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Variability analyses of functional domains within glucosamine-6-phosphate synthase of mycoses-causing fungi

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Abstract
The immunosuppressive individuals are highly prone to get afflicted with invasive opportunistic fungal infections such as Candidiasis, Aspergillosis, Histoplasmosis, Coccidioidomycosis, Blastomycosis, Penicilliosis, Cryptococcosis and Zygomycosis which are becoming a cause of concern to the mankind due to their high morbidity and mortality rates. The existing antifungal agents are not completely effective due to their severe side-effects and recurrent drug resistance in fungi. Hence, there is an urgent need to develop newer and better antifungal drugs. The enzyme Glucosamine-6-phosphate (G-6-P) synthase catalyzes the rate-limiting step of the fungal cell-wall biosynthetic pathway and targeting it can inhibit the growth of the fungus. The present study attempts to investigate the inherent variations in functional domain *viz*. Glutaminase (GATase II) and Sugar Isomerising (SIS) of Glucosamine-6-phosphate (G-6-P) synthase enzyme of mycoses-causing fungi**.** These domains may be identified as probable active site(s). Multiple sequence alignment performed using ClustalX2 and construction of phylogenetic tree of individual domains by MEGA v5.0 helped in the analyses of several variable amino acid sites within the domains suggesting their vital role in the pathogenesis of the fungi. Further, the online server ConSurf implied that mostly, the highly conserved residues of the domains were functional and exposed on the surface of the active site, making it an easy target for the drugs. Consequently, variable analysis of functional domains of target implicated the importance of target specific drug discovery for the treatment of invasive fungal infections or mycoses.

Keywords: Glucosamine-6-phosphate synthase, mycoses, variability analyses, active site, phylogenetics

Background:
An increasing number of immunocompromised patients **[1]** suffering largely from AIDS, cancer and undergoing organ transplants or immunosuppressive therapy are getting afflicted with invasive fungal infections, better known as mycoses. Such infections *viz.* Candidiasis, Aspergillosis, Histoplasmosis, Coccidioidomycosis, Blastomycosis, Penicilliosis, Cryptococcosis and Zygomycosis are caused by fungi belonging to different phyla, *i.e.* Ascomycetes, Basidiomycetes and Zygomycetes. The disease management involves treatment with antifungal drugs, and even surgery at times **[2]**. However, drugs such as Amphotericin B, which is the first-line drug of choice for invasive mycoses is nephrotoxic and hepatotoxic. Besides, the fungi are acquiring recurrent resistance against the existing antifungal agents, rendering them less effective. Therefore, there is a rapid requirement for the development of effective antifungal drugs for mycoses at large, for which a potential drug target could be found in the housekeeping genes which are highly conserved throughout species and could be studied further to carry out thorough analyses of the catalytic active site before a lead compound can be selected by the drug discovery process.

The enzyme, Glucosamine-6-Phosphate (G-6-P) synthase [EC 2.6.1.16] is ubiquitously present and highly conserved in mycoses-causing fungi. It is known to catalyze the rate-limiting step of the fungal cell-wall biosynthetic pathway and inhibiting it can delimit the growth of the fungus. The enzyme consists of two domains, the Glutaminase (GATase II) domain and the Sugar Isomerising (SIS) domain where the probable active site(s) can be identified. For instance, previous studies in *E. coli* have suggested the active sites to lie within the two domains of G-6-P synthase **[3]**. The drug discovery protocols frequently consider selecting the highly conserved functional motifs against which drugs could be designed. But the increasing ineffectiveness, toxicity and fungal resistance towards existing antifungal drugs have raised an issue to find inherent amino acid variations within the domains and its influence on the active site(s) for mycoses-causing fungi. The amino acid sequences of various mycoses-causing fungi were collected from various databases followed by their phylogenetic analysis subsequent to multiple sequence alignment and further detection of their functional residues. Consequently, this study will aid drug discovery for mycoses-causing fungi in the near future. The availability of sequenced fungal genomes has greatly contributed to this study.

Methodology:
Amino acid sequences of G-6-P synthase of mycoses-causing fungi as obtained from World Health Organization, listed in **Table 1 (see Supplementary material)** were retrieved from Broad Institute of MIT and Harvard fungal

database **[4]**, NCBI fungal database **[5]** and The DOE Joint Genome Institute fungal database. A total of 20 sequences were retrieved and compiled from these databases. The online server PFAM was used to investigate protein domains present within the enzyme. Subsequently, the sequences were manually separated according to the domains predicted by PFAM. Further, the protein sequences were aligned using the offline software CLUSTALX2 **[6]**. MEGA v5.0 **[7]** was employed to infer the evolutionary history using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated. Additionally, the functionally important amino acids were identified using ConSurf webserver.

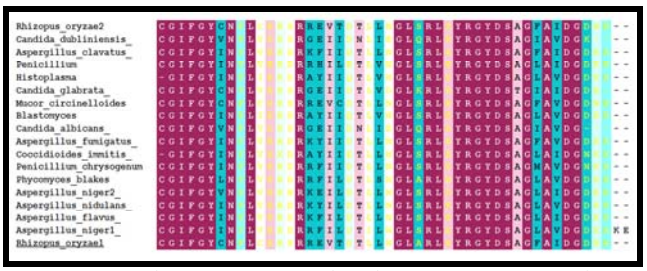

Figure 1: ConSurf color-coded MSA of the first GATase II domain of G-6-P synthase using amino acid sequences of mycoses-causing fungi. The pink to blue color gradient represent highly conserved to variable regions of the domain. The yellow marked amino acids indicate that the calculation for the particular site was performed on less than 10% of the sequences.

Discussion:

The online server PFAM yielded the inherent protein domains lying within G-6-P synthase *viz.* two GATase II domains and two SIS domains in mycoses-causing fungi where the probable active sites are found. Subsequently, the MSA results (**Figure 1** showing partial results) obtained from ConSurf showed few variable sites within the highly conserved regions which are being investigated in the present study, along with the more evolutionary affected regions. The GATase II domains comprised 48 and 140 amino acid residues and the SIS domains comprised 127 and 146 residues. Position 18 of the first GATase II domain and position 51 of second SIS domain observed zygomycetous fungi having isoleucine replaced with a valine and valine replaced with isoleucine, respectively. Hence, for drugs targeting zygomycosis, it should be able to bind to these sites apart from the highly conserved residues for a better efficacy. At position 21 of the first GATase II domain, threonine has been replaced by asparagine in the case of *C. albicans* and *C. dubliniensis.* During analysis of the second GATase II domain, it was found out that lysine has taken the place of serine in all *Candida* species at position 24. Position 8 and 123 has asparagine instead of serine and glycine instead of alanine, respectively for all *Candida* species except *C. glabrata.* Since, Candidiasis is the most prevalent antifungal infection which is mainly caused by *C. albicans*, it is possible that threonine, lysine, asparagine and glycine may play a pivotal role in the pathogenesis of *Candida* species. *C. tropicalis* and *Cryptococcus neoformans* do not encompass the first GATase II domain. Furthermore, the only commonality found between zygomycetes and basidiomycetes is at position 12 and 73 of the MSA of second GATase II domain, where methionine and valine replaced isoleucine, respectively and at position 38 of second SIS domain where isoleucine is present instead of valine. Sites 66 and 107 of second GATase II domain and sites 35, 70 and 72 of second SIS domain showed that all A*spergillus* species share common residues with *Penicillium* species, *H. capsulatum, Blastomyces* and *C. immitis.* Aspergillosis has been known to be a fatal disease after Candidiasis, but it is not the case with Penicilliosis, Blastomycosis, Histoplasmosis and Coccidiomycosis; hence, these sites might not have a lethal role in the fungus. The first SIS domain is observed to be a highly conserved region among all species with no variability sites within the conserved regions. Hence, targeting this site can be upheld to the drugs' efficacy. According to literature, cysteine 1 of G-6-P synthase in *E. coli* **[3]** and cysteine 2 of the same enzyme in *Volvariella volvacea* **[8]** have significant role in the enzyme catalysis. The online server ConSurf predicted the functional amino acid residues for *R. oryzae* RO3G_04247.3. Results reveal that cysteine residue at position 1 is highly conserved and exposed on the active site, making it easily targetable by drugs. Thus, it can be seen that conserved residues generally comprising the active site are mostly functional and central to drug binding.

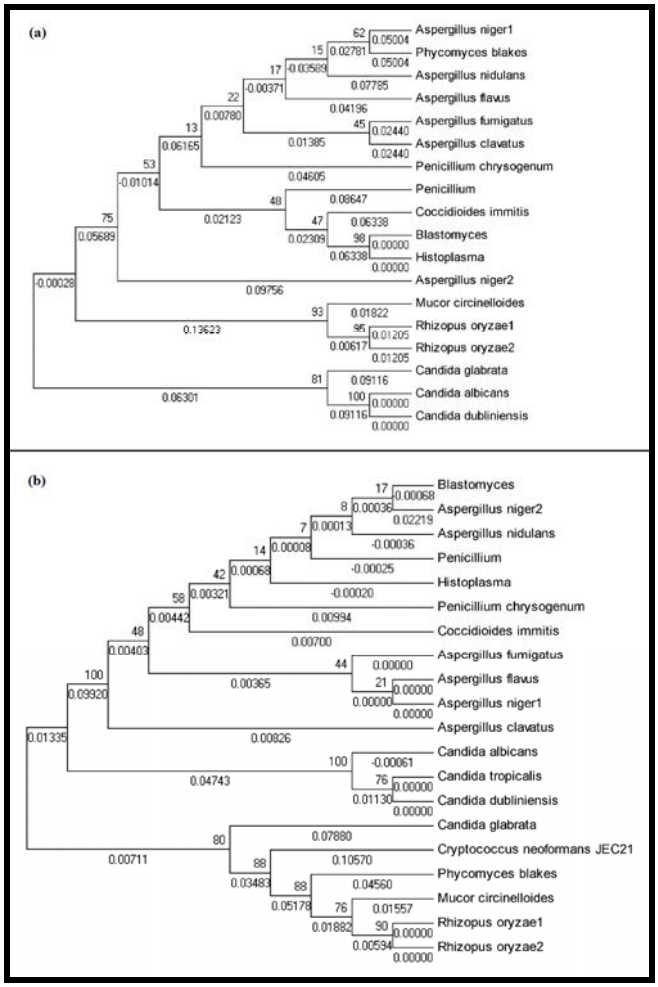

Figure 2: A Neighbour-Joining phylogenetic tree from amino acid sequences of **(a)** first GATase II domain and **(b)** first SIS domain of mycoses-causing fungi, constructed using MEGA v5.0 with 1000 bootstrap replicates. The evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated.

Moreover, phylogenetic tree for individual domains was constructed as well **(Figure 2)** using MEGA v5.0 with 1000 bootstrap replicates which helped in understanding the evolutionary relationship of the various functional domains in mycoses-causing fungi. All the four trees constructed individually for each domain grouped *Candida* species together, with *C. albicans* and *C. dubliniensis* being closer to each other than the rest suggesting that all the *Candida* species could be targeted with one effective drug. *A. niger 2* lies quite separately from the rest of the *Aspergillus* species, implying that it might be more resistant to antifungal drugs. Consequently, variations within the G-6-P synthase of *A. niger 2* could reveal answers to increasing resistance to drugs, and accordingly, newer lead compounds could be designed. Zygomycetes, *R. oryzae* and *M. circinelloides* are grouped at the bottom of the tree and single drug needs to be developed to be effective against zygomycetes causing zygomycosis.

Conclusion:

Domain variability analyses of G-6-P synthase in mycoses-causing fungi were carried out because of increasing fungal resistance towards antifungal drugs, and consequently, there is a requirement for better and more effective drugs. The current study was aimed to investigate the variable regions within the conserved domains of the enzyme which might affect its active site and subsequently, alter the drug binding, since a single amino acid residue can augment or reduce the drug binding to the target enzyme. Our study revealed specific residues which might play significant roles in the fungal pathogenesis. Hence, variant analyses implicate the binding efficacy between the receptor and ligand and direct the new direction in effective drug development through pharmacophore modelling and structure-activity relationships. The work may

be enhanced by incorporating the ensemble approach of variability analysis on target site for mycoses-causing fungi.

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Supplementary material:

Table 1: List of mycoses-causing fungi used in the present study as obtained from World Health Organization

Fungi name	Database ID	Protein size
*Rhizopus oryzae*_1	RO3G_04247.3 (Broad institute of Harvard and MIT)	688 aa
*Rhizopus oryzae*_2	RO3G_14807.3 (Broad institute of Harvard and MIT)	688 aa
Mucor circinelloides	JGI ID: 50270	688 aa
Phycomyces blakesleeanus	JGI ID: 37053	692 aa
Candida albicans	NCBI-GeneID: 3636557	713 aa
Candida tropicalis	NCBI-GeneID: 8301698	686 aa
Candida dublinieasis	NCBI-GeneID: 8046592	711 aa
Candida glabrata	NCBI-GeneID: 2890320	723 aa
Aspergillus nidulans	NCBI-GeneID: 2872076	898 aa
Aspergillus fumigatus	NCBI-GeneID: 3508772	694 aa
Aspergillus flavus	NCBI-GeneID: 7919759	693 aa
*Aspergillus niger*_1	NCBI-GeneID: 4990250	694 aa
*Aspergillus niger*_2	NCBI-GeneID: 4980582	702 aa
Aspergillus clavatus	NCBI-GeneID: 4705325	694 aa
Coccidioides immitis	NCBI-GeneID: 4562337	716 aa
Cryptococcus neoformans	NCBI-GeneID: 3256900	706 aa
Penicillium chrysogenum	NCBI-GeneID: 8315998	694 aa
Penicillium marneffei	NCBI- GeneID: 212543329	694 aa
Blastomyces dermatitidis	BDBG_01706.1	694 aa