ORIGINAL ARTICLE

Clinical Profile of Patients and Antifungal Susceptibility Pattern of Invasive *Cryptococcus neoformans* Isolates from Pakistan

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Abstract

Background
Cryptococcosis affects immunocompromised as well as immunocompetent individuals. A wide spread improper antifungal use has resulted in antifungal resistance. Data regarding clinical characteristics and antifungal susceptibilities of *Cryptococcus neoformans* from Pakistan is limited.

Objective
To determine clinical characteristics of patients and antifungal resistance in invasive *Cryptococcus neoformans*isolates.

Method
This study was conducted at Aga Khan University Hospital, Karachi, Pakistan from 2009-2016. Forty-nine *Cryptococcus neoformans* strains were isolated from various clinical specimens. Clinical data for inpatients was collected through medical records and for outpatients, it was collected by interviews via phone calls to patients or attendants. Antifungal susceptibilities were evaluated for amphotericin B, flucytosine and fluconazole by broth microdilution (BMD).

Results
Cryptococcosis was seen in immunosuppressed states and in conjunction with chronic infections like hepatitis B (n=2), hepatitis C (n=2) and tuberculosis (n=3). Outcomes were not known for 67% (n=37) of patients. Incidence was higher in adult patients 82% (n=40), and 67% (n=37) cases occurred in males.

For all isolates, MIC50 and MIC90 were within the epidemiological cut off values (ECVs) for 5-flucytosine and fluconazole. One isolate had MIChigher than ECV for amphotericin B.

Conclusion
Invasive cryptococcal infections are seen in immunocompromised population as well as in association with certain risk factors such as chronic infections, diabetes and steroid use. Thorough evaluation of all patients must be done for risk factors to ensure better clinical outcomes. Since antifungal resistance is on the rise globally and our findings also show isolated amphotericin B resistance, it is important to perform susceptibility testing of all clinical strains to optimize therapy and for continuous surveillance.

Key words
Cryptococcosis, *Cryptococcus neoformans*, antifungal susceptibilities

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Introduction

Cryptococcosis is an opportunistic infection caused by *Cryptococcus neoformans*, an encapsulated yeast which has a predilection for central nervous system (CNS), but may also cause invasive infections at other sites including lower respiratory tract. It can also present as a disseminated infection particularly in immunocompromised population. More alarmingly, there is a surge of cryptococcal infections in immunocompetent population as well. In 1995, Mitchell *et al* reported 35 out of 118 (30%) cryptococcal infections in immunocompetent individuals in Australia, however, in 2005 Chen *et al* from China reported 91 out of 129 (71%) cases in immunocompetent population.[1,2] Similarly, another study from China by Weng *et al* conducted between 1997-2007 showed that majority of study population,103 out of 154 (70%) patients was apparently healthy.[3]

Invasive cryptococcal infections result in significant morbidity and mortality, with an incidence of nearly one million cases per year globally.[4] The increased incidence of these infections and an overall increase in immunosuppressed population, has led to increased use of antifungal agents for treatment as well as prophylaxis among susceptible populations like HIV/AIDS patients. Subsequently, there are reports of emerging antifungal resistance in *Cryptococcus neoformans*.[5, 6] So far, no study has been conducted regarding clinical characteristics of patients presenting with cryptococcosis in our country. Moreover, antifungal susceptibility profile of clinical isolates of *Cryptococcus neoformans* from Pakistan is also not known.

Hence, we conducted a study to describe the clinical characteristics of patients with proven invasive cryptococcosis and determine the susceptibility profile of *Cryptococcus neoformans* isolated at a major clinical microbiology laboratory of Pakistan from 2009-2016.

Material & methods

This retrospective analysis was conducted at the clinical microbiology laboratory of Aga Khan University Hospital, Karachi, Pakistan from 2009-2016. A total of 49 non-duplicate strains of *Cryptococcus neoformans* were isolated from various clinical specimens from all over the country. All isolates were identified by microscopy, colony morphology, and biochemical analysis. This identification was confirmed by commercially available identification panel API 20CAUX (bioMe´rieux, France). Details of clinical information were obtained from inpatients (n=22) through medical records and by telephone for outpatients (n=27) in the course of clinical reporting of cultures. Hence, the study was exempted from ethical approval by the Ethical Research Council of AKU (ERC No: 1373-Path-ERC- 09).

Broth microdilution plates were prepared according to the CLSI M27-A2 method[7]; using antifungal powders for amphotericin B, flucytosine and fluconazole (Sigma-Aldrich, St Louis, MO,USA).Antimicrobial susceptibility profile of these strains was evaluated for resistance against by broth microdilution (BMD) as per methodology recommended by the Clinical Laboratory Standards Institute (CLSI). Since CLSI interpretative break points for susceptibility are not available, the susceptibilities were interpreted using epidemiological cutoff values (ECVs) from previously published literature.[8,9] *Candida parapsilosis* ATCC22019 and *C. krusei* ATCC6258 were used as quality control strains with each batch of antifungal susceptibility testing.

Data Analysis

The data was coded and analyzed by using Microsoft® Excel 2010 software. Frequency and percentages of the categorical variables i.e. age, source of specimen and results of antimicrobial susceptibility (MIC_{50} and MIC_{90}) for three antifungals, amphotericin B, flucytosine and fluconazole were determined.

Results

Among49 clinical isolates of *C. neoformans* 35 (71%) were isolated from cerebral spinal fluid, 10 (20%) from blood and 4 (8%) from miscellaneous samples like tracheal aspirate, bone marrow, urine and ascitic fluid. All isolates were from major urban centers like Karachi (37/49), Lahore (9/49), two from Hyderabad and one from Peshawar. Amongst the patients presenting with invasive cryptococcal disease, 33 (67%) were male. According to the age, patients were categorized into three groups: 2 (4%) cases belonged to pediatric age group (0-18 years), 40 (82%) were adults (19-60 years) and 7 (14%) were elderly (>60 years) patients. The clinical data was collected retrospectively, and details of clinical information could not be obtained for as many as 20 (41%) cases. Of the 29 cases in which history was obtained, invasive cryptococcal disease was seen in immunosuppressed patient population, in conjunction with various chronic infections (Table1). Amongst cases in which history could be obtained, 11 (38%) were HIV reactive, one patient (3%) was found to be HIV non-reactive, whereas HIV status was unknown in 17 (57%) cases. Details of other opportunistic infections could not be obtained in HIV patient population; however, two cases had concomitant pulmonary tuberculosis. Multiple comorbids were seen in HIV non-reactivepatient population as well as patients with HIV status unknown, who presented with invasive cryptococcal infection. In this population, cases of viral hepatitis (n=6), diabetes mellitus (n=6) and those with history of steroid use (n=5) were seen. Hepatitis C was the most common viral hepatitis (3 out of 6 cases). Four cases presented with history of malignancy; hematological malignancies being the most common, with one case each of Hodgkin's lymphoma, Non-Hodgkin's lymphoma and T-cell lymphoma, and one case of hepatocellular carcinoma was also seen. Three patients with history of solid organ transplant were also included; all were renal transplant recipients.

Eighteen out of 29 (62%) patients received an antifungal, however, outcomes were available for 15 cases only. Out of these, 9 patients expired eventually, 4 were treated successfully, and 2 patients were discharged after treatment but were lost to follow up. Outcomes were not known for 33 (67%) patients.

Table 1: Summary of associated clinical conditions in 29 clinical cases diagnosed with culture proven disseminated cryptococcal infections.

HIV status	Comorbidity/ risk factors	Number of cases (%)
*HIV reactive		
[n=11(38%)]	Without Tuberculosis/OI~	9 (31)
	With tuberculosis/OI	2 (6.8)
HIV non-reactive		
[n=1 (3%)]	Viral hepatitis, steroid use	1 (3.4)
HIV status unknown		
[n=17(58.6%)]	Steroid use	5 (17.2)
	Viral hepatitis	5 (17.2)
	Diabetes	6 (20.6)
	Malignancy	4 (13.7)
	Hypertension	3 (10.3)
	IVDU#	1 (3.4)

*HIV: Human Immunodeficiency Virus
#IVDU: Intravenous drug use
~ Opportunistic Infections

For all 49 *Cryptococcus neoformans* isolates, MIC_{50} and MIC_{90} were found to be within the epidemiological cut off values (ECVs) for 5-flucytosine and fluconazole. However, one isolate had MIC (2 µg/mL) higher than the ECV for amphotericin B (Table 2).

Table 2: Susceptibilities of *Cryptococcus neoformans* isolates (n=49) against amphotericin, fluconazole and flucytosine

Antifungal	Epidemiological cutoff values (ECVs) (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	No. of agents susceptible isolates
Amphotericin B	1	0.5	1	98
Fluconazole	16	8	16	100
Flucytosine	16	4	8	100

Discussion

This study shows that clinical characteristics in Pakistani patients with invasive cryptococcal infection are consistent with literature published from other part of the world, not only among those suffering from HIV infection, but also with in those having a variety of other immunocompromised states and chronic infections. It is reassuring that almost all isolates are found susceptible to azoles, polyenes and flucytosine, except one which showed higher MIC to amphotericin B.

Invasive cryptococcal infections are seen in immunocompromised as well as immunocompetent population.[10, 11] Targeted therapy with appropriate antifungal agent is required for clearance of infection, failure of which causes considerable morbidity and mortality.[12] As a commonly known fact, mainly affected populations include people suffering from HIV/AIDS, with an estimated 1 million cases of cryptococcal infections per year globally.[4] Studies have shown a decline in incidence of cryptococcal infections in AIDS patients in the US in 1990s, mainly due to early diagnosis, prophylactic antifungal therapy and treatment initiation for HIV.[13] A review regarding serious fungal infections in Pakistan estimates the annual burden of cryptococcal meningitis in HIV infected patients to be about 794 cases.[14] Rates of cryptococcal meningitis have been reported as 2.5% and 9% in two studies in HIV infected population in Pakistan.[15, 16]

This study included local cases of cryptococcal infections, found more in adult patients, with 67.3% of cases occurring in males. The patient distribution is consistent with literature published previously.[17, 18] A study was conducted on 62 cases of cryptococcosis in Brazil in 2009-2010 and similar findings were seen with regards to demographic profile. Majority of the patients from the Brazil study were 20-40 years of age and a higher incidence was seen in male patients (74.2 %), but most patients diagnosed with cryptococcosis had HIV (85%). In this study, other underlying illnesses were candidiasis (30.6%), leprosy (8.1%), toxoplasmosis (12.9%) and tuberculosis (8.1%).[19] Another case series from Karnataka, India, which studied clinical profile of 12 patients with disseminated cryptococcosis, all cases were HIV positive and did not have any other risk factor.[20]

Our data shows that HIV and cancer are not the only underlying comorbids in patients with cryptococcosis in Pakistan. In this study, 4 patients had concomitant malignancies, mainly lymphomas. A study from the largest cancer referral center in Pakistan showed 2 out of 24 (8.3%) culture positive meningitis caused by *C. neoformans* in cancer population.[21] Besides immunosuppressive states, cryptococcosis was seen in association with infections such as hepatitis B, hepatitis C and tuberculosis, which is understandable as their burden is high in the community. In a study done in China by Zhong *et al*, a higher association of hepatitis B was seen in patients with cryptococcal meningitis.[22] Association of tuberculosis and cryptococcosis is seen in certain studies which can be attributed to defective T-cell immunity.[19, 23, 24]

In this study, all clinical isolates were found susceptible to fluconazole and flucytosine, however one isolate was found to have higher MICs against amphotericin B. Literature review from regional countries like India and China and countries like Cambodia and Brazil show that antifungal resistance against *Cryptococcus* species is on the rise.[25-28] Datta *et al* reported higher MICs for fluconazole and itraconazole in 16% and 7% of *C. neoformans* isolates from India.[25] In Brazil, a study by Figueiredo *et al* showed 20% resistance to itraconazole.[28] In general, antifungal resistance is seen mainly with azoles, however, there are reports of resistance against amphotericin B as well.[29, 30] Continuous surveillance of antifungal susceptibility patterns is needed to monitor the changes in sensitivity profile of *Cryptococcus* species.

This study is the first of its kind from Pakistan. Major strength of the study is that a considerable number of cases of invasive cryptococcal disease were included. The collection of isolates was from different parts of the country and susceptibility testing was performed on by CLSI recommended BMD method. One of the limitations of this study is that the strains of *Cryptococcus* isolates were not identified by molecular methods. Another limitation was lack of availability of clinical information for 41% of cases including HIV status which was known only in 12 patients.

Due to lack of access to healthcare facilities and availability of diagnostic modalities, the actual burden of cryptococcal infections in our population is likely to be underestimated. At times diagnosis is not considered as HIV status is not known or other risk factors are not taken into consideration. Further studies with larger number of cases and details of clinical presentations may be able to better highlight the risk factors involved in invasive cryptococcal infections.

Conclusion

In summary, our finding suggests that in Pakistan, empirical

use of antifungal drugs can be used as per available guidelines in invasive cryptococcosis as clinical isolates are susceptible to azoles, polyenes and flucytosine. Since, isolated resistance to amphotericin B is seen in one of the study isolates, routine susceptibility testing for all invasive cryptococcal isolates is recommended. Routine surveillance of antifungal resistance is imperative to ensure optimum therapy and eventual clearance of infection as well as to monitor trends of antifungal resistance in invasive cryptococcal strains. To ensure early diagnosis and therapy, there is a need to keep a high index of suspicion and exploration of risk factors, not only in HIV/AIDS patients but also those with impaired T-cell immunity or apparent immunocompetent status.Studies with a larger sample size and details of clinical information will be helpful to further ascertain predisposing risk factors of invasive cryptococcal infections in our population.

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