–3 h. Before the PCR tubes were placed into the thermocycler (Hain Lifescience, Nehren, Germany), they were mixed slightly and spun down for 5–10 s in a minicentrifuge at 10,000 x g. The PCR machine was heated up to 95°C for 15 min to denature the DNA and at 95°C respectively for 30 s and subsequently run for: (1) 2 min at 58°C for 30 cycles for the annealing of primers; (2) 40 s at 70°C for extension and elongation; (3) 25 s at 95°C for 20 cycles to further denature the DNA; (4) 40 s at 53°C for the re-annaling of primers, and (5) 40 s at 70°C for extension and elongation.

**Hybridization**

The hybridization procedure was performed directly after the amplicons were removed from the thermocycler. Hybridization and stringent washing buffers were preheated to 45°C. Next, 20 μL of denaturation buffer was mixed thoroughly in a plastic well tray with 20 μL of amplified sample and incubated at room temperature for 5 min. Hybridization buffer (1 mL) was added to each well and mixed. Prelabeled test strips were added to each well, and the wells were incubated for 30 min at 45°C after labeling. The solution was completely aspirated following incubation. To each strip, 1 mL of stringent buffer was added and incubated at 45°C for 15 min, and subsequently, 1 mL of rinse buffer was also added to each strip and incubated at room temperature for 1 min. The rinse buffer was removed. Next, 1 mL of dilute conjugate buffer was added to each strip and incubated for 30 min at room temperature. The solution was removed after incubation, and the test strips were rinsed twice with washing buffer solution for 1 min and also with distilled water for 1 min. The strips were then placed into the thermocycler (Hain Lifescience, Nehren, Germany) and incubated for 5 min at 95°C in a water bath. Subsequently, 100 μL of neutralization buffer was added to the lysate. The mixture was vortexed and centrifuged for 5 min at 14,000 x g. The extracted DNA was kept in the freezer at −20 or −80°C. For LiPA, 5 μL of the supernatant (extracted DNA) was used.
distilled water. The test strips were dried and then taped to the MTBDRplus assay worksheet for interpretation.

**Polymerase chain reaction interpretation**

For the identification of *M. tuberculosis*, 22 probes are located on the DNA strip. This zone hybridizes the amplicons generated from *M. tuberculosis*. Five controls (conjugate control, amplification control, and rpoB, katG, and inhA loci control zones) were set on the DNA strip. The rpoB, katG, and inhA loci control zones detect a region specific for the respective loci and stain positive. The conjugate control area shows the efficiency of conjugate binding and the development of substrate reaction. One probe is complementary with *M. tuberculosis* complex-specific region of the 23S rRNA gene (tub). Eight rpoB WT probes (WT1 to WT8) encompass the region of the rpoB gene coding for amino acids 505–534. Four other probes rpoB MUT1, MUT2, MUT3, and MUT4 are specific for the most common mutations D516V, H526Y, H526D, and S531L, respectively. Three probes are specific for the codon 315 region of katG: katG WT, katG MUT1, and katG MUT2. katG WT is the WT probe, whereas katG MUT1 and katG MUT2 are designed for the AGC-to-ACC (S315T) and AGC-to-ACA (S315T2) mutations, respectively. Six probes are designed for the promoter region of the inhA gene. Two WT probes, inhA WT1 and WT2, and four others, inhA MUT1, MUT2, MUT3A, and MUT3B, detect mutations of C15T, A16G, T8C, and T8A, respectively. rpoB predicts RIF resistance, katG predicts high-level INH resistance, and inhA predicts low-level INH resistance.

The MTBDRplus assay results were interpreted as described by Hain protocol (Hain Lifescience, Nehren, Germany). When all WT probes gave a positive signal and all mutation probes reacted negatively, the isolate was considered susceptible. When at least one negative signal was obtained with WT probe, the isolate was considered resistant. When the resistance was due to mutations described above, a positive reaction was obtained with one of the mutation probes and was always accompanied by a negative reaction with the corresponding WT probe.

**RESULTS**

A total of 384 sputum samples from patients who had completed the intensive phase of the first-line four-drug anti-TB treatment were studied. Out of the 384 sputum samples, 165 (43.0%) were from male and 219 (57.0%) from female patients. The mean age of the patients was 34 ± 7.38 years, and 24 (6.3%) were from male and 219 (57.0%) from female patients. The number of patients were HIV-positive [Table 1].

RIF resistance with the GenoType MTBDRplus assay was evaluated with the hybridized band profile of the rpoB gene, and INH resistance was evaluated by hybridization to the katG open reading frame and the inhA promoter sequence. Mutations conferring resistance to RIF and INH were detected in 12 (66.7%) and 6 (33.3%) samples, respectively, of the 18 DNA samples extracted from the sputum. The number of katG and inhA mutations was 3 (16.7%) each. In all the 12 (66.7%) DNA samples where RIF resistance was detected, mutations in katG and/or inhA genes were also detected indicating that they were INH-resistant also, indicating MDR. An isolated rpoB mutation was not detected. The prevalence of monodrug resistance and MDR among the follow-up patients was 6/384 (1.6%) and 12/384 (3.1%), respectively, giving an overall prevalence of drug resistance TB of 18/384 (4.7%). Table 2 shows the distribution of drug resistance based on the demographic variables. Transmission dynamics of DR-TB was not significantly (*P* > 0.05) dependent on demographic characteristics [Table 2].

**DISCUSSION**

In public hospitals in Nigeria, pretreatment resistance testing is not routinely performed and is only offered to patients with suspected treatment failure. Therefore, a significant number of patients with primary DR-TB are erroneously commenced on the first-line treatment, and this may have fueled the emergence of MDR-TB. We found a prevalence of 4.7% of DR-TB, of which 66.7% were MDR-TB, among the follow-up patients after the intensive phase of first-line anti-TB treatment.

Drug resistance in *M. tuberculosis* isolates arises from spontaneous genetic mutations mostly enhanced by poor adherence of patients to anti-TB drugs.[8] The fact that all our patients were following DOTS strategy made on-treatment resistance unlikely because it has been shown that DOTS increases compliance and significantly decreases the risk of emergence of resistance mutation.[9] It is alarming, therefore, that these cases would have had primary drug resistance that

| Table 1: Baseline characteristics |
|----------------------------------|
| Variables                        | n (%)     |
| Gender                           |           |
| Males                            | 165 (43.0)|
| Females                          | 219 (57.0)|
| Employment status                |           |
| Employed                         | 284 (74.0)|
| Unemployed                       | 100 (26.0)|
| Educational status               |           |
| Educated                         | 334 (87)  |
| Not educated                     | 50 (13)   |

| Table 2: Association between drug-resistant tuberculosis and demographic variables |
|----------------------------------------------------------------------------------|
| Variables                        | DR-TB | No DR-TB | P (CI)       |
| Males                            | 7     | 2        | 0.50 (0.184-20.760) |
| Females                          | 11    | 5        | 0.08 (0.003-1.573)  |
| Employed                         | 8     | 6        |                |
| Unemployed                       | 10    | 1        |                |
| Educated                         | 6     | 3        | 0.50 (0.081-6.18)  |

DR-TB: Drug-resistant tuberculosis, CI: Confidence interval
was not picked at initial evaluation of the patients. This has great consequences as it may lead to poor treatment outcome and further spread of MDR-TB in the community. The prevalence rate of MDR-TB in this study falls within the World Health Organization (WHO) reported figure of 1.9% and 9.3% for new and previously treated patients, respectively. Since resistant bacteria are more likely to be less fit than sensitive bacteria,[11-13] and therefore, cause paucibacillary disease, the use of AFB-sputum smears using the Ziehl–Neelsen stain for initial screening in our study may represent an underestimate of drug resistance.

Resistance to RIF was relatively low compared to the other first-line drugs.[14] Previous studies in Nigeria have also shown similar trends.[15,16] We found no RIF monodrug resistance in this study, which is similar to that reported by Otu et al.[17] in Calabar, Nigeria. Similar results have also been reported in the United Kingdom among secondary TB cases.[18] The prevalence of MDR-TB in this study is similar to the WHO estimates of 3.1% among new TB patients in Nigeria.[19] Similar results were reported in several studies across Nigeria.[20,21]

RIF resistance is recommended by WHO as a “surrogate marker” for detecting MDR-TB because monodrug resistance to RIF in M. tuberculosis is rare.[22] Because 95%–98% RIF resistance occurs through genetic mutations in 81-bp region of the rpoB gene,[23,24] it is more amenable for genotypic drug susceptibility testing (DST) than INH, which is more complex and requires assessment of mutations in multiple genes. Furthermore, at least 90% of all RIF-resistant clinical isolates are also resistant to INH.[1] Similarly, the incidence of MDR-TB in Thailand was found to be the same as that of RIF resistance.[25] Therefore, the genotypic analysis of rpoB for RIF resistance is thought to be sufficient for evaluating the public health threat of DR-TB. However, recent reports indicate that this remains controversial.[26] The absence of RIF monodrug resistance in this study also supports the use of GeneXpert MTB/RIF assay for evaluating MDR-TB in resource-limited countries. However, this finding should be treated with caution because of the small sample size.

This study is limited by small sample size and the use of AFB for the initial screening of the patients. Nevertheless, the presence of resistance to anti-TB drugs among the follow-up TB patients after the intensive phase of anti-TB treatment has highlighted the need for all TB patients to have access to culture and DST services so that appropriate treatment can be prescribed to patients from the outset and prevent the amplification of resistance.

This challenge can be overcome in developing countries with the recent introduction of newer molecular technologies, such as LiPA and the recently introduced GeneXpert assay, which are rapid technologies that can detect resistance to INH and/or RIF, the most potent anti-TB drugs. These new technologies will greatly assist in the appropriate diagnosis and subsequent appropriate management of patients with DR-TB.

**Conclusion**

There is a need to introduce a routine screening of primary DR-TB before commencing treatment in Nigeria. This will be achieved by strengthening the laboratory capacity for diagnosis and DST and making the services available, affordable, and accessible to the patients who need them.

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**Conflicts of interest**

There are no conflicts of interest.

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