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Effect of Reduced Oxygen on the Antifungal Susceptibility of Clinically Relevant Aspergilli

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The influence of hypoxia on the *in vitro* activities of amphotericin B, azoles, and echinocandins against *Aspergillus* spp. was evaluated by comparing MICs, minimal fungicidal concentrations (MFCs), and epidemiological cutoffs (ECOFFs). Changes of MIC distributions due to hypoxia largely depend on the method, the species, and the growth ability under hypoxia. The activities of antifungals were not significantly altered under hypoxia, except for *Aspergillus terreus*, for which the activity changed from fungicidal to fungistatic.

At sites of infection, microenvironmental factors influence the growth of fungal pathogens and most likely also the efficacy of antifungal drugs (1). Hypoxia is one microenvironmental stress that occurs during pulmonary fungal infections *in vivo* (2) and has a significant impact on antifungal targets such as ergosterol biosynthesis or β-glucan in *Aspergillus fumigatus* (3, 4). Simulating the host environment in *in vitro* susceptibility testing will contribute to a better understanding of how these conditions influence antifungal activity.

In this study, the *in vitro* activities of amphotericin B, triazoles, and echinocandins against *Aspergillus* spp. under hypoxia were evaluated by using the Etest (bioMérieux, France) and broth microdilution method according to EUCAST guideline 9.2 (5). Epidemiological cutoff values (ECOFFs) were established and set two dilution steps higher than the modal MIC (6). Both methods were chosen to verify the different impacts of oxygen on surface (exposure to 1%) or liquid cultures, where the oxygen concentration might also vary in normoxic cultures. Putative changes from fungicidal to fungistatic activity were determined based on minimal fungicidal concentrations (MFCs) (7), defined as the lowest drug concentration resulting in 99.9% killing.

All clinical isolates tested ($n = 49$) were identified by internal transcribed spacer sequencing, according to the methods of White et al. (8). The strain set comprised *A. fumigatus* ($n = 25$), including five azole-resistant isolates with a mutation in *cyp51A* (9), *A. terreus* ($n = 16$), and *A. flavus* ($n = 8$). Hypoxic conditions were set to 1% O_2, 5% CO_2, 94% N_2 (C-Chamber and Pro-Ox, Pro-CO_2 controller; Biospherics), and all experiments were done in parallel under normoxia (~21% O_2). To check for a normal distribution, the D'Agostino and Pearson omnibus normality test was performed. The Kruskal-Wallis test was applied, since data were not normally distributed. *P* values of ≤0.05 were regarded as statistically significant. For supplemented media, ergosterol or cholesterol (25 μM) was mixed with coenzyme Q_{10} (5 μM) and added to RPMI agar. Additionally, Etests were conducted on blood agar (25% [vol/vol]). To compare fungal growth under both oxygen conditions, radial growth assays were performed according to methods described previously (10).

With the Etest, the influence of hypoxia on the susceptibility profile demonstrated a species- and drug-dependent manner (Fig. 1; Table 1). Among all *Aspergillus* spp. tested, *A. fumigatus* isolates exhibited the lowest oxygen-dependent changes in MICs for all antifungals tested. A significant reduction of the MIC distribution was observed for amphotericin B, while no alterations in MICs for azoles and echinocandins were detected. *A. fumigatus* strains carrying a mutation in the *cyp51A* gene did not show differences in azole susceptibility. *Aspergillus terreus* isolates, a species that is intrinsically resistant to amphotericin B (11, 12), exhibited susceptibility under hypoxia, with a significant decrease in the MIC distribution (12 $\log_2$ dilutions). Lower MICs were mainly due to the missing *mycelium sterilium* zone (Fig. 1). For the azoles, a significant reduction in the MIC distribution was observed under hypoxia while, as for *A. fumigatus*, no alteration was detected for echinocandins. The same results were found for *A. flavus*. Reductions in MICs under hypoxia were abrogated by addition of ergosterol, cholesterol, or whole blood to the medium (Fig. 2). MIC changes under hypoxia correlated with impaired growth under hypoxia; the *in vitro* susceptibilities of fungi that were less sensitive to low oxygen concentrations were less affected (Fig. 3).

In broth microdilution assays, MICs of voriconazole and posaconazole were not altered under hypoxia for all *Aspergillus* spp. tested (Table 2). For only amphotericin B was a stepwise decrease in MICs (≤2 $\log_2$ dilutions) under hypoxia prominent for *A. terreus* and *A. flavus* strains, while no difference was detected for *A. fumigatus* strains. Minimal effective concentrations (MECs) for caspofungin were not significantly influenced by hypoxia.

MFCs demonstrated no alterations between hypoxic and normoxic conditions for *A. fumigatus* and *A. flavus* strains (Table 2), and this correlated with already published data (7). For *A. terreus* strains, either increased or no MFCs were detected for azoles under hypoxia. Similarly, significantly more colonies were able to recover from cultures treated with amphotericin B under hypoxia,

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although no MFCs could be determined under either oxygen condition.

So far, only a few studies have investigated the effect of hypoxia on antifungal susceptibility of *Aspergillus* spp., focusing either on some antifungal agents (13) or on one standard *in vitro* test method (7). Similar to what was shown for anidulafungin (13), MIC/MEC readings were much easier to obtain under hypoxia, as typical "trailing" (microcolonies within the inhibition zone [14]) was less pronounced for echinocandins. The observed reductions in the MICs, being more pronounced with the agar-based method than in liquid assays, matched results obtained by Warn et al. (7) and might have been due to oxygen depletion in microtiter plates, even under normoxia. Increased susceptibility to antifungals that target ergosterol itself or its biosynthesis (oxygen-dependent pathway [3]) indicated that the fungus has to cope with two stressors: antifungal pressure and maintenance of membrane stability, despite lacking oxygen as a cofactor for ergosterol biosynthetic enzymes. Additionally, MICs under hypoxia rose to the levels of those under normoxia when membrane compounds were available. Xiong et al. (15) demonstrated that cholesterol is integrated into fungal membranes to compensate for ergosterol depletion during azole treatment. Further, cholesterol can be used as a putative carbon source in filamentous fungi (16) and thereby enhance growth.

Except for *A. terreus*, MFCs were less influenced by oxygen than were MICs of surface cultures. This may even better reflect the actual situation in the host, as Rex et al. (17) already suggested that MFCs are more relevant for predicting the clinical outcome. For *A. terreus*, no MFCs were detectable, suggesting a shift to fungistatic activity under low-oxygen conditions. Slesonia et al. (18) showed that *A. terreus* is able to persist and survive without germination within acidified phagolysosomes due to the resistance against microbicidal enzymes. Also, conidia are more resistant to environmental conditions than are hyphae (19). Therefore, delayed germination, especially after diluting the antifungal agent by plating on agar, could contribute to enhanced resistance against antifungal drugs under hypoxia.

In conclusion, hypoxia influenced *in vitro* antifungal susceptibilities of *Aspergillus* spp. marginally, and observed differences were most pronounced with the Etest. Importantly, changes in antifungal activities against *A. terreus* strains under hypoxia might partially explain the high failure rate of antifungal therapy *in vivo* (12, 20).

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TABLE 1 *In vitro* susceptibilities to amphotericin B, azoles, and echinocandins of *Aspergillus* species, determined by Etest[a]

Agent[b]	*A. fumigatus* isolates (n = 26)						*A. terreus* isolates (n = 14)						*A. flavus* isolates (n = 8)					
	Normoxia			Hypoxia			Normoxia			Hypoxia			Normoxia			Hypoxia		
	MIC range	MIC_{50}	ECOFF	MIC range	MIC_{50}	ECOFF	MIC range	MIC_{50}	ECOFF	MIC range	MIC_{50}	ECOFF	MIC range	MIC_{50}	ECOFF	MIC range	MIC_{50}	ECOFF
AMB	0.125–3	0.75	0.75	0.047–0.38	0.19	0.25	0.19 to >32	>32	>32	0032–0.75	0.25	0.5	8 to >32	32	>32	1 to >32	4	8
VRC	0.064–8	0.125	0.19	0.032–4	0.064	0.094	0.032–0.125	0.064	0.125	0.008–0.032	0.016	0.032	0.19–0.38	0.25	0.5	0.016–0.064	0.047	0.094
POS	0.016–8	0.094	0.19	0.016–4	0.064	0.125	0.047–0.125	0.064	0.125	0.006–0.064	0.023	0.047	0.064–0.19	0.094	0.19	0.016–0.094	0.023	0.047
ITR	0.064–4	0.5	1	0.047–4	0.25	0.5	0.25–0.5	0.25	0.5	0.023–0.125	0.094	0.19	0.5–1	0.75	1.5	0.047–0.75	0.38	0.75
CAS	0.032–0.125	0.064	0.19	0.016–0.125	0.064	0.125	0.023–0.125	0.064	0.125	0.023–0.094	0.047	0.094	0.002–0.094	0.016	0.125	0.002–0.125	0.016	0.032
AND	0.002–0.047	0.016	0.032	0.004–0.032	0.016	0.032	0.002–0.006	0.002	0.004	0.002–0.006	0.002	0.004	0.002–0.002	0.002	0.006	0.002–0.002	0.002	0.004
MYC	0.002–0.19	0.002	0.004	0.002–0.094	0.002	0.004	0.002–0.008	0.004	0.008	0.002–0.016	0.006	0.012	0.002–0.19	0.002	0.006	0.002–0.125	0.002	0.004

[a] MICs (in μg/ml) were determined after growth for 48 h at 37°C under normal oxygen conditions or hypoxic growth conditions.

[b] AMB, amphotericin B; VRC, voriconazole; POS, posaconazole; ITR, itraconazole; CAS, caspofungin; AND, anidulafungin; MYC, micafungin.

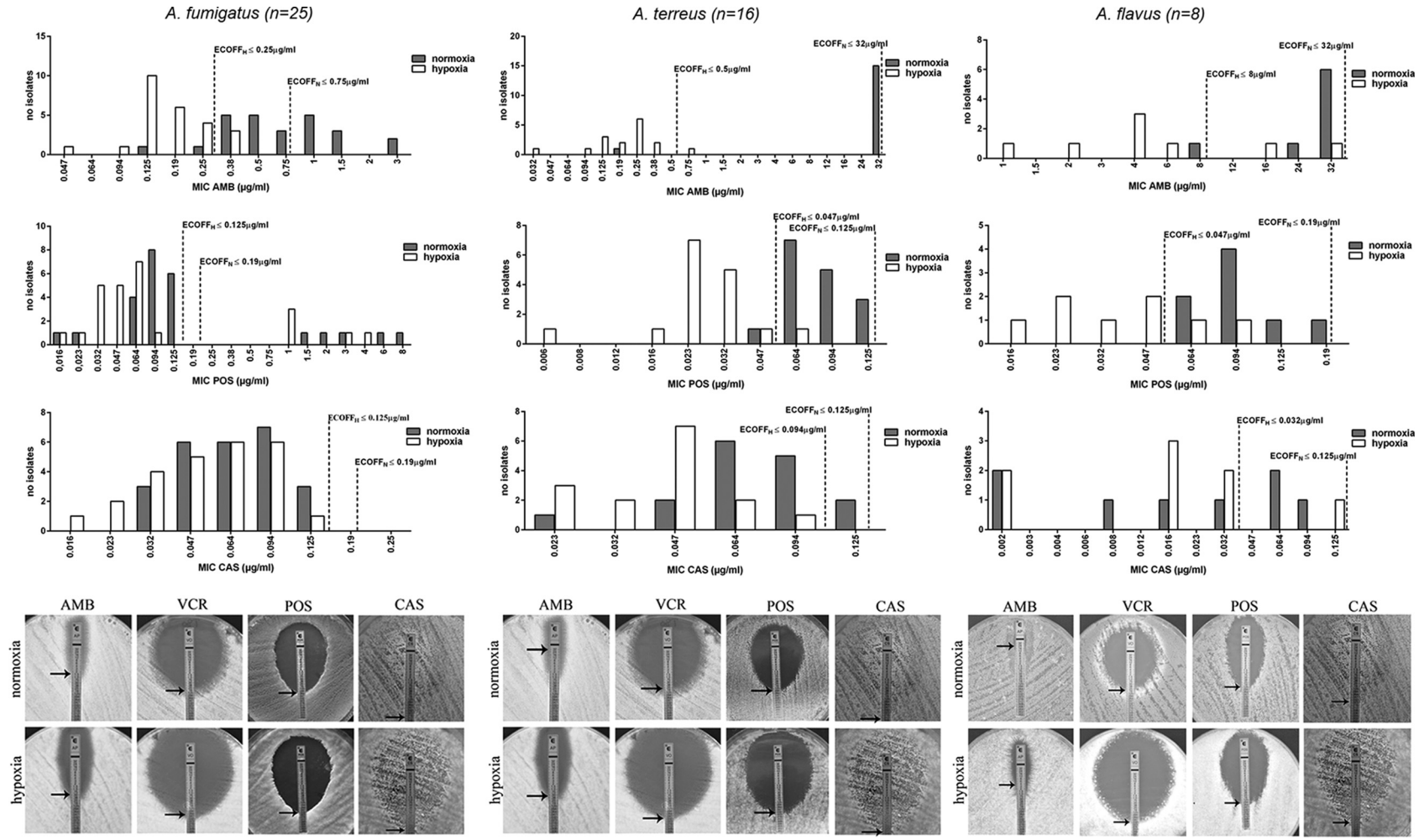

FIG 1 MIC distributions for amphotericin B (AMB), posaconazole (POS), and caspofungin (CAS) for *A. fumigatus* (left column), *A. terreus* (middle column), and *A. flavus* (right column) strains under normoxic (gray) and hypoxic (white) growth conditions. Antifungal susceptibility testing was performed via the Etest method, MICs were determined under both oxygen conditions after 48 h at 37°C, and ECOFFs were established for both conditions. Images present results for one representative strain of the species group.

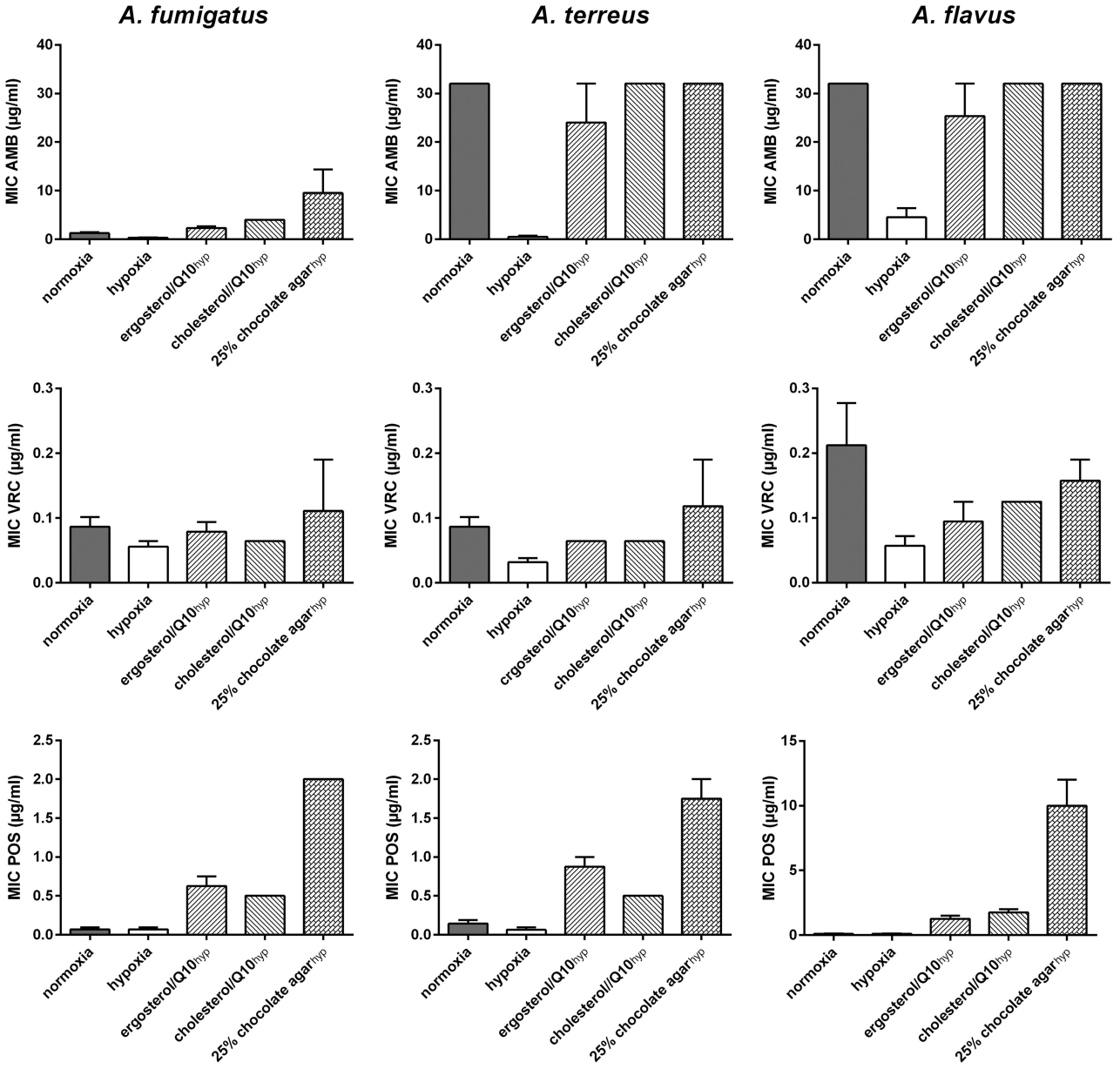

FIG 2 Supplementation of ergosterol/coenzyme Q10 (Q10), cholesterol/Q10, and blood enhances antifungal susceptibilities to amphotericin B (AMB), voriconazole (VRC), and posaconazole (POS) of *Aspergillus* spp. in hypoxia. Final concentrations of ergosterol or cholesterol and Q10 (Sigma-Aldrich, Germany) in RPMI 1640 agar were 25 μM for the sterols and 5 μM for Q10, respectively. Chocolate agar consisted of 25% (vol/vol) whole blood added to water agar, cooked for 30 min at 80°C. Bars represent MICs of one representative isolate of *A. fumigatus*, *A. fumigatus*, and *A. flavus*.

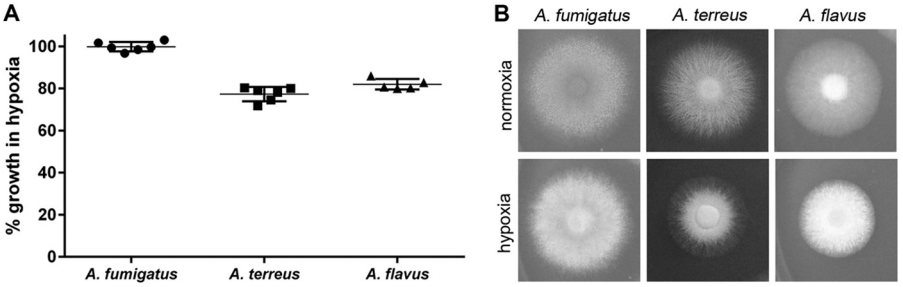

FIG 3 Hypoxia influences the growth of *Aspergillus* species. A total of 1×10^4 conidia were point inoculated on RPMI 1640 plates and incubated for 48 h at 37°C under normal oxygen and hypoxic growth conditions before colony diameter was determined. (A) Percentage of radial growth of hypoxic cultures normalized to normoxic growth (= 100%). (B) One representative example of six parallels.

TABLE 2 *In vitro* susceptibilities of *Aspergillus* spp., determined by the EUCAST method[a]

Species (*n*)	Agent	Normoxia						Hypoxia					
		MIC range	MIC_{50}	MIC_{90}	ECOFF	MFC range	MFC	MIC range	MIC_{50}	MIC_{90}	ECOFF	MFC range	MFC
A. fumigatus (25)	AMB	0.5–1	1	1	4	1–2	2	0.25–1	0.5	1	2	1–2	2
	VRC	0.125–8	0.5	6	2	0.25–1	0.5	0.125–4	0.25	2	1	0.25–0.5	0.25
	POS	0.25–8	0.5	6	2	0.5–1	1	0.125–8	0.25	6	1	0.125–0.5	0.5
	CAS[b]	0.125–0.5	0.25	0.5	1	16	>16	0.125–0.5	0.25	0.5	1	16	>16
A. terreus (16)	AMB	1–8	4	8	16	4–16	>16	0.5–2	1	2	4	16	>16
	VRC	0.25–0.75	0.5	0.5	1	4–8	8	0.125–0.25	0.25	0.25	0.5	8–16	>16
	POS	0.25–0.5	0.5	0.5	2	0.25–4	4	0.25–0.5	0.25	0.5	1	4–16	>16
	CAS[b]	0.06–0.5	0.125	0.25	0.5	16	>16	0.03–0.5	0.125	0.25	0.5	16	>16
A. flavus (8)	AMB	1–4	2	4	8	1–4	2	1–2	1	2	4	2–16	2
	VRC	0.5–1	1	1	4	0.5–2	1	0.25–1	0.5	0.5	2	0.5–1	1
	POS	0.25–0.5	0.5	0.5	2	0.25–1	0.5	0.125–0.5	0.25	0.5	1	0.25–0.5	0.5
	CAS[b]	0.06–0.5	0.25	0.25	1	16	>16	0.03–0.25	0.125	0.125	0.5	16	>16

[a] MIC/MEC, MFC, and ECOFF values for amphotericin B (AMB), voriconazole (VRC), posaconazole (POS), and caspofungin (CAS) (in μg/ml) were determined under normal oxygen conditions and hypoxic conditions.
[b] Data for caspofungin are MEC (minimal effective concentration) values rather than MIC values.

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