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Research Article

ANTIFUNGAL DRUG SUSCEPTIBILITY OF *CANDIDA ALBICANS* ISOLATES FROM PULMONARY TUBERCULOSIS PATIENTS

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ABSTRACT

Drug susceptibility studies of *Candida albicans* isolates from tuberculosis (TB) patients is not routinely done in India and other developing countries. Aim of our study was isolation and identification of *C. albicans* from tuberculosis patients; as well as testing sensitivity of these clinical isolates to commonly prescribed antifungal drugs. In vitro susceptibility testing of *C. albicans* isolates from pulmonary tuberculosis patients to five antifungal drugs was carried by the standard broth micro dilution method as per CLSI guidelines. Twenty two percentages of the *C. albicans* isolates were susceptible dose dependent (MIC 16 µg/ml) to fluconazole, while none was resistant. Thirty nine percentage of clinical isolates showed resistance to ketoconazole and clotrimazole. Fifty percentage of isolates were susceptible dose dependent (S-DD) to ketoconazole, while 33 % were found clotrimazole S-DD. All the *C. albicans* isolates showed susceptibility to amphotericin B and terbinafine. Caution need to be taken while prescribing azoles against candidiasis in TB patients, as occurrence of the drug resistant strains may result in failure of the treatment. Outcome of this in vitro study indicated need of antifungal susceptibility studies for better prophylaxis and treatment of *C. albicans* infections in immunocompromised patients in general, and in pulmonary tuberculosis patients in particular.

Keywords: Antifungal, Azoles, *Candida albicans*, Drug resistance, Opportunistic infections, Susceptibility, TB

INTRODUCTION

Tuberculosis (TB) causes significant morbidity and mortality throughout the world, particularly in developing countries in Asia and Africa [1, 2]. At present about one third of the human population is infected with *Mycobacterium tuberculosis* and every year two million persons die because of it [2]. Progress of the disease and prolonged treatment with antibiotics or immunosuppressive agents makes tuberculosis patients immunocompromised and hence susceptible to fungal infections [3- 6]. *Candida albicans* is the most commonly isolated fungal pathogen and may cause severe secondary infections in immunocompromised population, including tuberculosis patients [7- 10]. Nine to eighty percentages of pulmonary tuberculosis patients are infected by *Candida* species [3, 4, 9, 13, 14]. Options of the antifungal drugs available for the treatment of systemic and invasive candidiasis are restricted to polyenes, allylamines, azoles and echinocandin class of molecules [15, 16]. Undesirable side effects, toxicity and emergence of drug resistance are the limitations for the effective use of these drugs [17]. Emergence of drug resistance in *C. albicans* isolated from immunocompromised patients is reported from all over the world [15, 16, 18- 23]. Prevalence of *C. albicans* infections from pulmonary tuberculosis patients is being reported for long time; however not much is known about the drug susceptibility status of these isolates, except for one report [24]. In this communication, we are reporting the antifungal drug susceptibility status of *C. albicans* isolates from pulmonary tuberculosis patients.

MATERIALS AND METHODS

Clinical history and collection of clinical samples

Sputum samples were collected from patients suspected for pulmonary tuberculosis, from the Out and In Patient Departments of Dr. Shankarrao Chavan Government Medical College and Shri Guru Govind Singh Memorial Hospital, Nanded, of the Maharashtra state of India. Patients were having symptoms like marked cough, expectoration, dyspnea and fever. Two sputum samples, spot sample (i.e. at the time when patient was examined) and the next day morning sample were collected. To confirm the TB infections, sputum samples were examined for acid fast bacilli (tubercle bacilli). Positive patients were subjected for radiological study i.e. X-ray examination of chest and then confirmed as TB positive. About 100 tuberculosis patients suspected for fungal infections were examined for presence of *C. albicans*. In general, the patients were treated with three dosages of fluconazole, on alternate days. Patients which were not responding to this were given five dosages of fluconazole. Patients with systemic fungal infections were prescribed with amphotericin B, while superficial infections were treated with clotrimazole and ketoconazole. The study was carried over a period of year 2007-2008.

Isolation and Identification of *C. albicans*

Isolation and identification *C. albicans* was done as described earlier [25]. Briefly, all the clinical samples from tuberculosis patients were cultured on Sabouraud Dextrose Agar (SDA) containing 0.5% chloramphenicol, pH 6.5, for 24 hours at 37 °C. Creamy moist colonies were picked up and used for presumptive identification on HiCHROM agar- *Candida* (HiMedia Lab. Ltd., Mumbai, India). Plates were incubated at 35 °C for 24 hours. Green colored colonies developed on HiCHROM agar *Candida*, were identified as *C. albicans* (Fig. 1) [26]. Germ tube formation assay, carbohydrate assimilation test and Corn meal agar test were used as confirmatory tests. Formation of germ tube at 37 °C temperature in horse serum after 2 hours indicated the germ tube test positive [22]. In carbohydrate assimilation test, growth and fermentation profile on various sugars confirmed *C. albicans* [27]. Formation of chlamydospores on Corn meal agar plates, at 25 °C after 7 days was observed for *C. albicans* [27]. Media components and chemicals were purchased from HiMedia Lab. Ltd., Mumbai, India. Isolates were numbered depending on the number of sample from which they were isolated. All the pure cultures were maintained on Yeast Extract Peptone Dextrose agar (YPD) slants, at 4 °C temperature. A standard *C. albicans* strain, ATCC 90028 (MTCC 3017), obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India, was used as a control.

Susceptibility testing

Five antifungal drugs, fluconazole (Forcan, Cipla Ltd. India); ketoconazole (Nizoral, Johnsen & Johnsen Ltd. India); clotrimazole (Lotril, Gufic, Ltd. India); amphotericin B (Fungizole, Nicholas Piramal, India) and terbinafine (Terbofine, Ocho Labt., India); were obtained from local market. Antifungal susceptibility tests were performed by standard broth microdilution method (as per CLSI) with little modification, as described earlier by our group [28]. Briefly, various concentrations (ranging from high to low) of the selected drugs were prepared in RPMI- 1640 medium by double dilution in the 96-well plates. Each well contained an inoculum of 1×10^{-3} cells/ ml and the final volume of RPMI-1640 medium maintained in each well was 200 µl. The wells without addition of drugs served as a control. Microplates were incubated at 35 °C for 48 h and read spectrophotometrically at 620 nm, using a microplate reader (Multiskan EX; Thermo Electronics Corp., USA). The lowest

concentration of the drugs which caused a 50% reduction in absorbance compared to the control was considered the minimum inhibitory concentration (MIC). The antifungal drug concentrations used were- fluconazole (0.12 to 128 µg/ml), ketoconazole (0.03 to 8 µg/ml), clotrimazole (0.03 to 8 µg/ml), amphotericin B (0.03 to 8 µg/ml), and Terbinafine (0.125 to 32 µg/ml). All the experiments were performed in triplicates. Results obtained are the mean of the triplicate observations.

Interpretation of results

MICs for the antifungal drugs were read after 48 hours and the interpretive breakpoints were as suggested by CLSI. These were as follows- For fluconazole, MIC ≤ 8 µg/ml was considered susceptible, MIC in the range16 to 32 µg/ml as susceptible- dose dependent (S-DD), and ≥ 64 µg/ml as resistant. Breakpoints for ketoconazole, MIC ≤ 0.125 µg/ml as susceptible; 0.25 to 0.5 µg/ml SDD and ≥ 1 µg/ml resistant. For clotrimazole breakpoints were, susceptible ≤ 25 µg/ml; SDD if ≥ 0.5 µg/ml and resistant if ≥ 1 µg/ml. Amphotericin B susceptibility breakpoints - susceptible MIC ≤ 1 µg/ml; ≥ 2 µg/ml as resistant. Terbinafine susceptibility breakpoints as, ≤ 8 µg/ml susceptible; > 8 µg/ml resistant [19, 22, 29, 30].

RESULTS

Eighteen isolates were identified as *C. albicans* in samples from pulmonary tuberculosis patients with clinical manifestations like marked cough, expectoration, dyspnea and fever. Susceptibility testing for fluconazole revealed MICs ranging from 2 µg/ml to 16µg/ml (Table 1).

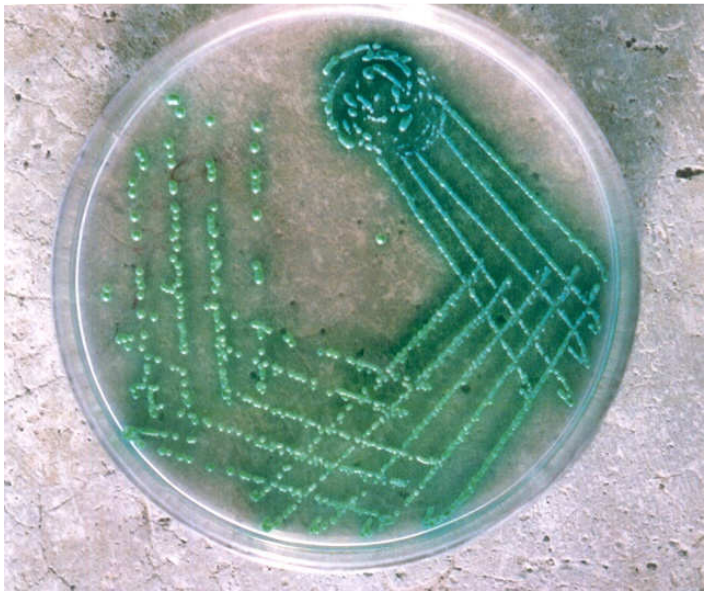

Fig. 1: Growth of *Candida albicans* colonies on HiCHROME-agar *Candida*. Note the characteristic green colored colonies.

Table 1: Drug susceptibility of eighteen *C. albicans* isolates from pulmonary tuberculosis patients to five antifungal drugs. (1- Fluconazole, 2- Ketoconazole, 3- Clotrimazole, 4- Amphotericin B, 5- Terbinafine)

S. No.	Isolate No./ Strain No.	MIC_{90} In µg/ml				
		FLC[1]	KET[2]	CLOT[3]	AMP B[4]	TER[5]
1	8	8	0.5	1	0.5	8
2	9	8	0.25	0.5	0.5	4
3	10	8	1	1	0.5	4
4	15	16	1	0.5	0.5	2
5	16	4	0.125	0.25	0.5	4
6	20	8	0.25	0.5	0.25	4
7	21	16	1	2	1	8
8	22	16	1	2	1	4
9	25	8	1	2	1	4
10	26	4	0.25	0.5	0.25	2
11	28	8	0.25	0.5	0.5	4
12	30	8	0.5	0.25	1	4
13	32	8	0.25	0.25	0.25	2
14	36	8	0.25	0.5	0.25	2
15	39	8	1	0.25	0.5	4
16	40	2	0.125	0.25	0.25	2
17	43	16	1	2	0.5	4
18	49	8	0.25	1	1	4

Seventy eight percentages of the isolates were susceptible to fluconazole. Twenty two percentage were susceptible dose dependent (MIC 16 µg/ml), while none were resistant to fluconazole (Fig. 2). Eleven percentages of the *C. albicans* isolates were sensitive to ketoconazole (MIC≤ 0.125 µg/ml), 50 % were found susceptible dose dependent (MIC 0.25 - 0.5 µg/ml) and 39 % exhibited resistance, with MIC 1 - 2 µg/ml. For the drug clotrimazole, 33.5 % isolates were S-DD (MIC 0.5 µg/ml). Resistance was exhibited by 39 % isolates, which required more than 1 µg/ml clotrimazole, for at least 50% inhibition of growth compared to that of control (Table 1 & Fig 2).

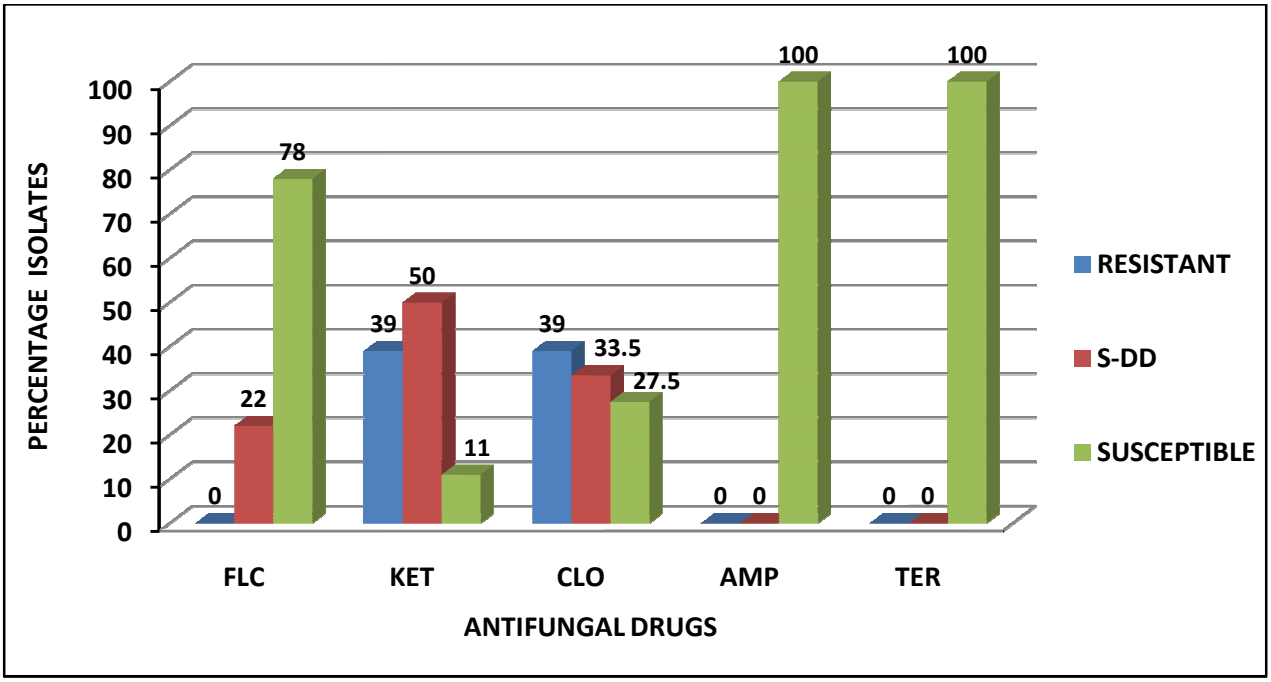

Fig. 2: Susceptibility status of eighteen clinical isolates of *C. albicans* to five antifungal drugs (FLC- Fluconazole, KET- Ketoconazole, CLO- Clotrimazole, AMP- Amphotericin B, TER- Terbinafine).

All the *C. albicans* isolates showed susceptibility to amphotericin B with MICs in the range of 0.25 to 1 µg/ml. Out of 18, none of the isolates was resistant to terbinafine and the MICs ranged between susceptible drug concentrations i.e. 2 to 8 µg/ml (Table 1). Twenty eight percentages of the isolates were resistant to both ketoconazole and clotrimazole (isolate numbers- 10, 15, 21, 22, 25 and 43). Interestingly, four of the ketoconazole and clotrimazole resistant isolates were fluconazole S-DD isolates. There is a possibility of occurrence of cross resistance among antifungal azoles, due to previous exposure to one of the drugs mentioned above [16]. S-DD isolates (from tuberculosis patients) observed in this study, may turn into azole resistant strains upon repeated exposure of the drug. Also, ketoconazole and clotrimazole resistant isolates may become resistant to fluconazole [16]. *C. albicans* ATCC 90028, which was used as a control strain, was sensitive to the five antifungal drugs included in this study; MIC values for fluconazole, ketoconazole, clotrimazole, terbinafine and amphotericin B were - 2, 0.125, 0.125, 2 and 0.5 µg/ml, respectively.

DISCUSSION

Weak immune status, destruction of lung tissues and lesions formed due to TB are the predisposing factors for fungal infections. Even after successful recovery from TB, prolonged treatment with antibiotics and corticosteroids makes the patients very much prone to opportunistic infections [31, 32]. Considering the huge population of TB patients, a large number of individuals are at the risk of fungal infections. Coexistence of *Candida* and tubercle *bacilli* is known since a long time [33- 35]. Both the organisms are frequently isolated from the sputum of patients. Various studies discussing prevalence of *C. albicans* in pulmonary tuberculosis patients are available [8, 9, 11-14]. Although *Candida* infections in pulmonary tuberculosis is not well recognized, in few cases it was shown to be associated with chronic secondary infections responsible for cough, expectoration, dyspnea, anaemia and fever which may prove fatal in severe cases [3, 4, 32]. Adequate measures need to be taken for the prevention and treatment of opportunistic infections in tuberculosis patients, as the current cost of health care systems is elevated. Options of antifungal drugs available to treat chronic candidiasis infections are limited; moreover resistance to the available drugs may result in failure of the treatment. Although prevalence of *C. albicans* in tuberculosis patients is documented, not much is known about its drug susceptibility status [3, 4, 7, 9, 13]. Only one study is available where the susceptibility of different *Candida* species associated with pulmonary tuberculosis revealed fluconazole resistance in 2 % and itraconazole resistance in 6% of *C. albicans* isolates [24]. The most common mechanisms responsible for drug resistance are, lowered accumulation of drugs into the cells due various drug efflux proteins, including multiple drug resistance (*MDR1*) and *Candida* drug resistance (*CDR1 and CDR2*) proteins. Another possibility is mutations or over-expression of the target gene, Erg 11. Mutation in the gene Erg11 leads to change in the structure of target enzyme 14-α- Demethylase which may result in alteration of the target and hence insensitivity towards azole drugs [15, 16, 22]. Invasive tissue infections by such drug resistant *C. albicans* may prove fatal in tuberculosis patients.

CONCLUSION

The study suggested that fluconazole may remain a drug of choice for the treatment of *C. albicans* infections in pulmonary tuberculosis patients; however care must be taken while prescribing it. Treatment with imidazoles may be ineffective when infections involve azole resistant strain of *C. albicans*. To avoid clinical failures, provisions of antifungal susceptibility testing procedures are important. The outcome of this *in vitro* study indicated need of antifungal susceptibility studies for better prophylaxis and treatment of opportunistic *C. albicans* infections in general and in pulmonary tuberculosis patients in particular.

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