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Clinical Effectiveness Of Antifungl Drugs For Treatement Of Cryptococcus Isolates

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Abstract

Cryptococcus spp., enhance pathogenicity to human beings, grow mechanism of adaptive survival within environment host of immune reaction. Most of the fungal infections to humans caused the immune response of pro-inflammatory not in Cryptococcus spp. We analyzed the susceptibility for antifungal drugs in150 HIV patients with meningitis. From them, 13 have Cryptococcal meningitis of which 10 were found to have primary episode and 3 have relapse episode. The microbroth dilution method and epsilon meter test were performed to test antifungal susceptibility; the isolates of C.neoformans and C.gattii were susceptible. We observed the MIC levels ofamphotericin-B by micro dilution method observed from 0.10- 0.4µg/ml and 0.016-0.256µg/ml in E test. The MIC for the drugs Fluconazole from 0.10 – 0.4 and 1.0-8.0 and Voriconazole also observed from 0.06-0.24 respectively.

Key words: Cryptococcal, treatment, microbroth, immune system.

Introduction

Cryptococcosis

Cryptococcal infectionis a dangerous fungal infection in both of HIV and non-HIV patients. Accordingly, from the recent taxonomy the fungus is divided into two types of species namely Cryptococcusneoformans and C. gattii). The name Cryptococcus means ‘hidden seed’, earlier less known pathogen came into public with the arrival of pandemic AIDS (Stephen et al., 2002). The Cryptococcus genus composed of above 70 species but infection in human is rarely formed by species other than C.neoformans and C.gattii.

According to WHO, Cryptococcosis leads to death at 13-44% in HIV infection correlated in particular countries. The mortality rate of HIV with CM is higher level from 10-25% and reached upto 65%. Due to Cryptococcus that allocated with tuberculosis caused >5, 00,000 deaths in developing countries. In India, the ubiquity of meningitis because of Cryptococcus in HIV patients had reduced range from 45-60% in the 21st century beginning to 3-16%. Cryptococcus spp., enhance to pathologic to human beings,

grow mechanism of adaptive survival within environment host of immune reaction. Most of the fungal infections to humans caused the immune response of pro-inflammatory not in Cryptococcus spp. (Kwon Chung, 1976).

Risk factor of Cryptococcosis

The major risk of Cryptococcosis is primary or secondary T-cell immune response impairment. In 1980s the most of the cryptococcal disease noted was iatrogenic, next to the large use of therapy for immunosuppressive (Deak and Park, 2011). Prior to 1980s, HIV was the main cause for depression for T-cell immune response with CM.

Amphotericin B is a fungicidal drug which binds toergosterol, forming pores in the membranes of yeasts. Flucytosineis a fungistatic drug, a base analog that intercalates intofungal RNA, preventing protein synthesis from occurring. Differentmechanisms of action for these drugs make it difficult forthe cells to develop resistance to both drugs during the course oftreatment. Fluconazole isalso fungistatic and acts by binding to and inhibiting the 14-demethylase, preventing a cell from producing ergosterol for thecell membrane, a mechanism of action which targets the same cellprocess as amphotericin B. The WHO also recommends Voriconazolefor preemptive treatment of asymptomatic cryptococcalantigen-positive persons with CD4 counts of 100 cells/l thathave early subclinical infection(Nigam et al., 2012).

Treatments of cryptococcosis

Prophylaxis

TheCryptococcosis prevention mostly for HIV patients and suggested oral fluconazole-based prophylaxis. Some patients were treated with chemotherapy without disease proof and had no mortality effect and some patient's benefits in decreased CM incidence.

The preventive measures on fluconazole-based pre-emptive therapy of symptomless patients of CrAg positive have screening of CrAg pre-ART with < 100 CD4 cells/ml. And also offered Fluconazole at a dose 800mg/day for two weeks then 400mg/day till ten weeks and 200 mg/day till immune reconstitution (Rajasinghamet al., 2017).

Curative

The treatment of CM depends on restricted medications are classified into three classes namely(amphotericin B [Am B]), azoles (fluconazole) and pyrimidine analogue (flucytosine [5FC]). WHO guidelines suggested CM treatment in three phases includes induction, consolidation and maintenance. The treatment of induction showed intravenous infusions of Am B at 1mg/kg/day and flucytosine at 100 mg/kg/day both in combination and administrated for 14 days. Consolidation therapy take up on fluconazole separately at 400 – 800 mg/day till 10 weeks continued by maintenance therapy of 200 mg/day till immune reconstitution (CD4 count >200). 16% and 32% of Am B-related caused anemia and impairment of kidney (Wadhwa et al., 2007). Onthis basis, we examined the MIC values for susceptibility from our clinicalisolates to analyze the current levels of susceptibility and to determine if could be a viable adjunctive preemptive treatment option.

Materials and method

Place of study

This work was accompanied on patient with HIV positive that they were admitted in the Government hospital, Thiruchurapalli, medicinal wards.

Study Period

The period of this study was lead from October 2020 to September 2021.

Sampling Method

The patients were hospitalized in the unit of inpatients of hospital at the time of study will be embraced.

Reference Population

Cryptococcal meningitis of patients having infection of HIV

Antifungal susceptibility testing

Minimum Inhibitory Concentration (MIC) determination was finished by using the method of microbroth dilution. Its performance was compared with the Epsilometer test with using microbroth dilution method.

Microbroth dilution method

Powder Amphotericin B was purchased from Hi media, Mumbai and voriconazole and fluconazole powders from Pharma Fabricon and their power for each was 750µg/mg.

$$\text{Weight (mg)} = \frac{\text{volume (ml)} \times \text{concentration (µg/ml)}}{\text{Assay potency (µg/ml)}}$$

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Stock solution

For water soluble drugs like fluconazole and water insoluble drugs like amphotericin B and voriconazole, dimethyl sulfoxide (DMSO) was used. Prepared the fluconazole 5200 µg/ml used as stock solution and other two drugs at 1600µg/ml. The drugs were dissolved into the stock solution of fluconazole or amphotericin B and voriconazole to obtain an intermediate solution. After it was dissolved into 1:10 ratio of fluconazole or 1:100 ratio of amphotericin B and voriconazole in RPMI.

Test medium used

The test medium used was RPMI- Rosewell Park Memorial Institute was purchased from Himedia, Mumbai.

Inoculum preparation

Isolates of C.neoformans from patients and sub cultured and incubated with Sabouraud Dextrose Agar for 48hours at 35^{o}C to obtain purity and capability. Candida krusei and C.parapsilosis were the control strains incubated for 24 hours. Suspended the colonies in 5ml saline and obtained the stock suspension of 1x106

- 5 x106 cells/ml and diluted it with broth medium of RPMI 1640 1:100 continued by dilution of 1:20 to get 5x102 - 2.5x103 cells/ml.

Incubation

The microtitre plates were incubated at 35^{o}C and study after 46-50 hours formation of Candida spand 70-72 hours for C.neoformans.

Interpretation

Minimum inhibitory concentration is the lower concentration of antifungal that forms the decreased visible growth of broth dilution in susceptibility test. The reduction of visible growth was analyzed by the each microdilution well numerical score.

Epsilometer (E) Test

This test was performed to determine the isolates of amphotericin- B and fluconazole to cryptococcal MIC and it was profound by Khyriemet al., 2009. For this E test, the strips were purchased from Hi Media, Mumbai. 90mm of disposable plates having RPMI and 2% of glucose agar to a 4mm depth used. The isolates of cryptococcal and C.neoformanswere sub cultured using SDA for 48hours. The suspension of meadow culture was constructing with sterile swab and E strips were put in the agar surface. The concentration for amphotericin- B was 0.002- 32µg/ml and fluconazole was 0.016-256 µg/ml. The plates were incubated for 72 hours at 35^{0}C and the intersection of MIC was recorded at the inhibition point of growth for amphotericicn B was 100% and 80% for fluconazole.

Comparison of E test and microbroth dilution test, the essential and Categorical agreement were measured. The essential agreement is the method that MIC dilution within ± one 2-fold and Categorical agreement is standard reference method. If the categorical disagreement occurs it is called as ‘minor error’ and reference method indicates the organism is susceptible is called as ‘major error’.

Statistical analysis

It was measured by the usage of Statistical Package for Social Sciences (SPSS) version 20.0. This study proportional data was recorded by analysis of Pearson’s Chi square test $\chi 2$.

Result and Discussion

A total of 150 CSF samples were collected during the study period from HIV positive patients presenting with features of meningitis. The age of the patients varied from 14 to 65 years with a mean age of 38 yrs. Only 30 (18.86%) of the 150 patients were females and the male patients were 120. Among these HIV patients, 13 patients were diagnosed with Cryptococcal meningitis (CM), of which, 10 were primary episodes and 3 were symptomatic relapse episodes.

The CSF culture on SDA observed the creamy coloured mucoid yeast like colonies in 48 hours in two samples and in 9 samples they found yeast like colonies in 48-72 hours (Table-1). The balance two samples growth were noted on 5th and 7th day. Cryptococcus delayed growth differs from 5-14 days and it was noted in earlier studies (Chuck and Sande, 1989).

Table-1: Growth of Cryptococcal isolates on SDA

Duration of growth on SDA	No. of isolates	Percentage
<48 hours	2	15.38
48- 72 hours	9	69.23
>72 hours	2	15.38
	13	**100**

The diagnosis of infection of HIV and primary episode of Cryptococcal meningitis was noted in most of the patient having 1-2 years. The eight patients have ART with first episode of CM and 3 patients were taking the T.fluconazole with ART. Most of the patients diagnosed the infection of HIV at 1-2 years. Three patients was affected by Cryptococcal meningitis was the AIDS and from them half of the patients taking ART with CM at the time of presentation. And also three patients were received the prophylactic measure of T.fluconazolein case of infections. Only one of three patients diagnosed with relapse does not taken the fluconazole prophylaxis and also not starting on HAART.

The insufficient availability of 5-flucytosine (5-FC), no one can taking the combination of amphotericin-B + 5-FC for the treatment of CM. out of 16, 13 cases had primary episode of CM that they were taking IV amphotericin-B, 6 patients taking in the form of monotherapy and 3 in combination with fluconazole.

The observed mortality rate for primary episodes of CM was 43.75% and the remained 9 cases were start to take secondary fluconazole prophylaxis but one case was discontinued and later present with relapse and died. This higher mortality rate was compared to the study of Vermes et al., 2000 and observed the 15% in their studies.

After the completion of 89 days the outset of HAART was diagnosed in two relapse patients because of formation of IRIS. Due to the quit of secondary fluconazole prophylaxis the only one patient had relapse. Most of the isolates of cryptococcal growed in the CSF within 72 hours on SDA. One isolates grew on 5^{th} day and another one on 7^{th} day.

None of the patients received 5-FC as part of primary therapy for Cryptococccal meningitis. 4 of the 10 patients died during the primary episode despite institution of amphotericin-B and 1 patient died even before starting amphotericin-B. But, 6 patients received intravenous amphotericin-B as monotherapy while 9 patients received intravenous amphotericin-B and oral fluconazole in combination. One patient died before starting amphotericin-B itself while 6 more patients died despite institution of amphotericin-B. In addition, another patient died during the relapse episode. The overall mortality rate observed was 42.1%.

At the period episode, the patients cave in to the infection and it was determined with absence of secondary prophylaxis, CM patients of 50-60% have the relapse disease. If controlled properly, the fluconazole with secondary prophylaxis was higher effective, increased relapse at six months over thirteen fold (Missoni et al., 2011).

Kwon Chung (1982) had investigated the EA of 82.2% for fluconazole in their study. For fluconazole the categorical agreement (CA) was100% was determined in the study of Larsen et al., 1989and he had found 77.4% of CA among E test and fluconazole for reference method. The difference in levels of MIC noted with the E test for both amphotericin-B and fluconazole make it essential that isolates of cryptococcal tested with the method of microbroth dilution as suggested by the CLSI.

The CLSI does not susceptible to amphotericin –B to cryptococcal strains (Table-2) but MIC >1µg/ml for any isolate was resistant to amphotericin-B. The amphotericin-B MIC differs from 0.12-0.5 µg/ml and E-test of amphotericin-B MIC differed from 0.016-0.256 µg/ml.C.neoformans isolates found that have low MIC level foramphotericin-B by E-test compared to microbroth dilution method. The EA (Essential Agreement) was 61.5% found in 8 of 13 isolates of cryptococcal MIC ranged from 0.064- 0.5 µg/ml in one 2 fold dilution observed by the reference method (0.12-0.5 µg/ml).

Table-2: Susceptibility pattern of Cryptococcal isolates by antifungal agents

Antifungal drug	Microbroth dilution method (Susceptible isolates in %)	E test (Susceptible isolates in %)
Amphotericin- B	100	100
Fluconazole	100	100
Voriconazole	100	-

The CA (categorical agreement) was 100% results to isolates of cryptococcal were allowed to amphotericin-B by microbroth dilution method (cf 746t) and E test. There was a significant coefficient among the isolates of cryptococcal E-test and amphotericin-B MIC values <0.012 µg/ml that were compared to microbroth dilution method (p= 0.002) (Table-3).On the report of CLSI M27-A3, isolates of cryptococcal was tested by microbroth dilution method and revealed susceptible MIC <8 µg/ml, dose dependant for 16-32 µg/ml and resistant to fluconazole >64 µg/ml. The MIC values differed from 0.5-2 µg/ml.

Table-3: Microbroth dilution method and E test for antifungal drugs

	Amphotericin B - microbroth dilution method MIC (µg/ml)

Test isolates (12)	**0.02**	**0.05**	**0.10**	**0.23**	**0.4**	**1**	**2**	**3**	**6**	**12**
C.neoformans (11)	-	-	2	8	1	-	-	-	-	-
C.gattii(1)	-	-	-	-	1	-	-	-	-	-
	Amphotericin B –E Test MIC (µg/ml)									
Test isolates (12)	**0.002**	**0.004**	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.5**	**1**
C.neoformans (11)	-	-	-	2	2	2	4	1	-	-
C.gattii (1)	-	-	-	-	-	-	1	-	-	-
	Fluconazole - microbroth dilution method MIC (µg/ml)									
Test isolates (12)	**0.02**	**0.05**	**0.10**	**0.23**	**0.4**	**1**	**2**	**3**	**6**	**12**
C.neoformans (11)	-	-	6	4	1	-	-	-	-	-
C.gattii (1)	-	-	-	-	1	-	-	-	-	-
	Fluconazole - E Test MIC (µg/ml)									
Test isolates (12)	**0.16**	**0.32**	**0.64**	**0.128**	**0.256**	**0.5**	**1**	**2**	**4**	**8**
C.neoformans (11)	-	-	-	-	-	-	3	3	4	1
C.gattii (1)	-	-	-	-	-	-	-	-	-	1
	Voriconazole-- microbroth dilution method MIC (µg/ml)									
Test isolates (12)	**0.03**	**0.06**	**0.12**	**0.24**	**0.5**	**1**	**2**	**4**	**8**	**16**
C.neoformans (11)	-	5	5	1	-	-	-	-	-	-
C.gattii (1)	-	-	2	-	-	-	-	-	-	-

C.neoformans and C.gattiiboth isolates were observed in an increased MIC forfluconazole by E test when compared to the microbroth dilution method. The cryptococcal isolates 10 of the 13 had essential agreement [EA] was 76.9% and MIC from 1- 4µg/ml within one fold dilution determined from reference method (0.5-2 µg/ml). cryptococcal isolates for categorical agreement [CA] is 100% susceptible to fluconazole for tests of microbroth dilution method and E test.Cryptococcal isolates testing was

significantly associated with E test and MIC values from >4µg/ml for fluconazole compared to dilution method (p=0.008).

The CLSI M27- A3, the isolates of cryptococcal tested by method of micro dilution and formed the susceptible values for MIC <1 µg/ml, susceptible for dose dependent at 2 µg/ml and resistant to voriconazole at >4 µg/ml. The voriconazole MIC differed from 0.06-0.25µg/ml and isolates of cryptococcal differed sensitive to voriconazole by microbroth dilution method with MIC<1 µg/ml.

The isolates of cryptococcal were delicate to voriconazole and their MIC values were 0.06-0.25µg/ml. The antifungal agents of cryptococcal isolates were observed to be 100% and now the C.neoformansresistance to agents of antifungal is very scarce (Jarvis and Harrison,2007) and in the global scenario only fitful reports of fluconazole resistance have been found.

Conclusion

Cryptococcal is the most prevalent laboratory-confirmed etiological agent among adultHIV-infected patients. Mortality rate in this populationremains unacceptably high. Improving diagnostic capacity and early treatment may help to decrease the mortality rate. The study concludes that this work calls attention tothe ongoing need for improved diagnosis and managementfor patients presenting with Cyptococcus in resource-limited settings.

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