Pathogenicity of *Ascotricha chartarum* for Albino Rats and Drug Sensitivity Testing: Study of A Clinical Isolate From Cerebrospinal Fluid of A Cancer Patient

Nidhi Warthe[1,2] , S.M. Singh[1,2] , S. R. Nawange [1'2] , Shruti Singh[3]

[1]*Medical Mycology Research Laboratory, Department of Biological Sciences, Rani Durgavati University, Jabalpur- 482001, Madhya Pradesh, India*

[2]*Center for Medical Mycology, Fungal Disease Diagnostic and Research Center, Jabalpur-482002, Madhya Pradesh, India*

[3]*Department of Biotechnology, Mithibai College, Vile Parle (W), Mumbai, Maharashtra*

Abstract - Ascotricha chartarum is rarely known to cause human infection.A single case of maxillary sinusitis patient is known so far. Case report .We here present a case of 50 year old male suffering from Cancer (Seminoma testis) third stage with complaints of frequent fever and pain in abdomen.The patient was randomly investigated for opportunistic fungal infection as secondary infection.The fungus Ascotricha chartarum was isolated from the cerebrospinal fluid of the patient.The isolated fungi was later tested on white albino mice to study its pathogenic potential.Both immunosuppressed and healthy animals were kept under the study design. Result - 80 % of mortality was seen among the immunosuppressed animals in comparision to 40 % mortality among the healthy group. Antifungal testing by three azoles (Fluconazole,Itraconazole and Ketoconazole) and one polyene (Amphotericin B) showed that Amphotericin B gave good results in-vitro followed by Ketoconazole and Fluconazole.

Keywords : Ascotricha chartarum , human fungal opportunist pathogen, pathology , antifungal susceptibility testing.

I. INTRODUCTION

Ascotricha chartarum belongs to Ascomycetes,one of the major groups of the kingdom fungi, characterized by the production of ascospores. Many species occur in nature and ascospores are a frequent component of outside air detected most readily in spore trap samples. Certain species are known to colonize outdoor environments and some (including *Ascotricha*, *Chaetomium*, *Eurotium*, *Myxotrichum, Petriella*, *Peziza*, etc.) are common indoor dwellers on wet building materials. Anamorph (asexual state): Dicyma. *Ascotricha* species comprise a small proportion of the fungal biota. This genus is most closely related to *Chaetomium.* No information is available regarding health effects, or toxicity. Allergenicity has not been studied.They are identified on surfaces by tape lifts, tease mounts from bulk samples, and in air by spore trap sampling. Spores are round, brown, and may be identified to genus if other structural elements (such as perithecial terminal hairs) are present. Otherwise, these spores may be placed in the spore category "smuts, Periconia, myxomycetes" or may be called "unknown brown." *Ascotricha* is cellulolytic, and sources of isolation include damp sheet-rock paper, woody and straw materials.However there are no reports of human infection by *Ascotricha chartarum,* except one in a patient of maxillary sinusitis reported in year 1996 (1). With the exception of *Cryptococcus neoformans*, fungi are less detected in cerebrospinal fluid obtained from patients having or suspected of having fungal meningitis. A review done by Mcginnis in 1983 revealed that several fungi have been either isolated, observed, or both in cerebrospinal fluid specimens These fungi include *Acremonium* species, *Aspergillus amstelodami, A. flavus, A. fumigatus, A. oryzae, A. terreus, Blastomyces dermatitidis, Candida albicans, C. tropicalis, C. viswanathii, Coccidioides immitis, Cryptococcus albidus, C. neoformans, Histoplasma capsulatum, Paecilomyces variotii* etc. (2).

Because few fungi are professional pathogens, fungal pathogenic mechanisms tend to be highly complex, arising in large part from adaptations of pre-existing characteristics of the organisms' nonparasitic lifestyles.(3) Over a period of time fungi devloped an arsenal to attack its hosts and hence lead to pathogenesis.Animal models of aspergillosis have been used extensively to study various aspects of pathogenesis, innate and acquired host-response, disease transmission and therapy (4).Several other examples are also given in literature for the experimental pathogenicity testing of fungi including from phaeohyphomycetes ,hyalohyphomycetes family and yeasts in animals models (5,6,7,8,9).

Antifungal susceptibility testing of yeasts can assist in treating patients with fungal infections as well as the ones with prior antifungal exposure in determining resistance or cross-resistance. Mold susceptibility testing is of limited

clinical benefit, however there are exciting new agents for treating difficult invasive fungal infections (10). The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) has developed a reference method for broth microdilution antifungal susceptibility testing of filamentous fungi (CLSI/NCCLS M38-A document)(11).

II. PROPOSED METHODOLOGY

i)Isolation and identification of the fungi - Cerebrospinal fluid was collected aseptically and was brought to the laboratory within few hours. The sample was centrifuged at 2000 rpm for 15 minutes .Later the sample was used for isolation of fungus by inoculating in culture media. After 7 days of incubation the culture was sub cultured and macro as well as micro morphological studies were done. The identification of the fungus was finally confirmed by Prof. J.Guarro (Spain).

ii)Pathogenicity studies –

(a) Animal models

Albino rats, out bred, disease free, young weighing between 20-30 g were used in the present study. Animals were housed I four per polypropylene cage and provided with food and water ad libitum. They were maintained in accordance with the guide for care and use of animals. Four groups of animals, viz i) Cortisone treated (CT),ii) Without cortisone treated (WCT),iii) Cortisone treated control and iv)Without cortisone treated control.For immunosuppression rats were injected intraperitoneally with Decadron (4% Dexomethasone sodium phosphate) 125 mg/kg of animal weight in 3 alternate doses before the experiment with the help of disposable 2 ml syringe with 24 gauge needle. All groups of rats were given oral dose of chloramphenicol (0.5 mg/ml) to prevent bacterial infection till the end of the experiment.

(b) Mortality/Survival Studies:

Mortality/survival of the animals due to fungi was determined following intravenous injection of 0.5 ml of inoculum with 1.5 X 10^6 cfu/ml through peripheral tail vein. Control animals were injected with 0.5 ml of sterilized normal saline. Animals were observed upto 30 days post infection and mortality/survival recorded for each group.Course of infection in animal as determined by pathological lesions, organ culture and direct microscopy The animals which died/survived during the course of infection were autopsied/sacrified and their visceral organs, viz. lungs, kidneys, liver, spleen, brain, heart and stomach were removed and studied for pathological lesions. Each organs were then cut into two equal halves. One half was fixed in 10% formaline for histopathological study. The other half of the organs was homogenized and cultured on SDA slants with 0.05 mg/ml chloramphenicol and incubated at 28°C for 2 weeks and growth of the pathogen recorded.

(c) Study of Virulence :

Time course distribution of the inoculated fungi was kept as a parameter to study the degree of virulence in the experimental animal in due course of time. Time interval in which the course of infection was studied was 3rd day, 5th day , 7th day, 9th day, 14th day and 28th day of post infection with respective etiological agent .The animals were sacrificed on the determined day and the parameter for study of infection were same as that in mortality studies that is culture study of organ homogenate on culture medium, micro-examination and histopathology of the organs that is , liver, lungs, kidney, spleen, brain and heart.

iii) Antifungal susceptibility testing

Antifungal drug – The following five antifungal drugs were used. Amphotericin B (AMFOCAN, Dabur India Ltd.), Ketoconazole (NIZRAL; Johnson and Johnson) Janssen Pharmaceutica, Regd. trademark of Johnson and Johnson, USA), Itraconazole capsules (CANDISTAT; E Merk India Ltd; Licensed user of T.M.), Fluconazole (Flustan^{TM}; Dr. Reddy's Lab. Ltd.)^{TM} Trademark under registration.

Broth micro-dilution test / Susceptibility testing procedure,incubation and determination of MIC's for moulds:

Sterile plastic microtitration plates with 96 flat bottom wells each were employed.These plates contained two – fold serial dilutions of the antifungal drugs and two drug – free medium wells for sterlity and growth controls.The trays were inoculated with 0.100 ml into each well.The plates were incubated at 35° C for 24 ,48 and 72 hrs in a humid atmosphere.Visual readings were performed with the help of mirror.

Statistical analysis – Student 't' test was employed to determine the significance of the difference between the geometrical means of MICs values. Statistically significance was set at $P< 0.05$. Survival analysis was done by Kaplein –Meier test . Test of significance at $p = 0.05$ was done using T-test.

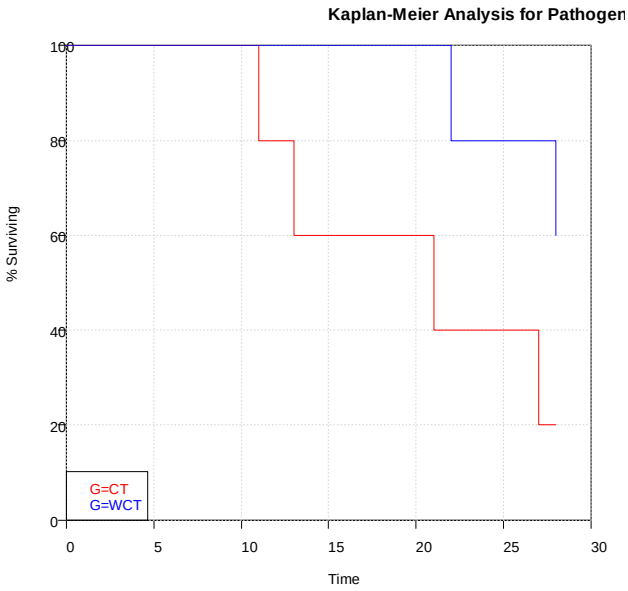
III. RESULT

i)Results of Survival /mortality

80 % of mortality was seen among the CT group of animals by 27th day of post infection whereas 40 % of animals died in the WCT group of animals. Spleenomegaly was seen among CT animals .Lesions were seen in spleen that were small in size and black in color.

Histopathological findings

In lungs congestion in alveoli and inter alveolar septa with peri bronchiolar inflammatory cells was seen. Bronchopneumonia was present. Congestion in glomeruli and interstitial tissue and tubular degeneration was seen in kidneys.Spleen section showed congestion in pulp and malpigian tubules.In brain tissues no change was recorded.



Chisq= 2.6 on 1 degrees of freedom, p= 0.105

ii) Results of time course distribution of the fungus in various organs of the animal model

Lungs – The CFU values increased upto 9th day and then subsequently got reduced upto 28th day. Similar pattern was observed in WCT group of animals but the values of CFU were much lower as compared to the CT group of animals, which was statistically significant.

Liver - In Liver of CT animals the CFU values continuously increased upto 14th day and the got reduced on 28th day. Significantly lower CFU's were seen in WCT animals except on 9th day.Though the titer has reduced but there was no marked difference in due course of time and the organ was found to be grossly affected.

Kidneys - Amongst the CT group of animals the titer was found to increase with the time and reaced its highest value on 9th day and then decreased markedly until the end of experiment.As in other organs here also the WCT group of animals were less affected but statistically no significant difference could be evaluated on 3rd ,5th and 28th day.

Spleen - The spleen of the animal was found to be most affected as compared to other organs.Here aswell the CFU values tend to increase upto 9th day and the got reduced upto 28th day.The WCT animals were again less affected as shown by the CFU values which were statistically significant also.

Brain - The brain was not affected at all as there was no isolation of fungus from the organ.

Heart - Heart was least affected organ. Though here again the CT group of animals were more affected compared to the WCT animals. On an average there was no significant difference in isolation of the organism with respect to time.and the CFU values were somewhat similar until the end of experiment.

Histopathologically all these findings were confirmed and congestion in the organs was a major finding.

Results of antifungal drug sensitivity testing by CLSI method-

Statistically it was found that there was significant difference in Amphotericin B (MIC 0.0625) and Fluconazole (MIC 16 – 32 µg/ml). As an outcome of the susceptibility testing Amphoterin B was the most effective drug followed by Fluconazole.However the organism was found resistant towards Ketoconazole and Itraconazole.

Antifungal drug sensitivity							
Amphotericin B		**Ketoconazole**		**Itraconazole**		**Fluconazole**	
MIC in µg/ml	Category	MIC in µg/ml	Category	MIC in µg/ml	Category	MIC in µg/ml	Category
0.0625	S	0.5-2	R	2-4	R	16-32	SDD

IV. CONCLUSSION/DISCUSSION

There has been continuous change in the scenario of fungal infections with the time. Less common and emerging fungal pathogens are often resistant to conventional antifungal therapy and may cause severe morbidity and mortality in immune-compromised hosts Apart from the usual and contemporary fungal organisms we have seen the emergence of new and rare fungal pathogens. This article deals with one such description where *Ascotricha chartarum* was isolated from cerebrospinal fluid.This fungi belongs to ascomycetes family xyalareacea and genus Aschotricha berk .To the date only one report of ascotricha has been given by Singh *et al* in the year 1996 from a patient of maxillary sinusitis(1).This fungi has been rarely identified and isolated but few reports are there of its isolation as fungal contaminants(12)other reports have shown isolation of the same genus as air contaminants capable of potential opportunistic pathogen (13).

Animal models of fungal infections are always been the most important aspect in the advancement of the medical mycology. Over a period of time different types of animal models of fungal infection have been developed,right from murine models to gunea pigs and several other newly applied models, however murine models are the most frequently used, for studies of pathogenesis, virulence, immunology, diagnosis, and therapy. Potential pathogenicity of the fungi was tested in murine model and histopathology, reverse fungal culture was done to confirm the ability to produce the infection.(14,15,16,17,18,19,20,21,22,23,24).Setting the experimental design with both immunocompetant and immunosuppressed model was done but as always it is difficult to exactly mimic the human infection ,also use of laboratory animals is quite questionable.(25,26,27)

Our findings suggested that except brain all the organs under the study design were affected. Liver and spleen being most grossly affected.This justified their pathogenic potentials towards the murine model.Since there is no other pathogenicity testing work related to this particular organism some other closely related organisms were reviewed.It was found that *Chaetomium* extracts when injected in mice three organs were remarkably affected and those were liver,spleen and kidney.(28,29) .Several other filamentous fungi have also been studied for their pathogenicity in murine models like acremonium.(30,31).

In the presented study we have also found that Amphotricin B was the most effective drug when compared to other azole drugs.MIC as low as 0.0625 µg /ml was reported as compared to MIC values of other azole drugs. In some earlier works mucormycetes and other rare hyaline moulds exhibit variable susceptibilities to antifungals, and hence antifungal testing is valuable. In on such study the in vitro antifungal susceptibilities of 159 clinical isolates of Candida species from patients with invasive candidiasis in Kuala Lumpur Hospital, Malaysia, were determined against amphotericin B, fluconazole, voriconazole, itraconazole and caspofungin. The most common species were Candida albicans (71 isolates), Candida parapsilosis (42 isolates), Candida tropicalis (27 isolates) and Candida glabrata (12 isolates). Amphotericin B and voriconazole showed the best activities against all the isolates tested, with MIC(90) values of ≤1 µg/ ml(-1) for all major species. (32,33,34) .

Several new pathogens are being reported as they evolve from non pathogenic to pathogenic forms. In the year 2014 opportunistic fungal pathogen Kodamaea ohmeri was reported in two new cases though this yeast rarely causes life-threatening human infections.. The nature and treatment of K. ohmeri infections using minimum inhibitory concentrations of antifungal agents was studied and it was found that the organism is more susceptible to amphotericin B compared to fluconazole [15/25 isolates (60%) vs. 25/25 isolates (100%), respectively].So it can be said that Amphotericin B is found to be effective in new emerging fungal isolates. (3).The nature of our findings also suggest similar outcomes in results and open a gate to further investigate the unattended fungal entities.

V. FURTHER SCOPES

Recently fungal infections are considered as an important aspect of disease diagnosis and and has been given a status at par to bacterial,viral and other high risk infections by the clinicians. Even today much less is understood about host immunity towards fungi as compared to bacterial and viral infections. Studies such as these are to be undertaken in order to bring out a proper arsenal against the fungal infections which sometimes turn out to be silent killers.There is an urgent need to find the proper connection between emerging fungi in medicine and changes in ecology or human behaviours.

VI. ACKNOWLEDGMENT

The authors wish to thank the Vice-Chancellor, Rani Durgavati University Jabalpur and Principal Government Model Science College Jabalpur for providing laboratory facilities. The Director-General, Indian Council of Medical Research (ICMR), New Delhi for awarding Senior Research Fellowship and Research Associateship to one of the author (SRN). Prof. J.Guarro .Dept.of Microbiology,Medicine Universty of Revira,Spain for strain identification. Mr. Arvind Kavishwer Biostatitican RMRC, Jabalpur (M.P.) for the analyzing of the statistical data of anti-fungals .Late Prof.John C Pezzullo,PhD

Associate Proffesor Georgetown University Medical Center.Washington D.C. U.S.A.(He passed away on June 6, 2016.I dedicate this paper to him.)

REFERENCES

1.Singh SM, Naidu J, Jain S, Nawange SR, Dhindsa MK. Maxillary sinusitis caused by Ascotricha chartarum Berk. (anamorph Dicyma ampullifera Boul.): a new phaeoid opportunistic human pathogen. J Med Vet Mycol. ;34(3):215-8,1996

2. McGinnis MR Detection of fungi in cerebrospinal fluid. Am J Med. 28;75(1B):129-38, 1983.

3. Mcginnis MR .Laboratory handbook of medical mycology.New York : Academic Press ; 1980

4. Van Burik JA,Magee PT. Aspects of fungal pathogenesis in humans. Annu RevMicrobiol.;55:743-72,2001.

5.Mayayo E, Pujol I, Guarro J. Experimental pathogenicity of four opportunist Fusarium species in a murine model. J Med Microbiol. ;48(4):363-6, 1999

6.Okeke CN, Gugnani HC, Onuigbo WI. Potential pathogenicity of Cladosporium tennuisimum, Phaeoisaria clematidis and Ramichloridium subulatum in a mouse model. Mycopathologia.;114(2):65-70,1991.

7.Alture-Werber E, Edberg SC .An animal model of Curvularia geniculata and its relationship with human disease. Mycopathologia ;89(2):69-73, 1985.

8.Cermeno-Vivas JR, Torres-Rodriguez JM, Corominas JM, Madrenys N. Pathogenicity of Hansenula anomala in model of immunocompromised mice. Mycopathologia;144(2):67-71, 1999.

9.Rao CY, Burge HA, Brain JD. The time course of responses to intratracheally instilled toxic Stachybotrys chartarum spores in rats. Mycopathologia;149(1):27-34,2000

11. National Committee for Clinical Laboratory Standards.. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard. NCCLS document M38-A. Clinical and Laboratory Standards Institute, Villanova, Pa,2002.

12 Yonesu K, Ohnuki T, Ono Y, Takatsu T, Nara F.Ascotricins A and B, novel antagonists of sphingosine-1-phosphate receptor 1 from Ascotricha chartarum Berk. SANK 14186. J Antibiot (Tokyo). 2009 ;62(7):359-64. Epub 2009 May 22.

13. Filipello Marchisio V, Cassinelli C, Piscozzi A, Tullio V, Mischiati P.A preliminary survey of cycloheximide-resistant airborne fungi in Turin, Italy. Mycopathologia;123(1):1-8, 1993

14. Hawksworthe DL.Arevision of genus Aschotricha Berk. Mycological paper no 126.commenwealth Institute,Kew ,Surrey,England, ,pp.1-9,1971

15. Asmundsdóttir LR, Erlendsdóttir H, Agnarsson BA, Gottfredsson M. University of Iceland, Faculty of Medicine, Reykjavik, Iceland. The importance of strain variation in virulence of Candida dubliniensis and Candida albicans: results of a blinded histopathological study of invasive candidiasis Clin Microbiol Infect,;15(6):576-85, 2009

16 .Yang RY, Wang WL, Ao JH, Hao ZF, Zhang J, Wang CM. Pathogenicity of Trichosporon asahii in a murine model of disseminated trichosporonosis. Chin Med J (Engl). 20;121(24):2557-60, 2008 .

17. Brito MM, Conceição-Silva F, Morgado FN, Raibolt PS, Schubach A, Schubach TP, Schäffer GM, Borba CM. Comparison of virulence of different Sporothrix schenckii clinical isolates using experimental murine model. Med Mycol;45(8):721-9,2007

18. Arendrup M, Horn T, Frimodt-Møller N. In vivo pathogenicity of eight medically relevant Candida species in an animal model. Infection.;30(5):286-91, 2002.

19. Okeke CN, Gugnani HC, Onuigbo WI. Department of Microbiology, University of Nigeria, Nsukka. Potential pathogenicity of Cladosporium tennuisimum, Phaeoisaria clematidis and Ramichloridium subulatum in a mouse model. Mycopathologia.;114(2):65-70, 1991.

20 .Yegres F, Richard-Yegres N, Nishimura K, Miyaji M.. Virulence and pathogenicity of human and environmental isolates of Cladosporium carrionii in new born ddY mice. Mycopathologia.;114(2):71-6, 1991.

21.Whitcomb MP, Jeffries CD, Weise RW. Curvularia lunata in experimental phaeohyphomycosis Mycopathologia. 7;75(2):81-8, 1981

22. Tuttobello L, De Bernardis F, Morelli L. Experimental pathogenicity of Cryptococcus cereanus in mice. Boll Soc Ital Biol Sper. 28;60(2):355-61, 1984

23. Khan ZU, Misra VC, Randhawa HS, Damodaran VN Pathogenicity of some ordinarily harmless Sabouraudia.;18(4):319-27. yeasts for cortisone-treated mice, 1980.

24. López-Martínez R, Toriello C, Mier T, Ximénez-García C, Martínez A, Fernández-Diez J. Study of the pathogenicity of Conidiobolus coronatus in experimental animals.Mycopathologia. 29;66(1-2):59-65, 1978.

25. Seok et al., J. Seok, H.S. Warren, A.G. Cuenca, M.N. Mindrinos, H.V. Baker, W. Xu, D.R. Richards, G.P. McDonald-Smith, H. Gao, L. Hennessy, C.C. Finnerty, C.M. Lopez, S. Honari, E.E. Moore, J.P. Minei, J. Cuschieri, P.E. Bankey, J.L. Johnson, J. Sperry, A.B. Nathens, T.R. Billiar, M.A. West, M.G. Jeschke, M.B. Klein, R.L. Gamelli, N.S. Gibran, B.H. Brownstein, C. Miller-Graziano, S.E. Calvano, P.H. Mason, J.P. Cobb, L.G. Rahme, S.F. Lowry, R.V. Maier, L.L. Moldawer, D.N. Herndon, R.W. Davis, W. Xiao, R.G. Tompkins Genomic responses in mouse models poorly mimic human inflammatory

diseases. Proc. Natl. Acad. Sci. U.S.A., Volume 110, pp. 3507–3512. 2013.

26. Ortoneda et al., 2004,M. Ortoneda, J. Guarro, M.P. Madrid, Z. Caracuel, M.I. Roncero, E. Mayayo, A. Di Pietro Fusarium oxysporum as a multihost model for the genetic dissection of fungal virulence in plants and mammals.Infect. Immun., Volume 72, , pp. 1760–1766,2004.

27. Gupta, J., Pathak, B., Sethi, N., and Vora, V. C. Histopathology of Mycotoxicosis produced in Swiss albino mice by metabolites of some fungal isolates. Appl Environ Microbiol. 41[3], 752-757, 1981.

28. Guarro, J., Soler, L., and Rinaldi, M. G. Pathogenicity and antifungal susceptibility of Chaetomium species. Eur J Clin Microbiol Infect Dis. 14[7], 613-618, 1995.

29 Fernández-Silva F1, Capilla J, Mayayo E, Sutton D, Guarro J. Experimental murine acremoniosis: an emerging opportunistic human infection.. Med Mycol.;52(1):29-35. doi: 10.3109/13693786.2013.797610, 2014 .

30. Schäfer K1, Di Pietro A1, Gow NA2, MacCallum D2. Fusarium PLoS One. Murine model for Fusarium oxysporum invasive fusariosis reveals organ-specific structures for dissemination and long-term persistence.2014 27;9(2):e89920. doi: 10.1371/journal.pone.0089920. eCollection 2014.

31. K.V. Clemons, D.A. Stevens.The contribution of animal models of aspergillosis to understanding pathogenesis, therapy and virulenceMed. Mycol., Volume 43, Issue Suppl 1, S101-10pp. 2005

32. Amran F, Aziz MN, Ibrahim HM, Atiqah NH, Parameswari S, Hafiza MR, Ifwat M. In vitro antifungal susceptibilities of Candida isolates from patients with invasive candidiasis in Kuala Lumpur Hospital, Malaysia. J Med Microbiol. 2011;60(Pt 9):1312-6. doi: 10.1099/jmm.0.027631-0. Epub 2011

33. Drogari-Apiranthitou M, Mantopoulou FD, Skiada A, Kanioura L, Grammatikou M, Vrioni G, Mitroussia-Ziouva A, Tsakris A, Petrikkos G.In vitro antifungal susceptibility of filamentous fungi causing rare infections: synergy testing of amphotericin B, posaconazole and anidulafungin in pairs. J Med Microbiol. 2011;60(Pt 9):1312-6. doi: 10.1099/jmm.0.027631-0. Epub 2011.

34. Shang ST, Lin JC, Ho SJ, Yang YS, Chang FY, Wang NC. The emerging life-threatening opportunistic fungal pathogen Kodamaea ohmeri: optimal treatment and literature review. J Clin Diagn Res.; 9(3): DD01–DD03, 2015.