CHAPTER 2

CLINICO-EXPERIMENTAL STUDIES
2.1 INTRODUCTION

The yeast - *Candida albicans* typically exists in a symbiotic relationship with humans\(^1\). In this event, however, when the host is immunologically debilitated upon challenge by an underlying disease or long-term immunosuppressive therapy. *C. albicans* being a commensal can transform into a virulent pathogen of the host. For many years, this micro-organism has gained increasing prominence as an opportunistic pathogen in hospital acquired infections in the compromised patients. Candidiasis causes a chronic bronchitis leading to complications such as cough, production of sputum, and median to coarse basilar rales with linear fibrosis or peribronchial thickening seen on radiologic examination. The etiologic significance of *C. albicans* in this disease is difficult to determine. Some studies have shown that the organism occurs, sometimes in a considerable number in essentially all chronic pulmonary conditions\(^2\). The fungal propagules may be visualized in a considerable number in direct smear preparation of sputum/bronchial aspirate or by culture of sputum. Bronchoscopy may not infact assess the extent or degree of candidal colonization of the bronchial tree. In advanced disease, small, white, curdlike patches similar to those observed in a thrush or oral or vaginal infestations are seen. Ulcerative lesions may occur in advanced cases but are very rarely described\(^3\). Recently, more attention has been paid to the role of *Candida* species in inciting allergic-type diseases. The spread of disease is often progressively fatal being pulmonary in origin. The candidiasis due to *Candida* species is a very common disease in immunocompromised patients and the contribution of the yeast to the human pathology is firmly established.

Pulmonary Candidiasis, as a primary disease is often rare. The diagnosis of this disease must rest on adequate evidences that rules out all other etiologies of the pathologic process\(^4\). *Candida* readily colonizes preexisting pathologic conditions attributable to other infectious agents such as tuberculosis (TB). In these cases, the organisms may be present in numbers to \(\times 10^6\, \text{ml}^{-1}\) and are easily visible in the sputum smears. If their multiplication continues, the *Candida* may contribute to the pathology and may cause severe distress and death. Often such patients have some underlying condition such as leukaemia,
lymphoma, diabetes and in those patients where derangement of immunity occur such as in Acquired Immunodeficiency Syndrome (AIDS) and AIDS-like complexes (ARCs)\(^5\).

The unprecedented increase in pulmonary mycosis in tubercular patients is a global threat particularly in India where more than 1 crore people suffer from TB of which 5,00,000 people die per annum\(^6\). The impact of mycosis on the morbidity and mortality of such patients has not yet been studied in India. Therefore, we initiated studies on the prevalence of fungal infections in hospitalised/OPD immunocompromised patients (ICP) in Lucknow, India.

2.2 MATERIALS AND METHODS

2.2.1 Selection of cases:

In the present study one hundred-and-eighteen (118) patients of pulmonary tuberculosis who were immunocompromised (ICP) due to primary underlying diseases such as diabetes, cancer, asthma and suspected cases of meningitis were studied among those attending the (OPD) and or those admitted to Kasturba Chest Hospital, Department of Tuberculosis and Chest Diseases, and other Departments of Gandhi Memorial and Associated Hospitals, King George's Medical College, Lucknow. The patients who showed clinical, radiological and bacteriological evidences of pulmonary tuberculosis were selected for the investigation of underlying pulmonary mycotic infections. The cases included both gender and all age groups. A detailed clinical investigation of each patient was made as follows:

2.2.1(i) Case History. (a) General interrogation, (b) Complaint of disease and duration, (c) Present history, (d) Treatment taken in past regularly or irregularly, (e) Past history of similar episodes of respiratory tract infection, or history of contact, or family history for pulmonary complications.

2.2.1(ii) Physical examination. (a) General examination—such as—general condition, general appearance, consciousness, conjunctiva, pupils, tongue, lips, teeth, gum, glands, nails, nutrition, bowel, micturition, sleep, appetite, temperature,
pulse rate, blood pressure and respiratory rate; (b) detailed examination of respiratory system such as - inspection, palpation, percussion, auscultation, and (c) systemic examination including central nervous system (CNS), cardiovascular system (CVS), abdomen and musculo-skeleton system.

2.2.2 Investigations.

2.2.2(i) Pathological examination - routine investigations such as - (a) hematological: hemoglobin (Hb), TLC, DLC and ESR, (b) urine examination for albumin, sugar, acetone and microscopic examination, and (c) stool examination for ova and or cyst.

2.2.2(ii) Bacteriological examination. (a) Repeated examination for sputum smear (at least 3 consecutive occasions) for acid fast bacilli and Gram's staining for other pathological organisms, (b) smear examination for fungal elements, and (c) sputum culture for fungus.

2.2.2(iii) Radiological examination. Chest X-ray, (PA view and other views as required).

2.2.2(iv) Routine biochemical examination. (a) Blood sugar, (b) Blood urea etc.

2.2.3 Mycological tests.

These tests were performed in all the sera of immunocompromised patients (ICP).

2.2.3(i) Collection and processing of clinical material. The sputa (approx. 10 ml) bronchial aspirate (approx. 2.5 ml), were collected in the sterile corning wide mouth glass tubes. The specimens were obtained from the patients early morning prior to breakfast by thoroughly rinsing the mouth and teeth with sterilised physiological saline (SPS, 0.85%), under aseptic condition. The specimens were processed for mycological examination within approximately 2 hrs. Gross examination of the sputum and the necrotic material was done as follows:
2.2.3(ii) Gross examination of sputum. The sputum specimen was spread on to a Petri dish (90 mm diam) and examined for the presence of suspicious foci infected with fungal propagules. Gross examination of sputum coloration, nodules or granules was made. The clinical specimen was treated with 10% KOH in sterile triple distilled water (TDW) for direct microscopic examination (300x, Nikon, Japan) for fungal elements.

2.2.3(iii) Concentration of sputum. The sputum specimens collected in the specimen containers as above were transferred to sterile 50 ml plastic, screw capped, graduated centrifuge tubes. The sputum (approx. 10 ml) specimen was transferred in centrifuge tubes as above and an equal volume (v/v) of freshly prepared digested N-acetyl-L-cysteine (NALC, Sigma) was added. The tubes were tightly capped and the material was mixed on a vortex mixture for 5-10 sec. Phosphate buffer (PB) (M 0.07, pH 7.0) was filled (20 ml) in tubes. The tubes were screw tightened with caps and the contents were swirled on a vortex mixture as above. The tubes containing the clinical material were centrifuged at 2,000 rpm (4°C), for 10 minutes. The supernatant was decanted and the sediment was treated with chloramphenicol (0.05-0.1 µg ml⁻¹). For dimorphic yeasts particularly Histoplasma capsulatum instead of chloramphenicol, penicillin (20 units ml⁻¹) and streptomycin (40 units ml⁻¹) was substituted. The material was mixed on a vortex mixture for 10 sec. and 0.2 ml of the sediment was spread on to the fungal isolation media (Himedia, Bombay), viz., Sabouraud chloromycetin agar (SCA), Sabouraud dextrose agar (SDA), Nutrient agar (NA), Niger seed (Staib) agar (NSA) and Brain heart infusion agar (BHIA) (Appendix). The cultures were incubated at 28±1°C and examined periodically for at least 4 weeks. The cultures which failed to grow during this period were considered negative and discarded. From the positive cultures, the fungal pathogen recovered were identified with the help of available literature.

2.2.4 Examination.

2.2.4(i) Direct microscopic examination. The smear of sputa/bronchial aspirate prepared in 10% KOH as described above
was examined within 2 hr for fungal (yeast and or mycelial elements) by the microscope (300x, Nikon, Japan).

2.2.4(ii) Cultural examination. The digested (NALC) specimens were inoculated (0.2 ml) and spread thoroughly by a glass spreader on to the Petri dish (90 mm diam) containing differential fungal and bacterial (Himedia, Bombay, India) media: SDA (pH 5.6), SCA (pH 5.6), BHIA (pH 7.4), NSA (pH 5.5) and NA (pH 7.4±0.2; Appendix). The plates were incubated at 28±1°C (for fungi) and 37±1°C (for bacteria) for atleast 48-72 hr. For recovery of dimorphic fungal infections the BHIA plates were kept for 3-4 wk at 37°C and examined periodically for fungal (yeast phase) growth. The smear of the organism was stained in cotton blue (Sigma) and the organism was identified (300x) by the microscope (Nikon, Japan) with the help of available literature. The microbial isolates were subcultured, maintained, and deposited at the Pathogenic Fungal Culture Collection of Medical Mycology, CDRI, Lucknow, India.

2.2.5 Preparation and storage of serum from whole blood.

The venous blood (approx. 10 ml) from the tuberculosis patients was collected in sterilised corning tube (2.5 mm diam) and after clotting (approx. 30 min) the serum was separated by the standard method from the whole blood by cold (-4°C) centrifugation (2,000 rpm for 10 min). In order to avoid loss of serum in the clot it was loosen from the glass surface before centrifugation by rotating the clot by a sterilised Pasteur pipette around the glass wall. The sera were transferred to sterile screw cap vials (5 ml).

The sera obtained from the blood of patients were added with 0.001% sodium azide (w/v; Sigma) and stored in closed ampules at -20°C. The reference antisera of C. albicans and A. fumigatus (obtained from hyperimmunized goat) were supplied by the Institute Pasteur Productions (France) or by the Meridian Diagnostics (USA), and stored in the same manner as described above.

2.2.6 Serological investigations.

The genus Candida and Aspergillus were detected as secondary fungal infections in tuberculosis patients. Therefore, efforts
were made to detect antigen/antibody (Ag/Ab) by the double Immunodiffusion (ID), Immunofluorescence (IF) and Enzyme Linked Immunosorbent Assay (ELISA) in the clinical specimens\textsuperscript{7,8}. The following antigens were prepared for fungal serology.

2.2.6(i) Preparation of antigens (Ags). Two types of fungal antigens—somatic (intracellular) and metabolic (extracellular) were prepared as follows:

2.2.6(ia) Candida albicans (Ags). \textit{C. albicans} (Ca-27, an isolate obtained from Indian tuberculosis patient) was grown for 24-48 hr on SCA slants at 28°C. The fungal growth was harvested and suspended in normal physiological saline (NPS) (0.85%), thus forming a stock concentration of blastospores $10^8$ cells ml\textsuperscript{-1}. One millilitre of the suspended growth was inoculated into the SCB (200 ml/1000 ml Erlenmeyer flask). Two sets of such inoculated flasks were prepared and one set was put on a gyrotatory shaker (300 g) and another set was kept as stable stationary culture for 7 days. For metabolic antigens the fungal growth was pelleted by cold centrifugation (4°C at 3000 g) for 10 minute and the supernatant was dialysed (dialysis tubing, cellulose membrane pore size—retaining most proteins of molecular wt of 12,000 or greater, flat width 43 mm, 1.7 inch, Sigma, USA) in NPS extensively for 3 days with successive changes of water 2 to 3 times per day under constant stirring at 4°C. The material was concentrated with PEG 6000 (Sigma), overnight and the antigen was lyophilised (Sico Lyophilizer) at -36°C. For the somatic antigens of \textit{C. albicans}, the blastospores pseudomycelia were crushed with white sterilised sand and NPS on a pestle mortar. The disrupted blastospores/pseudohyphae were centrifuged as above and the supernatant processed for the dialysis and concentration as described above. The protein content of the antigens (both metabolic and somatic) was determined by the Lowry’s method\textsuperscript{9}.

2.2.6.1(b) Aspergillus fumigatus (Ags). A four-day growth of \textit{A. fumigatus} (AF-5, isolated from an Indian patient of bronchopulmonary aspergillosis, BPA) on SDA at 28°C was harvested in NPS and 1 ml. (approx. $10^5$ spores ml\textsuperscript{-1}) was inoculated into the SDB in Erlenmeyer flask as described for Candida. The metabolic antigens were prepared from the stationary broth
culture grown at 28±1°C for a fortnight. The antigens were prepared from sporulating culture by a seitz filtration. For somatic or cellular antigens the mycelial lysates were obtained from shake flask cultures (2 wk old) at 28±1°C. The antigens were dialysed and concentrated before lyophilization as described earlier. Sodium azide 0.001% (w/v) was added to all the antigens stored at 4°C.

2.2.6(ii) Serological techniques. Following serological tests were employed for the fungal diagnostics.

2.2.6.(iiia) Immuno-precipitation technique (Ouchterlony's double diffusion test). Immunoprecipitation in agar medium (Oudin) is based on the appearance of one or more specific opaque precipitation lines corresponding to different antigen-antibody complexes. The plastic disposable Petri dishes (5 cm) were filled with melted agarose (Indubiose, 37 Industrie biologique Francaise, France) 1.5% in barbital buffer (pH 8.2) (Appendix). The agar gel was punched, a central well (3 mm diam.) for serum and six peripheral wells (3 mm diam) 6 mm away from the central well. The central well was filled with the serum (10 µl) and the peripheral wells with the antigens (10 µl, each well) and their dilutions in sterile triple distilled water. The plates were placed at a constant ambient temperature. The precipitating arcs were observed after staining with Coomassie blue (Sigma) (Appendix). Simultaneous controls were run with reference antigens/antisera. The appearance of one or more precipitating lines corresponding to antigen/antibody complexes indicated positive reaction for infection.

2.2.6(iiib) Immunofluorescence (IF) technique. The fluorescent antibody technique is a very important technique in biological sciences for tracing antigen with labelled antibody. The method provided useful information in the investigation of various infectious pathogens as well as in the detection of infectious agents in different materials even in the early stage of their development, thus, enabling an early and rapid diagnosis. The antigens present in cells, in cultures, reacts specifically with its antibody. A specific antiserum is prepared against the gammaglobulin fraction of the serum of a particular species of
animal. Complexes formed by binding with antigens to antibodies are insoluble. These complexes become visible under the fluorescent microscope as greenish-yellow spots when the gammaglobulin fraction of the antiglobulin serum being pre-labelled with fluorescin isothiocyanate (FITC; Sigma).

In our experiments, the indirect method of immunofluorescence was used following the method described by Drouhet. The slides (3x8 mm) were precoated with a suspension of approximately 5x10^6 yeasts of C. albicans, serotype A, strain (CA-27), and A. fumigatus (AF-5) air-dried and fixed by heat. Serial dilutions of patients serum 1/20, or 1/40 etc. and upto 1/2560, and 1/100 fluorescein isothiocyanate (FITC) labelled rabbit serum antihuman globulin (Institute Pasteur Productions) were added. The slides were observed under the fluorescent microscope (400x, Nikon, Japan) and the fluorescent antibody titre was determined. Suitable controls were also run simultaneously with standards.

2.2.6(iic) Enzyme linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assay (ELISA) is one of the group of enzyme immunoassays which was first described by Engvall and Perlmann and later applied to microbiological diagnosis by Voller et al. ELISA has the advantage over immunofluorescence (IF) technique due to increased sensitivity since the enzyme acts catalytically and objectively which enables the optical density (OD) of the colour end-point to be measured. Enzymes are safe, inexpensive and stable when compared with the radio-isotopes used in radioimmunoassay (RIA).

In the ELISA test soluble antigens or antibody are rendered insoluble by adsorption to a solid phase such as polystyrene tubes or microtitre ELISA plates. Adsorption is dependent on time, temperature and pH. Overnight coating of plates at 4°C in a buffer at a high pH (9.6) is done. The antigens or antibody are conjugated to an enzyme without loss of activity. The ELISA test used for the determination of C. albicans and A. fumigatus antibodies was based on the method of Voller et al. using polyvinyl microtitre (96 wells) plates (No. 1-220-29, Dynatech Laboratories). In subsequent test, each well was first coated with C. albicans or A. fumigatus antigens (100 µl of antigen solution containing 1 µg of protein) for 1 hr at 37°C. The diluent used was 50 mM carbonate buffer (pH 9.6, Appendix). The
plates were incubated overnight at 4°C. The coated wells were washed thrice for 5 minutes each with PBS containing 0-10% (v/v) Tween-20 (PBST, pH 7.4). PBST (200 µl) containing 1% bovine serum albumin (BSA, Sigma) was added to each well and the plates were kept for 2 hr at 37°C. The titre wells were again washed with PBS 3 times for 5 minutes each.

Patient serum was diluted 1:1000 with PBST and duplicate volumes were added to antigen-coated wells and incubated for 2 hr at 37°C. The plates were again washed with PBST three times for 3 min each. Heavy chain specific antiserum to human IgG was horseradish peroxidase (HRPO) conjugate (Sigma), diluted to 1:1000 fold with PBST and 100 µl was added to each well. The plates were left at room temperature for 2 hr, washed three times for 5 min, each with PBST and 100 µl of a substrate solution of 0.01% 0-phenylenediamine (Sigma) and 0.006% H₂O₂ in 150 mM citrate buffer (pH 5.0) was added in dark to each well. This mixture was left standing at room temperature in dark for 30 minutes. Finally, the reaction was stopped by adding 50 µl of 7% H₂SO₄ to each well. Absorbance was recorded at 492 nm with a titertek multiskan (Flow Labs., U.K.).

Wells of microtiter plates were coated with 0.10 ml of the IgG fraction (20 µg/ml) of rabbit antibody to H. capsulatum in 0.10M Tris-HCl buffer, pH 7.0, incubated for 1 hr at 37°C, aspirated and washed. Two wells were left uncoated to serve as blanks. Further 0.10 ml of 5% BSA in 0.01M Tris HCl, pH 7.0, was incubated in each well for 1 hr at 37°C, after which the wells were emptied and washed. Then, 0.10 ml of 1:1000 diluted serum, was incubated in each well for 1 hr at 37°C. The wells were then aspirated and washed. Two coated and two uncoated wells were left as blanks (control) without receiving the specimens.

The Histoplasma polysaccharide antigen that adhered to the solid-phase antibody was measured by incubation at 37°C for 1 hr with 0.10 ml of rabbit IgG antibody to H. capsulatum conjugated to alkaline phosphatase (AP) that was diluted in 50% BSA in 0.10 M Tris-saline, pH 8.0. All specimens were tested in duplicate.

2.2.7 Physiological tests.

The following tests were performed for the identification of Candida species isolated from the patients.
(i) Germ tube test, (ii) Chlamydosporo test or Cornmeal agar (CMA) test, (iii) Carbohydrate assimilation test, (iv) Fermentation test and (v) Urease test.

2.2.7(i) Germ tube test. The serum was separated from the blood (10 ml) of healthy rhesus monkey (CDRI) as discussed earlier. A colony of each Candida strains (isolated from tuberculosis patients) was inoculated separately in 200 µl of serum stored in appendorf microtube and incubated at 37°C for 2-3 hr. The germination of the yeast blastospores for C. albicans in each strain was recorded by the microscopic examination (200x)\textsuperscript{12}. Appropriate controls were run simultaneously.

2.2.7(ii) Chlamydosporo test or cornmeal agar test. The chlamydosporo test\textsuperscript{13} was performed as follows.

Cornmeal agar (CMA) with 1% Tween-80 (Appendix) was plated (15 ml) in sterilised Petri dishes (90 mm diam). Each plate was divided into 4 quadrates with the help of a marker pen. The growth of different strains of Candida spp. was streaked (approx. 5 cm long and 1.2 cm apart) separately on to the surface of agar medium. A flame sterilized cover glass (22 mm square) was placed gently over the streaked marks. The plates were incubated at 28°C for 24 hr. The cover glass was removed gently after 24 hr with the help of a sterilised forcep and placed onto the clean slide. The chlamydosporo formation was observed under the microscope (200x). The results were expressed as positive or negative for chlamydosporo formation. Suitable controls were maintained under identical conditions.

2.2.7(iii) Carbohydrate assimilation test. Assimilation test is the utilization of carbon source by the yeast in presence of oxygen. A positive assimilation reaction is usually read either in the presence of growth or as an acid pH value in the growth medium. The aerobic oxidation (assimilation) of a sugar results in the formation of carbon-dioxide and water. The basic reaction is:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

Several techniques have been developed for determining the ability of a yeast to utilize various sources of carbon
aerobically for energy and growth. The most commonly employed techniques for conducting assimilation studies is the liquid medium technique designed by Wickerham and Burton.\textsuperscript{14} Auxanographic procedure was designed by Weijerink\textsuperscript{15}, in which solid agar medium is used. The basal agar medium (Appendix) was poured in the sterilised Petri plates (90 mm diam.) and allowed to solidify. An inoculum ($10^6$ ml\textsuperscript{-1}) per plate was poured and then transferred to next successive plates. The experiment was performed in triplicate. The plates were dried down and kept at room temperature, for approx. 20-30 minutes. The wells in solidified agar were punched with a sterilised cork borer (3 mm diam). The recommended quantity of each sugar in sterilised TDW were sterilised by millipore filter (0.02 mm diam) and poured into the corresponding well. The sugars were allowed to diffuse adequately in the agar and the plates were incubated at 28°C for 24-72 hr. The observations were made for assimilation of sugars. Simultaneous controls were run accordingly. A positive result was indicated by the growth of yeast and an indicator colour shift from purple to yellow. A negative reaction was indicated by no growth of the yeast and no indicator colour shift i.e., the colour remains purple.

2.2.7(iv) Fermentation reaction test. Alcoholic fermentation is the process by which a carbohydrate, such as glucose is fermented anaerobically to form ethanol and carbon dioxide. Fermentation is read as the production of gas not as an acid pH change. The fermentation process requires, that the yeasts have enzymes such as alcohol dehydrogenase and pyruvate decarboxylase, and a transport mechanism for getting the carbohydrate across the plasma membrane under anaerobic conditions. If these are absent, the yeast will be unable to ferment the carbohydrate being tested, while if a carbohydrate is fermented, it is also assimilated but the converse is not necessarily true. The stock solutions of the sugars [dextrose (D), maltose (M), sucrose (S), lactose (L), galactose (G) and trehalose (T), Appendix] were prepared along with the yeast fermentation broth in tubes and to each fermentation tubes (for each strain of Candida spp.) contained sterilized inverted Durham vial 0.2 ml of the yeast suspension (seeded broth) was added. The tubes were incubated at 28°C for 24-48 hr. Suitable controls were maintained under
identical conditions. The observations showed that the gas production (evident by a gas bubble in the Durham tube) is the indication of yeast fermentation. A change in colour of broth from purple to yellow indicates acid production and carbohydrate assimilation\textsuperscript{12}.

2.2.7(v) Urease test. Yeasts possessing the enzyme urease are capable of splitting urea to form an alkaline reaction. The yeasts (strains) to be identified were lightly inoculated ($10^4$ blastospores) to a slant of Christensen's urea agar (Appendix) and incubated at $28^\circ C$ for 24-72 hr. The colour shift of the agar pink or red showed the positive reactions, whereas, the amber medium unchanged or yellow indicated negative reaction\textsuperscript{12}. Appropriate controls were run simultaneously. The experiment was done in triplicate of tubes.

2.2.8 Statistical analysis.

The data obtained in the present investigation were analysed statistically by the Student-t test for significance between the two groups, coefficient correlation determination between the two variables, and Chi Square Test to find the significance among different categories.

2.3 RESULTS

In the present investigation 118 immunocompromised patients (ICP) with pulmonary tuberculosis were studied for fungal infections. Initially, the underlying disease of the ICP patients and their number was categorised as of diabetes (40), cancer (32), asthma (30) and suspected meningitis (16). The fungal infection in cases of diabetics were 34 (85%), cancer 28 (88%), asthma 3 (10%) and of suspected meningitis 1 (6%). Overall, out of 118 (ICP) cases of proven tuberculosis, 66 (47%) cases were found positive concomitantly with fungal infection (Table 1).

Of the 118 pulmonary tuberculosis patients (ICP) studied, cough was the predominant symptom of pulmonary tuberculosis (92%) followed by expectoration (84%), fever (81%), hemoptysis (57%), breathlessness (40%) and chest pain (16%, Fig. 1). The direct microscopic examination of the smear of sputa/bronchial aspirate
revealed that 44 (37%) cases were positive for fungus, whereas, 74 (63%) were negative. Of the patients, 66 (56%) showed positive culture for fungal infection while 52 (44%) were negative for fungus (Table 2). The sputum/bronchial aspirate digested with N-acetyl-L-cysteine (NALC) revealed, blastospores and lymphocytes in sputum smear (300x, Ph) (Fig. 2a). The C. albicans blastospores (budding cells) were observed in sputum culture (Figs. 2b, 300x Ph, 2c, 600x). The demographic data showed that of the 118 ICP patients 87 were male and 31 female (Table 3). There was a significant (P<0.01) predominance of fungal infection in female 26 (84%) than in male 40 (46%). The cases of urban (63) and rural (55) population, was also categorised of which urban population was more susceptible 37 (59%) than the rural 29 (53%) population. However, the difference was non-significant (P>0.05). It was also evident from the results that the age played an important role in the disease since as the age increased the possibility of fungal infection in pulmonary tuberculosis cases also increased. The maximum number of fungal positive cases 39 (56%) was observed in the age group of 26-35 years although the difference in age criteria was non-significant (P>0.05). There was no significant difference in patients of mycotic infections which were found more 40 (59%) in untreated cases of tuberculosis than the treated 26 (52%) cases. Further, among treated cases (50) there was no significant correlation (P>0.05) in increase of fungal infection with respect to prolongation of antitubercular treatment (6-13 months and above). The maximum number of cases 18 (62%) were positive for fungus, where antitubercular treatment was given for more than one year, however, the difference was non-significant (P>0.05). It was also clear from the results that there was no significant correlation between duration (below 6 months and above) of illness and fungal positivity. A high frequency of positive cases was observed where the duration of pulmonary tuberculosis was more than 6 months. In non-cavitary type of lesions (77 cases), there was a high frequency, 44 cases (57%) of occurrence of fungal superinfection compared to cavitary lesions (41 cases, 54%), however, the difference was non-significant. Of the 118 subjects, 40 were diabetic with fungus positivity of 85%, 32 cases were of cancer patients where the positivity of fungal infection was 88%, whereas, 10% positivity was shown by
asthmatic patients (30), and 6% by meningeal patients (16). The difference between these groups was statistically significant (P<0.01) (Table 3).

Sabouraud chloromycetin agar (SCA) was the best media which recovered repeatedly 80% of fungal infection from sputa/bronchial aspirate followed by Sabouraud dextrose agar (SDA) (64%) or niger seed (Staib) agar (NSA) (63%) particularly for Cryptococcus neoformans with BCE. The brain heart infusion (BHI); and nutrient agar (NA), however, recovered only 38% and 21% fungi respectively (Figs. 3 and 4). The culture recovery of fungal species from pulmonary tuberculosis patients (ICP) showed that the maximum frequency was of Candida spp. 30% (35 cases) followed by Aspergillus spp. 27% (32 cases). Penicillum rubrum was encountered in 2.54% (3 cases), whereas, Fusarium oxysporum, Paeclomyces lilacinus and mycelia sterila were detected in approx. 2% (2 cases each). The species of Histoplasma capsulatum, Alternaria alternata, Cryptococcus neoformans, Cladosporium cladosporioides, Rhodotorula glutinis and non-candida (yeast) were recovered from 1 case (0.85%) each (Table 4; Figs. 5 and 6).

The serological test in the patients sera were done with our own prepared metabolic (extracellular) and somatic (intracellular) fungal antigens of A. fumigatus and C. albicans separately and with mycelial antigens of H. capsulatum adopting immunodiffusion (ID), immunofluorescence (IF) and enzyme linked immunosorbent assay (ELISA). The reference antigens from Meridian diagnostics (USA), and Institute Pasteur Productions (France) for Candida and Aspergillus infections were used for a comparative activity with our own laboratory (CDRI) antigen preparations. The protein value of prepared antigens was determined by the Lowry's method. The protein value of C. albicans somatic antigens was 100 µgml⁻¹ and of metabolic 450 µgml⁻¹, and that of A. fumigatus somatic antigens 170 µgml⁻¹ and of metabolic antigens 500 µg ml⁻¹.

Of the positive cases (ICP) of tuberculosis (118), 24 cases (20%) were positive for Candida by both the reference antigens from Meridian diagnostics and Institute Pasteur Productions, while with our own laboratory (CDRI) antigens the positive cases were 21 (18%) by the immunodiffusion (ID) test (Table 5). Figure 7 shows distinct precipitating arcs (1-4) in the patient sera
out of 35 Candida positive cases. Of those sera 18 showed 1 precipitating arc, 4 sera with 2 arcs, and 1 serum each with 3 and 4 and precipitating arcs (Fig. 7 and 8). With the IF test the positive cases were 31 (26%) by both reference antigens while with CDRI antigens it was 28 (24%). For determination of IF titre the reference antigen used was of Institute Pasteur Productions. Nineteen sera gave IF titre (1:160), 9 sera with 1:320, 2 sera with 1:640, and one serum with a titre of 1:1280 (Fig. 8). In case of A. fumigatus the positive cases were 21 (18%) by ID, and with IF and ELISA it was 22 (19%) and 28 (24%) by both reference antigens respectively. With laboratory antigens the positivity was 18 (15%), 19 (16%) and 25 (21%) by ID, IF and ELISA respectively (Table 5). For A. fumigatus, out of 32 sera, 21 sera showed precipitating arcs (1-5) of which 8 sera showed 2-3 arcs, where, 3 sera revealed 4 arcs and 2 sera with 5 arcs. These arcs showed identity of precipitating bands in most cases. For IF 22 sera showed positive results with IF titre range 160-640 of which maximum sera (17) showed positive at 1:160 (Fig. 9).

In the ELISA, positive cases were 33 (28%) with each reference antigens, whereas, with laboratory antigens it was 31 (26%) (Table 5). Of the 118 sera used for antibody by ELISA detection against C. albicans the highest titre was found in the OD range (1.81-1.90, 13 sera) and the lowest titre was in the range of 1.91-2.0 (1 serum), while in those of A. fumigatus the higher titre was found in range of (1.61-1.80, 17 sera) and the lowest titre in the range of 1.41-1.60 and 2.0-2.20 (1 serum each). For H. capsulatum only 1 case showed positive ELISA antigen titre (OD 1.94). Specimens yielding results of <1.0 EU were considered negative only if the results were reproducible (Fig. 10). For H. capsulatum both ID and ELISA tests detected only 1 case (0.8%) by the Meridian antigens (Table 5).

The physiological tests (based upon carbohydrate assimilation and fermentation tests) were done for characterisation of Candida spp. isolated from pulmonary tuberculosis patients. To differentiate C. albicans from other non-albicans species germ tube test was performed. Only two species showed positivity to germ tube test i.e., C. albicans and C. stellatoidea (Fig. 11). The highest % prevalence of Candida spp. encountered in tuberculosis patient was C. albicans 15 (43%) followed by C. tropicalis 7 (20%), C. stellatoidea, C.
guillermondii 4 each (11%), C. rugosa 3 (9%) and C. parapsilosis 2 (6%, Table 7). For further differentiation among these species carbohydrate assimilation and fermentation test were performed (Fig. 12). C. albicans utilised sucrose, whereas, C. stellatoidea did not. Urease test was negative against all Candida strains (Fig. 13). The chlamydospor test was found positive in C. albicans, C. stellatoidea and C. tropicalis, while with other Candida spp. viz., C. guillermondii, C. parapsilosis, and C. rugosa, it was negative (Table 8).

C. stellatoidea did not utilize sucrose compared to C. albicans, while, C. tropicalis and C. guillermondii did not utilize lactose. C. rugosa did not utilize other sugars except dextrose, mellibiose and xylose. Lactose and mellibiose were not utilized by C. tropicalis. Regarding cellobiose it was assimilated by C. tropicalis (partially), and by C. guillermondii. Inositol was not utilized by any of the yeast as shown in Table 8, while xylose was universally utilized by all the yeast. Raffiinose was only utilized by C. guillermondii and, trehalose was not utilized by C. rugosa compared to other species. Regarding fermentation test lactose was not fermented by C. albicans, while, dextrose, maltose, sucrose and galactose produced both acid and gas, while, trehalose produced only acid. Further details of carbohydrate assimilation and fermentation is shown in Table 8.

2.4 DISCUSSION

Recent decades have witnessed an alarming increase in fungal infections posing potential health problem. Several intrinsic (physiologic and pathologic) and extrinsic (indiscriminate use of antibacterials, corticosteroids and cytostatic agents), medico-surgical interventions and physical factors predispose the host for opportunistic mycoses especially those suffering from primary infection such as tuberculosis16, 17. Fungal superinfection in the pulmonary tuberculosis patients have been reported to cause complications since tuberculosis is a chronic destructive disease causing caseation, necrosis and fibrosis leading to formation of cavities and bronchiectatic changes. The opportunistic fungi tend to settle in these cavities and cause destruction of dilated
bronchi predisposing the immunocompromised (ICP) host for fungal superinfection. In the present investigation 118 immunocompromised patients, (ICP) have been studied for pulmonary fungal infection. Of those cases the underlying disease was diabetes, cancer, asthma and suspected meningitis. The diabetic patients were 40, cancer 32, asthma 30 and suspected meningitis 16. The maximum number of positive fungal infections was found in diabetic 34 (85%), followed by cancer 28 (88%), asthma 3 (10%) and suspected meningitis 1 (6%) (Table 1). All these patients although had an underlying disease, however, they were diagnosed as proven cases of pulmonary tuberculosis (Table 1, Fig. 1).

Out of 118 immunocompromised (ICP) cases the fungi were recovered in 66 (56%) cases by culture of sputa/bronchial aspirate on differential fungal media (Fig. 4), whereas, by microscopic smear examination the fungal elements (Fig. 2) were detected in 37% cases (Table 2). None of these cases revealed any specific feature which could suggest etiology for mycosis. Our results are in accordance with the findings of other workers who reported similar findings particularly for candidiasis.

The demographic pattern of fungal infections in ICP showed that the fungal infection was influenced by the area of population, age group, duration of pulmonary tuberculosis and type of lesion (Table 3). The sex and underlying disease such as cancer and diabetic status, however, showed a significant correlation (P<0.01) for fungal infection in such patients. Candida species although considered as commensals of mouth and gastrointestinal tract and had frequently been responsible for bronchopulmonary disease. In our study we found slightly high incidence (30%) of Candida spp. (Table 4) compared to other reports by Shome et al. (9%), Khanna et al. (26%) and Jain et al. (24%). The occurrence of Candida spp. concomitantly with tuberculosis is of paramount significance as C. albicans enhances the virulence of Mycobacterium tuberculosis. The anticancer or immunosuppressives (steroids etc.) and antibacterial drugs of non-specific action used in patients with destructive forms of pulmonary tuberculosis promote the growth and frequency of Fungal flora and aggravate the course of underlying factors responsible for tissue destruction. In the
present investigation the cases which were positive (30%) for Candida spp (Table 4), there was no statistical difference in the age group (Table 3), since, Candida species are known to exist in all age groups as commensal. The sputum examination does not reflect the correct picture of pathogenic fungal organisms residing in the lower respiratory passage, therefore, we studied bronchial aspirate of some patients which showed high counts (>10^6 cfu ml⁻¹) of Candida spp. in repeated specimens.

In the present study Aspergillus spp. were encountered in 32 (27%) cases, P. rubrum in 3 cases (2.54%), F. oxysporum, P. lilacinus and cases due to mycelia sterila 2 cases (1.69%) each and A. alternata, non-candida (yeast), C. neoformans, C. cladosporioides and R. glutinis and H. capsulatum 1 case each (0.85%, Table 4). The superinfection of healed tuberculosis lesions by "a typical" mycobacteria or Aspergillus species particularly A. fumigatus is well documented. Pulmonary tuberculosis is the commonest antecedent disease leading to the development of aspergilloma as opportunistic infection in immunocompromised patients. Of the positive cases of Aspergillus we observed 4 cases of allergic broncho-pulmonary aspergillosis (ABPA). These cases have not been discussed in details since these cases do not come under the perview of the thesis on candidiasis. Some cases of pulmonary aspergilloma, however, have been reported from India²⁵, ²⁶. Singh et al. ²⁷ reported aspergilloma radiologically in 49 cases of tuberculosis as underlying disease. In a report from the Research Committee of the British Tuberculosis Association, the maximum prevalence of Aspergillus spp. infection occurred in cavitary tuberculosis of 7-11 years duration²⁸.

Cryptococcosis although has its predilection for the central nervous system but the portal of entry of the causative agent is through inhalation of C. neoformans spores²⁹. We found only 1 case of pulmonary cryptococcosis due to detection of encapsulated yeast in India ink smear of bronchial aspirate and culture on Staib agar with specific Brown Colour Effect (BCE) ³⁰. There are reports of pulmonary cryptococcosis in patients of tuberculosis from India³¹. Khan et al.³² reported cryptococcosis concomitantly with M. tuberculosis and emphasised the diagnostic difficulties in such cases. In the present study we found 1 case of Histoplasma capsulatum confirmed by yeast culture at 37°C of
bronchial aspirate on BHI agar after 2-4 weeks. Although, histoplasmosis is not a frequently reported infection from India, Teewari et al.\textsuperscript{33} reported a case of pulmonary histoplasmosis associated with tuberculosis. The similarity of its clinical and radiological features with pulmonary tuberculosis often cause diagnostic dilemma when two occur concomitantly. The fungal infection in cases of pulmonary tuberculosis multiply the complexity of problem both for the diagnosis and management of disease. The patients of pulmonary tuberculosis who are immunocompromised due to certain other underlying diseases suffer concomitantly with fungal infections. These fungal infections are either undiagnosed or misdiagnosed on account of paucity of awareness to mycosis and/or lack of adequate diagnostic facilities in the Indian hospitals. From the data (Tables 3 and 4) it is evident that there is an urgent need to pay attention to the problem of fungal infection in patients of pulmonary tuberculosis who are initially immunocompromised due to primary life-threatening diseases for public health.

For the diagnosis of ICP patients for fungal infections serological tests are useful adjuncts to mycological diagnosis\textsuperscript{7}. These tests are based on the detection of hosts humoral immune response to fungal infection or on the detection of fungal antigens. The usefulness of these test system depends on the method of testing and the reagents used. The precipitin or agglutinin test are useful in the diagnosis of fungal infection particularly in patients capable of producing normal levels of immunoglobulins but are less helpful in the diagnosis of immunocompromised host\textsuperscript{34}. Therefore, more sensitive and quantitative test for detection of antibodies or antigen offer the greatest promise in the diagnosis of candidiasis. Immunoassays play a major role in the serodiagnosis of fungal infection. Immunofluorescence (IF) test has been used in the detection of antibodies and antigens for fungal infections\textsuperscript{7, 36}. In our studies we have used precipitin test, IF and ELISA for the diagnosis of candidiasis in the ICP of tuberculosis. The immunodiffusion (ID) or precipitin test revealed that it can detect the \textit{C. albicans} infection in 24 (20\%) cases by using the kit of either Meridian diagnostics or of Institute Pasteur Productions. Our own (CDRI) prepared antigen showed comparable activity by ID in 21 (18\%) cases (Table 5). Our data showed that
out of 35 Candida positive cases 10 sera showed precipitating arcs (1-4) due to C. albicans, and 14 sera (1-3 arcs) with non-albicans (Fig. 7 and 8); and 2-5 arcs in Aspergillus (Fig. 9), and 1 arc in H. capsulatum by ID test. Likewise, in IF test the IF titre for C. albicans was 160-1280 (4 sera) and for non-albicans (20 sera) with 160-640 (Fig. 7), whereas, with A. fumigatus the IF titre was 160-640 (22 sera, Fig. 9). The indirect immunofluorescence technique for the detection of antibodies against the surface antigens appears more sensitive specific and rapid than the ID technique. Drouhet\(^7\) reported that in IF a titre higher than 1/80 can be considered as the sign of Candida infection. Further a "fluorescent" antibody titre of 1/160 and 1/1280 or more is considered as chronic mucocutaneous candidiasis. In our studies we found IF titre more than 1/80 i.e., 160-1280 (Fig. 8). Thus, there is every likelihood that these ICP cases may be considered as cases of chronic mucocutaneous (pulmonary) infection. The increase in IF titre may be accompanied by 1-3 precipitating arcs by counter immunoelectrophoresis (CIE)\(^7\). Our data showed that higher IF titre was accompanied by multiple arcs in both Candida and Aspergillus. Likewise, positive cases in A. fumigatus were 21 (18\%) by both of the reference antigens while with our preparations it was 18 (15\%) (Table 5). The usefulness of ID or agglutination test for candidiasis in patients with neoplastic disease has been reviewed by Filice et al.\(^35\), however, they reported an inconsistent correlation of precipitin test results with invasive infections. A double microimmunodiffusion test in agar for antibody to Candida spp. and Aspergillus spp. has been used recently in Memorial Sloan Kettering Cancer Centre, New York, for precipitins to C. albicans. Sera from less than one-half of the patients with disseminated or invasive gastrointestinal candidiasis exhibited positive ID reaction with titres of agglutinating antibody more than 1:16 or four-fold rise in titre of agglutinins\(^35\). The ID test and titres of agglutinating antibody may not correlate with invasive Candida infection since false positive and false negative reactions may occur\(^36\). There have been some investigations on the reliability of ID as an aid in the diagnosis of systemic candidiasis. Taschidjian et al.\(^37\) reported in an autopsy study of 33 patients of which 24 patients were found to have disseminated candidiasis
on post-mortem examination, and 21 had antibodies to *Candida*
sonicated extracts by ID test. None of the 9 patients without
candidiasis had ID preexisting and none had high agglutinin
titre. Priestler et al.\textsuperscript{38} also found ID reaction positive in
patients with superficial candidiasis and Pepys et al.\textsuperscript{39} found
positive ID reaction in patients with asthma. Stickle et al.\textsuperscript{40}
reported that the occurrence of multiple precipitin lines and
high LA titres was suggestive of prognosis for severe
candidiasis. Wheat\textsuperscript{41} reported that serological tests with
*Candida* does not appear to be useful since *Candida* antibodies
have been falsely negative in 50\% of ICP patients with serious
candidiasis. Accurate assays for *Candida* antigens are not
available for latex agglutinatids test (LAT) like CALCAS for
cryptococcosis for clinical testing\textsuperscript{41}. For histoplasmosis
serologic tests have been considered as valuable diagnostic
adjuncts. The complement fixation (CF) test is reported to be
more sensitive than ID test in histoplasmosis\textsuperscript{42-45}. Only about
1\% of patients with histoplasmosis with negative CF tests can be
identified by ID\textsuperscript{44,45}. The ID test for histoplasmosis is
considered more specific but less sensitive than CF and fewer
than 20\% demonstrate H bands\textsuperscript{44,45}. In a study of outbreak of
histoplasmosis, the ID test was found to be uniformly negative at
3 weeks after exposure, positive in 27\% of cases at 4 weeks and
49\% at 6 weeks\textsuperscript{46}. In a comprehensive investigation using well
standardised antigens and procedures, false positive results were
rare by ID and CF\textsuperscript{44,46}. The H and M precipitins and CF titres of
1:16 or greater to the mycelial antigen and of 1:32 or greater to
the yeast antigen are highly suggestive of histoplasmosis. In
our study, 1 patient showed positive ID test for histoplasmosis
revealing a single well defined precipitin band in the serum.
Radioimmunoassay and Enzyme Linked Immunosorbent Assay (ELISA)
have attractive features and are beginning to be used for
antibody and antigen reaction. Although, commercial kits are
available for *Candida*, *Cryptococcus*, *Histoplasma* and other
dimorphic fungi and *Aspergillus* for the detection of
immunoglobulins (IgG, IgM, IgA and IgE) etc. and polysaccharide
antigens. ID test is the only widely available serodiagnostic
test for aspergillosis particularly in cases of aspergillomas or
allergic aspergillosis. ID has been reported to be positive in
75-100\% of such cases. It has been suggested that precipitating
antibodies to *Candida* occur only in response to disease that invades tissue. The antigens which were prepared in our laboratory were comparable with activity provided by reference antigens (Table 5). Since, there is a likelihood of getting false positive and false negative results we studied IF test which is more sensitive and less time consuming than ID test. The indirect immunofluorescence for detection of antibodies against the surface antigens is more sensitive and more specific than the ID technique. We found that of the 118 cases 31 (26%) cases were positive for *C. albicans* by IF in both the reference antigens, whereas, with our own prepared antigens the positive results were slightly below 28 (24%) cases. For *A. fumigatus* the IF test was positive in 22 (19%) by both the reference antigens while with our antigenic preparations it was 19 (16%) cases. For *H. capsulatum* we did find only one positive case (0.8%) by ID (Meridian Kit) or ELISA (Table 5). With IF there was an increased positivity of cases for *C. albicans* when compared to ID test (Table 5). Drouhet\(^7\) reported increased sensitivity and higher titre for *Candida* infection in patients. Polonelli et al.\(^47\) reported the significance of *in vivo* IgA coated yeast cells for the diagnosis of candidiasis by IF in patients with or without clinical symptoms. The diagnostic potential of IF test has been reported to be most significant in the culturally positive and symptomless patients. The evaluation of significance of immunofluorescence non-invasive rapid assay may permit appropriate clinical and therapeutic decisions. Moreover, these serological assay particularly in immunocompromised patients would be much useful. Recently role of aspartic proteinase antigen was demonstrated by IF in mycotic human lung\(^48\).

The application of ELISA methods for detection of antibodies to fungal antigens has been described elsewhere\(^49,50\). These investigations demonstrated a good correlation between ELISA and other serological test. The ELISA method is less time consuming compared to agar gel double diffusion method. In our experiments we found that in case of *C. albicans* 33 (28%) cases were positive for antibodies while with our prepared (CDRI) antigens, positive results were found in 31 (26%) cases (Table 5). The maximum percent (11%) of sera showed ELISA antibody titre (IgG) in the absorbance range of 1.81-1.90 EU and the minimum (1%) in
the range of 1.91–2.0 EU (Fig. 10). The prevalence of IgG antibodies to \( C. albicans \) mannan in the normal human population\(^5\) suggests that the detection of free mannan antigen in infected patients might be more difficult than had at first been supposed. Lew et al.\(^5\) have found that normal human serum contains sufficient interfering factors to reduce the lower limit of detection for mannan as much as 1000-fold in an ELISA test. The major interfering factor has been identified as antibodies to \( C. albicans \) mannan\(^5\).

Warren et al.\(^5\) reported the first clinical application of ELISA for the detection of circulating fungal antigen. With microtiter plates coated with anti-\( C. albicans \) serum raised in rabbits and the same serum conjugated with alkaline phosphatase, rising titres of \( C. albicans \) related antigen were detected in sera from 3 patients with suspected invasive candidosis. The antigen detected was not characterized. Araj et al.\(^5\) have reported an ELISA for detection of circulating somatic antigens of \( C. albicans \). This method detected elevated antigen concentrations in 7 cancer patients with disseminated candidosis, and in 9 of 13 similar patients with suspected disseminated candidosis. Patients with other fungal or bacterial infections and normal subjects had much lower antigen concentrations. These findings are interesting and suggest that the detection of circulating somatic antigens rather than mannan might offer a useful approach to diagnosis.

In \( A. fumigatus \) like \( C. albicans \) better results were found with ELISA when compared with ID or IF. There were 28 (24%) cases which were positive compared to our prepared antigens which provided positive results in 25 (21%) cases. In case of \( H. capsulatum \) of the cases tested for serology only 1 (0.8%) case was positive by the kit of Meridian productions (Table 5). The data showed that ELISA test is much more sensitive and less time consuming as compared to ID or IF test. Richardson et al.\(^8\) studied the effect of shortening the incubation times in ELISA for IgG antibodies to \( C. albicans \) in patients with suspected invasive candidosis. It has been reported that although agar diffusion test (ID) and IF are simplest and more specific serological methods for establishing a diagnosis of various forms of aspergillosis\(^45\). In case of aspergillosis ID test is positive in 75–100% cases. False positive results may occur with other
fungal diseases, tuberculosis and in some patients with non-infectious lung diseases. Both the number and strength of precipitin lines are significant for the degree of involvement of *A. fumigatus* but determination of elevated levels of IgG or IgE in cases of ABPA (allergic bronchopulmonary aspergillosis) has been useful for diagnostic purposes. It is doubtful whether ELISA will replace precipitin method for the diagnosis of different forms of aspergillosis in non-compromised host but precipitin detection has been less successful with specimens from immunocompromised patients in invasive aspergillosis. In our studies we found higher percentage of positivity by mycological tests than with serological tests in *C. albicans*, *A. fumigatus* and *H. capsulatum* (Table 6).

Thus, it is clear from our results, although immunodiffusion (ID) test is simple, specific and inexpensive but it is relatively less sensitive than immunofluorescence (IF) test. ELISA test nevertheless is more rapid reliable and sensitive for the diagnosis of fungal infections. However, the serological test are considered as adjuncts to other means of fungal diagnostics.

Since candidiasis is one of the most common opportunistic infectious disease in immunocompromised patients and more commonly in AIDS/ARCs. A decrease in CD4+ lymphocytes is one of the hallmarks of this disease. CD4+ lymphocytes are important for resistance to a number of fungal diseases in which cell mediated immunity (CMI) is thought to be critical for host defense. Cryptococcal meningitis and pneumonia and other fungal diseases are common complications of HIV infection and are usually associated with CD4+ lymphocytopenia. Decreased CD4+ cell counts and candidiasis have been frequently associated with the evolution of human immunodeficiency virus-induced immunosuppression. Although CD4+-helper/inducer lymphocytes are critical for the generation of a CMI response, it is unlikely that they are direct effector cells against *C. albicans*. Possible effector cells for protection against mucosal candidiasis include polymorphonuclear cells and macrophage. CD4+ lymphocytes secrete a variety of soluble lymphokines, which include activators of polymorphonuclear cells and macrophage (IFN-γ), T-cell autocrine factors (IL-2) and B-cell stimulator factors (IL-4 and IL-6). Depletion of CD4+ lymphocytes
could result in a reduction of all these signals and thus impair candidacidal activity of phagocytic cells. Although, in the present investigation the clinical history of immunocompromised patients, underlying primary complication in those subjects, and anticancer or corticosteroid therapy could be responsible for immunosuppression, but a clear picture of analysis of lymphocyte population especially CD$_4^+$ T cells counts could be more conclusive in terms of immunosuppressed status of host.

2.5 CONCLUSION

For diagnosis of pulmonary candidiasis concomitantly with tuberculosis in the immunocompromised patients, mycological and serological tests may offer adequate evidences for candidiasis, however, serological tests are considered as adjuncts to other means of fungal diagnostics.
REFERENCES


26. Sandhu, D.K., V.N. Sharma, R.S. Sandhu, V.N. Damodaran and


Table 1. Fungal infections in immunocompromised patients (ICP) with pulmonary tuberculosis

<table>
<thead>
<tr>
<th>Underlying disease</th>
<th>Number of cases</th>
<th>Positive cases</th>
<th>Negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
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</tr>
<tr>
<td>Diabetes</td>
<td>40</td>
<td>34 85</td>
<td>1 3</td>
</tr>
<tr>
<td>Cancer</td>
<td>32</td>
<td>28 88</td>
<td>2 6</td>
</tr>
<tr>
<td>Asthma</td>
<td>30</td>
<td>3 10</td>
<td>6 20</td>
</tr>
<tr>
<td>Suspected Meningitis</td>
<td>16</td>
<td>1 6</td>
<td>15 94</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>66 47</td>
<td>24 31</td>
</tr>
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</table>

Table 2. Fungal infections in immunocompromised patients (ICP) of pulmonary tuberculosis based upon smear examination and culture recovery

<table>
<thead>
<tr>
<th>No. of cases diagnosed</th>
<th>Pulmonary tuberculosis (Criteria)</th>
<th>Positive</th>
<th>Negative</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of cases</td>
<td>% positive</td>
</tr>
<tr>
<td>Direct Microscopic examination*</td>
<td>44</td>
<td>37</td>
<td>74</td>
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<tr>
<td>118</td>
<td>Fungal infections (Culture)</td>
<td>66</td>
<td>56</td>
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*Smear was prepared in 0.5% N-acetyl-L-cysteine
Table 3. Demographic status of patients (ICP) for fungal infections

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Number of Fungus positive cases</th>
<th>Statistical analysis</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
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<tr>
<td>Sex</td>
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<td>Female</td>
<td>31</td>
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<td>Age (group) years</td>
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<td>15-25</td>
<td>26</td>
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<td>26-35</td>
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<td>36-45</td>
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<td>46 and above</td>
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<td>Population</td>
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<td></td>
</tr>
<tr>
<td>Rural</td>
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<td>Urban</td>
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<tr>
<td>Duration of illness</td>
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<td>0-3</td>
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<td>4-6</td>
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<td>13 and above</td>
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<td>Diabetes mellitus</td>
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<td>Suspected meningitis</td>
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<td>102</td>
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**[Contd. table 3]**

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<th>Pulmonary lesion</th>
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<tbody>
<tr>
<td>Cavitary</td>
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<td>22</td>
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</tr>
<tr>
<td>Non-cavitary</td>
<td>77</td>
<td>44</td>
<td>57</td>
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**Anti-tubercular treatment (months)**

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<tbody>
<tr>
<td>0-6</td>
<td>18</td>
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<td>33</td>
</tr>
<tr>
<td>7-12</td>
<td>6</td>
<td>2</td>
<td>33</td>
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<tr>
<td>13 and above</td>
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<tr>
<td>Total</td>
<td>50</td>
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<table>
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<tr>
<th>Untreated</th>
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<tr>
<td></td>
<td>68</td>
<td>40</td>
<td>59</td>
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</table>

$t = 0.31 (**P > 0.05)$

$t = 1.96 (**P > 0.05)$

$t = 1.23 (**P > 0.05)$

$t = 0.27 (**P > 0.05)$

$t = 0.78 (**P > 0.05)$

*Each group comprised of ICP cases (n=118) of pulmonary tuberculosis*

**Highly significant**

***Not significant***
Table 4. Culture recovery of fungal species from sputum/bronchial aspirate of pulmonary tubercular patients

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Number of cases</th>
<th>% positive</th>
<th>Media of choice</th>
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<tbody>
<tr>
<td>Candida spp.</td>
<td>35</td>
<td>30.0</td>
<td>SCA</td>
</tr>
<tr>
<td>Aspergillus spp.</td>
<td>32</td>
<td>27.0</td>
<td>SCA</td>
</tr>
<tr>
<td>Alternaria alternata</td>
<td>1</td>
<td>0.85</td>
<td>SCA</td>
</tr>
<tr>
<td>Non-Candida (yeast)</td>
<td>1</td>
<td>0.85</td>
<td>SCA</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>1</td>
<td>0.85</td>
<td>BHIA</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>1</td>
<td>0.85</td>
<td>NSA</td>
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<tr>
<td>Fusarium oxysporum</td>
<td>2</td>
<td>1.69</td>
<td>SCA</td>
</tr>
<tr>
<td>Cladosporium cladosporioids</td>
<td>1</td>
<td>0.85</td>
<td>SDA</td>
</tr>
<tr>
<td>Penicillium rubrum</td>
<td>3</td>
<td>2.54</td>
<td>SDA</td>
</tr>
<tr>
<td>Paecilomyces lilacinus</td>
<td>2</td>
<td>1.69</td>
<td>SDA</td>
</tr>
<tr>
<td>Rhodotorula glutinis</td>
<td>1</td>
<td>0.85</td>
<td>SCA</td>
</tr>
<tr>
<td>Mycelia sterila</td>
<td>2</td>
<td>1.69</td>
<td>SDA</td>
</tr>
</tbody>
</table>

*SCA: Sabouraud chloromycetin agar
SDA: Sabouraud dextrose agar
BHIA: Brain heart infusion agar
NSA: Niger Seed Agar
Table 5. Fungal serological tests in immunocompromised cases* of pulmonary tuberculosis

<table>
<thead>
<tr>
<th>Antigens used</th>
<th>C. albicans</th>
<th>A. fumigatus</th>
<th>H. capsulatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meridian Diagnostics, U.S.A.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>**Institute Pasteur Productions, France</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>**CDRI (prepared), India</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID IF ELISA ID IF ELISA ID IF ELISA ID IF ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. % No. % No. % No. % No. % No. % No. % No. % No. % No. % No. % No. %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meridian Diagnostics, U.S.A.</td>
<td>24 20 31 26 33 28 21 18 22 19 28 24 1 0.8 ND 1 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>**Institute Pasteur Productions, France</td>
<td>24 20 31 26 33 28 21 18 22 19 28 24 ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>**CDRI (prepared), India</td>
<td>21 18 28 24 31 26 18 15 19 16 25 21 ND ND ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Total number of sera examined (118 cases)
**Both metabolic and somatic antigens (pooled)
ID = Immunodiffusion test, IF = Immunofluorescence test, ELISA = Enzyme Linked Immunosorbent Assay
ND = Not done
Table 6. Comparative results of mycological and serological tests for fungal infection in tuberculosis patients

<table>
<thead>
<tr>
<th>Total No. Tests of cases performed</th>
<th>Organisms</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. albicans</td>
<td>A. fumigatus</td>
<td>H. capsulatum</td>
<td></td>
</tr>
<tr>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycological*</td>
<td>35 (30%)</td>
<td>32 (27%)</td>
<td>1 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>Serological**</td>
<td>33 (28%)</td>
<td>28 (24%)</td>
<td>1 (0.8%)</td>
<td></td>
</tr>
</tbody>
</table>

*Details as given in Table 4
**Details as given in Table 5

Table 7. Percent prevalence of Candida species based on physiological tests

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Number positive</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>15</td>
<td>43</td>
</tr>
<tr>
<td>C. stellatoidea</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>C. rugosa</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 8. Physiological tests for Candida spp. (35) isolated from immunocompromised patients of pulmonary tuberculosis

<table>
<thead>
<tr>
<th>Organisms isolated</th>
<th>No. of strains positive</th>
<th>Carbohydrate Assimilation Test</th>
<th>Fermentation Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D M S L G MB C I X R T D M S L G T P CH GT U BP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>15</td>
<td>+ + + - + - - + - A/G A/G A/G - A/G A</td>
<td>+ve +ve +ve -ve -ve</td>
</tr>
<tr>
<td>C. stellatoidea</td>
<td>4</td>
<td>+ + - - - - - + + A/G A/G - - A/G -</td>
<td>+ve +ve +ve -ve -ve</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>7</td>
<td>+ + + - - + + - + A/G A/G A/G - A/G A</td>
<td>+ve +ve -ve -ve -ve</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>4</td>
<td>+ + + - + + + + + + A/G A/G A/G - A/G A</td>
<td>+ve -ve -ve -ve -ve</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>2</td>
<td>+ + + - - - - + + A/G A/G A/G - A/G A</td>
<td>+ve -ve -ve -ve -ve</td>
</tr>
<tr>
<td>C. rugosa</td>
<td>3</td>
<td>+ - - - - + - - - A - A</td>
<td>+ve -ve -ve -ve -ve</td>
</tr>
</tbody>
</table>

D = dextrose, M = Maltose, S = sucrose, L = lactose, G = Galactose, MB = Mellibiose, C = Cellobiose, I = inositol, X = xylose, R = rafinose, T = trehalose, P = pseudomycelium, CH = chlamydospores, GT = Germ tube, U = urease, BP = brown pigment (caffeic acid agar)
A = acid reaction; G = gas production
+ = sugar utilized, - = sugar not utilized, ± = partially utilized
Figure 1. Predominant symptoms of pulmonary tuberculosis in immunocompromised patients (n=118), (Co-Cough, Ex-Expectoration, Fe-Fever, He-Hemoptysis, Br-Breathlessness, Ch-Chest pain)
Figure 2a. Sputum (digested with N-acetyl-L-cysteine) smear showing blastospores of *C. albicans* and lymphocytes (300 x, Ph)

Figure 2b. *C. albicans* blastospores with budding cells from sputum culture (300 x, Ph)

Figure 2c. *C. albicans* blastospores with budding cells from sputum culture (600 x)
Figure 3. Fungal culture recovery from sputa of pulmonary tuberculosis patients (n=118) on synthetic media (SCA-Sabouraud dextrose agar, NSA-Niger seed agar, BHIA-Brain heart infusion agar, NA-Nutrient agar)
Figure 4. Photograph showing culture recovery of the pathogen (C. albicans) at 48hr from the sputum on different fungal media (upper row). The lower row indicates control plates of the media, (L to R)- Brain heart infusion agar, Niger seed (Staib) agar, Sabouraud chloromycetin agar, Sabouraud dextrose agar and Nutrient agar.

Figure 5. Photograph showing culture of C. albicans and non-albicans (2-days), and H. capsulatum (7-days) recovered from sputa/bronchial aspirate on Sabouraud dextrose and Brain heart infusion agar slants respectively. (1. C. albicans, 2. Rhodotorula sp., 3. Cryptococcus neoformans, 4. H. capsulatum (Y-phase), and H. capsulatum (Y to M-phase transition)

Figure 7. Immunodiffusion (ID) in agar showing precipitating (antigen and antibody complex) arcs. The Central well(s) contains serum and the peripheral wells (a-f) contain different concentrations of the Candida antigens.

Figure 11. Microphotograph of germ tubes (in serum) of C. albicans (600 x, ph)
Figure 8. Fluorescent and precipitant antibodies in sera of patients (ICP) with suspected pulmonary candidiasis
Figure 9. Fluorescent and precipitant antibody titre in suspected cases of aspergillosis and histoplasmosis in patients (ICP)
○ Aspergillus Sp.
○ Histoplasma Capsulatum
Figure 10. Percent sera positive by ELISA (based on OD) for suspected candidiasis and aspergillosis
Figure 12. Photograph of carbohydrate fermentation test at 48hr of *C. albicans* (strain 18) revealing fermentation of dextrose (1), lactose (2), maltose (3), galactose (4), sucrose (5), trehalose (6) control broth (7) and broth plus inoculum (8), by the presence of gas within the inverted Durham tubes.

Figure 13. Photograph showing urease negativity (Christensen’s urea agar) of *C. albicans* pathogenic strains (*C_1* = medium control, *C_2* = *Cryptococcus neoformans* pathogenic strain for comparison for urease positivity) indicating colourshift pink.
Figure 14. Chest X-ray PA view shows multiple nodular shadows which are distributed bilaterally symmetrically predominantly involving the upper zone (Fungal pneumonia)