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In Vitro Antifungal Susceptibility Profile and Correlation of Mycelial and Yeast Forms of Molecularly Characterized *Histoplasma capsulatum* Strains from India

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The antifungal susceptibility profiles of the mycelial and yeast forms of 23 *Histoplasma capsulatum* strains from pulmonary and disseminated histoplasmosis patients in India are reported here. The MIC data of this dimorphic fungus had good agreement between both forms for azoles, amphotericin B, and caspofungin. Therefore, the use of mycelial inocula for *H. capsulatum* antifungal susceptibility testing is suggested, which is less time-consuming vis-à-vis the yeast form, which requires 6 to 8 weeks for conversion.

Histoplasma capsulatum is the causative agent of histoplasmosis, a disease that is endemic in the Americas, Asia, and Africa, with sporadic cases reported worldwide (1). The clinical manifestations range from acute pulmonary to disseminated histoplasmosis, with histoplasmosis developing in immunocompromised patients (2). Therapy with amphotericin B and itraconazole is commonly used for the disseminated and mild to moderate forms of histoplasmosis, respectively (3). However, treatment with itraconazole and amphotericin B is limited by variable absorption/metabolism and toxicity, respectively. Treatment is often noncurative, and relapse and recrudescence can occur even while patients are on therapy (4). Furthermore, *in vitro* antifungal susceptibility testing of this dimorphic fungus remains unstandardized. In addition, no consensus exists regarding testing of the pathogenic yeast form, which occurs in tissues, or the saprotrophic mold form present in the environment, which causes infection by inhaling microconidia. Differences in the antifungal susceptibilities of the two forms of this dimorphic pathogen were suggested in a solitary report of 4 isolates of *H. capsulatum*, revealing low MICs for fluconazole and high MICs for micafungin with the yeast form vis-à-vis the mycelial form (5). We report here the *in vitro* antifungal susceptibility profiles against 8 antifungals of both the mycelial and yeast forms of 23 molecularly characterized *H. capsulatum* strains from patients in India with pulmonary and disseminated histoplasmosis. Further, we correlated the MICs of *H. capsulatum* with clinical outcome.

Twenty-one *H. capsulatum* var. *capsulatum* isolates originating from 19 (1.9%) patients were cultured from 1,261 clinical specimens of 952 patients with pulmonary or systemic infection from tertiary care hospitals in northwestern India during 2010 to 2013. The clinical specimens comprised blood, bone marrow, bronchoalveolar lavage fluid, lung and skin biopsy specimens, lymph node aspirates, and sputum. The isolates, along with 2 reference strains (*H. capsulatum* CDC B-5324 and ATCC 66368), were subjected to yeast conversion at 37°C on brain heart infusion (BHI) slants containing glutamine. The isolates were serially transferred onto fresh BHI slants every 5 to 6 days, and after 8 to 10 transfers, they revealed small (2 to 5 μm in diameter) yeast-like cells with narrow-based budding in 6 to 8 weeks. Sequencing of the D1/D2 large subunit (LSU) region of the isolates was done using the NL-1 and NL-4 primers, as described previously (26). GenBank searches of the sequences showed 99% identity with *Ajellomyces capsulatus* (GenBank accession no. HM595605.1).

Antifungal susceptibility testing (AFST) of both forms was determined by broth microdilution using CLSI documents M27-A3 and M38-A2 (6, 7). The 8 to 10 days of hyphal growth on Sabouraud's dextrose agar (SDA) at 28°C was used for preparing the inoculum for the mycelial form, which was adjusted to an optical density at 530 nm of 0.20 to 0.24, diluted 1:10 in RPMI 1640 medium to obtain 2.5×10^5 to 5×10^5 conidia/hyphal fragments per ml. The plates were incubated at 28°C for 96 to 120 h. The yeast inocula, prepared from growth on BHI agar, were adjusted to 5 McFarland standard, diluted 1:100 in RPMI 1640 to obtain 1×10^5 to 2.5×10^5 CFU/ml, and the plates were incubated at 37°C for 72 to 96 h (8). The antifungals tested were amphotericin B (Sigma, St. Louis, MO, USA), fluconazole (Pfizer, Groton, CT, USA), itraconazole (Lee Pharma, Hyderabad, India), voriconazole (Pfizer), posaconazole (Merck, Whitehouse Station, NJ, USA), isavuconazole (Astellas, USA), 5-flucytosine (Sigma), and caspofungin (Merck). The final concentrations of the drugs ranged from 0.125 to 64 μg/ml for fluconazole and 5-flucytosine, 0.03 to 16 μg/ml for amphotericin B, itraconazole, and voriconazole, and 0.015 to 8 μg/ml for posaconazole, isavuconazole, and caspofungin. The CLSI-recommended reference strains *Aspergillus fumigatus* ATCC 204305 and *Paecilomyces variotii* ATCC 3630, were included with each test of the mycelial form, and *Candida krusei* strain ATCC 6258 and *Candida parapsilosis* strain ATCC 22019 were used for testing the yeast form. The MIC endpoints were read visually and for azoles and 5-flucytosine were defined as the lowest concentration yielding 80% inhibition of growth for the yeast form and 50% inhibition of growth for the mycelial form compared with the

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TABLE 1 *In vitro* antifungal susceptibility profile of mycelial and yeast forms of 23 clinical strains of *H. capsulatum* against 8 antifungal drugs

H. capsulatum form	Parameter(μg/ml)	Data for antibiotic[a]:							
		AMB	ITC	VRC	POS	ISA	CAS[b]	FLU	5-FC
Mycelial	GM	0.11	0.043	0.102	0.05	0.055	0.097	7.05	64
	MIC_{50}/MEC_{50}	0.125	0.03	0.125	0.06	0.06	0.125	8	32
	MIC_{90}/MEC_{90}	0.25	0.125	0.25	0.125	0.25	0.25	16	>64
	Range	0.03 to 0.25	<0.03 to 0.125	<0.03 to 0.25	0.015 to 0.125	0.015 to 0.25	0.015 to 0.5	2 to 32	8 to >64
Yeast	GM	0.13	0.051	0.17	0.085	0.04	0.17	4.56	48.2
	MIC_{50}	0.125	0.06	0.25	0.06	0.06	0.25	4	16
	MIC_{90}	0.25	0.125	0.5	0.25	0.125	0.5	8	64
	Range	0.03 to 0.5	0.03 to 0.25	0.03 to 0.5	0.03 to 0.5	0.015 to 0.125	0.03 to 1	2 to 8	8 to 64
Essential agreement (%)[c]		100	100	100	87	100	96	100	100

[a] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole; CAS, caspofungin; FLU, fluconazole; 5-FC, flucytosine.
[b] For caspofungin, the minimal effective concentration (MEC) was read for testing the mycelial form.
[c] MIC discrepancies of more than two dilutions were used to calculate the essential agreement.

growth of the drug-free control wells. For amphotericin B, 100% inhibition of growth was considered for both forms. For caspofungin, the minimal effective concentration (MEC) against the mycelial form was defined as the lowest concentration of drug that led to the growth of small, rounded, and compact hyphal forms, whereas the MIC of the yeast form was defined as 50% inhibition. The geometric mean of MICs/MECs (GM) and MICs/MECs at which 50% (MIC_{50}/MEC_{50}) and 90% (MIC_{90}/MEC_{90}) of the tested isolates were inhibited for each drug were determined (8, 9). Furthermore, viability testing of the mycelial growth was done by streaking 20 μl of each suspension from the well, which showed complete inhibition, as well as the preceding two lower dilutions on SDA plates, and incubation at 28°C. The MICs/MECs of the quality control strains were in the CLSI-recommended ranges, and for all isolates, the drugs tested revealed reproducible MICs/MECs when performed by different personnel on two occasions, revealing only 1-fold difference in the dilutions. All procedures involving sporulating cultures of *H. capsulatum* were performed inside a class II biological safety cabinet under conditions of biosafety level 3 (BSL3) containment (10).

The results of AFST and the essential agreement between the two forms of *H. capsulatum* are presented in Table 1. The mycelial and the yeast forms of the isolates were inhibited by itraconazole, posaconazole, voriconazole, amphotericin B, and the new drug isavuconazole. Previously, a solitary study reported isavuconazole MICs of the mold form of 28 *H. capsulatum* isolates (9). Additionally, 13% of the yeast forms revealed high caspofungin MICs of ≥0.5 μg/ml, which is in consonance with Kohler et al. (11), who demonstrated that caspofungin is not effective against this fungus. All the isolates in this study had high MICs for fluconazole and 5-flucytosine. Although the established treatment options for *H. capsulatum* include azoles, amphotericin B, and echinocandins, no comparative trials of these agents have been performed. Fluconazole treatment failure has been reported in cases of histoplasmosis, being partially attributed to isolates that demonstrated drug MICs of >5 μg/ml (8). However, in a mouse model, divergent observations suggesting fluconazole to be more efficacious against histoplasmosis caused by a fluconazole-resistant strain than by a fluconazole-susceptible strain have been reported (12). Further, there are studies reporting disagreements between *in vitro* and *in vivo* activities against *H. capsulatum* for caspofungin using either the yeast or mold form (11, 13). Notwithstanding the fact that the host immune status plays a vital role in patient recovery, susceptibility testing of dimorphic fungi yields basic information pertaining to its resistance and estimation of clinical value of the therapeutic agent. Therefore, emphasis on a standardized method for *in vitro* susceptibility testing and *in vivo* studies correlating both growth forms with the therapeutic outcome in dimorphic fungi is needed.

Of the 19 histoplasmosis cases, the therapy and outcome records of 18 patients were available, which included 12 disseminated, 4 chronic, and 2 acute pulmonary histoplasmosis cases. Out of the 12 disseminated cases, 6 patients died due to a misdiagnosis of tuberculosis with multiple courses of antituberculosis therapy. Of the remaining 6 disseminated cases, 2 patients died after treatment with amphotericin B deoxycholate (AMB) (1 mg/kg of body weight) for 1 to 2 weeks. The remaining 4 were treated with AMB for 2 weeks, followed by 200 mg itraconazole twice daily for 6 to 12 months. The 6 pulmonary histoplasmosis cases were treated with 200 mg itraconazole twice daily for 3 months and 12 months for acute and chronic cases, respectively. These patients had no recrudescence after 12 to 18 months of follow-up.

The AFST data for *H. capsulatum* so far have been reported predominantly from the United States and Mexico, and a single report is from Brazil (Table 2) (8, 9, 11, 12, 14–22). Although the disease is endemic in Asia, including India, there are a paucity of data on AFST and the treatment outcome of the pathogen from this part of the world. Unlike in North and South America, disseminated disease in India is predominantly seen in HIV-negative patients who are often misdiagnosed as having tuberculosis and, consequently, are not correctly treated (23, 24). In the present study, all disseminated cases were HIV negative. Notably, working with the mycelial morphotype, which is the infective form, requires BSL3 containment conditions, which are cumbersome (10). AFST studies for testing the mycelial form are limited, and no comparison has been made in the susceptibility differences with the parasitic yeast form (5, 11, 12, 14, 20, 21). The mycelial phase transforms to the yeast phase within 48 h of infection, suggesting that outcome relates more to the antifungal activity to the yeast phase than to the mycelial form (5). However, the mycelial form of a dimorphic fungus can coexist with yeast cells *in vivo* and

TABLE 2 *In vitro* antifungal susceptibility profile of *H. capsulatum* isolates reported globally

Location of isolation by continent	Yr of isolation	No. investigated (+ no. of reference strains investigated) (dimorphic form)[a]	Method used (source)	MIC/MIC range (μg/ml) for[b]:								Reference or source
				AMB	ITC	VRC	POS	ISA	FLU	CAS/MFG/AFG[c]	RAV	
North America												
St. Louis, MO	1990	8 + 4 (Y)	Broth microdilution using SDB[d]	0.30 to 1.04					2.95 to >1,000			12
Indiana	1997	1 (Y)	Broth macrodilution (NCCLS)		0.004				0.62 (parent), 1.25 (8th wk), 2.5 (12th wk), 20 (16th wk)			14
Virginia	1998	5 (M)	Broth microdilution (NCCLS)	0.25 to 0.5	0.06	0.06						15
Virginia	1998	5 (M)	Broth microdilution (NCCLS)				0.04			CAS, 1.3; AFG, 3.6		16
Indiana	2000	20 (Y)	Modified broth microdilution (NCCLS M-27A)	0.5 to 1						8 to 32		11
Texas	2000	100 (M)	Modified broth microdilution (NCCLS M-27A)	<0.03 to 2	<0.03 to 0.5	<0.03 to 2						17
Indiana	2001	65 (M)	Modified broth microdilution (NCCLS M-27A)		0.019 to 0.077				0.31 to 10			8
Virginia	2003	4–5 (M)	Broth macrodilution (NCCLS M38-A)	0.06 to 0.5	<0.01 to 0.03		<0.03 to 0.06		1 to 16	CAS, 0.5 to 4; AFG, 2 to 4; MFG, >0.03 to 0.06		18
Mexico	2005	28 (M)	Broth macrodilution (NCCLS M38-A)		0.06 to 1	0.06 to 2	0.03 to 2		2 to 32		0.125 to 2	19
Indiana	2006	17 (median MIC) (Y)	Broth microdilution (NCCLS M-27A)			0.015	0.007		1		0.007	20
Mexico	2009	28 (M)	Broth macrodilution (CLSI M38-A)	0.06 to 0.25	0.25 to 2	0.06 to 2	0.03 to 2	0.125 to 2	4 to 32			9
South America												
Brazil	2012	68 (M)	Modified broth microdilution (NCCLS M38-A)	0.007 to 0.5	0.001 to 0.031	0.0078 to 0.5			3.9 to 125	0.016 to 32		21
		8 (Y)	Modified broth microdilution (CLSI M27-A2)	0.06 to 0.5	0.0039 to 0.03	0.002 to 0.03			3.9 to 7.8	1 to 4		
Asia												
Japan	2010	3 (M)	Modified broth microdilution (CLSI M38-A)	0.013 to 0.05	<0.0004	0.006 to 0.025		0.003 to 0.006	0.55 to 1.2			22
India	2014	21 + 2 (M)	Modified broth microdilution (CLSI M38-A2)	0.03 to 0.25	<0.03 to 0.125	<0.03 to 0.25	0.015 to 0.125	0.015 to 0.25	2 to 32	0.015 to 0.5		Present study
		21 + 2 (Y)	Modified broth microdilution (CLSI M27-A3)	0.03 to 0.5	0.03 to 0.25	0.03 to 0.5	0.03 to 0.5	0.015 to 0.125	2 to 8	0.03 to 1		

[a] M, mycelial form; Y, yeast form.
[b] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole; FLU, fluconazole; CAS, caspofungin; RAV, ravuconazole; MFG, micafungin; AFG, anidulafungin.
[c] For caspofungin, MEC were defined for testing the mycelial form.
[d] SDB, Sabouraud's dextrose broth.

might be more virulent and invasive, leading to dissemination (25).

The present comprehensive study reports the MIC data of a large number of isolates and revealed good essential agreement with the MICs of both the forms, which were 87 to 100% for azoles, 100% for amphotericin B, and 96% for caspofungin. The use of mycelial inocula for *H. capsulatum* AFST testing in future studies is suggested, considering that testing of the mycelial form is less time-consuming (12 to 15 days) vis-à-vis that for the yeast form (6 to 8 weeks), which requires cumbersome conversion and is prone to contamination.

Nucleotide sequence accession numbers. The LSU sequences of 21 *H. capsulatum* isolates are deposited in GenBank with accession no. KJ653230 to KJ653245 and KJ939255 to KJ939260.

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