

Molecular Diagnosis of Multidrug Resistant Pulmonary Tuberculosis among Patients in a Nigerian Teaching Hospital

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Received: March 27, 2018; **Published:** April 20, 2018

Abstract

Tuberculosis (TB) is considered to be a major cause of ill health and death globally with a number equating to nine million people contacting the disease and 1.5 million deaths in Nigeria which is reported to be one among 30 countries that is burdened with TB, Human Immunodeficiency Virus (HIV)/TB and Drug Resistant Tuberculosis (DRTB). A clustered sampling technique was adopted to assay 85 pulmonary samples out of which 28.24% were co-infected with HIV. Highest Pulmonary TB-HIV burden was 16.47% among age group 33 - 48 years. Lowest Pulmonary TB-HIV co-infection was 1.18% among age group 1-16 years. Multidrug Resistant TB among the study participants was 4.71%. Mono-resistance for rifampicin and isoniazide was 18.82% and 10.50% respectively. Generally, a high drug resistant pulmonary TB burden was noticed in the study area. Our results may represent an underestimate of drug resistance because of the low sample size however, the results of this study indicates that transmission of drug-resistant TB has become a more serious problem than earlier imagined. It is high time for Nigeria to adopt a strict committed approach by visiting families in the communities to identify those coughing and collecting their sputum samples for TB diagnosis. After all delay in diagnosing TB is a thing of the past with the advent of the GeneXpert and Line Probe Assay (LPA). Educating them that TB is not as abominable as they taught hence behavioral change and adherence to policies binding the TB control can actually prevent it and also informing them that those that are already infected can be cured.

Keywords: *Molecular; Diagnosis; Pulmonary TB; Multidrug Resistant Tuberculosis; TB-HIV Co-infection*

Introduction

Tuberculosis (TB) has been seen to be a major cause of ill health and death globally, with a number equating to nine million people contacting the disease and 1.5 million deaths in 2013 which equals to 4100 deaths daily [1]. Nigeria, is reported to be one among 30 countries that is burdened with TB, HIV/TB and DRTB. According to the WHO, the burden of TB in Nigeria accounts to 322 in every 100 000 people among which only 15% of the number has a record in 2015 [21]. TB is caused by an acid fast bacterium known as *Mycobacterium tuberculosis* that most often affects the lungs. A form of TB that is only targeted at the lungs is called Pulmonary TB which can otherwise spread from one person to another through the air contaminated with a droplet nuclei of an acid fast bacilli discharged by an infected person either by coughing, sneezing, talking, singing, shouting or spitting. A mere inhalation of a few of these bacilli renders one to become infected with the TB disease especially if the immunity has already been compromised with other wasting diseases. A person infected with the latent form cannot transmit the disease whereas the active form of it presents clinical manifestation of the symptoms which may be mild hence can be transmitted to other people who eventually become infected [2]. TB that is diagnosed drug-sensitive is under normal

circumstances treated with four anti TB drugs namely Rifampicin, Isoniazid, Ethambutol and Streptomycin which is accompanied with information given by the care giver under close supervision as provided by the directly observed treatment short course program (DOTS).

World Health Organization in 2015 reported that at least a one-third fraction of people living with Human Immunodeficiency Virus (HIV) worldwide were infected with TB bacteria. It has been noted from other studies that People living with HIV are many times more likely to develop active TB disease than people without HIV. HIV together with TB form a catalyst in disease progression causing a devastating proportion of mortality and morbidity in the world at large. A growing death rate emanating from TB-HIV co-infection calls for a proactive strategic fight because people living with HIV are at risk of drug-resistant TB. The tendency of diagnosing PTB becomes delayed and difficult as they are unable to produce sputum readily for diagnosis and if otherwise only produce sputum that is salivary. Invariably, these patients begin to develop multidrug-resistant (MDR) Pulmonary TB (PTB) and finally end up as a major challenge to public health [14].

The emergence of the multidrug resistant TB (MDR-TB) poses a major problem by diluting the efforts of the international TB control. MDR-TB can be said to be TB that is able to resist the activity of rifampicin and isoniazid anti TB drugs [3]. At the moment, the drugs prescribed for multidrug resistant tuberculosis requires 18-24 months treatment out of which rifampicin had been seen to be more potent [4]. Drug toxicity, prolonged treatment, undetected TB and late detection of TB which increases the risk of transmitting the disease to others, having poor health outcomes, or that the patients and their family will suffer distress and economic hardship, progress in controlling TB and mitigating its consequences needs to be expedited through early diagnosis and treatment hence the need for a technology that has a shorter turnaround time.

Molecular techniques have been seen to be very effective in the diagnosis of TB irrespective of their limitations such as the need for constant power and skilled human resource. The LPA can diagnose patients with MDR-TB and rifampicin-resistant TB within few days [5,6]. Also the GeneXpert technology can analyze and detect *M. tuberculosis* and rifampicin resistance directly from sputum specimen in less than two hours [7-9]. The benefits of these molecular assays outweighs the limitations especially in a country like Nigeria where prompt case detection is a challenge. The conventional culture method used in diagnosing TB takes a minimum of 4 - 10 weeks. It has a feature of delaying rapid case finding and patient management. The two molecular assays used in this study has the capacity to provide accurate results which can enable care givers to initiate patient treatment as quickly as possible until when the culture and Drug Susceptibility Testing (DST) results are released [12].

Materials and Methods

Sample Collection

Samples included in the study were received from different States of the country which were transported in cold chain using triple packaging system to the South-South Tuberculosis Zonal Reference Laboratory (SSTBZRL) in University of Port Harcourt Teaching Hospital (UPTH), Nigeria. Patients only on baseline investigation were included in the study. They were counseled to wash their mouth with clean water firstly, then breath in air for three to five times before expectorating sputum into the sterile containers designated for sputum collection. Sputum containers had been previously labeled with patient name and on getting to the SSTBZRL, they were examined macroscopically before assigning a Laboratory identification number. Only samples that met the Laboratory sample collection criteria were included in the research. Samples that did not meet the sample collection criteria were logged into the sample rejection register and destroyed using standard method. Blood samples were collected for HIV screening from patients which was done at various sites and results entered into patients forms before collecting sputum samples.

Study Area: The study area of this research covers the following states Rivers, Bayelsa, Edo, Delta, Akwaibom, Calaber, Ebonyi, Enugu, Abia, Anambra, and Imo in Nigeria.

Study Design: A qualitative descriptive study design was used in this research.

Conceptual Framework

Roger’s Diffusion of Innovation theory has been used extensively in the last 20 years to understand the process of transferring new knowledge gained in research into clinical practice [19]. This theory provides the framework for the current study and its interpretation. The stages presented in the framework (knowledge, persuasion, decision to adopt an innovation, implementation and confirmation) represent a non-linear pathway toward change in practice. Rogers further proposes specific attributes of innovations that are important in whether practice change is ultimately adopted and successfully implemented: relative advantage, compatibility, complexity, trialability and observability (Rogers, 1995). Relative advantage is the degree to which the innovation is thought to be better than usual care; compatibility is the degree to which the innovation is compatible with existing values; complexity refers to whether the innovation is difficult to understand or implement; trialability is whether the innovation can be implemented on a limited or trial basis; and observability is whether the result of the innovation is apparent. A further consideration in the ultimate success of the translation of research to practice is fidelity or how close the practice innovation, once implemented, matches the methods and intent of the research protocol [15] and the organizational context in which the innovation is implemented [16-18]. For this study, new research knowledge, and the influence of knowledge constitutes an innovation consistent with Rogers’ description of a new preventive health care practice. The dissemination of research, which occurs during the knowledge stage, can be viewed in this context as the analysis, interpretation, presentation and publication of the findings [20].

Sampling Technique

A clustered sampling technique was employed in this research. The samples from various sites were first clustered together. Thereafter, they were categorized under diagnosis and follow up. The follow up samples were further categorized into zero month and other months. The samples for zero months that were Rifampicin resistant were included in the study. Also samples that were only MTB detected, Rifampicin resistant in the diagnosis category were included in the study. Furthermore, the sex and HIV status were selected according to age groups.

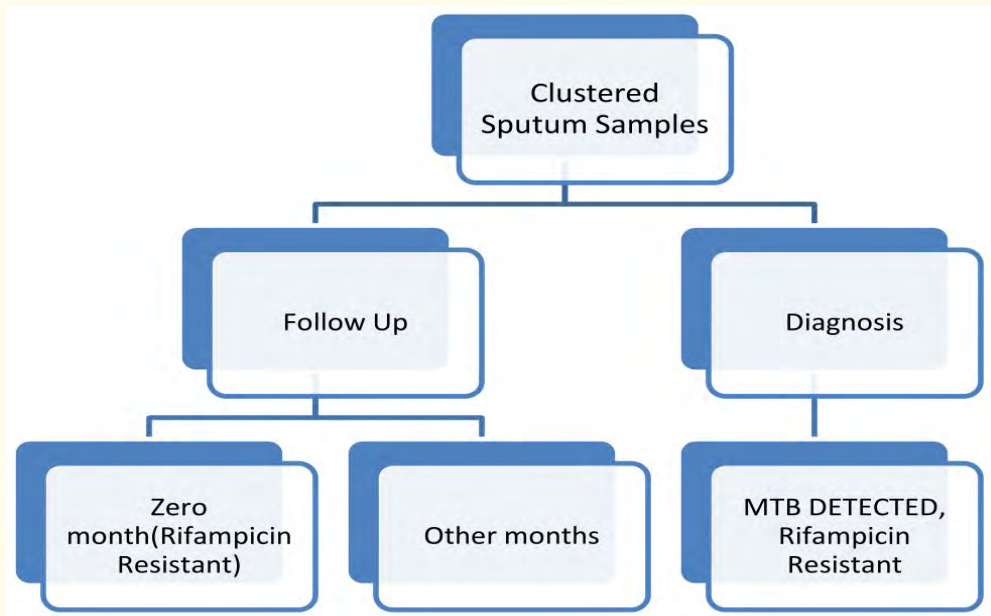


Figure 1: Flow chart of sampling technique.

Eligibility Criteria

All patient samples confirmed by GeneXpert MTB detected, rifampicin resistant were selected into the study including patient samples that were on follow up month zero.

Laboratory Analysis

Ziehl-Nelson's Smear Microscopy Method

Once the sputum samples are received from the transport officers, the sample reception/rejection criteria are checked to ensure compliance. Samples that met the Laboratory standard criteria were accepted. The samples were given Laboratory identification numbers which were written on the samples containers and the patient forms before logging into the sample reception register. The macroscopy of the samples were written on the forms before making smears on grease free slides labeled with patient identification number. The smears were stained using Ziehl-Neelson's standard method.

GeneXpert MTB/RIF Assay

The GeneXpert Dx system version 4.8 was used to analyze the sputum samples. The sputum samples were decontaminated with NaOH isopropanol which was commercially prepared. This reagent decontaminates the sample in the ratio of 1:2. Two mls of sample was agitated with 4mls of isopropanol for 5minutes under the biosafety cabinet. It was allowed to stand for 10minutes and then agitated again for another 5 minutes and further allowed to incubate for 10minutes. 2mls of the solution was transferred into the Xpert cartridge with a disposable pipette and sample identification number written on the cartridge. The Xpert camera was used to capture the cartridge. Patients information were logged into the Xpert monitor and the Xpert machine was commanded to start. All GeneXpert results were recovered from the Xpert machine.

Digestion, Decontamination and Concentration of Sputum Samples

The Sodium Hydroxide Citrate NALC standard method was used following Laboratory Bio-safety practices. The bio-safety cabinet was prepared by using 10% Lysol to decontaminate the Bio-Safety Cabinet (BSC). Thereafter 70% alcohol was used to wipe it. Smoke test was done also to certify the flow of air in the BSC. Sample decontamination was carried out by opening one tube at a time. An equal amount of fresh working NALC-NaOH solution was added to all tubes, rotate and invert tube ensuring that the mixture coats the entire interior surface. The mixture was vortexed for 10 - 15 seconds. This step was repeated for each specimen in the batch. The timer was set for 15 minutes after adding the solution to the first tube in the batch. The samples were allowed to stand for 15minutes. During the incubation time each specimen was checked by slightly tilting the tube and observing for liquefaction. If a specimen was seen to be very mucoid with no change during these checks, a small amount of NALC was directly added to the tube, vortex and allowed to stand until the end of this incubation time.

After the digestant has remained in the first tube for 15 minutes, beginning with the first specimen, the first tube was filled to the 50 ml mark with phosphate buffer saline (PBS) by slowly pouring the buffer down the side of the tube avoiding splashing or contamination. The cap was tightened and the outside of each tube was wiped with the disinfectant soaked towel, then the tube is inverted several times to mix thoroughly. This step was repeated on each of the remaining specimens in the batch, mixing well after each addition. The tubes were then taken to the refrigerated centrifuge to be centrifuged at 3000RM for 15 minutes. The tubes were brought out, supernatant was decanted and body of the tubes wiped with paper towel soaked with 10% Lysol. 2mls of PBS was expelled into the tubes using a graduated Pasteur pipette to keep the organisms viable and tubes were tightly capped.

Extraction of DNA

DNA was extracted from decontaminated samples by transferring samples into micro tubes and centrifuged for 15 minutes in a micro centrifuge at 10,000 rpm. Thereafter, lysis buffer was added to the pellet and incubated for 5 minutes at 95 degrees centigrade. Neutralization buffer was then added. The supernatant was transferred into another micro tube which serves as the DNA.

Line Probe Assay (LPA)

LPA was conducted by firstly preparing the master mix. This was taken to the sample preparation room. Prepared DNA was passed through the polymerase chain reaction according to Standard method provided by Hain Life Science.

Results

The results of the study are as follows:

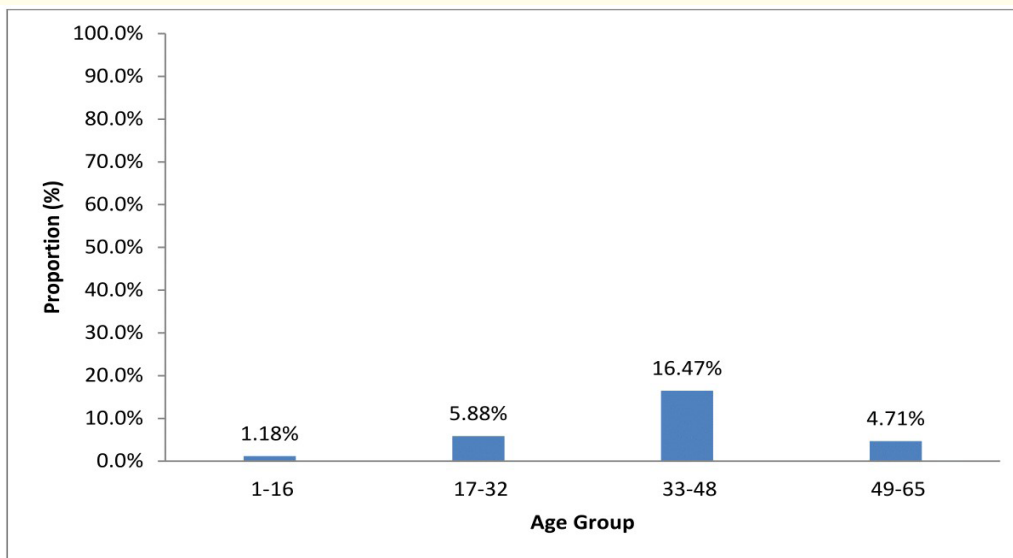


Figure 2: Prevalence of Pulmonary TB-HIV Co-infection among Age groups.

Table 1: Distribution of TB-HIV Co-infection among sexes in relation to age group.

*Statistically significant (p < 0.05) (p = 0.001)

Age group (years)	No Tested	HIV (Positive)	Sex		GENEXPERT (Positive) Rifampicin Resistant	Smear (AFB Positive)	TB-HIV Co-infection
			M	F			
1-16	11 (12.94)	2 (2.5)	6 (7.0)	5 (5.88)	1 (1.18)	2 (2.35)	1 (1.18)
17-32	21 (24.71)	10 (11.76)	14 (16.47)	7 (8.24)	5 (5.88)	5 (5.99)	5 (5.88)
33-48	34 (40.0)	21 (24.71)	22 (25.88)	12 (14.12)	14 (16.47)	14 (16.47)	14 (16.47)
49-65	19 (22.35)	7 (8.24)	15 (17.65)	4 (4.71)	4 (4.71)	4 (4.71)	4 (4.71)
Total	85 (100.0)	40 (47.06)	57 (67.06)	28 (32.94)	24 (28.24)	25 (29.41)	24 (28.24)
X ² (p-value)							16.86 (0.001)*

Table 2: Prevalence of Multidrug Resistant Pulmonary TB.27

Age group (years)	No Tested	LPA (Resistance)		
		First Line Drugs		
		RIF only	INH only	INH/RIF
0 - 16	11 (12.94)	0 (0.0)	0 (0.0)	
17 - 32	21 (24.71)	3 (3.5)	3 (3.5)	1 (1.18)
33 - 48	34 (40.0)	10 (11.76)	5 (5.88)	2 (2.35)
49 - 65	19 (22.35)	3 (3.5)	1 (1.18)	1 (1.18)
Total	85 (100.0)	16 (18.82)	9 (10.5)	4 (4.71)

Discussion

The incidence and prevalence of Pulmonary Tuberculosis (PTB) and HIV co-infection in developing countries, such as Nigeria, remains worrisome while the cure rate is still low. The prevalence of multidrug PTB-HIV co-infection among age groups was studied. In this study, highest prevalence was seen among age group 33-48 years (16.47%) and lowest prevalence was seen among 1-16 age group (1.18%) (Figure 2). Bassett., *et al.* reported a PTB-HIV co-infection prevalence among adults to be 19% in Durban, South Africa, which is closely higher than our PTB-HIV co-infection frequency of 16.47% [34]. Worldwide prevalence of PTB-HIV is reported to be 0.18% [35], which is lower than the 16.4% in our study. Aweke., *et al.* [36] reported the prevalence of TB/HIV co-infection in adults on ART in his study to be 27.7% which is slightly higher than that reported in our study. It was a little lower than 32.8% reported from Nigeria [41] and 33% reported from Ethiopia [42]. However, the findings of this study were higher compared to studies conducted in Ethiopia (7.5%), Nigeria (7.8%) and Tanzania (8.5%) [25,43,44]. The differences in PTB prevalence rates in other populations compared to ours may somewhat be true. Measures taken to diagnose TB in children are routinely accompanied with challenges especially when it is co-infected with HIV. This can otherwise be attributed to the difficulty encountered in expectorating sputum for the diagnosis of TB among children. From the forgoing, it was observed that a few number was studied in this age group because of the fore mentioned challenge. The results of this study differs from the one reported by WHO which is 10 - 60% among HIV children, in countries with moderate to high TB [22] burden. Another studies reported 10.0% from West Africa [25]. A much lower proportion than the former 19.5% was reported [26] from Abuja in Nigeria. Also 17.0% was reported among HIV [27] hospitalized children in Peru, and even far lesser than 48.8% found among HIV positive children in [28] Benin, Nigeria and 48.0% reported from a study in [29] South Africa. Nevertheless it is closely comparable to 2% [23,24] from HIV children at Nnewi, Nigeria 3.0% and 5.5% [30,31] reported from US and UK respectively. The disparity in the proportions seen from various countries could be as a result of difference in technology, study sites, expertise, storage temperatures of reagents, sample transportation methods, and criteria for sample acceptance and storage. Nigeria is one of the countries of the sub region that have continued to bear the greatest burden of paediatric [24] HIV/AIDS. It was observed from our study that more than half of people living with TB reside in the sub-region where the TB bacilli can thrive faster and HIV becomes an advantage to [28] developing TB due to compromised immune system.

The overall distribution of PTB-HIV co-infection in our study appears to be high (28.28%) (Table 1). This is somewhat closely higher than that reported by [37]. TB-HIV co-morbidity was higher in males than in females in this study with a statistical significance P = 0.001. This result is in contrast to the finding published by [37] but in accordance to the findings reported by [38] where there was no sex difference. Other studies [45,46] recorded a close margin compared to the findings of our report in this study having a higher HIV-TB burden in the male population than in females. A close association of infection was seen in this study between HIV and TB. This finding differ from a study [46] where the incidence of HIV-TB co-existence was 9.6% but similar to that of the national HIV infection rate among adult TB patients in Nigeria which was also estimated to be 27% by the WHO [47]. For instance, Borno, Plateau and Benue were also reported by

the Federal Ministry of Health to have HIV-TB prevalence of 27%, 30%, and 35% respectively [48]. The emergence of the ancient disease has been attributed strongly to the destruction of the human defense system, poor personal hygiene, overcrowding, ignorance, and poverty among others [50].

Prevalence of DR-PTB was also evaluated in our study (Table 2). A mono drug resistance and multidrug resistant pattern was seen to be 18.82% for Rifampicin (RIF), 10.5% for Isoniazide (INH) and 4.71% for Isoniazide and Rifampicin (RIF/INH). This is higher than that reported by [52] where 1.4% for INH mono-resistance and 2.8% for RIF mono-resistance. The rifampicin resistance in this study is closely comparable to [14] where the proportion of resistance among the study participants is 1%. Generally, a high drug resistant pulmonary TB burden was noticed in the study area. Our results may represent an underestimate of drug resistance because of the low sample size however, the results of this study indicates that transmission of drug-resistant TB has become a more serious problem than earlier imagined. The molecular technology used in this study has the potency to correctly identify mutations with a high concordance rate. Also, the ability of the *rpoB* gene which codes for RIF resistance is thought to be sufficient for evaluating the public health threat of drug-resistant TB. Nevertheless, this fact remains for further investigation [51]. Lack of laboratory capacity in high TB burden countries, notably Nigeria is a barrier to diagnosis DRTB. Adequate skilled workforce is required in the diagnosis and management of MDR-TB. Government and external funders should live up to the task of ensuring that human resource is adequately deployed to facilities where TB is diagnosed and managed. Policy makers should widen the scope of technology and facilities with training and retraining Health workers to discourage contacting and spreading this deadly disease to family members. This is crucial as some care givers and health workers have become victims of this deadly disease. They automatically become a potential incubating source for the TB bacilli if they are not catered for. The concept of screening MDR-PTB at time of diagnosis seems to be a novel approach for detecting MDR-TB in Nigeria. However, constraints do exist. Firstly, the available facilities for conducting the rapid tests using DNA based line probe assay could be a challenge in far flung areas and also at the district hospital level where the infrastructure, availability of trained and skilled manpower is a problem. Transportation of the samples in high temperature climate is again another challenge. This is also highlighted by another study where specimen transport and specimen contamination issues pose a challenge in prompt diagnosis of DR-TB in developing countries [13].

Conclusion

The increasing trend towards pursuit for greener pastures put all countries for potential targets of outbreaks of MDR- TB. Nigeria is not an exception as the growing number of people with MDR-TB and emerging evidence from the expanding use of molecular technologies for TB diagnosis points towards increasing case detection. The TB epidemic in Nigeria rides on the HIV-co-morbidity to stimulate the progression of latent TB infection to active TB disease. I believe that Nigeria can make rapid progress in the control of TB if she adopts a strict committed approach by visiting families in the communities to identify those coughing and collecting their sputum samples for TB diagnosis. After all delay in diagnosing TB is a thing of the past with the advent of the GeneXpert and LPA. Educating them that TB is not as abominable as they taught hence behavioral change and adherence to policies binding the TB control can actually prevent it and also informing them that those that are already infected can be cured.

Ethical Approval

Ethical approval was obtained from the Rivers State Hospitals Management Board, Nigeria.

Conflict of Interest

There was no conflict of interest.

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Volume 7 Issue 5 May 2018

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