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"SPECTRUM OF FUNGAL INFECTION IN A DIAGNOSED CASES OF TUBERCULOSIS IN A TERTIARY CARE HOSPITAL BIHAR,,

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ABSTRACT **BACKGROUND**: Pulmonary tuberculosis is one of the most important health concerns. pulmonary fungal infections have clinical and radiological characteristics similar to tuberculosis which may be easily misdiagnosed as tuberculosis, Fungal pulmonary infection can be acquired in tuberculosis, immunodefi- ciency patients, and other chronic diseases. Many physicians missed fungal pulmonary infection because it does not show specific clinical manifestations. The aim was to identify the presence of overlapping fungal infections in tuberculosis patients.**MATERIALS AND METHODS**: The present study was conducted on 50 tuberculosis patients,Department of microbiology at IGIMS patna Bihar, who were subdivided into: Group I consisted of 30 cases of multidrug resistance tuberculosis, Group II consisted of 10 fresh cases and Group III consisted of 10 relapse cases.**RESULTS:** Aspergillus spp., was the only fungus detected in 24% of cases, Group I showed the highest percentage (26.6%) with statistically significant difference compared to Group II and III (20%) for each. Aspergillus fumigatus was the predominant spp. identified followed by Aspergil- lus niger and Aspergillus flavus. Mixed infection was identified in 4 cases in Group I. A statistical significant association between fungal detection and MDR-TB, diabetic patients, smoker, being male, presence of haemoptysis and toxic manifestations, presence of cavitary lesion or abscess and severity of X-ray finding.**CONCLUSION**: detection of mycotic infection represents a rapid diagnostic tool helping early diagnosis of fungal co-infection and pulmonary TB. MDR-TB patients carry the risk of higher percentage of fungal infections and more liable for acquiring mixed fungal pathogens. Presence of male sex, smoking, DM and far extent of lesion must attract physicians' attention for fungal co- infection with pulmonary TB.

KEYWORDS : pulmonary tuberculosis, fungal infection, immunocompetent

INTRODUCTION

Tuberculosis (TB) which is an infectious disease, is sti a serious threat for the population, Fungal pulmonary infection has been emerging recently due to widely used broad spectrum antibiotics and steroids. It can be acquired primarily or secondarily in tuberculosis (TB), immunodeficiency patients, other chronic diseases such as diabetes mellitus or malignancy, and may worsen the primary disease. TB is principally a disease of poverty, with 95% of cases and 98% of deaths occurring in developing countries. Glob- ally, about 3% of all newly diagnosed patients have multidrug resistance tuberculosis (MDR-TB). Egypt is ranked among the mid level incidence countries in tuberculosis. In 2007 the an- nual risk of infection was calculated mathematically by WHO it was (24/1,00,000). Egypt is estimated to have 15,000 TB cases and a rate of 18 cases per 1,00,000 of popula- tion. The percentage of mycotic infections increase in pul- monary tuberculosis patients. Mainly four types of fungi, i.e. *Aspergillus niger, A. fumigatus, Histoplasma capsulatum* and *Cryptococcus neoformans* were recorded, which causes severe infection in lungs in patients suffering from pulmonary tubercu- losis. Many physicians missed fungal pulmonary infection because it does not show specific clinical manifestations and usu- ally hindered by other diseases and cause high rates of morbidity and mortality. There is an increasing awareness amongst clinicians and microbiologists pertaining to importance of infection caused by opportunistic fungi. Therefore there is an acute need for proper diagnosis of the opportunistic fungal pathogen especially in tuberculosis patients. The conventional identification of pathogenic fungi based on phenotypic features and physiological tests is time-consuming and, therefore, often imperfect for the early initiation of an antifungal therapy. DNA microarrays were introduced for the rapid and simultaneous identification of different fungal species at the same time. Based on pan fungal internal transcribed spacer (ITS) primers directed at the conserved regions between the 18S and 28S rRNA, which were shown to correlate well with culture results. Aim of the work: Is to identify the presence of overlapping fungal infections in tuberculosis patients, using high multiplex- ing capacity of DNA microarray which may help in correct diagnosis of these diseases that may increase the cure rate. there are many different types of pulmonary and extra pulmonary disease related to TB, which might have serious problems in differential and therapeutical diagnosis.

MATERIALS AND METHODS

The present study was conducted on 50 tuberculosis patients, Department of microbiology, at Indira Gandhi institute of medical sciences Patna Bihar. randomly selected amongst the diagnosed pulmonary tuberculosis cases admitted in TBDC Hospital Agam kuan Patna, They were divided into the following groups:

Group I consisted of 30 cases diagnosed as MDR-TB.
Group II consisted of 10 cases diagnosed as new cases (fresh cases).
Group III consisted of 10 cases diagnosed as relapse cases.

Case definition by previous anti TB treatment.(9) A patient who has never had treatment for TB or who has taken anti-TB drugs for less than 4 weeks.*Relapse*A patient who has been declared cured of any form of TB in the past by a physician after one full course of chemotherapy, and has become sputum smear-positive.

MDR-TB

It is defined as resistance to any combination of anti-TB drugs that include INH and rifampicin.

Cases were radiologically classified based on chest X-ray (CXR) findings according to National Tuberculosis Association of USA (196)1into:

Patients Samples

Informed consent was obtained from each participant prior to specimen collection. Early morning sputum was collected in a sterile dry wide-necked, leak-proof container from each case and transported to the laboratory.Then sample was homogenized and liquefied using NALC—Na OH method that involves use of *N*-acetyl-L-cys- teine (NALC) according to Colle et al.. DNA extraction was done using Qiamp DNA Mini Kit (Qiagen, Izasa, Madrid, Spain), the yield of total DNA obtained was determined spec- trophotometrically. Universal fungal primers were used for amplification of the ITS1 and ITS2 regions. The sequence of primers is ITS1: 5^0-TCCGTAGGTGAACCTGCGG-3^0 (posi- tion 36–54) and ITS 4: 5^0-TCCTCCGCTTATTGATATG-3^0(position 601–620), as described by White et al.. The se- quence of the forward primer ITS1 is complementary to a con- served region at the end of the 18S rRNA gene, and the sequence of the reverse primer ITS 4 binds to a conserved re- gion at the beginning of the 28S rRNA gene. DNA ampli- fication was performed in parallel with positive and negative controls. The positive control strain was isolated from clinical sample. The clinical isolate was identified by standard methods according to Colle et al.. The negative control consisted of an equal volume of water replacing the DNA template. A total reaction volume of 50 ll was prepared for PCR. The mixture contained 5 ll of 10· reaction buffer (100 mM Tris, 500 mM kcl; pH 8.3), 3 ll of 25 mM $MgCl_2$, 1 ll of 10 mM PCR Nucle- otide mix, 2.5 ll of each primer (20 lM), 0.2 ll of Taq DNA Polymerase (5 unit/ll) Biogenet – Korea, 500 ng of template DNA and DEPC treated water. The amplification was per- formed. An initial denaturation step (94 °C for 5 min) was followed by 35 cycles (with each cycle consisting of DNA denaturation at 94 °C for 30 s, primer

annealing at 57 °C for 30 s, and elongation at 72 °C for 1 min) and a final extension step at 72 °C for 7 min . Amplified DNA products were separated by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg/ml); the running buffer was TAE (40 mM Tris acetate [pH 8.0], 1 mM EDTA). A 100-bp DNA ladder was used as a molecular size marker (Promega – USA). DNA bands were visualized by UV transillumination.

Then, DNA was denaturated for 5 min at 95 °C and stored at 20 °C. Conserved regions served as targets for probes which are able to discriminate between *C. neoformans*, *H. capsulatum* and *Aspergillus* (genus-specific probes.

Table 1 Oligonucleotide probes sequence for identification of selected fungal species.

Probes sequences	Name	Position
ACA AGA GAC GAC GGT AGC TTC ACG	*H. capsulatum*	663–686
GAACCCACCGCCCTCTTC	*C. neoformans*	540–565
GGAGACACCACGAACTCTGT	*A. flavus*	175–194
CCAACACGAACACTGTCTGA	*A. niger*	114–133
CCGACACCCAACTTTATTT	*A. fumigatus*	502–520

Table 2 Prevalence of fungal infection among the studied groups.

Patients group	Number of positive cases	%	Chi-square test value	Significance
Group I (MDRTB)	8	26.6	$v^2(1)$ 2	$P < 0.05$ (S)
Group II fresh TB	2	20	$v^2(2)$ 2	$P < 0.05$ (S)
Group III relapse TB	2	20	$v^2(3)$ 0	$P > 0.05$ (NS)

Table 3 Prevalence of fungal species among the studied groups.

	A. fumigatus	*A. niger*	*A. fumigatus* and *A. niger*	*A. fumigatus* and *A. flavus*
GroupI MDR-TB	2	2	2	2
Group II fresh TB	2			–
Group III relapsed TB	2			–

Table 4 Prevalence of fungal infection among the studied groups in relation to risk factors and clinical manifestations.

Variable	Group (MDR-TB) (30)		Group II fresh TB (10)		Group III relapsed TB (10)	
	No.	No. of (+)ve fungal cases	No.	No. of (+)ve fungal cases	No.	No. of (+)ve fungal cases
Smoking	12	6	4	2	6	2
(DM)	4	3	0	0	2	2
Haemoptysis and toxic manifestations	12	6	6	2	4	2

RESULTS

The present study was conducted on 50 tuberculosis patients admitted to TBDC Hospital Agam kuan Patna Bihar. Their age was ranging from (25 to 65) years. The patients were divided into three groups: Group I consisted of 30 cases diagnosed as MDR- TB (60%) (20 males and 10 females). Group II consisted of 10 cases fresh diagnosed TB (20%) (8 males and 2 females). Group III consisted of 10 cases with relapse of TB (20%) (8 males and 2 females).The presence of fungal infection in pul- monary tuberculosis patients was detected by DNA micro- array which allowed rapid and simultaneous identification of many fungal species at the same time. *Aspergillus* spp., was the only type detected in this study, while other fungal species as *H. capsulatum* or *C. neoformans* were not identified. Twelve out of 50 cases (24%) were positive for *Aspergillus* spp., 8 cases from Group I (26.6%), 2 cases from Group II (20%) and 2 cases from Group III (20%). Comparison between the studied groups shows statistically significant difference between Group I and both Groups II and III, while no statistically significant difference was found between Group II and III.

A. fumigatus was the predominant Aspergillus spp. identi- fied in all the patients followed by *A. niger* and *A. flavus*. Dis- tribution of the isolated fungal spp. among the studied groups was as follows:

A. fumigatus was identified in 2 cases of Group I, 2 cases of Group II and another 2 cases of Group III. While *A. niger* was identified in 2 cases of Group I only. Mixed infection was iden- tified in 4 cases in Group I only (two of them were *A. fumigatus* with *A. niger* and the other two were *A. fumigates* with *A. fla- vus*) as shown in Table3.

DISCUSSION

Tubeculosis is a serious health and treatment problem which occurs in all countries over the world, Fungal infections remain a leading cause of infectious mortality and morbidity in heavily immunosuppressed patients. For diagnosis of fungal infection, establishing cultures from blood and bronchoalveolar lavage (BAL) samples is of- ten unsuccessful due to the low yields of CFU, and in the case of immuno-compromised high-risk patients who are febrile, pulmonary tuberculosis, neutropenic, thrombocytopenic, and often seriously ill, tissue biopsy specimens, in general, are not available. Early initiation of effective antifungal ther- apy and reversal of underlying host defects remain the corner- stones of treatment for pulmonary fungal infections. More sensitive and rapid detection assays of mycotic infections in pulmonary tuberculosis patients have been established by use of the PCR method. However, traditional methods in molecular biology generally work on a "one gene in one experiment" basis. Recently, DNA microarray has attracted tremendous interests among biologists as it promises to monitor the whole genome on a single chip. An experiment with a single DNA chip can provide researchers information on thousands of genes simultaneously. The current study was carried upon 50 pulmonary tuberculosis patients; the presence of fungal infection in pulmonary tuberculosis patients was detected by DNA microarray. *Asper- gillus* spp., was the only type detected in this study in (24%) of cases, while other fungal species as *H. capsulatum* or C. *neo- formans* were not identified. Njunda et al. found that the prevalence of *Aspergillus* spp. in the sputum of patients sus- pected of pulmonary tuberculosis was 15%, also, Kurhade et al. reported that the prevalence of *Aspergillus* spp. was (16.26%). Ekkena et al. noticed that the most common fungal isolates were *Aspergillus* spp. (42.9%). tuberculosis stimulate the growth and virulence of infecting fungus by destruction of competing bacterial flora. Another explanation for high susceptibility of MDR-TB patients to fungal infection could be clarified by understanding the immunological changes associated with multi-drug resistant tuberculosis. It was confirmed that the most important host defenses against fungi are neutrophils and alveolar macrophages. IFN-c produced by the T lymphocytes increases the production of nitric oxide and other nitrogen and oxygen-reactive radicals of macrophages. It was observed that patients with MDR-TB show low IFN-c production when compared with patients with non-resistant tuberculosis before and after treatment. It was noticed that the MDR group (Group I) carry the risk of higher percentage of fungal infections and it was the only group which was harboring mixed species. As treatment of MDR group necessitate the use of different antibiotics and anti-metabolites for long duration which may influence the incidence of fungal infection.

CONCLUSION

Tuberculosis coinfection and mycotic infection are not common in patients without evidence of comorbidities or immunosuppressive disease.MDR-TB patients carry the risk of higher percentage of fungal infections and more liable for acquiring mixed fungal pathogens. Presence of male sex, smoking, DM and far extent of lesion must attract physicians' attention for fungal co-infection with pulmonary tuberculosis.

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