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Characterization of mycobacterial isolates from pulmonary tuberculosis cases with HIV seropositivity

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Abstract
Background: Tuberculosis is the most common opportunistic infection in HIV patients, which may be caused by either Mycobacterium tuberculosis or non-tuberculous Mycobacterial species. There is scarcity of data enumerating the proportion of different mycobacterial species causing pulmonary Tuberculosis in HIV patients in North east India.
Aims & Objectives: (1) Understanding the proportion of Pulmonary TB co-infection in the HIV seropositive patients (2) Characterization of different mycobacterial isolates in such cases.
Materials & Methods: Sputum samples from 50 HIV cases clinically and radiologically suspected with TB were tested for presence of Mycobacterial species by concentrated smear microscopy with ZN staining and conventional solid and liquid culture. MPT 64 Ag detection assay, LPA and CBNAAT were also performed.
Results & Conclusion: Out of the 50 cases, 24 cases were smear positive and 18 cases showed culture positive; of which, 13 isolates were of Mycobacterium Tuberculosis and 5 isolates were of Non- tuberculous Mycobacteria.

Keywords: HIV-TB coinfection, Mycobacterium tuberculosis, non-tuberculous Mycobacteria, Line probe assay, CBNAAT, MPT64 Ag, GeneXpert TB/RIF

1. Introduction
HIV/AIDS pandemic has caused a resurgence of Tuberculosis (TB) resulting in increased morbidity and mortality worldwide. HIV and *Mycobacterium tuberculosis* have a bidirectional and synergistic interaction, each accentuates progression of the other. Clinical presentation of TB in early HIV infection resembles that observed in immunocompetent persons. In late HIV infection, however, TB is often atypical in presentation, frequently causing extra-pulmonary disease. HIV-infected patients respond well to the standard 6-month anti-tuberculosis treatment regimens, although mortality is high [1]. People who are HIV-positive and infected with TB are 20 to 40 times more likely to develop active TB than people not infected with HIV living in the same country [2]. TB is the commonest HIV-associated opportunistic disease in the world and the leading cause of death in such patients [3].
Pulmonary involvement occurs in about 75% of all HIV/TB co-infected patients [4]. It was observed that the most frequent extra-pulmonary form of TB in HIV-positive people is with involvement of the lymph nodes, with cervical region being the commonest. Co-infection of HIV and TB also results in more rapid development of MDR-TB. It is associated with increased risk of HIV disease progression to AIDS, overlapping toxicities, drug interactions and complications as well as reducing HIV treatment efficacy. There is also increased risk of death regardless of CD_4 count. Diagnosis is difficult because of smear negativity, extra pulmonary TB and disseminated forms [5].
On the basis of pathogenesis and the severity of the disease caused by them, mycobacteria are divided into two broad groups- *M. tuberculosis* (the causative agent of tuberculosis) and non-tuberculous mycobacteria(NTM) also known as mycobacteria other than tuberculosis (MOTT), environmental mycobacteria or atypical mycobacteria[6]. The incidence of NTM as pathogens in an immuno-compromised host is increasing. A large number of NTM are frequently found in the environment and can easily colonize in HIV/AIDS patients and cause disease, particularly in the advanced AIDS stage. Among the NTM, the most common opportunistic pathogen isolated from HIV/ AIDS patients is the *Mycobacterium avium* complex (MAC) which is associated with disseminated disease [7]. *M. kansasii* is the second most common NTM to affect patients with HIV/AIDS. Other NTM that are less frequently encountered include *M. fortuitum, M. chelonae, M. simiae,* etc [7-12].
The North eastern states of India have shown a rising trend in Tuberculosis infection among HIV affected patients over the years especially the states of Nagaland and Manipur. Assam, although as a lower prevalence of HIV-TB coinfection, is highly vulnerable due to its geographical location and various demographical factors.

2. Materials & methods
2.1. Study Setting
The study was carried out in the Department of Microbiology, Gauhati Medical College & Hospital and Intermediate Reference Laboratory, GMCH, Assam for a period of 1 year from September 2013 to August 2014 after availing due permission from NACO and ethical clearance from Institutional Ethical Committee, GMCH.

2.2. Type & Design of the study
The present study has been a hospital based randomized observational study. Mycobacterial coinfection was searched for in sputum samples of HIV seropositive patients by concentrated smear microscopy with ZN staining and conventional cultures done in both solid (LJ media) and liquid (MGIT 960) media. In the culture positive cases, the Mycobacterial colonies were tested for positivity to MPT64 Antigen using *Capilia TB assay*. The smear positive samples were additionally subjected to Line probe assay (LPA) testing using *GenoType MTBDRplus* for identification and sensitivity pattern to Isoniazid and Rifampicin as per the RNTCP protocol followed in the Intermediate Reference Laboratory (IRL), GMCH. The smear negative cases were confirmed by conventional cultures and by using the *GeneXpert MTB/RIF assay.*

2.3. Study Population
A total of 50 individuals of different socio-economic status diagnosed as HIV positive in various ICTCs (Integrated Counseling and Testing Centre), having pulmonary symptoms and suspected of Pulmonary Tuberculosis either clinically or radiologically, attending the Antiretroviral Therapy Plus Centre (ART-Plus Centre), GMCH were taken up for the study. The samples of all those HIV seropositive patients sent to the Intermediate Reference Laboratory (IRL), GMCH from different DTCs and TUs for confirmation of Pulmonary Tuberculosis were also included in the study.

2.4. Subject Selection Criteria
Subjects fulfilling the following criteria were selected for the study.
Inclusion Criteria
-- HIV positive patients on Highly Active Antiretroviral Therapy (HAART) having pulmonary symptoms irrespective of ATT status
-- HIV patients newly diagnosed with pulmonary TB by sputum smear microscopy whose confirmation by Mycobacterial culture has not been done.

2.5. Sample Collection & Transport
Two sputum samples (one morning and the other spot), wherever possible, were collected from each symptomatic patient in two different sterile vials according to revised RNTCP guidelines after obtaining the informed consent of the participants. All the specimens collected were transported immediately to the Intermediate Reference Laboratory for further processing, maintaining the reverse cold chain. At times when immediate transfer was not possible, the specimens were refrigerated, but not for more than 72 hours.

2.6. Sample processing
All sputum samples were screened for the presence of acid fast bacilli (AFB) by Ziehl Neelsen (ZN) staining. The samples processed by N-acetyl L-cysteine- Sodium citrate- Na-hydroxide (NALC- Na-citrate-NaOH) method of homogenisation and decontamination. The pellet obtained was suspended in 2ml phosphate buffer saline (PBS).

2.7. Culture
All the processed samples irrespective of smear microscopy results were subjected to conventional culture by both solid (LJ) and liquid (MGIT 960) methods. The cultures showing positive growths were subjected to rapid immunochromatographic tests for detection of MPT64 TB Ag (Capilia TB assay) and differentiation of Mycobacterium tuberculosis from NTM. All the cultures positive for M. tuberculosis were subjected to LPA.

2.8. GenoType MTBDRplus assay and reporting
The GenoType MTBDRplus assay version 2.0 (Hain Life sciences, Germany) based on DNA-strip technology was carried out on the processed samples as per manufacturer's instructions. DNA extraction, master mix preparation, DNA amplification and hybridization were done after thorough cleaning in dedicated rooms. The result of DNA strips was interpretated with the help of reporting card as resistant or sensitive for RIF or INH or invalid according to the kit insert. The presence of mutation bands and the absence of sensitive bands were also recorded. M. tuberculosis H37Rv (ATCC 27294) was run as positive control and sterile molecular grade water was run as negative control.

2.9. GeneXpert MTB/RIF assay
All the smear negative cases were confirmed by the GeneXpert MTB/RIF assay (Cepheid GeneXpert System) which is a semi-quantitative nested real time PCR for *in-vitro* detection of *Mycobacterium tuberculosis* complex DNA in sputum sample (smear positive or negative) and the detection of rifampin resistance associated mutations of the *rpoB* gene.

2.10. Statistical analysis
The data obtained were analysed with the help of statistical software **Epi Info 7**. Chi square test and Fischer's extract were used to determine the statistical significance of the variables. p value < 0.05 was considered significant.

3. Observations & Results
3.1. Study population:
Out of the 50 cases, 43 (86%) were males and 7(14%) were females. No transgender were present in the study population. Maximum of 24(48%) cases were in the age group of 31-40 yrs and minimum of 1(2%) case each in the age groups of 11– 20 yrs, 51 -60 yrs and 80 yrs & above.

3.2. Sputum smear microscopy
Out of the 50 HIV seropositive cases suspected of Pulmonary Tuberculosis, the ZN stained sputum smears were positive for 24 (48%) cases and negative in the remainder of 26 (52%) cases.

3.3. Culture
Out of the total 50 cases, only 18 cases were found to be culture positive, 11 cases showed positive growth in both solid and liquid cultures. 5 out of 18 culture positive cases showed only liquid culture positivity and 2 cases showed only solid culture positivity. A total of 13 cases showed positive result in solid culture (LJ media) and the rest 37 cases showed no growth in LJ media. Out of these 13 solid culture positive cases, 12 were

positive for sputum smear microscopy and the remainder 1 was negative. Now, out of the 37 solid culture negative cases, 12 were smear positive and 25 were smear negative.
Out of the total 50 cases, 16 cases showed positive result in Liquid MGIT 960 culture and the remainder 34 cases were negative. Now, out of these 16, MGIT 960 positive cases, 13 were positive for smear microscopy and the remainder 3 cases negative. Out of the 34 MGIT 960 negative cases, 11 were smear positive and 23 were smear negative.

3.4. Analysis of Sputum microscopy v/s culture result

Out of the 24 smear positive cases, 12 were positive by solid culture and 12 were solid culture negative. Of the smear negative 26 cases, 1 case showed solid culture positivity. This analysis showed a significant correlation between the two variables as the p-value was <0.05.

Out of the 24 smear positive cases,13 showed liquid culture positivity and 11 showed negative by liquid culture. Out of the 26 smear negative cases, 3 showed positivity by liquid culture and 23 were liquid culture negative. This analysis showed a significant correlation between the two variables as the p-value was <0.05.

3.5. Mycobacterial isolates from cultures:

Of the 50 cases, 18 cases showed culture positivity. Out of which 13 isolates were of *Mycobacterium tuberculosis* (MTB) and the remaining 5 isolates were of Non-tuberculous Mycobacteria (NTM), whose genotyping were not done in this study.
Liquid culture yielded the growth of 11 MTB isolates and 5 NTM isolates
Solid culture yielded the growth of 12 MTB isolates and 1 NTM isolate

Table 1: Comparision between solid and liquid culture

Mycobacterial Isolate	Liquid Culture In Mgit 960		Solid Culture In Lj Media	
	No Growth	Growth	No Growth	Growth
Mtb	2	11	1	12
Ntm	0	5	4	1
Total	2	16	5	13

3.6. Mycobacterial Isolate V/S Present Art Status

This analysis shows no significant correlation between the type of Mycobacterial isolates and the present ART status of the cases. Out of the 13 MTB isolates, 12 were isolated from patients on ART and 1 from patient not on ART. Similarly, out of the 5 NTM isolates, 4 were isolated from patients on ART and 1 from patient not on ART.

3.7. Smear Microscopy Result v/s Present Art Status

This study showed no significant correlation between smear microscopy and present ART status. Out of the 46 case on ART, 22 were smear positive and 24 were smear negative. Out of the 4 cases not on ART, 2 were smear positive and 2 smear negative

3.8. Mycobacterial Isolate v/s Anti Tuberculosis Treatment Status

Out of the 50 cases, only 7 cases were yet to start ATT (Anti tubercular Treatment), the rest 43 were under ATT at the time of sample collection. Out of the 18 cases showing positive culture results, 15 cases were on ATT while 3 cases were yet to start ATT. Now, out of those 15 cases on ATT, 11 isolates were MTB and 4 isolates were NTM. Of those 3 cases not on ATT, 2 isolates were MTB and 1 NTM

3.9. LPA Pattern of the Mycobacterial Isolates

All the 24 smear positive cases were subjected to LINE PROBE ASSAY testing using GenoType MTBDR*plus*, of which 12 showed positive results with drug sensitivity pattern. A total of 3 different drug sensitivity patterns were obtained from the 12 LPA positive MTB isolates

Table 2: LPA drug sensitivity pattern

Lpa Drug Sensitivity Pattern	Frequency	Percent	Cum. Percent	95% Ci Lower	95% Ci Upper
Inh Resistant Rif Resistant	5	41.67%	41.67%	15.17%	72.33%
Inh Resistant Rif Sensitive	1	8.33%	50.00%	0.21%	38.48%
Inh Sensitive Rif Sensitive	6	50.00%	100.00%	21.09%	78.91%
Total	12	100.00%	100.00%		

3.10. Genetic makeup of isolates from LPA

The genetic makeup of the 12 MTB isolates by LPA and the frequency of each type

Table 3: Genetic makeup of isolates from LPA

GENETIC MAKEUP OF ISOLATE	Frequency	Percent
rpoB WT +ve MUT -ve; katG WT-ve MUT1+ve; inhA WT+ve,MUT-e	1	8.33%
rpoB WT+ve,MUT-ve; katG WT+ve,MUT-ve; inhA WT+ve,MUT-ve	6	50.00%
rpoB WT2,7,8-ve,MUT-ve; katG WT-ve,MUT1+ve; inhA T+ve,MUT-ve	1	8.33%
rpoB WT8-ve,MUT3+ve; katG WT-ve, MUT1+ve; inhA T+ve,MUT-ve	4	33.33%
TOTAL	12	100.00%

3.11. Results of Genexpert MTB/RIF Assay

Only 16 cases were processed in GeneXpert MTB/RIF. Smear and culture negative cases, diagnosed clinically and radiologically as Pulmonary Tuberculosis and on Anti Tubercular Treatment were considered for GeneXpert

Table 4: Results of GeneXpert MTB/RIF assay

GeneXpert MTB/RIF assay	Frequency	Percent
TUB +ve RIF resistant	1	6.25%
TUB -ve	15	93.75%
TOTAL	16	100.00%

4. Discussion

The present study was a hospital based observational study and was undertaken with a purpose to study the proportion of Pulmonary Tuberculosis coinfection in HIV infected individuals and to characterize the different mycobacterial isolates involved in this so called "cursed duet". A total of 50 cases from different socio-economic strata, who fulfilled the inclusion criteria of the study, were taken up for this purpose.

Out of the 50 cases, the highest number of 48% of cases were in the age group of 31- 40 yrs, followed by 24% of cases in the age group of 41 – 50 yrs and the lowest 2% of cases each in the age groups of 11 – 20 yrs, 51 -60 yrs and 80 yrs & above. There were no cases in the age groups of 0 – 10 yrs, 61 – 70 yrs and 71 – 80 yrs**.** 86% of the cases were males and 14% were females. No transgender were present in the study population. These findings have some resonance with studies performed by Deshmukh *et al* (1999) [13], Dhungra *et al* (2008) [15]. 46% of cases were from the lower middle class, followed by 30% cases from the lower class. 2% of cases were from the upper middle class. These statistics have some resonance with the study performed by Kamath *et al* (2013) [14].

Of the 50 HIV seropositive cases considered for this study, 46(92%) cases were still on ART, whereas 4(8%) cases were yet to start ART. Out of the 46 cases on ART, 22 were smear positive and 24 were smear negative. Out of the 4 cases not on ART, 2 were smear positive and 2 smear negative.

Of the 50 HIV seropositive cases suspected of Pulmonary Tuberculosis, the ZN stained sputum smears were positive for 24(48%) cases and negative in the remainder of 26(52%) cases. The smear positivity rates were 45.83% for the lower middle class and 29.17% for the lower class.

Out of the 18 cases (13 MTB and 5 NTM) showing culture positivity, the maximum number of 8 cases (4 MTB and 4NTM) were from the Lower socio-economic class, followed by 7 cases (7 MTB and no NTM) from the Lower middle class and the minimum 1 case (1NTM and no MTB) was from the upper middle class. 4 out of the 5 NTM isolates were found in the Lower class.

Out of the 50 cases, only 7 cases were yet to start ATT(Anti tubercular Treatment), the rest 43 were under ATT at the time of sample collection. Out of the 18 cases showing positive culture results, 15 cases were on ATT while 3 cases were yet to start ATT. Now, out of those 15 cases on ATT, 11 isolates were MTB and 4 isolates were NTM. Of those 3 cases not on ATT, 2 isolates were MTB and 1 NTM

Out of the 50 cases, a total of 13 cases showed positive result in solid culture (LJ media) and the rest 37 cases showed no growth in LJ media. Out of these 13 solid culture positive cases, 12 were positive for sputum smear microscopy and the remainder 1 were negative. Now, out of the 37 solid culture negative cases, 12 were smear positive and 25 were smear negative.

A total of 16 cases showed positive result in Liquid MGIT 960 culture and the remainder 34 cases were negative. Now, out of these 16 MGIT 960 positive cases,13 were positive for smear microscopy and the remainder 3 cases negative. Out of the 34 MGIT 960 negative cases, 11 were smear positive and 23 were smear negative. These statistics are comparable with the studies by, Deshmukh *et al* (1999) [13], Kamath *et al (*2013) [14], Dhungana *et al* (2008) [15], C. Padmapriyadarshini *et al* (2011) [16]

All the 50 cases were inoculated in both solid and liquid MGIT960 media, of which a total of 18 cases showed culture positivity. 11 cases showed positive growth in both solid and liquid cultures, 5 cases showed positivity only in Liquid culture whereas 2 cases showed positivity only in solid culture.

Solid culture in LJ media: Out of the 13 solid culture positive cases, 11 were positive by Liquid MGIT 960 culture and 2 were negative. Out of the 37 solid culture negative cases, 5 were positive by Liquid MGIT 960 culture.

Liquid culture in MGIT960: Out of the 16 cases showing Liquid MGIT960 positivity, 11 were positive by solid culture and the remainder 5 cases were solid culture negative. Of the Liquid MGIT960 negative 34 cases, 2 were positive by solid culture and remainder 32 were solid culture negative.

These findings can be compared with studies by Deshmukh *et al* (1999) [13], *Dhungana et al* (2008) [15],Wankhede A.B. *et al* (2011) [18]

Of the 50 cases, 18 cases showed culture positivity. Out of which 13 isolates were of Mycobacterium tuberculosis (MTB) and the remaining 5 isolates were of Non-tuberculous Mycobacteria (NTM), whose genotyping were not done in this study. Liquid culture yielded the growth of 11 MTB isolates and 5 NTM isolates while Solid culture yielded the growth of 12 MTB isolates and 1 NTM isolate. These statistics can be compared to studies by *A. Somoskovi et al* (2002) [17], Wankhede A.B. *et al* (2011) [18].

All the 24 smear positive cases were subjected to LINE PROBE ASSAY testing using GenoType MTBDR*plus,* of which 12 showed positive result with drug sensitivity pattern. A total of 3 different drug sensitivity patterns were obtained from the 12 LPA positive MTB isolates. INH sensitive RIF sensitive in 6 cases, INH resistant RIF resistant in 5 cases, INH resistant RIF sensitive in 1 case.

5. Conclusion

The present study shows the proportion of Pulmonary Tuberculosis in HIV patients attending Gauhati Medical College & Hospital. Tuberculosis is the commonest co-infection in HIV patients and it has been established that not only *Mycobacterium tuberculosis* but also the Non-tuberculous Mycobacteria are involved in such cases. The findings of this study that out of 18 isolates, 13 are MTB and 5 are NTM also supports this fact. The findings of this study and various other studies show that developing countries and HIV seropositive individuals from low socio-economic status are more vulnerable to Tuberculosis co-infection. Keeping this in mind, there is a need to take up more such studies involving a larger study population and of longer time frame to understand various facets of the HIV-TB co-infection including genotyping of the isolates, immune responses, early diagnosis, prevention and treatment. This will help us to provide a better care and longer life to all the HIV infected individuals who have a very high probability of co-infection with TB.

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