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Role of HIV Infection in Multi-Drug Resistant Tuberculosis in Parts of Benue State, Nigeria

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ABSTRACT

The pathogenesis of *Tuberculosis* shows that *M. tuberculosis* target and persist within phagocytes including T-lymphocytes in blood circulation. As a result, the possibility of cellular interaction between *M. tuberculosis* and HIV, especially for patients that are co-infected with HIV and TB, and subsequent exchange of genetic material via transduction needs to be investigated. Three hundred and eighty sputum samples mostly from suspected rifampicin-resistance patients were collected from Nigerian Airforce (NAF) Hospital Makurdi, and Federal Medical Centre (FMC) Makurdi. In vitro culture of sputum samples, Drugs Susceptibility Testing (DST) of M. tuberculosis isolates, and transduction protocol were carried out at the National Tuberculosis and Leprosy Training Centre (NTBLTC) Zaria, Nigeria. Statistical analysis was carried out using Student's t-test. Minitab version 14.0 statistical software was used for data analysis. P-values < 0.05 were considered significant. Twenty-six (9.7%) cases of Multi-drug resistant tuberculosis (MDR-TB) were detected (retreated cases 7.1%; treatment naive 2.6%). Twenty-one (80.8%) were males and 5(19.2%) were females. There was statistical difference in MDR-TB between male and female in Benue State (P<0.05). The mean age group 35-45 years had the highest cases of MDR-TB accounting for 35% of MDR-TB. Human-immunodeficiency virus and Tuberculosis co-infected patients (category I) had the highest MDR-TB incidence of 10(38.5%). There was no significant difference between category-1 and category-III [patient with only TB disease (P>0.05)]. However, there were elevated cases of MDR-TB in category-III patients from 7(26.9%) to 10(38.5%) following transduction protocol. Multi-drugs resistant tuberculosis is prevalent in Benue State, affecting the most economically active youths within the age group of 35-45 years, as a result the need to direct more attention on molecular basis for *M. tuberculosis* drugs resistance is

INTRODUCTION

The genus *Mycobacterium* are non-motile, non-sporulating, weakly Gram-positive, acid-fast bacilli that appear microscopically as straight or slightly curved rods measuring 0.2 to 0.4 μm in length (Willey *et al.*, 2011).

Mycobacteria are within the order Actinomycetales, which it shares with bacteria such as *Corynebacterium*, *Norcadia* and *Rhodococcus*. Mycobacteria have been divided into two major groups based on fundamental differences in epidemiology and association with disease. Those belonging to the *Mycobacterium tuberculosis* complex (MTBC) (*M. tuberculosis*, *M. bovis*, *M. africanun*, *M. canetti*, and *M. microti*, with *M. laprae* and *M. pinnipedii* considered variants of *M. bovis*), and those referred to as the non-tuberculous mycobacteria (NTM) such as *M. avium* complex, *M. haemophilum*, *M. ulcerans*, M. leprae (nonculturable), and the potentially pathogenic species such as *M. smegmatis* and *M. abscessus* (Forbes *et al.*,2002).

A very important and unique characteristic of Mycobacteria is that the organisms grow more slowly than most other human pathogenic bacteria because of their hydrophobic cell surfaces (Forbes *et al.*,2002).

Tuberculosis is a common, and in many cases fatal, infectious disease caused by various strains of mycobacteria, usually *M. tuberculosis* (Kumar *et al.*, 2007). Tuberculosis is an airborne disease that affects the lungs (pulmonary TB), but can also affect other parts of the body (extra pulmonary TB) such as the larynx, the lymph nodes, the pleura, the brain, the kidneys, or the bones and joints (Bardarov *et al.*, 2002).

Based on clinical presentation, TB can be categorized into active TB disease characterized by chronic cough with blood-tinged sputum, fever, night sweats and weight loss (the latter giving rise to the formerly common term consumption), most infections do not have classical symptoms and are thus referred to as latent TB infection (LTBI). Persons with LTBI have *M. tuberculosis* in their bodies, but do not have TB disease and cannot spread the infection to other people (Bardarov *et al.*, 2002).

It is estimated that one-third (at least 2 billion people) of the world's human population is infected (Willey *et al.*, 2011). Of the 212 countries and territories in the world, 202, (99.6% of the world's population) reported TB cases in 2007; the World Health Organization (WHO) reported 9.2 million new TB cases, with approximately 7.7% being HIV positive (Willey *et al.*, 2011).

Today, TB is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent, in 2012 for instance, 8.6 million people fell ill with TB and 1.3 million died from TB, with over 95% of TB deaths reported in low and middle-income countries (WHO, 2014), tuberculosis is still among the top three causes of death for women aged 15 to 44 years (WHO, 2014).

The emergence of MDR-TB, first reported in the late

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1980s (Cegielski, 2010), and present in virtually all the countries surveyed has posed a great obstacle to effective TB control at both national and global levels.

In 2010, the World Health Organization (WHO) estimated that globally there were 290, 000 cases of MDR-TB among reported cases of pulmonary TB (WHO, 2011). The World Health Organization also reported an estimated 650, 000 cases of MDR-TB among the world's 12 million prevalent cases of TB with Nigeria alone accounting for 95 reported cases of MDR-TB (WHO, 2011). Resistance to TB-drugs is said to arise from both service and patient related factors in the management of Tuberculosis ranging from poor compliance, inadequate supervision, inadequate dosing, wrong drug combination, lengthy duration of treatment and poor training of health personnel (Federal Ministry of Health FMOH, 2005).

Despite reasonable degree of successes and achievements recorded in the stop TB targets of the millennium Development Goals (MDGs) number-6, target-8 of reducing the global burden of TB disease (death and prevalence) by 50% by 2015 (WHO, 2011), the second component of the stop TB strategy to address TB/HIV and MDR-TB remains a global concern.

The pathogenesis of Tuberculosis shows that *M. tuberculosis* is often acquired early in life with acute infection and with developing immunity, granuloma formation, and calcification. This is followed by a long latent period, which continues until reactivation occurs in a proportion of individuals. At this period, the organism target and persist within phagocytic monocytes, macrophages, polymorphonuclear neutrophils and T-lymphocytes in blood circulation (Van Crevel *et al*., 2002). As a result, there is a possibility of cellular interaction between *M. tuberculosis* and HIV, especially for patients that are co-infected with HIV and TB, and subsequent exchange of genetic information via transduction. It has therefore become necessary to determine the role of HIV in the emergence of MDR-TB in HIV/TB co-infection dynamics. Considering the fact that HIV plays a major role in infectious diseases generally, and TB in particular (Bardarov *et al*., 2002), the need to focus attention on understanding the molecular basis of TB pathogenesis especially MDR-TB with particular emphasis on HIV/TB co-infection has become a top priority.

MATERIALS AND METHODS

A total of 380 sputum samples determined by Raosoft sample size calculator (Raosoft, 2015) were collected from a wide spectrum of TB patients from two geographically distinct sites: three hundred and three sputum samples were collected from Nigerian Airforce (NAF) Hospital/CDC laboratory, while the remaining seventy-seven sputum samples were collected from Federal Medical Centre (FMC)/APIN laboratory, in Makurdi Benue State. Between three to ten millilitres of sputum samples were collected in cotylpyridinium chloride (Bromide) containing universal bottles, and immediately refrigerated at 2-8°C. Patients who test positive for Acid Fast Bacilli (AFB) after two months of intensive phase TB DOTS therapy (suspected Rifampicin resistance) was enrollment criteria. Demographic information of subjects was obtained from medical records. Data was collected all age groups. The study design was approved by Research and Ethics committee, Benue State Ministry of Health.

Out of 380 sputum samples analysed, 130 (34.2%) were from TB patients co-infected with HIV, and who were positive for AFB after more than two months' intensive phase of TB DOTS therapy (Category I Patients). Another 130 (34.2%) were from HIV negative TB patients who were positive for AFB after more than two months of intensive TB DOTS therapy (Category II Patients), while 120 (31.6%) sputum samples were collected from HIV negative TB patients who yet to commence TB DOTS therapy (treatment naïve)-Category III Patients). Detection of Rifampicin resistance was by *GeneXpert* Technology. All Sputum samples were plated on Lowenstein-Jensen (LJ) medium for pure cultures of *M. tuberculosis* and confirmed by biochemical tests. Pure cultures of *M. tuberculosis* were preserved by refrigeration as stock cultures at -20°C (David, 1970). Anti-TB Drugs Susceptibility Testing (DST) of *M. tuberculosis* isolates was carried out using the proportion method on *BACTEC MGIT 960* TB system (Becton and Dickinson, New Jersey USA). Stock cultures of Human T-cell Lymphotrophic Virus-3 (HTLV-III) polyclonal unconjugated preparation procured from *GENTAUR Molecular GenWay* products, U.S.A, were used as specialized transducing phage in transduction protocols. Drugs Susceptibility testing of transductants was also carried out by proportion method on *BACTEC MGIT 960* TB system (Becton and Dickinson, New Jersey USA). All procedures were carried out at Biosafety Level two (BSL-11).

GeneXpert Procedure

Xpert MTB-RIF Assay G4 Version 5 (Cepheid, USA) was used. Briefly, 2.0 ml of Sputum sample was added into 4.0 ml of *Xpert* reagent in a ratio of 1:2. The closed specimen was manually agitated twenty times and incubated at room temperature (20-25°C) for 15 minutes. Two millilitres of the reagent-sample mixture was transferred to Xpert test cartridges and inserted into the *Xpert* device. Results (for M. tb either detected or not, and with or without Rifampicin resistance) were obtained in exactly 110 minutes (Cepheid, 2014).

Processing of Sputum for *M. tuberculosis* Culture

Modified Petroff's method 2012 for culture of *M. tuberculosis* was used. Between 3-5 ml sputum was homogenized in a shaker using an equal volume of 4% NaOH and centrifuged at 3000rpm for 15 minutes. Then 0.067M phosphate buffer (pH 6.8) was added to the digested-decontaminated sample (deposit) up to the 45.0ml mark to reduce the continued action of NaOH, while the sediment was re-suspended in 2.0ml of buffer. The sediment was now ready for inoculation unto LJ slants, or refrigerated at 2- 8°C (Joshua *et al*., 2013).

Culture of Processed Sputum Sample for *M. tuberculosis*

Lowenstein-Jensen medium (Oxoid Biologicals, Canada) was used. Lowenstein-Jensen slants (egg based) were prepared according to manufacturer's instructions and stored at 2-8°C in the dark. Three drops of processed sputum sediment were added to each Lowenstein-Jensen tube using a sterile plastic pipette. The inoculum was spread over the surface of the slant by gently rolling the liquid over the slant and incubated at 35-37°/7 days in a slanted position with loose screw for even distribution and adsorption of inoculum. After one week, inoculated slants were incubated at 35-37°C, and examined respectively at three and seven days of incubation to allow early detection of contaminants (or rapidly growing mycobacteria). Thereafter cultures were examined weekly for growth of *M. tuberculosis*. Negative cultures were discarded after 8 weeks of incubation. Pale cream colonies which were granular, rough or dry were suggestive of *M. tuberculosis*. Ziehl Neelsen (ZN) staining confirmed growth of mycobacteria. Growth was reported as none (no visible growth), contamination (C), <50 colonies (actual count), 50-100 colonies (1+), 100-200 colonies (2+) >200 colonies (3+), confluent growth (4+) (Joshua *et al*., 2013).

Conventional biochemical tests such as catalase test, growth on P-Nitro Benzoate (PNB), and Nitrate reduction were used to distinguish and differentiate *M. tuberculosis* from other mycobacteria. Pure cultures of *M. tuberculosis* were inoculated on Lowenstein-Jensen broth and preserved as stock cultures in 2ml cryovials at -20°C for drugs susceptibility testing and transduction protocols.

Staining Mycobacterial Isolates from LJ Slants

Ziehl Neelson (ZN) staining method was used. One drop of distilled water was placed in the middle of a clean grease free slide. Growth from the LJ slope was scrapped off and emulsified in saline on the slide using a sterile disposable loop. The smear was allowed to air dry thoroughly and was fixed by passing the reverse three times through a blue flame. A negative control slide was also prepared from a *genexpert* negative sample as earlier described. The slides were arranged on a staining rack, flooded with carbol fuschsin working solution and heated to steam for five minutes without drying or boiling. The slides were washed gently with tap running water to remove excess carbol fuschsin and flooded with 3% acid alcohol for three minutes to decolorize completely, and washed under running water for one minute. The slides were flooded with Methylene Blue and counterstained for 1 minute. The slides were rinsed with tap running water, drained and air dried, examined under oil immersion magnification (x100). Acid Fast Bacilli appeared as pink or red bacilli while the negative control slide appeared blue (Joshua *et al*., 2013).

Preparation of mycobacteria growth indicator tube (MGIT)

Plastic caps from the Streptomycin Isoniazid Rifampicin and Ethambutol (SIRE) supplements were removed. Caps from MGIT were also removed, and 0.8ml supplements were aseptically dispensed into each MGIT using sterile pipette. The tubes were immediately recapped. The procedure was repeated using PZA supplement. Five MGIT (7.0ml) were labeled with SIRE supplement for each test isolate as (growth control), S(SM), I(INH), R(RIF), and E(EMB). Two MGIT (7.0ml) were labeled with PZA supplements for each test isolate as C (growth control), S(SM), I(INH), R(RIF), and E(EMB). Two MGIT (7.0ml) were labeled with PZA supplement for each test isolate as C (growth control), and PZA.

Micropipette was used to aseptically pipette 100µL working drug concentrations into each of the appropriately labeled MGIT. No antibiotics were added to MGIT control tubes. 0.5 mL of the organism suspension was aseptically dispensed into each of the five tubes containing drugs (SM, INH, RIF, EMB, PZA). 1:10 growth control suspension was prepared by aseptically adding 0.5 ml of the organism suspension into 4.5 mL of sterile saline. The 1:10 suspension was mixed thoroughly, and 0.5ml inoculated into MGIT-PZA control tube, 0.5ml was further diluted with 4.5 ml sterile saline from the previous 1:10 growth control suspension to produce 1:100 dilutions. The 1:100 suspensions were thoroughly mixed and 0.5 ml was inoculated into the MGIT control tube.

The Tubes were tightly recapped, thoroughly mixed by gently inverting three to four times and were wiped with disinfectant, loaded into the appropriate DST carrier. SIRE was loaded into a five-carrier holder, while PZA was loaded into a two-carrier holder. One drop of the organism suspension from the 1:100 control tube was streaked on a Blood Agar Plate (BAP), sealed with paraffin and incubated at 35-37°C for four days. The BAP was read daily, for up to four days, for bacterial contamination. The DST was allowed to proceed only for BAP that showed no growth during the four days monitoring (Joshua *et al*., 2013).

Drug Susceptibility Test (DST) for *M. tuberculosis*

BACTEC MGIT 960 TB system (Becton and Dickinson, New Jersey USA) was used. The system monitors continuous growth of microorganisms in both drug-containing and control tubes to determine susceptibility or resistance, and automatically interprets and reports results of tests. The following anti-TB drugs (SIRE) were reconstituted to the following concentrations: Streptomycin 1.0 µg/ml, Isoniazid 0.1 µg/ml, Rifampicin 1.0 µg/ml, Ethambutol 5.0 µg/ml, Pyrazinamide 100 µg/ml (PZA). Preserved stock cultures of 0.1ml *M. tuberculosis* were sub-culturedm to *Mycobacterium* Growth Indicator Tube (MGIT) McFarland standard (1.0 suspension) equivalent to 3.0 x 108 cfu/ml. Reconstituted 0.8 ml SIRE supplements were aseptically dispensed into each MGIT with a pipette and sterile tips. MGIT was immediately recapped Interpretations of DST results were read

between days four and thirteen SIRE, PZA test. Control tubes that flagged positive before day four were repeated. Similarly, control tubes that remained negative after day thirteen were also repeated. The DST for the drugs under consideration was reported as "sensitive" when the control tubes reached growth unit (GU) of 400, while the drug tubes had growth units (GU) of less than 100. The results were reported as resistant with a rise in GU equal to or greater than 100 and growth in the control tube equivalent to 400 GU (Siddiqui and Rusch-Gerdes 2006). *M. tuberculosis* strains that exhibited combined resistance to rifampicin and isoniazid were reported as MDR-TB (Willey *et al*., 2011).

Transduction Protocol

Transduction protocol was carried out as described by Bardarov and co-researchers (Bardarov *et al*., 2002), with slight modifications. Preserved cultures of *M. tuberculosis* from category III patients were used. One milliliter of *M. tuberculosis* stock was inoculated into 10ml LJ broth in 30ml plastic culture bottles, and incubated at 37°C in an incubator shaker. The *M. tuberculosis* strains were grown to optical density OD of 600 ~ 0.8-1.0(6.0 x 108 c.f.u ml-1). Ten milliliters of the culture were centrifuged at 2500g for 5minutes and re-suspended in 10ml washing medium of 1% tween 80 phosphate buffered saline (PBS-TW pH 7.0) and incubated as a standing culture (37°C; 24hr). After the preparation period, the cells were again centrifuged at 2500g for five minutes and re-suspended in 10ml LJ broth, pre-warmed at 37°C and mixed with a specialized transducing phage (Human T-cell Lymphotrophic Virus-3) in a 10ml: 1ml v/v ratio. The cell/phage mixture was inoculated into 50ml LJ broth and incubated at 37°C. Outgrowth of the cultures was performed for 24hr at 37°C. Cells were then pelleted by centrifugation at 2500g for 15minutes and re-suspended in one millilitre PBS-TW (1.0% tween 80 in phosphate buffered saline). DST of transductant was also carried out using BACTEC MGIT 960TB system earlier described. The results of DST for transductants were interpreted and reported using the format for pure *M. tuberculosis* isolates earlier described.

Analysis of Data

The results were analyzed using minitab version 14.0 statistical softwares. Student's t-test was used to compute frequencies and proportions, P-values <0.05 were considered significant at 95.0 % confidence level.

RESULTS

Out of the 380 sputum samples collected and analyzed, 268(70.5%) yielded positive *Mycobacterium tuberculosis* cultures. The results of DST carried out on the 268 *M. tuberculosis* strains showed that MDR-TB (combined resistance to Rifampicin and Isoniazid) was detected in 26(9.7%) strains. Twenty-one (80.8%) cases were male, while five (19.2%) cases were female. There was statistically significant difference in MDR-TB between males and females (P<0.05) in the study population. The Results of DST for the three categories of patients is shown in table 1.

The results of DST for Category I patients showed that MDR-TB was detected in 10(38.5%) cases while rifampicin resistance was detected in 32(11.9%) of cases as shown in table 2.

Table 1: Overall Results M. tb of Culture and Anti-TB Drugs Susceptibility Testing (DST)

Age Group (Years)	M. tb + Cultures	CAT I		CAT II		CAT III		Total MDR-TB Detected	%
		M	F	M	F	M	F		
<1 – 10	2	0	0	0	0	0	0	0	0
11 – 20	8	0	0	1	0	1	0	2	7.69
21 – 30	70	1	1	1	0	1	0	4	15.38
31 – 40	106	2	0	2	0	2	0	6	23.08
41 – 50	37	3	1	3	1	1	1	10	38.46
51 – 60	31	1	1	1	0	1	0	4	15.38
61 – 70	9	0	0	0	0	0	0	0	0
≥ 71	5	0	0	0	0	0	0	0	0
Total	**268**	**7**	**3**	**8**	**1**	**6**	**1**	**26**	**100**

Key: M. tb = Mycobacterium tuberculosis, MDR-TB= Multi-drug Resistant Tuberculosis, M=Total Male, F=Total Female, +=positive M. tb cultures, CAT I = category 1, CAT II = Category 2, CAT III = Category 3

Results of DST for Category II patients showed a slight decline in cases of MDR-TB from 10(38.5%) to 9(34.6%) with a corresponding decrease in the total number of Rifampicin resistance of 26(9.7%) detected as shown in table 3

The least cases of MDR-TB of 7(26.9%) were detected in category III patients while Rifampicin resistance was detected in 24(90%) of cases as shown in table 4.

There was no statistical difference in MDR-TB between Category I and Category III (P>0.05). However, cases

Table 2: Sputum Culture and Anti-TB DST (Category I Patients)

Age (years)	No. of M. tb + Cultures	RIF		INH		S		PZA		EMB		MDR-TB Detected	M	F
		R	S	R	S	R	S	R	S	R	S			
<1 – 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11 – 20	3	0	3	0	3	0	3	0	3	2	1	0	0	0
21 – 30	26	9	17	2	24	7	19	2	24	16	10	2	1	1
31 – 40	38	15	23	2	36	7	31	1	37	17	21	2	2	0
41 – 50	14	5	9	4	10	2	12	0	14	8	6	4	3	1
51 – 60	5	2	3	2	3	0	5	0	5	5	0	2	1	1
61 – 70	1	1	0	1	0	0	1	0	1	1	0	0	0	0
≥ 71	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**87**	**32**	**55**	**11**	**76**	**16**	**71**	**3**	**84**	**49**	**38**	**10**	**7**	**3**

t = 800.00, df = 14, p< 0.05

Key: EMB = Etambutol, M. tb = Mycobacterium tuberculosis, MDR-TB= Multi-drug Resistant Tuberculosis, R = Resistant, S = Susceptible, RIF = Rifampicin, INH = Isoniazid, S = Streptomycin, PZA = Parazinamide, M=Total Male, F=Total Female, +=positive M. tb cultures, DST= Drugs Susceptibility Testing.

Table 3: Sputum Culture and anti-TB DST (Category II Patients)

Age (years)	No. of M. tb + Cultures	RIF		INH		S		PZA		EMB		MDR-TB Detected	M	F
		R	S	R	S	R	S	R	S	R	S			
<1 – 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11 – 20	1	1	0	1	0	0	1	0	1	0	1	1	1	0
21 – 30	18	6	12	1	17	2	15	5	12	8	11	1	1	0
31 – 40	31	11	20	2	29	3	28	6	25	11	20	2	2	0
41 – 50	7	4	3	4	3	1	6	0	7	2	5	4	3	1
51 – 60	14	2	12	1	12	1	13	1	15	2	12	1	1	0
61 – 70	6	2	4	0	6	0	6	0	6	1	5	0	0	0
≥ 71	2	0	3	0	3	0	3	0	3	0	3	0	0	0
Total	**79**	**26**	**54**	**9**	**70**	**7**	**72**	**12**	**69**	**24**	**57**	**9**	**8**	**1**

t = 885.44, df = 10, p< 0.05

Key: EMB = Etambutol, M. tb = Mycobacterium tuberculosis, MDR-TB= Multi-drug Resistant Tuberculosis, R = Resistant, S = Susceptible, RIF = Rifampicin, INH = Isoniazid, S = Streptomycin, PZA = Parazinamide, M=Total Male, F=Total Female, +=positive M. tb cultures, DST= Drugs Susceptibility Testing

Table 4: Sputum Culture and anti-TB DST (Category III Patients)

Age (years)	No. of M. tb + Cultures	RIF		INH		S		PZA		EMB		MDR-TB Detected	M	F
		R	S	R	S	R	S	R	S	R	S			
<1 – 10	2	0	2	0	2	0	2	0	2	0	2	0	0	0
11 – 20	4	3	1	1	3	0	4	1	3	0	4	1	1	0
21 – 30	26	6	20	1	25	5	21	2	24	4	22	1	1	0
31 – 40	37	4	33	2	35	4	33	8	29	6	31	2	2	0
41 – 50	16	6	10	2	14	2	14	6	10	4	12	2	1	1
51 – 60	12	3	9	1	11	2	10	1	11	3	9	1	1	0
61 – 70	2	1	1	0	2	0	2	0	2	0	2	0	0	0
≥ 71	3	1	2	0	3	0	3	0	3	1	2	0	0	0
Total	**102**	**24**	**78**	**7**	**95**	**13**	**89**	**18**	**84**	**18**	**84**	**7**	**6**	**1**

t = 632.46, df = 10, p< 0.05

Key: EMB = Etambutol, M. tb = Mycobacterium tuberculosis, MDR-TB= Multi-drug Resistant Tuberculosis, R = Resistant, S = Susceptible, RIF = Rifampicin, INH = Isoniazid , S = Streptomycin, PZA = Parazinamide, M=Total Male, F=Total Female, +=positive M. tb cultures, DST= Drugs Susceptibility Testing

of MDR-TB were statistically higher in Category I than Category III patients.

The age group 41-50 years had the highest cases of MDR-TB followed by the age group 31-40 years (mean age group 35-45 years) both of which accounted for 10(38.5%) and 6(23.1%) cases respectively, thereby placing the two age groups at relatively higher risk of developing MDR-TB. Demographic characteristics of the 268 *M. tuberculosis* strains analyzed in Tables 2 and 3 (re-treated cases) and Table 4 (treatment naive) recorded 19(7.1%) and 6(2.6%) cases respectively.

Following in-vitro transduction (induced mutation) of *M. tuberculosis* strains from Category III patients, there was an increase in the number of MDR-TB detected in this Category of patients from 7(26.9%) to 10(38.5%) cases as shown in Table 5. However, there was no statistical significance in the number of MDR-TB detected in Category III before and after transduction protocols (P>0.05).

Overall results of susceptibility for all the tested anti-TB drugs shows a cumulative susceptibility of 1012(84.3%) and resistance of 189(15.7%). Etambutol showed the highest resistance of 91(34.0%) followed by rifampicin with 82(30.6%) of cases, the least cases of resistance were

Table 5: Anti-TB DST of Category III (control group) Patients after HTLV-3 transduction protocol

Age (years)	No. of M. tb + Cultures	MDR-TB detected	RIF		INH		S		PZA		EMB	
			R	S	R	S	R	S	R	S	R	S
<1 – 10	1	0	0	1	1	0	0	1	0	1	0	1
11 – 20	2	1	2	0	1	1	0	2	0	2	0	2
21 – 30	13	2	5	8	2	11	9	4	3	10	9	4
31 – 40	18	3	7	11	3	15	11	7	4	14	11	7
41 – 50	8	2	2	6	3	5	4	4	1	7	4	4
51 – 60	6	2	2	4	4	2	2	4	0	6	2	4
61 – 70	2	0	0	2	0	2	0	2	0	2	0	2
≥ 71	2	0	0	2	1	1	0	2	0	2	0	2
Total	**79**		**26**	**54**	**9**	**70**	**7**	**72**	**12**	**69**	**24**	**57**

t=-300.00, df =14, p<0.05

Key: EMB = Etambutol, M. tb = Mycobacterium tuberculosis, MDR-TB= Multi-drug Resistant Tuberculosis, R = Resistant, S = Susceptible, RIF = Rifampicin, INH = Isoniazid, S = Streptomycin, PZA = Parazinamide, M=Total Male, F=Total Female, +=positive M. tb cultures, DST= Drugs Susceptibility Testing

produced by isoniazid which recorded 27(10.1%) making it the most effective anti-TB drug in the study population. 266(99.3%) of the *M. tuberculosis* isolates produced various degrees of susceptibility and resistance to the five tested anti-TB drugs. However, 2(0.7%) showed 100% susceptibility to all the five tested anti-TB drugs with no trace of drugs resistance. Meanwhile all the *M. tuberculosis* strains were resistant to at least one or more anti-TB drugs thereby implying complete absence of mono-drug resistant tuberculosis in the study population. It is equally noteworthy that no strain was completely resistant to all the tested anti-TB drugs as summarized in Table 6. t = -148.48, df = 13, p< 0.05

Results of *Genexpert* for all the 380 sputum samples showed rifampicin resistance was detected in 62(16.3%) cases. Male accounted for 36(58.1%) while female accounted for 26(41.9%) of the cases as contained in Table 7.

Table 6: Cumulative Results of anti-TB DST

Category	RIF No (%)		INH No (%)		S No (%)		PZA No (%)		EMB No (%)	
	R	S	R	S	R	S	R	S	R	S
CAT I	32(11.9)	55(20.5)	11(4.1)	76(28.4)	16 (5.0)	71(26.5)	3(1.1)	84 (31.3)	49 (18.3)	38 (14.2)
CAT II	26(9.7)	54(20.1)	9(3.4)	70(26.1)	7 (2.6)	72(26.9)	12(4.5)	69 (25.7)	24 (9.0)	57 (21.3)
CAT III	24(9.0)	78(29.1)	7(2.6)	95(35.4)	13 (4.9)	89(33.2)	18(6.7)	84 (31.3)	18 (6.7)	84 (31.3)
Total (%)	**82 (30.6%)**	**187 (69.8%)**	**27 (10.1%)**	**241 (89.9%)**	**36 (13.4%)**	**168 (62.7%)**	**33 (12.3%)**	**237 (88.4%)**	**91 (34.0%)**	**179 (66.8%)**

Key: Cat I = Category I, Cat II = Category II, Cat II = Category III, RIF = Rifampicin, INH = Isoniazid, S = Streptomycin, PZA = Parazinamide, EMB = Etambutol, R = Resistant, S = Susceptible, No. = Number, % = Percentage, DST= Drugs Susceptibility Testing.

Table 7: Age and sex distribution for M. tuberculosis rifampicin Resistance by geneXpert

Age (years)	Males No. (%)	Females No. (%)	Total No. (%)
<1 – 10	-	-	-
11 – 20	4 (6.5)	1 (1.6)	5 (8.1)
21 – 30	18 (29.0)	9 (14.5)	27 (43.5)
31 – 40	11 (17.7)	10 (16.1)	21 (33.9)
41 – 50	3 (4.8)	5 (8.1)	8 (12.9)
51 – 60	-	-	-
>61 – 70	-	1 (1.6)	1 (1.6)
Total	**36 (58.1%)**	**26 (41.9%)**	**62 (16.3%)**

t = - 148.48, df = 13, p< 0.05

DISCUSSION

The results obtained from this study attest to the fact that MDR-TB, which is an emerging epidemic, is not just prevalent in parts of Benue State, but it is on the increase as compared to the estimated 5.3% rate of global MDR-TB (WHO, 2011). The prevalence of MDR-TB in the study population was 9.7% (retreated cases 7.1%; treatment naive 2.6%) out of the 268 isolated strains of *M. tuberculosis*. There was a higher proportion of MDR-TB within the age group 31 – 40 and 41 – 50 (mean age limit 35 – 45). This age range represents the most economically productive and viable workforce both in the private and public sectors. As a result, the need to urgently attend to the menace of MDR-TB in Benue State cannot be over emphasized. Other research groups also reported that anti-TB drugs resistance peaks within the age range of 25 – 35 years (Lawson *et al*., 2011; Uzoewulu *et al*., 2014). Results of this study with strong, significant statistical differences between male and female MDR-TB resistance rates, with male patients having more resistant strains agree with the work by Uzoewulu and co-workers (Uzoewulu *et al*., 2014). This underscores the enormous role of male patients in the epidemiology of drug resistant tuberculosis in the study population. This may be due to the fact that most females are economically disadvantaged in seeking appropriate medical attention, in addition to social and cultural beliefs that place women under movement restrictions. Other studies reported stigma as a principal factor (Uzoewulu *et al*., 2014).

The rate of MDR-TB 9.7% in this study is higher than the estimated 5.3% rate of global MDR-TB (WHO, 2011). The rate of MDR-TB 2.6% in newly diagnosed TB cases has no statistical difference between current estimated rate of MDR-TB 2.2% for new cases in Nigeria and 2.9% new cases in current national survey, although slightly lower, but MDR-TB of 7.1% in previously treated cases is lower compared to the estimated rate of MDR-TB 9.4%(WHO, 2012) and even grossly lower than the 14% from the current national survey on MDR-TB in Nigeria (WHO, 2011), but is within the trend for African countries in which 3.9 – 5.0% was reported for new TB cases and 16.7% in previously treated cases (WHO, 2006). These results closely agree with the report by Uzoewulu and co-reseachers. (Uzoewulu *et al*., 2014), who reported MDR-TB of 7.7%. Kolo, Idigbe and co-reseachers, Lawson and co-reseachers, and Akaninyene and co-reseachers (Kolo, 1991; Idigbe *et al*.,1992; Lawson *et al*., 2011; Akaninyene *et al*., 2013) all reported similar findings.

Comparative analysis of the three categories of patients revealed that category I patients (patients with HIV-TB co-infection that tested AFB positive after 2 months of TB treatment) recorded higher cases of MDR-TB compared to category II patients (patients with only TB infection, that still test sputum AFB positive after 2 months of TB treatment). Both categories account for 10 (38.5%) and 9 (34.6%) cases of MDR-TB respectively. Statistical analysis shows no significant difference (P>0.05).

However, MDR-TB detected in category I is statistically higher than MDR-TB detected in category III patients (patients with only TB infection, and are yet to commence TB treatment), in which MDR-TB was detected in only 7(26.9%) cases. The relatively higher number of MDR-TB detected in category I patients underscores the enormous impact of HIV as one of the key factors underlying an approximately 1% annual increase in the global TB incidence as reported by Lawn and Gavin, (2014) in a retrospective study on the epidemiology of HIV-associated tuberculosis (HIV-TB) from 2007-2008. Findings in this study are also consistent with the report by Dean and co-researchers (2014), who reported a positive association between HIV infection and MDR-TB disease using data of member states of the World Health Organization (WHO, 2014). Eleven out of the 24 countries for which analysis was performed, HIV-positive TB patients had significantly higher odds (P<0.05) of MDR-TB disease than HIV negative TB patients. For almost all of these 11 countries, the prevalence of MDR-TB among newly diagnosed TB cases was higher than the estimated global average of 3.6% (95% CI 2.1 – 5.1%).

Rifampicin resistance of 82(30.6%) cases detected by in-vitro culture and DST is statistically higher than the 62(16.3%) cases detected by *Genexpert* automated machine. Although this underscores the diagnostic advantage and higher sensitivity of in-vitro culture over *Genexpert* machine, there was no statistically significant difference between the two testing methods (P>0.05), *Genexpert* is however faster and much easier to perform

even in remote areas.

Apart from various evidences suggesting HIV infection as a risk factor for MDR-TB, it has been specifically associated with acquired rifampicin resistance (Jenny-Avital, 1997; Munsiff *et al*., 1997). This is suggestive of a critical overlap between HIV and the global multi-drug resistant TB (MDR-TB). Although, it is yet unclear whether HIV is driving a disproportionate increase in MDR-TB cases at a population level, results of in-vitro *Mycobacterium tuberculosis* transduction protocol (induced mutation) in the current study recorded an increase from 7(26.9%) to 10(38.5%) cases in 50% of the study population. This may be attributed to mutation in the nucleotide sequence of the *Mycobacterium* tuberculosis, which may confer antibiotic resistance on the organism. If such genetic changes are scientifically proven, they may be rightly attributed as contributing to the emergence of mono and multiple drug resistance in the genome of *Mycobacterium* tuberculosis. Several studies have reported that *Mycobacterium tuberculosis* does not exhibit an elevated mutation rate relative to most other bacteria under in-vitro conditions (David, 1970; SiddiMizrahi, and Andersen, 1998; Ford *et al*., 2011). It is not entirely clear, though, whether a relatively low mutation rate is sufficient to account for the elevated rates of acquired drug resistance observed clinically (McGrath *et al*., 2013). Studies by Ford and co-researchers (2011), using whole genome sequencing (WGS) technology to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infections in non-human primates reported a slightly elevated but not significant increase in drug resistance in vivo. Sun and co-researchers (2012), in their research utilized more sensitive WGS technology to track genome changes in serial sputum samples obtained from three patients over the course of anti-TB treatment and reported a higher degree of diversity in the serial clinical specimens, an observation that is consistent with the idea that mutation rate in vivo might be higher than previously reported. Thus the levels of genetic diversity identified in the studies above imply that *M. tuberculosis* might have an elevated mutation rate within the host compared to that calculated in vitro, thereby highlighting the need for further in vivo studies to truly ascertain the role of HIV in the emergence of MDR-TB especially for TB-patients co-infected with HIV/AIDS.

CONCLUSION

In conclusion, results of this genetic study on the role of HIV in the emergence of MDR-TB, promise to offer useful, effective, and ground breaking molecular approach in the fight against mono, multiple, and extensively-drug resistance (XDR). Drug resistance has hampered many public health targets and interventions such as "Stop TB"-target of achieving 70% case detection and 85% cure rate by 2005, and "Stop TB"-target of 50% reduction in the global burden of TB disease (deaths and prevalence) by 2015 (WHO, 2011). Previous studies have shown that anti-TB drugs resistance arise from patient/service related causes such as poor patient adherence/compliance, wrong regimens, inadequate supervision, and lengthy duration of treatment. The need to direct more attention on molecular aspects of anti-TB drugs resistance however is fast becoming a top priority, especially since MDR-TB is an emerging public health epidemic, requiring novel TB drugs to adequately combat it. This is necessary if the "Stop TB" targets of less than one patient per million populations by year 2050 must be achieved.

Recommendations

Based on the results of the present study, we hereby recommend that this genetic study should be stepped up under in vivo conditions using the actual Human Immunodeficiency Virus (HIV) as the transducing phage in order to ascertain the true nature of mutation conferring antibiotics resistance. The nature of mutation conferring anti-TB drugs resistance most especially MDR-TB should be well defined and documented especially that the DNA of *Mycobacterium tuberculosis* has been fully sequenced. In-depth understanding of the molecular biology of *Mycobacterium tuberculosis* genetic dynamics can be very useful to pharmaceutical industry as targets in the design of novel anti-TB drugs. Design of newer automated systems in TB diagnosis and antibiotics susceptibility testing that includes other anti-TB drugs such as isoniazid, parazinamide and ethambutol in addition to rifampicin is strongly recommended. Automated systems offer quicker turnaround time (TAT) and should be employed in routine clinical practice while in vitro sputum culture for TB that takes longer time should be restricted to research purposes.

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