ANNEXURE
ANNEXURE- 1

QUESTIONNAIRE TO COLLECT SOCIO-BIOLOGICAL DETAILS OF PATIENTS

SOURCE : _________________________

DATE : _________________________ SAMPLE NO: _________________________

1) NAME : _________________________

2) SEX : _________________________

3) AGE : _________________________

□ MALE □ FEMALE

4) RELIGION

□ HINDU □ MUSLIM □ CHRISTIAN □ OTHERS

5) MARITAL STATUS

□ SINGLE □ MARRIED □ WIDOW □ OTHERS

6) PLACE OF RESIDENCE : _________________________

7) CONTACT NUMBER : _________________________

8) OCCUPATION : _________________________

9) INCOME : _________________________

10) NATURE OF JOB : FIXED □ ROAMING □

11) QUALIFICATION : _________________________

12) KNOWLEDGE ABOUT TUBERCULOSIS

13) CLINICAL SYMPTOMS OBSERVED
**Gram's stain**

Gram's staining kit was obtained from Hi-Media and used for the identification of yeast cells.

**Inoculation on SDA media**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabouraud’s dextrose agar</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>2 g</td>
</tr>
</tbody>
</table>

SDA was obtained from Hi-Media and stored at room temperature.

**Chloramphenicol stock solution**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>5 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

The stock solution was filter sterilized and stored at 4°C. The working solution of (0.5mg / ml) was utilized for preparation of SDA media.

**Cycloheximide stock solution**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>5 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

The stock solution was filter sterilized and stored at 4°C. The working solution of (0.5mg / ml) was utilized for SDA media.

**Inoculation on Hi Chrom Candida agar Plate**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hichrom Candida agar</td>
<td>21.36 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>
The media was obtained from Hi-Media and stored at room temperature. 0.5 g of extra agar was added to get proper solidification of the medium.

**Carbohydrate fermentation test**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>90ml</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>10ml</td>
</tr>
<tr>
<td>Sterile carbohydrate solution (20%)</td>
<td>0.3ml</td>
</tr>
</tbody>
</table>

(Glucose, maltose, sucrose and lactose) Each carbohydrate solution was added to the prepared fermentation broth medium separately and used for inoculation.

**Carbohydrate assimilation test**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast nitrogen base</td>
<td>12.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

The media was prepared and filter sterilized. Stored at 4 °C for avoid contamination.

**Preparation of sugar discs**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate solution</td>
<td>20 g</td>
</tr>
</tbody>
</table>

(Glucose, maltose, sucrose and lactose )

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Whatmann filter paper disc
The carbohydrate solution was filter sterilized and stored at 4°C. Whatmann Filter paper discs were soaked into the carbohydrate solution and dried. The discs were stored in a sterile petriplate for carbohydrate assimilation test.

**Germ tube test**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

*Candida albicans* used as positive control and *Candida tropicalis* used as negative control. The serum was preserved at -20°C.
Organisms and Media

The clinical sample were oral cavity swab taken from the patients of tuberculosis.

The Candida strain (CA1) was used in this study.

Candida culture

The Fluconozole resistant Candid albicans isolated from the tuberculosis patients had the age group of 41-50 was used for the isolation of genomic DNA.

TE buffer

10mM Tris HCl [pH 8.0]

1mM EDTA [pH 8.0]

Take 1ml of 1M Tris HCl [pH 8.0], 0.5ml of 0.5M EDTA in 98.5ml of distilled water.

SDS Solution

Dissolve 10g SDS in 100ml of distilled water. This solution was freshly prepared during the isolation of the DNA.

Phenol : Chloroform [50 : 50]

Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1M Tris .Cl [pH 7.6]. Store the equilibrated mixture under an equal volume of 0.01M Tris .Cl [pH 7.6] at 4°C in dark glass bottles.

Isopropanol

70% Ethanol
3M Sodium acetate [pH 5.2]

Dissolve 123g sodium acetate in 450ml of distilled water. Adjust the pH to 4.8-5.2 with glacial acetic acid. Make the volume to 500ml with distilled water.

Proteinase K

| Proteinase K | 20mg |
| Distilled water | 1 ml |

This solution was used to remove the protein debris from the precipitate of the DNA. It was stored at 4 °C.

Agarose gel electrophoresis

50x TAE buffer (Electrophoretic buffer)

| Tris base | 242g |
| Glacial acetic acid | 57.1ml |
| 0.5M EDTA | 100ml |

Make up the solutions with distilled water to 1000ml. The buffer was used after 1X conversion.

Sample buffer

| 0.1M EDTA (pH 8.0) | 2ml from 0.5M stock |
| 0.01M Tris HCl (pH 8.0) | 0.1ml from 1M stock |

Bromophenol blue

| Bromophenol blue | 25mg |
| Distilled water | 100 ml |
Glycerol (v/v) - 45 ml

2.9 ml distilled water was added to dilute the sample buffer.

**Ehidiium bromide**

Ehidiium bromide - 5 mg
TE buffer - 10 ml

This solution was prepared as a stock and stored in a brown colour bottle. 50µl was added to 100 ml of agarose and shaked well for complete dissolving before pouring to the boat.

**Electrophoretic apparatus**

A student model Electrophoresis units with all accessories was obtained from Biotech Pvt Ltd., Yercaud.

**Agarose**

Agarose was obtained from Himedia with quality of low EEO and preserved at room temperature.

**Marker (Fermentas SM1153)**

The marker was obtained from Fermentas, USA, and preserved at -20 °C.

**Primer Designing**

Primer 3 software was first invented by Eli and Edythe Broad, MIT, Harvard. Primer3 is a tool used to choose primers for PCR reactions. The primers were designed for the gene of our concern, MDR1 and ERG2.
DNA Amplification (Thermo cycler)

My Gene™ Peltier Thermal Cycler, Model MG25+ was used for polymerase chain reaction.

DNA Sequencer

ABI Prism 3100 automated sequencer was used to detect the sequences of the amplified product.
Prevalence of opportunistic candidal co-infections among patients of pulmonary tuberculosis

*Alagusundaram M. and *Ranjith Singh

Department of Advanced Zoology & Biotechnology, Sri Paramakalyani College, Alwarkurichi, Tirunelveli, Tamilnadu, 627412.

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Corresponding Author
M. Alagusundaram
Email: parambualagu@gmail.com

Abstract

Five hundred clinical specimens of oral swabs were collected from hospitalized tuberculosis patients for the isolation of Candida species. The patients were categorized in two Groups. Group A included male tuberculosis patients with the age group between 30-40 and between 41-50 taking antituberculosis treatment. Group B included female tuberculosis patients with the age group between 30-40 and 41-50 taking antituber culosis treatment. The Candida species were isolated and identified on the basis of morphological, cultural and biochemical characteristics. 18.8% of Candida species were isolated from (94/500) the specimens. The incidence rate of Candidal co-infection was higher in Group A patients (16.1%) as compared to Group B patients (13.8%). Among the Candida species, Candida albicans (8.4%) predominated over Candida tropicalis (6.4%) and Candida glabrata (4%). Furthermore, the incidence of Candidal infection was higher in male patients (16.3%) with the age group of 41-50 as compared to female patients (13.9%) between the age 41 and 50.

Key words: Candida spp, co-infection, opportunistic infection, tuberculosis, T

Introduction

Diseases nowadays multiply rapidly carrying proportionate complication. Concordantly in recent years a dramatic worldwide increase in the number of immunocompromised hosts has resulted in an increase in opportunistic fungal infections leading to morbidity and mortality rise (1,2). Due to immunodeficiency or suppression in tuberculosis, this group of patients are also vulnerable to opportunistic fungal infections (3,4). It has been reported that patients with tuberculosis have several disfunctions in macrophages, monocytes and T cells as well as chemotaxis that predispose them to opportunistic fungal infections. (5,6). When the host resistance is impaired, these unrecognised opportunistic fungi may affect the progress of the disease or may even become fatal. These etiological agents some of which are otherwise incapable of causing disease in healthy individuals. These opportunistic fungi are potential pathogen in the immunocompromised patients, patients with some preexisting disease and patients with a long history of antibiotics (7,8). The rate of opportunistic infections in tuberculosis patients is also very high. The reasons for increased prevalence are lowering of immune system due to tuberculosis and the use of antituberculosis drugs of non specific action which promote the growth and reproduction of fungus flora (9). Among the fungal pathogens, Candida albicans is a common yeast isolated from tuberculosis patients and it is responsible for causing severe secondary infections in such patients (10). Besides, a syntropic relationship between Candida albicans and Mycobacterium tuberculosis has also been reported in a number of studies, where tubercle bacilli were found to enable Candida albicans to grow on Lowenstein Jensen’s medium (11). Moreover Candida albicans also stimulated the growth of Mycobacterium tuberculosis has also been reported in a number of studies, where tubercle bacilli were found to enable Candida albicans to grow on Lowenstein Jensen’s medium (11). Moreover Candida albicans also stimulated the growth of Mycobacterium tuberculosis of reduced viability (12). Another study confirmed the effect of polysaccharide fraction of Candida albicans for enhancement of growth as well as reduction of the generation time of tubercle bacilli (13). Keeping in view the role of Candida in aggravation of tuberculosis, the present study was undertaken to determine the prevalence of Candidal co-infections and the pattern of distribution of Candida species against the sex and age of the tuberculosis patients.

Materials and Methods

The study comprised 500 tuberculosis patients, admitted as inpatients and outpatients at Government Thoracic Disease and Hospital, Chennai, Tamilnadu, India. These patients were further categorized in two groups on the basis of their age and sex. Group A and Group B. Group A: included male tuberculosis pa-
Table 1: Prevalence of Candidal infections in tuberculosis patients with respect to sex

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Patients co-infected with Candida species</th>
<th>Total n=500</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male n=310</td>
<td>Female n=190</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>35</td>
<td>11.2</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>26</td>
<td>8.3</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>18</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>25.3</td>
</tr>
</tbody>
</table>

Percentage have been calculated from corresponding values of n.

Table 2: Cross tabulation (Percentage analysis) of relationship between positivity of co-infection and sex

<table>
<thead>
<tr>
<th>Sex</th>
<th>No of positively infected patients</th>
<th>No of patients not infected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>79 R%=25 C%=84</td>
<td>231 R%=75 C%=57</td>
<td>310</td>
</tr>
<tr>
<td>Female</td>
<td>15 R%=8  C%=16</td>
<td>175 R%=92 C%=43</td>
<td>190</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>406</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 3: Chi-square test of Prevalence of co-infection among male and female patients

<table>
<thead>
<tr>
<th>Sex</th>
<th>No of patients coinfected</th>
<th>No of patients not infected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>79</td>
<td>231</td>
<td>310</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>175</td>
<td>190</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>406</td>
<td>500</td>
</tr>
</tbody>
</table>

Calculated value of Chi-square = \( \Sigma (O-E)^2/E = 23.74 \). Null hypothesis is rejected. There is significant relationship between gender and positivity of infection.

Table 4: Prevalence and positivity of co-infection among the age of both sex groups

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of patients coinfected</th>
<th>No. of patients not infected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-40</td>
<td>22</td>
<td>243</td>
<td>265</td>
</tr>
<tr>
<td>41-50</td>
<td>72</td>
<td>163</td>
<td>235</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>406</td>
<td>500</td>
</tr>
</tbody>
</table>

Calculated value of Chi-square = \( \Sigma (O-E)^2/E = 40.7 \). Null hypothesis is rejected. Age and positivity of infection are dependent.
tients (inpatients and outpatients) in the age group of 30 to 40 and group 41-50 taking antituberculosis drugs. Group B: included female tuberculosis patients (inpatients and outpatients) age ranging between 30 and 40 and 41 and 50. All the tuberculosis patients were taking antituberculosis drugs during the study collection of specimens: Clinical specimens; Oral swabs were collected from the patients. Direct smear examination: All the clinical specimens were Gram stained and observed under the microscope for the yeast cells. Culture for primary isolation: Sabouraud dextrose agar with Chloramphenicol (0.04 mg/ml) and Cycloheximide (0.5 mg/ml) (14) was used for primary isolation of Candida species. The culture plates were incubated at 37°C for 24 hours (15). Characterization of organisms: All pure cultures were characterized to species level using different tests confirming to required standard identification criteria. The criteria included morphological and cultural characteristics, C. albicans and other species of Candida were further distinguished by biochemical reactions, in which formation of acid and/or gas under anaerobic condition was noted in different sugars i.e. glucose, maltose, sucrose and lactose. Germ tube test also done (16). Statistical Method: To notice the significance of data achieved. Cross tabulation and Chi-square (x2) were implicated, the results of which were presented underneath.

Results

A total of 500 tuberculosis patients (AFB positive); 310 males and 190 females were included in the study. The clinical specimens collected from these patients were processed for the isolation of Candida species. The study revealed 18.8% (94/500) of the total tuberculosis patients co-infected with Candida species. Of these, C. albicans ranked high with an incidence rate of 8.4% (42/500) as compared to C. tropicalis 6.4% (32/500) followed by C. glabrata 4% (20/500) (Table 1). The relationship between the incidence of Candidal co-infection and sex of the tuberculosis patients were analyzed by percentage analysis (cross tabulation). The positivity of co-infection in male patients was 84% as compared to female patients was 16% (Table 2).

The relationship between gender and co-infections were analyzed by Chi-square test which revealed that there was a relationship between gender and positivity of co-infection (Table 3). Furthermore the relationship between age and positivity of infections was analysed by Chi-square Test. Age and co-infection are dependent in tuberculosis patients. (Table 4).

Discussion

Candida is a component of the normal microflora of the alimentary tract and mucocutaneous membrane of a healthy host. However, the slight alteration in the physiological state can turn normally harmless commensal yeast into aggressive pathogen causing mucosal, superficial or even life threatening systemic infections in the immunocompromised host pointing to the pathogenic potential of Candida species (17-19). The prime target of these opportunistic Candidal infections are patients who are critically ill and are at medical and surgical intensive care units. Candida species are well recognized in nosocomial infections and have been reported as the sixth most common nosocomial pathogen (20). The role of Candida species as secondary invader of lungs, kidneys and other organs of patients having some pre-existing disease like tuberculosis and cancer, have also been documented (21). The present study which was conducted to determine the incidence rate of Candidal infections among tuberculosis patients (inpatients and outpatients) in the hospital revealed that 18.8% was co-infected with Candida species. The isolation of the fungi C. albicans (26.36%) was the most common species in patients with larger cavities, diabetics and in those who had prolonged antituberculosis chemotherapy. The study finding is parallel with another study conducted in Pakistan which revealed 15.2% Candidal infections among hospitalized patients having tuberculosis and had a severe complications, C. tropicalis ranked high with an incidence rate of 8.4% (42/500) as compared to C. albicans (6.8%; 34/500) (22). The sex-wise distribution of these co-infected patients exhibited higher trend of Candidal infections among male tuberculosis patients (inpatients and outpatients) in the hospital revealed that 18.8% was co-infected with Candida species. The isolation of the fungi C. albicans (26.36%) was the most common species in patients with larger cavities, diabetics and in those who had prolonged antituberculosis chemotherapy. The study finding is parallel with another study conducted in Pakistan which revealed 15% Candidal infections among hospitalized patients having tuberculosis and had a severe complications, C. tropicalis ranked high with an incidence rate of 8.4% (42/500) as compared to C. albicans (6.8%; 34/500) (22). The sex-wise distribution of these co-infected patients exhibited higher trend of Candidal infections among male tuberculosis patients (inpatients and outpatients) in the hospital revealed that 18.8% was co-infected with Candida species. The isolation of the fungi C. albicans (26.36%) was the most common species in patients with larger cavities, diabetics and in those who had prolonged antituberculosis chemotherapy. The study finding is parallel with another study conducted in Pakistan which revealed 15% Candidal infections among hospitalized patients having tuberculosis and had a severe complications, C. tropicalis ranked high with an incidence rate of 8.4% (42/500) as compared to C. albicans (6.8%; 34/500) (22). The sex-wise distribution of these co-infected patients exhibited higher trend of Candidal infections among male tuberculosis patients (16.3%; 44/270) as compared to female tuberculosis patients (15.9%; 32/230). Similar pattern has also been observed from another study which reported 15% Candidal infections among non-immunocompromised critically ill patients (23). In our study we found C. albicans in 19 (8.4%), C. tropicalis in 32 (6.4%) and C. glabrata in 20 (4%) amongst a total of 500 cases. The main pathogen involved in these infections were Candida species (24).

As far as the sex-wise distribution of Candidal infections is concerned, it is evident from literature that the colonization with Candida species occur in equal numbers of males and females (25). However, in the present study Candidal infections were found more prevalent in male tuberculosis patients as compared to females. This might be attributed to more exposure of male to external environment the habit of using some addictive substances (26). In the present study, the species-wise distribution of Candidal infections revealed...
the predominance of C. albicans over C. tropicalis and C. glabrata. The role of C. albicans in causing severe secondary infections in tuberculosis patients has also been reported in a study where, despite success

ful completion of antituberculosis chemotherapy, patients suffered from chronic cough, sputum or occasional hemoptysis (27). All these above findings correlated with the results of present study where 18.8% of the tuberculosis patients were co-infected with Candida species and manifested different complications in their primary disease. Candidal infections may enhance the primary disease such as tuberculosis. In addition, it is evident from the literature that, 62.6% of the tuberculosis patients having candidal co-infections among pulmonary tuberculosis patients 86% oral Candidiasis were HIV positive (28). The higher incidence of fungus positivity in the treated group cases than the fresh group and also the incidence of positivity was significantly higher (78%) in cases who had had antitubercular treatment for more than one year than those who had for less than one year (39%) (29). In developing countries like India these problems are further increased by preponderance of pulmonary tuberculosis which may result in unavoidable complications of unwarranted chemotherapy or surgery.

References