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Original Research Article

Prevalence and Etiology of Tuberculosis from Sputum of Patients using Geno-Type Mycobacterium Tuberculosis Complex (MTBC) in Niger State, North Central, Nigeria

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Abstract

This study aimed at the prevalence of Patients infected with Tuberculosis and their causative Organisms and this was carried out from Six General Hospitals of Niger State, North Central, Nigeria. Three sputum samples and 2ml of blood were collected from1920 Patients that have cough of two weeks and above. Their HIV status was determined and Viral antibody detection was carried out using World Health Organization approved kits called "Determine, Unigold and Star pack. Their sputum samples were cultured after routine microscopy. Sputum samples were processed using Ziehl -Neelsen (ZN) reagents. The sputa were cultured on Loewenstein-Jensen egg –based medium and incubated at 370C for eight weeks. All the Acid-fast bacill (AFB) positive smears and culture positive isolates were tested for genetic identification using Mycobacterium tuberculosis complex (MTBC) (GenoType MTBC (HainLifescience)) to confirm the isolates to species level. The results reviewed that out of the one thousand Nine hundred and twenty (1920) Patients that were screened for HIV and TB, four hundred and sixteen (416) (23.1%) had *MTB complex* infection: four hundred and thirteen (413) (99.2%) had *Mycobacterium tuberculosis*; two (2) (0.4%) had *Mycobacterium africanum*; and one (1) (0.2%) had *Mycobacterium bovis*; fourty six samples were contaminated while seventy four samples were non tuberculosis mycobacterium (NTM) and were removed from the research, one thousand eight hundred Patients were used for the research. The most affected age group was between elevem to fourty (11-40) years and male had the highest prevalence of tuberculosis infection with two hundred and twelve (212) (50.9%) compared to the female with two hundred and four (204) (49.0%), statistical analysis shows that there was an association between gender and also between the age group with P< 0.05.Five hundred and fifteen (515) (28.6%) patients were screened positive for HIV and one hundred and eighty six (186) (36.1%) Patients had HIV and TB co-infections.

Keywords: Mycobacterium species, Loewenstein-Jensen, identification, GenoType MTBC.

1. Introduction

The TB pathogens are immobile, obligate aerobic, acid-fast bacilli that belong to the family *Mycobacteriaceae*. The genus *Mycobacterium* comprises numerous species, which includes members of the nontuberculous Mycobacteria and are considered opportunistic pathogens and the TB causing *Mycobacterium tuberculosis* complex (MTBC). The *Mycobacterium tuberculosis* complex is composed of the closely related the species *M. Tuberculosis* human pathogens, *M. bovis*, *M. Africanum* (human pathogens), *M. Microti* (rodents), and "*M. canetti*." (Human pathogens) *M. Bovis* (cattle) comprises *M. Bovis* subsp. *bovis*, *M. Bovis* subsp. *Caprae* (sheep and goats), *pinnipedii* (seals and sea lions), and the *M. bovis*-derived BCG vaccine strain; *M. Africanum* includes two subtypes, I and II; *M. microti*is the vole strain; and "*M. canetti*" may be merely a subspecies of *M. tuberculosis* [1][2].

Mycobacterium tuberculosis is often isolated from clinical specimen followed by *M. Bovis* and sometimes other members of the complex.

The epidemic of *M. tuberculosis* has affected about one third of the world's population creating an adverse impact socially and economically in developing countries [3]. TB, caused by the bacterium *M. tuberculosis*, is believed to enhance the progression of the HIV disease [4].

Little is known about the epidemiology of *MTB complex* species associated with pulmonary TB in Nigeria due to limited facilities for TB culture and molecular assays until the recent introduction of U.S President's emergency program for AIDS relief (PEPFAR) and the Global Funds. A better understanding of the circulating *MTB complex* species and their resistance to drugs is essential to guide diagnostic and therapeutic measures aimed at controlling this major public health burden in Nigeria especially with the increase in TB

cases due to the prevailing HIV epidemic. Over 3 million people live with HIV/AIDs in Nigeria with a national prevalence of disease estimated at 4.1% in 2010, as released by the country's National Agency for the Control of AIDS (NACA) in its Global AIDS Response Progress Report (GARPR) of 2012.[5]

Pulmonary disease caused by different *MTB complex* species is clinically similar, making surveillance and tracking of specie related to an epidemic a challenge. For example, pulmonary TB caused by *M. Bovis* is similar to that caused by *M. tuberculosis* in clinical, pathological, and radiological features [6]. However, in growth media, *M. Bovis* tends to have a colony appearance that is distinct from that of *M. tuberculosis* and produces entirely different biochemical reactions including its failure to produce niacin or to reduce nitrate [7] Conversely, *M. Bovis* exhibits striking similarities with *M. Africanum* in both morphological appearance and biochemical reactions including its failure to produce niacinor show any positive reaction for nitrate reduction. They both produce similar colonies with poor seeding that maybe hard to distinguish [7]. Misclassification errors are therefore likely to occur in environments where *M. Bovis* and *M. Africanum* are known to coexist. Newer molecular testing techniques are now available for the isolation and characterization of members of the *MTB complex*, including a Genotype MTBC (Hain assay) that enables rapid identification and differentiation of members of *MTB complex* using growth positive samples or direct clinical specimen with higher sensitivity and specificity when compared to conventional methods [8]. Identification of mycobacteria by conventional methods is a laborious and time-consuming procedure. The selection of adequate criteria as well as their evaluation by means of numerical techniques has been described for most clinically important mycobacteria.

GenoType MTBC (Hain Life science, Nehren, Germany) is a recently developed commercial DNA-strip assay for differentiating MTBC strains isolated from cultured material. The procedure involves isolating DNA from cultured material, multiplex amplification with biotinylated primers and reverse hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes. The resulting banding pattern indicates the species of the isolated mycobacterium.

2. Materials and Methods

2.1 Ethical letter

Ethical letter was obtained from Niger State Hospitals Management Board for permission to collect samples from all the said Hospitals Minna, Kontagora, Bida, Suleja, Lapai and Wushishi. Also an Ethical approval letter was also obtained from the Management of National Tuberculosis and Leprosy Training Centre, Saye, Zaria Kaduna State to enabled the research to be performed in the centre

2.2 Materials

Materials that were used includes Ziehl-Neelsen (ZN) stain reagent (carbolfuchsin, acid alcohol, methylene blue), 4% Sodium Hydroxide (NaOH), N-acetyl L-cysteine (NALC) Lowenstein- Jensen Medium (Drug free), Lowenstein- Jensen Medium (Anti Tb Drugs) Lowenstein- Jensen Medium with Sodium Pyruvate (SP), Isoniazid, Rifampicin, Light Microscope, Distilled water plant, Electronic weight balance, Water bath, autoclave, Twincubator, Genotype Mycobacteria complex kit (Genotypes MTBC), Biosafety cabinet, Molecular base water, Micro Pipette, Vortex, N95 respirator, sonicator, Inspissator and Amplifier.

2.3 Methods

Determination of Sample Size

The sample size of the samples collection for the research work was determined by the formula below, one thousand nine hundred and twenty samples were collected.

$$n = \frac{t^2 \times p(1-p)}{m^2}$$

2.4 Study Area

The study areas include six General Hospitals where tuberculosis cases were attended to in Niger State and were determined statistically by randomly selection method using geographical zones. The six General Hospitals include Bida, Suleja, Kontagora, Minna, Lapai and Wushishi General Hospital. These Hospitals have Directly Observed Treatment short course (DOTS) where TB Patients are attended to and receive treatment.

2.5 Collection of Samples

Two microliter of Blood and three Sputum samples was obtained from all Patients with cough of more than 2 weeks and was tested for human immunodeficiency virus (HIV) antibodies and tuberculosis using Determine, Unigold and Star pack. Patients Sputum that were find to be positive for tuberculosisafter smearing, staining and viewing through the Microscope (using the conventional AFB staining method) were stored at 4°C for up to 3 days and transported to the Provincial Diagnostic Mycobacteriology Laboratory in Zaria (National Tuberculosis and Leprosy Training Centre Zaria, Kaduna State).

2.6 Testing for human immunodeficiency virus (HIV) antibodies

Two microliter of blood was collected from all the patients with the help of new sterile two miles syringes and needle and transferred into a sterile test tube for centrifugation. With the aid of sterile transferring pipette, two drops of serum was placed on Determine HIV kits and the result was obtained after five minutes, Unigold and star pack was also used according to the National algorithm approval for HIV testing positive patient. The sputa of the HIV positive Patients were given a separate numbers to differentiate them from the negative Patients

2.7 Staining of the Sputum Samples Using Acid Fast Bacilli (AFB)

Ziehl-Neelsen (ZN) staining method was used to performed staining and light-emitting diode (LED) microscopes was used to view the smear.

Principle of acid fastness

The cell wall of Acid Fast Bacilli contains fatty acids known as mycolic acids, which makes them resistant to the action of many chemicals. Because of this, the bacilli cannot be stained easily like in Gram's stain. Strong dye concentration, application of heat, addition of phenol and longer staining time are required to stain the bacilli. Once stained, it is difficult to distains them. This property is used to differentiate the Acid Fast Bacilii (AFB) bacteria from non AFB bacteria. The bacteria cell walls was decolorized by the action of strong acid or acid-alcohol, leaving the acid fast bacilli stained with primary stain, which is basic fuchsin in case of ZN staining method.

2.8 Culturing of the isolates

Culturing of the isolates was performed using Lowenstein-Jensen (LJ) media. LJ media containing glycerol favours the growth of *M. tuberculosis* while LJ media without glycerol but containing Pyruvate encourages the growth of *M. bovis*as well as drug resistant strains of *M. tuberculosis*. The malachite green suppresses the growth of non-acid fast organisms and L-Asparagine was used for nitrogen source.

2.9 Specimen preparation

All direct sputum specimen from Patients that were positive for AFB were subjected to a treatment called N-acetyl cysteine and Sodium Hydroxide (NALC and NaOH) method of decontamination, digestion and concentrations under the biosafety cabinet according to Centre for disease control guide lines (CDC) for Public Health Mycobacteriology), a guide line for the Level 11 Laboratory.

2.10 Decontamination of sputum

Decontamination of sputum was done by adding 1ml of 4% sodium hydroxide (NaOH) to the 1ml of sputum to make 2ml of solution the caps of the McCartney bottles were Tightened and mixed well using vortex machine inside the biosafety cabinet and allowed to stay for 15 minutes. 2ml of sterile water was added to the two 2ml of the solution (sputum and NaOH plus sterile water) to make up to 4ml, these were centrifuged at 3000 revolution per minute for 15 minutes. At the end of 15 minutes, McCartney bottles were removed from the centrifuge without shaking. The supernatant were discarded into a Lysol bin.

2.12 Line probe Assay (Geno Type MTBC)

Geno-Type MTBC is a molecular Genetic Assay for the differentiation of the *Mycobacterium tuberculosis* complex from cultured materials. It is a qualitative in vitro test from cultured materials for the identification of species or strains belonging to the *Mycobacterium tuberculosis* complex (MTBC**).**The complex are *Mycobacterium tuberculosis/M. canettii, M. bovis, M. africanum, M. microti, Sub species of bovis, M. caprae, M. bovis BCG.*

Procedure involved in Genotype MTBC Hain molecular line probeassayincludes the following method DNA Extraction, DNA Amplification and Hybridization of the amplified DNA and these was done according to the manufacturer instructions.

2.13 Statistical analysis

Statistical Package for the Social Sciences (SPSS package) 19.0 version was used for data analysis.

3. Results

This study was carried out to determine the prevalence of Patients infected with Tuberculosis and their causative Organisms from Six General Hospitals of Niger State, A total of 1800 sputum and blood were collected from Patients attending the Hospitals. In this study, 416(23.1%) Patients were infected with mycobacterial species out of the 1800 Patients that were screened using Ziehl -Neelsen (ZN) staining method, culture and different ion with Genotype MTBC Hain molecular line probe assay, the results(Table 1.0) revealed the Culture and Genetic Differentiation of the isolates from all the study General Hospitals and the percentage prevalence of *Mycobacterium tuberculosis* isolated was 413(99.2%), followed by *Mycobacterium africanum* 2(0.4%) and the least was *Mycobacterium bovis* 1(0.2%) this was illustrated in Table 1.

The distribution of *mycobacterium tuberculosis* based on gender and age groups in Table 2 shows that from the 416 Patients that were TB positive, 212(50.9%) of them were males while 204(49.1%) were females, also the distribution based on the age group shows that ages less than 40years with the prevalence of 356(85.6%) are more prone to tuberculosis than ages above 40years with 60(14.4).Similarly, 515(28.6%) were HIV seropositive out of the 1800 Patients that were enrolled, 263(31.0%) of the Patients were HIV seropositive from the 847 males that were screened also 252(26.4%) were HIV seropositive out of the 953 females that were screened.

Furthermore, the study revealed that 88(21.1%) of the participants infected with TB have history of tuberculosis and 41(8.0%) out of the HIV seropositive have history of TB while 93(22.3%) of the TB Patients smokes cigarette and 72(14.0%) out of the total (515) Patients that were HIV seropositive have history of TB.

Based on the occupational activities of the Patients, Business people with 179(43.0%) have the highest TB prevalence followed by Farmers with the prevalence of 67(16.1%) and least was found among the commercial sex workers with the prevalence of 5(1.2%). Similarly the Artisans with 175(33.9%) have the highest prevalence of HIV followed by the Farmers with the prevalence of 98(19.4%) and the HIV seropositive was found among the commercial sex workers with the prevalence of 7(1.4%).

Co-infection of HIV/TB based on the occupation shows that the Artisans with 48(25.3%) have the highest HIV/TB co-infection followed by the Business people with 44(23.2%) and the least co-infection of HIV/TB was found among under parental care with the prevalence of 2(1.1%) and this was shown on Table 3.

Table 1: Culture and Genetic Differentiation of the isolates

Locations	Total Positive for AFB	*M. tuberculosis*	*M. bovis*	*M. africanum*	Contaminated Samples	Non *tuberculosis mycobacterium*
GHB	70	70	0	0	8	15
GHM	89	89	0	0	7	16
GHS	64	64	0	0	6	12
GHK	77	76	1	0	8	13
GHL	46	44	0	2	9	7
GHW	70	70	0	0	7	12
Total	416	413	1	2	46	74

Keys; GHB=General Hospital Bida GHM=General Hospital Minna, GHS=General Hospital Suleja, GHK=General Hospital Kontagora, GHL=General Hospital Lapai, GHW=General Hospital Wushishi.

Table 2: Group-specific prevalence and risk factors for TB infection among Patients in the study area

Total	416		1384		515		189	
Characterization	TB+ve	%	TB-ve	%	HIV+ve	%	HIV/TB	%
Age in years								
<40	356	85.6	1202	86.9	439	85.2	166	87.8
>40	60	14.4	183	13.1	76	14.7	23	12.2
Gender								
Male	212	50.9	635	45.9	263	51.1	99	45.4
Female	204	49.1	749	54.1	252	48.9	90	54.6
History								
Yes	88	21.1	55	4.0	41	8.0	32	16.9
No	328	78.8	1329	96.0	474	92.0	157	83.1
Cigarette smoker								
Yes	93	22.3	88	6.4	72	14.0	44	23.3
No	32.3	77.7	1296	93.6	443	86.0	145	76.7

Keys < =Less than, > = greater than, TB+ve =Tuberculosis Positive, TB-ve =Tuberculosis Negative HIV+ve = HIV Seropositive

Table 3: Occupation of patients with Tuberculosis, HIV and Co-infection of TB/HIV

Occupation	Total Patients	TB positive	percentage	HIV positive	percentage	Co-infection of TB/HIV	Percentage
BUS	356	179	43.0	65	12.6	44	23.2
C/S	430	45	10.8	98	19.0	34	17.9
CSW	12	5	1.2	7	1.3	3	1.6
FMR	405	67	16.1	98	19.0	25	13.2
H/W	45	23	5.5	20	3.8	9	4.7
LDD	48	36	8.6	27	5.2	11	5.8
STU	152	17	4.0	48	9.3	13	6.8
UPC	81	10	2.4	19	3.6	2	1.1
ART	271	34	8.1	175	33.9	48	25.3
	1800	416		515		189	

Keys: BUS- Business, C/S- Civil servants, CSW- Commercial sex workers, FMR- Farmers, H/W- House wives, LDD- Long Distance Drivers, STU- Students, UPC- Under parental care, ART-Artisans

4. Discussion

Previous studies have reported that the most isolated species was *Mycobacterium tuberculosis* from the mycobacterial family, Neonakis *et al* [9] reported 119(99.17%) of *Mycobacterium tuberculosis* as the highest isolated species from other mycobacterial species, Other studies from the neighboring west African countries of Ghana [10] reported 73% for *Mycobacterium tuberculosis*, 3% for *Mycobacterium bovis* and 13% for *Mycobacterium afrcanum* also, in Mali, Traore *et al* [11] whose research was on Molecular strain typing of Mycobacterium tuberculosis complex in Bamako, and found out that the highest isolated species was *Mycobacterium tuberculosis* with 71.4% followed by *Mycobacterium africanum* with 27.8% and the least isolated mycobacteria was *Mycobacterium bovis* with 0.8%. Similarly in Cameroun and Burkina Faso, Niobe-Eyangoh *et al* [12] and Gomgnimbou *et al* [13] have also reported a similar results with very low or absent of *M. Bovis* (0.2% from Cameroun, and none from Burkina Faso) and high proportions of *Mycobacterium tuberculosis* and some proportion of *M. Africanum.*

The low percentage prevalence of *M. bovis* found in this study could be attributed to the fact that the research was carried out in the Hospital and from the occupational background of this study there was no Patient reported to have come from or working with livestock or farms of slaughter houses because transmission of human *M. Bovis* is through ingestion and inhalation of droplet from an infected cattle.

Transmission among cattle, however, remains high, with over 95 percent transmission of *M. Bovis* occurring through direct contact between cattle. Only 1–5 percent of infected cattle shed the bacteria in their milk Menzies and Neill (2000) possibly explaining the low transmission to humans.[14]

In this study, males with the prevalence of 50.9% are more infected with tuberculosis than the female with the prevalence of 49.1% Statistically, there was no significant difference in the distribution tuberculosis and gender despite the differences, This is in agreement with the research of Alfred and Silas (2005)[15] whose study was on Epidemiology and Endemicity of pulmonary Tuberculosis (ptb) in Southeastern Nigeria Uyo, AkwaIbom State, Nigeria and reported that the prevalence of male to be 35.6% to that of the female 26.9%, Kehinde and Okesola (2010)[16] reported 76.0% for male and 24.0% for female in the of the Epidemiology of Clinical Isolates of *Mycobacterium tuberculosis* at Ibadan, Nigeria.

Age distribution of TB infected Patents been less than 40 years was also demonstrated by Christopher, Emeka and Vivien (2012)[17] whose research was on the Pattern of Presentation and Prevalence of Tuberculosis in HIV-Seropositive Patients Seen at Benin City, Nigeria and found that the age group distribution of TB infected individual was between 30-39 years. People living with patients that have history those that have been treated from tuberculosis cases and those that smokes cigarette have greater tendency of developing TB later in life. Based on the occupational background of the Patients, Artisans has the highest prevalence 179(43.0%) of Patients infected with TB more than other occupational status and the low percentage prevalence of TB found in under parental care (children) were also reported by other researchers. The present investigation has revealed the prevalence of tuberculosis in HIV seropositive was 189(45.4%) Patients in the selected General Hospital in the Local Government Area (LGA) of Niger State, Nigeria.

The recurrence of TB is seen most commonly in patients with HIV/AIDS. The percentage prevalence found in this study was higher than research performed by other researchers for example,13.9%was reported by Olaniran *et al* [18] whose research was on the Prevalence of Tuberculosis among HIV/AIDS Patients In Obafemi Awolowo University Teaching Hospital Complex Oauthc, Ile –Ife, this differences may be attributed to the fact that their investigation was done on only HIV patients and this research was done on new Patients attending the selected General Hospitals but this high prevalence found from this study is in agreement with the research of Kassu *et al* [19] that worked on Co infection and clinical manifestations of tuberculosis in human immunodeficiency virus-infected and - uninfected adults at a teaching hospital, northwest Ethiopia and found the percentage prevalence of Patients with TB, HIV co-infection to be 42%. The age distribution of Patients with co-infection of HIV and TB from this study revealed that the highest incidence was found between the age group 30-40yrs (43.7%) followed by 1-20 and 21- 30yrs (28.8%). This is in agreement with the research of Kamenju and Aboud (2011)[20] with 31-40yrs (43.7%) followed by 21- 30yrs (28.8.) as the percentage prevalence whose research was on Tuberculosis-HIV co-infection among patients admitted at Muhimbili National Hospital in Dares Salaam, Tanzania. The higher prevalence of HIV co-infection among TB Patients detected among younger age group in this study is in agreement with the findings of Kamenju and Aboud (2011)[20]. The age prevalence of TB co-infection among HIV Patients probably reflects the age-specific prevalence of HIV in the study areas. This may be connected to Patients being in a sexually active age group in which both TB and HIV prevail most [21]. The other possible reason for this may be their increased family, organizational, and societal responsibilities as people in this age group involve themselves in various extraneous daily activities in order to win the socio-economic hardship which increases the frequency of their contact with other patients in their society.

5. Conclusion

In conclusion, this result suggests that routine differentiation among members of the MTBC and NTM is necessary and also is required. NTM isolates accounted for 120(22.5%) of all isolates of mycobacteria identified in pulmonary specimens. Rapid speciation that distinguishes MTBC from NTM is an important prerequisite for the proper management of patients with mycobacterial infections, and will be introduced as a required standard into routine laboratory diagnostics of these infections in Niger State.

Similarly, the study showed no significant difference in the prevalence of HIV or TB with respect to age, sex, occupation and locations (P> 0.05). The prevalence of tuberculosis found in this study was 23.1% and commonest type of tuberculosis species was *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis* are found in some percentage. Co-infections of TB/HIV of 45.4% infection found in this study were high and sputum smear were atypical in most of the cases and therefore could be misleading in diagnosis. However, the results of this study have further highlighted the fact that TB and HIV infections are common in State, More studies are needed to isolate and differentiate MTBC from NTM using the clinical specimens for testing.

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