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Short report

In-vitro activity of active ingredients of disinfectants against drug-resistant fungi

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SUMMARY

The biocidal activities of peracetic acid and ethanol were tested against nine clinical fungal isolates and four reference strains. Ethanol was active (≥4.0 $\log_{10}$ reduction) against yeasts at a concentration of 50% v/v and against moulds at 80% v/v. Exposure times in both cases were 1 min. Peracetic acid was active as a 0.25% solution against yeasts and as a 0.5% solution against moulds; exposure times in both cases were 5 min. Compared with the reference strains, clinical isolates, including multi-drug-resistant strains, showed similar or higher sensitivity to the active ingredients of disinfectants *in vitro*.

Introduction

In Germany, approximately nine million patients suffer from fungal infections annually [1]. In medical mycology, two major trends have been identified during the past decade: the development of fungal resistance against commonly used antifungals (e.g. azole resistance in *Aspergillus fumigatus* and echinocandin resistance in *Candida glabrata* [2,3]); and an increased number of infections with emerging fungal pathogens such as Mucorales and *Scedosporium* spp., especially in immunosuppressed patients, which may be due to the increased use of antifungal prophylaxis. These species normally cause invasive pulmonary or soft tissue infections. Most commonly, the suspected mode of infection is through transmission from environmental sources on airborne particles or direct infection [4]. For these highly vulnerable patients, the prevention of such infections is essential, and proper disinfection of patients' surroundings may decrease abundance of these pathogens. Yeasts and moulds such as *Aspergillus* spp. or *Candida* spp. have demonstrated the ability to persist for up to 150 days on surfaces [5]. *Candida auris*, for example, was

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shown to cause nosocomial infections in healthcare settings [6]. The use of appropriate disinfectants with proven fungicidal activity on hands, surfaces and instruments is essential to prevent infections.

In Europe, the fungicidal activity of disinfectants is tested against the reference organisms *Candida albicans* and *Aspergillus brasiliensis*. If a product is active against *Candida albicans*, the product is classed as yeasticidal, meaning it is sufficiently active against all yeasts. If the disinfectant is active against both *C. albicans* and *A. brasiliensis*, it is labelled as fungicidal, denoting activity against all medically relevant fungi.

This study aimed to evaluate the continued suitability for disinfectant testing of the aforementioned reference strains in the current development of emerging drug-resistant yeasts and moulds. Commonly used active ingredients in chemical disinfectants (e.g. ethanol, peracetic acid) were tested to determine whether they exhibited sufficient activity against clinically relevant fungal species. Culture collection strains *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *A. brasiliensis* ATCC 16404 and *Aspergillus niger* ATCC 6275 were used as reference strains and will be referred to as such hereinafter. In addition, antifungal-resistant clinical isolates were tested with the aforementioned active ingredients, using antifungal-sensitive clinical isolates as control strains to compare efficacy.

Methods

Test strains

Four reference strains from the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) and nine yeasts and moulds from the clinical culture collection of the University Hospital Essen in Germany were included in this study (Table I).

Disinfectant solutions

A solution of peracetic acid was obtained from AppliChem GmbH (Darmstadt, Germany) and diluted to working concentrations of 0.05%, 0.1%, 0.25% and 0.5% with water of standardized hardness (EN 13624). Ethanol was obtained from Carl Roth GmbH & Co. KG, (Karlsruhe, Germany) and utilized at concentrations of 0.05%, 0.1%, 0.25% and 0.5% diluted in aqu bidest [v/v].

Suspension tests

The sensitivity of clinical isolates to the active ingredients (peracetic acid and ethanol) contained in commonly used disinfectants was tested under clean conditions (soiling 0.3 g/L bovine serum albumin) in a quantitative suspension assay

Table I
Chosen fungi for activity testing in a quantitative suspension test based on EN 13624:2013 with ethanol and peracetic acid

No.	Strain	Family	Origin	Special resistance
1	*Candida albicans*	Yeast	Reference strain DSMZ: ATCC 10231	No
2	*Candida tropicalis*	Yeast	Reference strain DSMZ: ATCC 13803	No
3	*Candida glabrata* (echinocandin-susceptible)	Yeast	Clinical isolate, blood culture	No
4	*Candida glabrata* (echinocandin-resistant)	Yeast	Clinical isolate, blood culture	Echinocandins: MEC 16 mg/L
5	*Exophiala dermatitidis*	Yeast	Clinical isolate, lung	Caspofungin: MEC >32 mg/L Micafungin: MEC 8 mg/L
6	*Exophiala dermatitidis*	Yeast	Clinical isolate, lung	Caspofungin: MEC >32 mg/L Micafungin: MEC 8 mg/L
7	*Aspergillus brasiliensis*	Mould	Reference strain DSMZ: ATCC 16404	No
8	*Aspergillus niger*	Mould	Reference strain DSMZ: ATCC 6275	No
9	*Aspergillus fumigatus* (azole-susceptible)	Mould	Clinical isolate, lung	No
10	*Aspergillus fumigatus* (azole-resistant)	Mould	Clinical isolate, lung	Itraconazole: MIC >16 mg/L
11	*Cunninghamella bertholletiae*	Mould	Clinical isolate, lung	Voriconazole: MIC >32 mg/L Amphotericin B: MIC >32 mg/L Caspofungin: MEC >32 mg/L
12	*Scedosporium prolificans*	Mould	Clinical isolate, lung	Voriconazole: MIC >32 mg/L Amphotericin B: MIC >32 mg/L Caspofungin: MEC >32 mg/L
13	*Rasamsonia argillaceae*	Mould	Clinical isolate, lung	Isavuconazole: MIC >32 mg/L Itraconazole: MIC >16 mg/L Voriconazole: MIC >32 mg/L Posaconazole: MIC 4 mg/L Echinocandines: MEC 0.03–0.25 mg/L

MEC, minimum effective concentration; MIC, minimum inhibitory concentration; DSMZ, German Collection of Micro-organisms and Cell Cultures GmbH.
All isolates were obtained from the University Hospital Essen in Germany.

based on EN 13624:2013. The durations of exposure to ethanol were 1 and 5 min. For peracetic acid, exposure times were 1, 5 and 15 min. The clinical isolates were prepared for testing based on the procedures described in EN 12353 and EN 13624:2013. For ethanol, 30 g/L polysorbate 80, 30 g/L saponin, 1 g/L histidine and 1 g/L cysteine in aqua dest were included in the suspension assay to act as neutralizers. For peracetic acid suspension tests, 30 g/L polysorbate 80, 30 g/L saponin and 5 g/L sodium thiosulfate in aqua dest were used. The neutralizers were validated for 80% and 50% ethanol and 0.5% peracetic acid with all tested organisms. After neutralization for 5 min, serial dilutions were performed, and aliquots of 1 mL were spread on malt extract agar plates and incubated at 30°C±1°C for 24–72 h, according to organism-specific growth characteristics. Colonies were counted after incubation and the number of colony-forming units (cfus) was calculated per millilitre and converted into a $\log_{10}$ value. All experiments were performed in triplicate. To indicate sufficient bactericidal activity, a $\log_{10}$ reduction $\geq$4.0 was required.

Analyses

Fungal cfus were quantified prior to and after completion of the suspension test to evaluate cell viability. Data analysis was performed using Excel 2013 (Microsoft Corp., Redmond, WA, USA). Graphical presentation was done using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

Identification of clinical isolates

Identification of clinical isolates was performed using morphological methods and sequencing of the internal transcribed spacer region, as described previously [7].

Susceptibility testing

Clinical mould isolates were tested for susceptibility by microdilution according to EUCAST Standard 9.3. Yeast susceptibility testing was performed according to EUCAST Standard 7.3.1.

Results

All yeasts and *Exophiala dermatitidis* could be inactivated significantly by concentrations of 50% v/v ethanol with an exposure time of 1 min (Figure 1). Yeasticidal activity against both echinocandin-susceptible and -resistant *C. glabrata* was observed at a lower concentration (37.5% v/v) and after a shorter exposure time compared with those of the reference strains *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803 and all other isolates (1 min vs 5 min; Figure 1).

All yeasts were inactivated by 0.05% peracetic acid in 5 min. Echinocandin-resistant *C. glabrata* and *E. dermatitidis* specimen N°. 6 could be inactivated by 0.25% peracetic acid in 1 min, whereas echinocandin-susceptible *C. glabrata* was only inactivated when exposed to peracetic acid at a concentration of

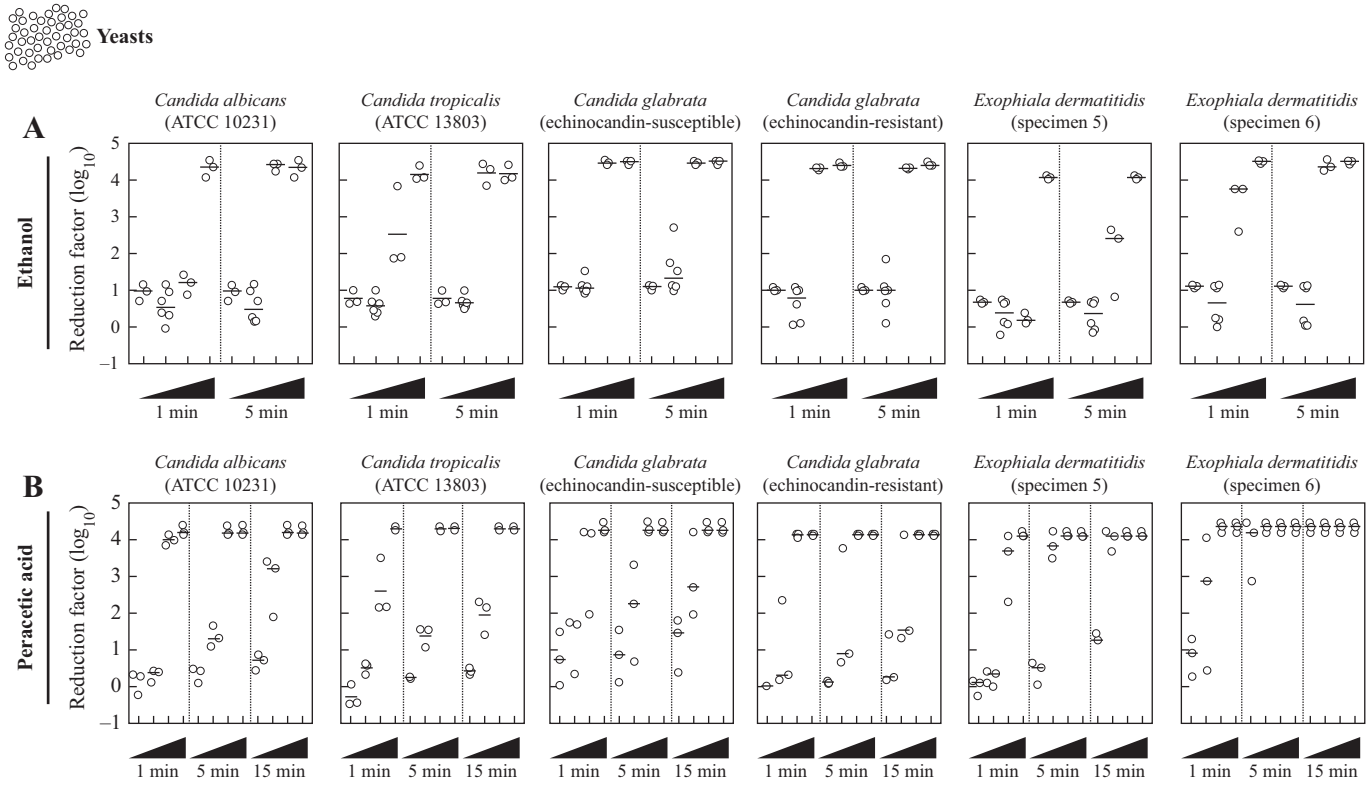

Figure 1. Results of the quantitative suspension tests with ethanol and peracetic acid for all yeasts. Ascending lines and corresponding x-axis steps indicate rising concentration of active agent (ethanol: 10% v/v, 25% v/v, 37.5% v/v, 50% v/v; peracetic acid: 0.05%, 0.10%, 0.25%, 0.50%). Exposure times are given for each concentration series. (A) Ethanol against yeasts. (B) Peracetic acid against yeasts. Circles describe individual data points. Bars show arithmetic means.

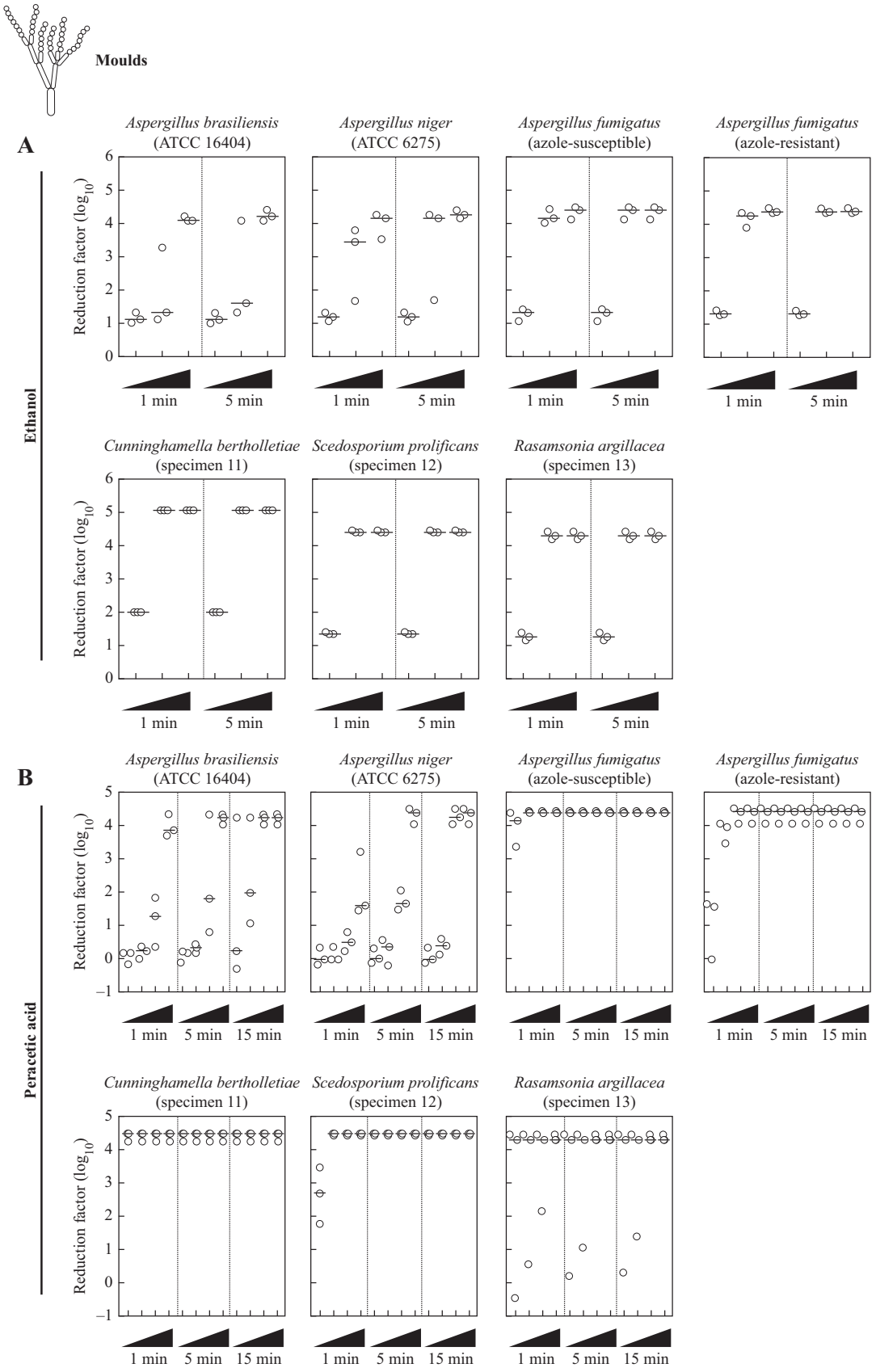

Figure 2. Results of the quantitative suspension tests with ethanol and peracetic acid for all moulds. Ascending lines and corresponding x-axis steps indicate rising concentration of active agent (ethanol: 25% v/v, 50% v/v and 80% v/v; peracetic acid: 0.05%, 0.10%, 0.25%, 0.50%). Exposure times are given for each concentration series. (A) Ethanol against moulds. (B) Peracetic acid against moulds. Circles describe individual data points. Bars show arithmetic means.

0.5% for 1 min. This response was similar to those of the reference strains *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 13803 (Figure 1). After 15 min of exposure, 0.05% peracetic acid was active against the aforementioned *E. dermatitidis* specimen N°. 6.

All tested moulds were inactivated with 80% v/v ethanol in 1 min (Figure 2). All clinical isolates were inactivated by 50% v/v ethanol, whereas reference strains *A. niger* ATCC 6275 and *A. brasiliensis* ATCC 16404 were inactivated by 80% v/v ethanol in 1 min. The tested *Cunninghamella bertholletiae* isolate was even reduced by 4 $\log_{10}$ using 25% v/v ethanol. There was no observed difference in efficacy between azole-resistant and azole-susceptible *A. fumigatus* when treated with ethanol. Both strains of *A. fumigatus* were inactivated with 50% v/v ethanol in 5 min by >4 $\log_{10}$. For *Scedosporium prolificans*, *Rasamsonia argillacea* and *C. bertholletiae*, similar results were observed: all three isolates were inactivated with 50% v/v ethanol in 1 min.

All mould isolates were inactivated with 0.5% peracetic acid in 1 min. The reference strains *A. brasiliensis* ATCC 16404 and *A. niger* ATCC 6275 were inactivated with 0.5% peracetic acid in 5 min. Peracetic acid was even effective at 0.05% in 5 min. *R. argillacea* was inactivated with 0.25% solution in 15 min, but was also reduced below the detection limit with 0.5% solution in 1 min. Azole-susceptible *A. fumigatus* and *S. prolificans* were inactivated with 0.10% peracetic acid in 1 min, whereas azole-resistant *A. fumigatus* was only inactivated at a concentration $\geq$0.25% in 1 min. *C. bertholletiae* was effectively inactivated in growth by 0.05% peracetic acid in 1 min.

Discussion

Ethanol and peracetic acid showed similar effects on all clinical yeasts and moulds compared with the reference strains. In most cases, clinical isolates required lower concentrations and/or shorter exposure times for significant reduction of fungal colony count.

E. dermatitidis specimen N°. 6 constitutes an exception to this trend, requiring at least 50% v/v ethanol for effective reduction. This black yeast-like thermophilic fungus is an opportunistic pathogen and can cause chromoblastomycosis, phaeohyphomycosis and invasive infection. Probably originating from tropical climate zones, *E. dermatitidis* can be found in dishwashers and sauna facilities as an environmental contaminant in Europe [8]. As *E. dermatitidis* has been shown to form biofilms [9], it may be more difficult to reach clinically effective concentrations of disinfectant on contaminated surfaces. Certain characteristics of biofilm may also confer extra robustness against the tested chemicals.

In contrast, echinocandin-resistant *C. glabrata* and azole-resistant *A. fumigatus* were inactivated by lower concentrations and shorter exposures to the tested active ingredients compared with those displayed by the reference strains, and expressed no relevant difference compared with the antifungal-susceptible strains. These strains were inactivated by both tested chemicals in 1 min, which is an exposure time often used in practice.

It should also be noted that the rare emerging fungi *S. prolificans* and *R. argillaceae* were inactivated by a lower concentration (50% v/v) of ethanol than the reference strains (80% v/v). For peracetic acid, *S. prolificans* showed similar data, but *R. argillaceae* was inactivated at the same concentration (0.25%) but after a shorter exposure time than that required for the reference strains (5 min vs 15 min).

The results of this study are somewhat limited by the design of the quantitative suspension test. EN 13624:2013 is the basis for testing all hand, surface and instrument disinfectants in Europe. However, yeasts and moulds in suspension tests are more susceptible to chemical disinfectants than those attached to surfaces [10]. A method developed to address this issue is EN 16615, also known as the 'Four Fields Test', which is designed to rate the efficacy of surface disinfection procedures. Testing of this alternative procedure could form the basis of a further study.

In the clinical setting, commercially available hand disinfectants often contain higher ethanol concentrations than 50%. In this study, all yeasts and moulds, with the exception of *A. brasiliensis* ATCC 16404 and *A. niger* ATCC 6275, were inactivated by 50% ethanol. This confirms that no increased tolerance has been developed by these clinical fungal isolates against ethanol-based disinfectants. Peracetic acid is used in different concentrations, but products used in a clinical setting are usually based on concentrations $\geq$0.5% in combination with other agents. It is mostly used for disinfection of medical instruments due to its reduced corrosive effect on metallic surfaces compared with other disinfectants [11].

To date, few data exist on the efficacy of peracetic acid and ethanol against *C. auris*. Peracetic acid was found to be effective at 2000 ppm (0.2%) [12], which is comparable with the present results for the tested *Candida* spp. Only one study has shown that 29.4% ethanol applied for 30 s was ineffective against *C. auris* in the American Society for Testing and Materials Standard Quantitative Carrier Disk Test Method (ASTM E-2197-02) [13]. Reproduction of these results and larger studies with *C. auris* should be performed to confirm the data gathered on peracetic acid and ethanol-based disinfectants.

Based on the in-vitro data from this study, it is concluded that currently used active agents have fungicidal activity against all tested clinical isolates of yeasts and moulds. In particular, emerging pathogenic fungi *S. prolificans* and *R. argillaceae*, and antifungal-resistant strains of *C. glabrata* and *A. fumigatus* appear to be highly sensitive, as ethanol and peracetic acid show sufficient activity even at lower concentrations compared with those required to inactivate the reference strains effectively. No relevant differences in sensitivity to the tested chemicals were found between the antifungal-susceptible and -resistant isolates.

As emerging antifungal resistance is a major problem in the clinical setting [2,14,15], the proper usage of chemical disinfectants with proven fungicidal activity will aid significantly in preventing transmission of these pathogens.

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Conflict of interest statement
None declared.

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