

Occurrence and susceptibilities to disinfectants of *Cryptococcus neoformans* in fecal droppings from pigeons in Bangkok, Thailand

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ABSTRACT. *Cryptococcus neoformans* is an opportunistic pathogenic yeast that causes meningoencephalitis and deep skin dermatitis in humans and animals. A hygienic strategy using disinfectants on environmental samples can reduce the risk to the public. The objectives were to survey the distribution of *C. neoformans* in pigeon fecal droppings collected in 11 districts in Bangkok during 2011–2012 and to evaluate the efficacy of three commercial disinfectant products (based on potassium monopersulfate, sodium hypochlorite and quaternary ammonium compounds, respectively). These were evaluated against pure *C. neoformans* and yeasts resuspended in sterile pigeon feces using the dilution-neutralization method [Europäische NORM (EN) 1656]. In total, 18 of 164 (11%) samples were positive for *C. neoformans*. These came from only three of the 11 districts, with a prevalence of between 13–56%. Using multiplex PCR, serotype A was the sole group found. For all disinfectants, *C. neoformans* mixed in feces was tolerated at a higher dose and time exposure than pure isolates. The most effective disinfectant in this study was a 0.12% quaternary ammonium compound that could rapidly eradicate the yeasts mixed in feces. This finding highlights the occurrence and distribution of *C. neoformans* in the capital city of Thailand and the need to prolong the duration of exposure to disinfectants with pigeon feces.

KEY WORDS: Bangkok, *Cryptococcus neoformans*, disinfectant, pigeon droppings, susceptibility

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Cryptococcus neoformans is a basidiomycetous encapsulated yeast that can cause systemic and cutaneous cryptococcosis in humans and animals, particularly those that are immunosuppressed [14, 18]. *C. neoformans* comprises three serotypes and two varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D and AD), whereas the *C. gattii* group comprises two serotypes: B and C [3]. *C. neoformans* has a worldwide distribution and has been associated with a variety of environmental sources, such as soil, decayed wood and bird excreta, especially pigeon fecal droppings [11, 20]. *C. gattii* typically is a cause of infections of the pulmonary and/or the central nervous system, and its favored ecological niche is in conditions of low moisture and low organic carbon content [8].

Cryptococcus neoformans variety *grubii* (serotype A) has been the major causative agent of cryptococcosis in Thai patients suffering from HIV/AIDS and has been shown to have a high prevalence in dove and pigeon excreta in Chiang Mai, Thailand [7, 25]. In general, *C. neoformans* is disseminated in the environment via fecal droppings of birds that can utilize ammonia as an assimilated nitrogen source within their digestive tracts [10]. Reports of cases of cryptococcosis have increased simultaneously with the increased number of HIV/AIDS patients: globally, estimated one million people

are infected each year, resulting in 620,000 deaths per year [19]. Consistent with this, in Thailand, there have been many cases of cryptococcosis in HIV/AIDS patients. Previously, the isolation rate of *C. neoformans* from pigeon fecal droppings in Thailand was found to be 9–24%, 16.4% and 10% in Bangkok and in Chiang Mai and Chonburi provinces, respectively [9, 24, 25, 28], and it was 45% in dove droppings in Chiang Mai [25]. Thus, areas of Bangkok with abundant wild pigeons may represent a risk of environmental contamination threatening the health of both immunocompromised and immunocompetent people. To date, the most contaminated districts in Bangkok have not been identified.

The disinfectants hydrogen peroxide, chlorine compounds, quaternary ammonium and potassium monopersulfate all potentially can serve as tools for reducing pathogen contamination. The use of either 1–2% quaternary ammonium compound for 10 min or 1% sodium hypochlorite for 15 min has been recommended to kill *C. neoformans* by membrane disruption and cell lysis [22]. The microorganisms were also able to resist disinfectants, because of intrinsic factors, including their cell capsule and biofilm production [29], and external factors, such as organic matter level, that can affect the time required for inactivation and the guideline concentrations [4, 15, 17]. Surveillance of *C. neoformans* in public areas has been conducted often, but little is known about decontamination of bird droppings. The objectives of this study were to determine the occurrence and distribution of *C. neoformans* in pigeon droppings in different areas of Bangkok and to evaluate the efficacy of common disinfectants against *C. neoformans* mixed in organic matter compared to pure isolates.

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MATERIALS AND METHODS

Sample collection and isolation: A total of 164 samples of pigeon fecal droppings were collected from 11 districts in Bangkok during 2011–2012 (Fig. 1 and Supplementary Table 1). These included 97 pools of partially dried droppings and 67 pools of fresh droppings that were collected at temples and public parks with high pigeon populations. Approximately 5–10 g of pooled dropping from 1 square meter area were collected into sterile sealable plastic bags, stored at 4°C and cultured within 24 hr. Yeast isolation was performed according to a previous protocol [5]. Briefly, one part of the sample was suspended in 9 parts of peptone saline diluents contained within a selective mixture consisting of 0.04% w/v chloramphenicol (Sigma-Aldrich, Carlsbad, CA, U.S.A.), 0.02% w/v of streptomycin sulfate (Sigma-Aldrich) and 0.01% w/v of biphenyl (Alfa Aesar, Lancashire, U.K.). The suspension was incubated for 24 hr at 37°C with agitation and then diluted ten-fold from 10^0 to 10^{-5} . A 100 μ l volume of supernatant was inoculated on Sabouraud dextrose agar (SDA) and on caffeic acid ferric citrate agar (CFCA) (Sigma-Aldrich) [31] and incubated at 37°C for 3–7 days. Suspected colonies were observed for typical features comprising cream-colored smooth mucoid colonies on SDA and brown colonies on CFAC. The presence of the organisms was confirmed by Indian ink staining, a urease test and sugar assimilations using sucrose, trehalose, lactose, maltose, galactose and dextrose [1]. *C. neoformans* CN 175 was used as the control strain.

Molecular identification and serotyping: Genomic DNA was extracted by the Wizard® Genomic DNA purification Kit (Promega, Madison, WI, U.S.A.) with a 0.5 mm diameter glass bead (Sigma-Aldrich). Briefly, the strains were grown on yeast extracts peptone dextrose broth (YPDB) at 37°C with agitation for 24 hr and then centrifuged for 5 min at 2,600 rpm. The packed cells were collected and re-suspended in 300 μ l of lysis buffer (1:1) with 300 mg glass beads. The samples were incubated at –80°C for 15 min and vortexed vigorously for 45 min three times before centrifuging at 15,000 rpm for 5 min. The aqueous phase was extracted using the purification kit according to the manufacturer's instructions (Promega).

A multiplex polymerase chain reaction (M-PCR) specific to *C. neoformans* and *C. gattii* was performed using primer sets: CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC3') / CNa-70A (5'-ATTGCGTCCATGTTACGTGGC-3') and CNb-49s (5'-ATTGCGTCCATGTTACGTGGC-3') / CNb-49A (5'-ATTGCGTCCATGTTACGTGGC-3'), respectively. The PCR conditions followed a previously described protocol [16]. For serotyping, the approved multiplex PCR using four primer pairs specific to the laccase gene fragment (*LAC I*) encoding capsule membrane variation was performed as previously described [16]. The PCR conditions were an initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation (94°C for 30 sec), annealing (47°C for 1 min) and extension (72°C for 1.5 min), and this was followed by final extension (72°C for 7 min). *C. neoformans* var. *grubii*, WM148, *C. neoformans*, WM629 and *C. neoformans* var. *grubii*/ var. *neoformans* hybrid, WM628 were used as the



Fig. 1. Presence on the Bangkok map of 11 districts where pigeon droppings were collected, represented by colors and numbers (1=Nong Khaem; 2=Samphanthawong; 3=Phra Nakhon; 4=Pathum Wan; 5=Khlong Toei; 6=Ratchathewi; 7=Dusit; 8=Lat Phrao; 9=Chatuchak; 10=Lak si and 11=Min Buri). Only districts 1, 5 and 8 yielded positive samples.

control strains.

Fungicidal efficacy evaluation: Three biocides: Product A [50% w/w potassium peroxymonosulphate, 5% w/w sodium dichloroisocyanurate and 15% w/w sulphamic acid] (Elanco, Saligo, Ireland), Product B [99.9% sodium hypochlorite] (Lab Valley, Bangkok, Thailand) and Product C [10.8% N-benzyl-N-dodecyl-N,N-dimethyl-ammonium chloride/N-benzyl-N,N-dimethyl-N-tetradecyl-ammonium chloride; benzalkonium chloride] (Laboratoire Huckert's International, Wavre, Belgium) were tested for their efficacies against pure isolates of *C. neoformans* and *C. neoformans* mixed in sterile pigeon droppings. A total of 8 strains comprising five *C. neoformans* strains: KT1/12, LP1/12, LP2/12, NK1/12 and NK2/12, obtained from pigeon dropping, one parrot strain (PR1/12), one feline strain (FL1/12) and one strain isolated from a human (WM148) were prepared on SDA at 37°C for 3 days. The yeast cells were harvested and suspended in Sabouraud dextrose broth and then adjusted to 0.1 at OD 530 nm ($1-5 \times 10^6$ cells/ml). For sterile organic matter preparation, a total of 20 g pigeon droppings were mixed with 80 ml sterile deionized water and then sterilized in an autoclave at 121°C for 15 min [13].

The protocol was modified from the dilution-neutralization method according to the Europäische NORM 1656 (EN 1656) recommendation (European Committee for Standardization, 2000) [26]. All disinfectants were diluted with sterile deionized water at the recommended concentration. Solutions of the three chemical disinfectant preparations were prepared at final serial two-fold concentrations from 1% to 0.03% for the pure *C. neoformans* test and from 2% to 0.06% for the *C. neoformans* mixed in sterile pigeon droppings test. For evaluation of disinfectant in organic matter, 1 ml pure *C. neoformans* suspension mixed with 1 ml sterile

Table 1. Sampling data including condition of the pigeon droppings, sun exposure and location, and risk in relation to the occurrence of *Cryptococcus neoformans*

Sources	Number of positive/total sample	P-value ^{a)}
Condition of pigeon droppings		
- Fresh	1/67	0.001
- Dried	17/97	
Sun exposure		
- Direct sunlight	13/99	0.204
- Shaded	5/65	
Location		
- Outdoors	10/110	0.200
- Indoors	8/54	

a) $P \leq 0.05$ is considered to be statistically significant by Chi-square.

pigeon droppings was filled up to 10 ml in each dilution of each disinfectant (in hard water prepared according to the EN1656 standard) and shaken thoroughly. The mixture was incubated at room temperature for 10 sec, 30 sec, and 1, 3, 5, 10, 15 and 30 min of exposure time. Afterward, 1 ml of the suspension was transferred into 9 ml of Dey/Engley neutralizing broth (Sigma-Aldrich) containing 0.5% pancreatic digest of casein, 0.25% yeast extract, 1% dextrose, 0.1% sodium thioglycollate, 0.6% sodium thiosulfate, 0.25% sodium bisulfite, 0.5% polysorbate 80, 0.7% lecithin and 0.002% bromocresol purple, and then incubated at room temperature for 5 min. Then, 100 μ l of each suspension was cultured onto SDA by the pour plate method and incubated at 37°C for 3 days. Colony-forming units were counted to determine the number of yeast that survived the disinfection test. At the same time, the former protocol and interpretation were determined with 1 ml pure *C. neoformans* without sterile pigeon droppings mixed with 9 ml disinfectant and assigned as group 2. The non-antimicrobial effect of neutralizer was confirmed by using the standard protocol [26].

Statistical analysis: Yeast enumeration was performed with 20–200 colonies grown on the agar. *C. neoformans* distribution associated with different factors including dropping condition, indoor and outdoor location and sun exposure (Table 1) were analyzed by the Chi-square test using SPSS version 17, (IBM., Manhattan, NY, U.S.A.). A value of $P \leq 0.05$ was considered to be statistically significant. The bacteriocidal efficacies of the three disinfectants at different dilutions and time-kill were compared by analysis of variance.

RESULTS

Of the 164 pools of samples analyzed, 18 (11%) were positive for *C. neoformans* by both selective culture and multiplex PCR. All isolates grew at 37°C, and all showed a mucopolysaccharide capsule-like appearance as presented by Indian ink staining. The occurrence and distribution of *C. neoformans* in Bangkok are shown in Table 1, Fig. 1 and Supplementary Table 1, with isolates only obtained from the districts of Nongkhaem (No 1), Latphrao (No. 5), and Khlongtoei (No. 8).

The number of yeast cells within one gram of feces ranged from 0.94 to 2.60×10^5 CFU/g with an average of $(1.79 \pm 0.54) \times 10^5$ CFU/g. For species identification, all product sizes were the same as the *C. neoformans* specific product at 695 bp, as well as those of the control strain of *C. neoformans*. For serotyping, all strains were grouped in *C. neoformans* var. *grubii* (serotype A). As shown in Table 1, 17 positive samples were obtained from 97 dried pigeon droppings samples, whereas only one was obtained from 67 fresh samples ($P < 0.001$). There was no difference associated with the location (indoors or outdoors) or sun exposure.

The susceptibilities of the pure *C. neoformans* isolates and the yeast mixed with sterile pigeon droppings to the three disinfectants are shown in Figs. 2 and 3. Regarding the endpoint of the fungicidal effect, Products A and B could kill all *C. neoformans* cells at 1% within 1 min while 0.50% killed within 3 min, while *C. neoformans* mixed with sterile pigeon droppings was completely eradicated at 2% within 3 and 1 min for Products A and B, respectively. Product C could rapidly kill all *C. neoformans* at 0.5% and still was effective at 0.03% for 1 min, while the yeast in organic matter was rapidly killed at 1% and the product maintained its efficiency to 0.062% within 30 min. All 8 tested strains from different sources showed similar results for effective concentration and time exposures.

DISCUSSION

This study was not the first surveillance in Bangkok, but it is the most recent for this capital city. Eleven districts were studied to represent a wide cross section of the city. Temples and public parks were prioritized areas due to their high density of pigeon flocks that were likely to represent the highest risk of contamination to the public. Overall, the occurrence of *C. neoformans* (11%) was at a lower percentage than reports for Chiang Mai, Thailand (16.4–45%), Seoul, Korea (25%) and Grand Canary Island (24.45%) [2, 21, 25]. In Bangkok, the distribution was focal, and of the three positive districts, the rate was substantially higher in Nongkhaem (9/16, 56.2%) and Latphrao (7/16, 43.7%) than in Khlongtoei (2/15, 13.3%). The reason for the differences in prevalence

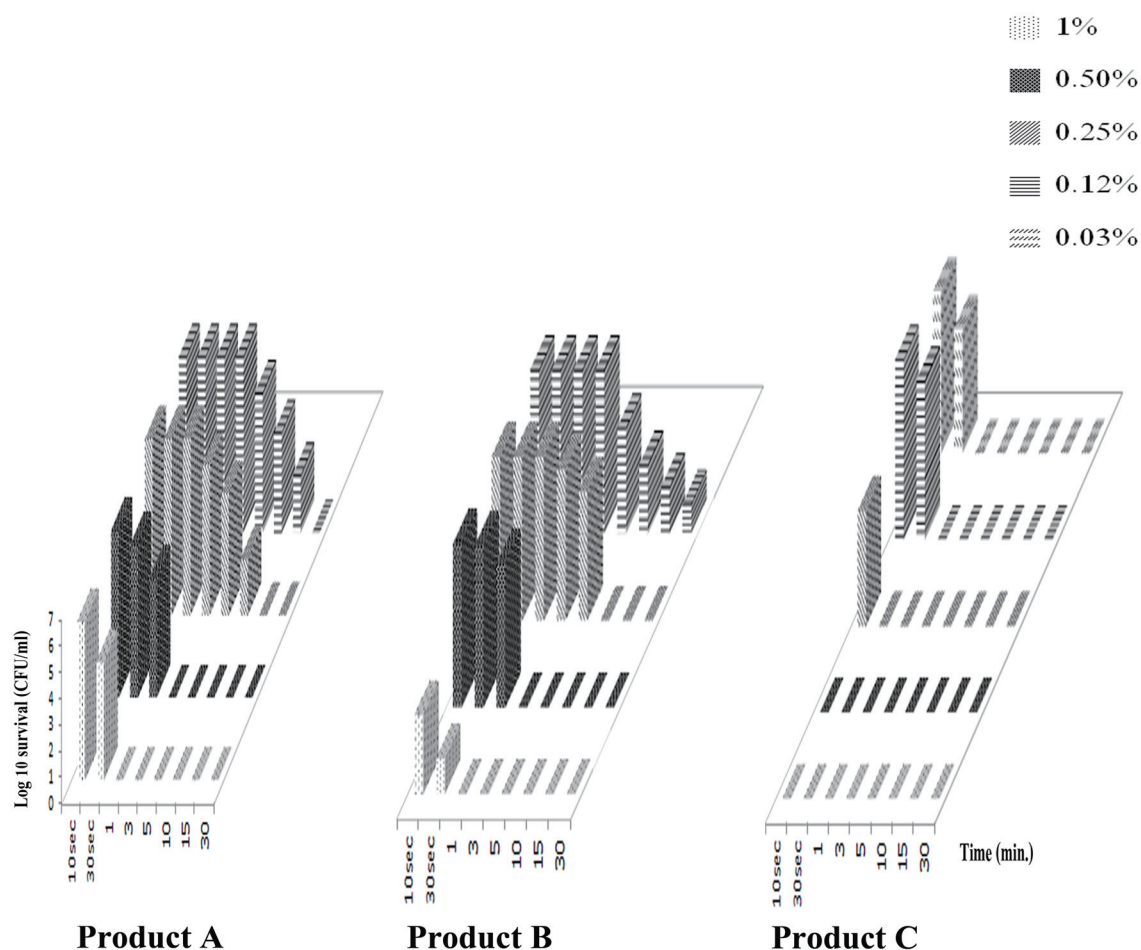


Fig. 2. Comparison of survival rate of the pure *C. neoformans* isolates after exposed to different concentrations of the three disinfectants for different times (see text for identification of the products).

is uncertain, but might reflect the habitat of bird flocks and their feed type in each area. However, bird migration also may contribute to the transmission of infection. In our study, the average number of yeast contamination in dropping was about 10 times less than in a previous report [30] and would presumably represent a lower risk for transmission.

Previously, serotype A was the most common group found in Bangkok as well as in Seoul, Korea [2], but only one human clinical isolate in Chiang Mai was serotype AD [25]. Genetic similarities recently have been shown between isolates from animal sources and human patients in Thailand [6]. This study strongly confirmed that dried droppings are more likely to contain yeast spores than fresh droppings [2]. The incidence of *C. neoformans* in the environment previously has been reported to depend on abiotic components, including the presence of protein, carbohydrate and creatinine, and physical conditions including light, temperature and moisture [4, 15, 17].

The inoculum concentration used for disinfectant evaluation was similar to the average number of organisms contained in bird droppings and also was within the acceptable

range according to the standard protocol. Therefore, this testing regimen should mimic the situation in the environment. This study selected strains from various sources and locations to reduce confounding factors arising from differences in *C. neoformans* virulence and resistance, such as cell wall thickness, the presence of a capsule, melanin and bio-film production [15]. However, all tested strains showed a strong consistence in their susceptibility to all disinfectants. In relation to the concentration of each disinfectant, the starting concentration (1%) for the pure *C. neoformans* isolates and (2%) for yeast mixed with sterile pigeon droppings were chosen because of the manufacturers' recommendations, and a two-fold reducing concentration was performed until the endpoint of the time-kill and doses of pure inoculum were reached. Sterile pigeon droppings were designed to represent organic matter that may interfere with the efficacy of the disinfectants [27].

Products A and B were effective in the range of the manufacturers' recommendations. At the same time, product C was the strongest fungicidal disinfectant for *C. neoformans* in pigeon droppings. This report is the first making recom-

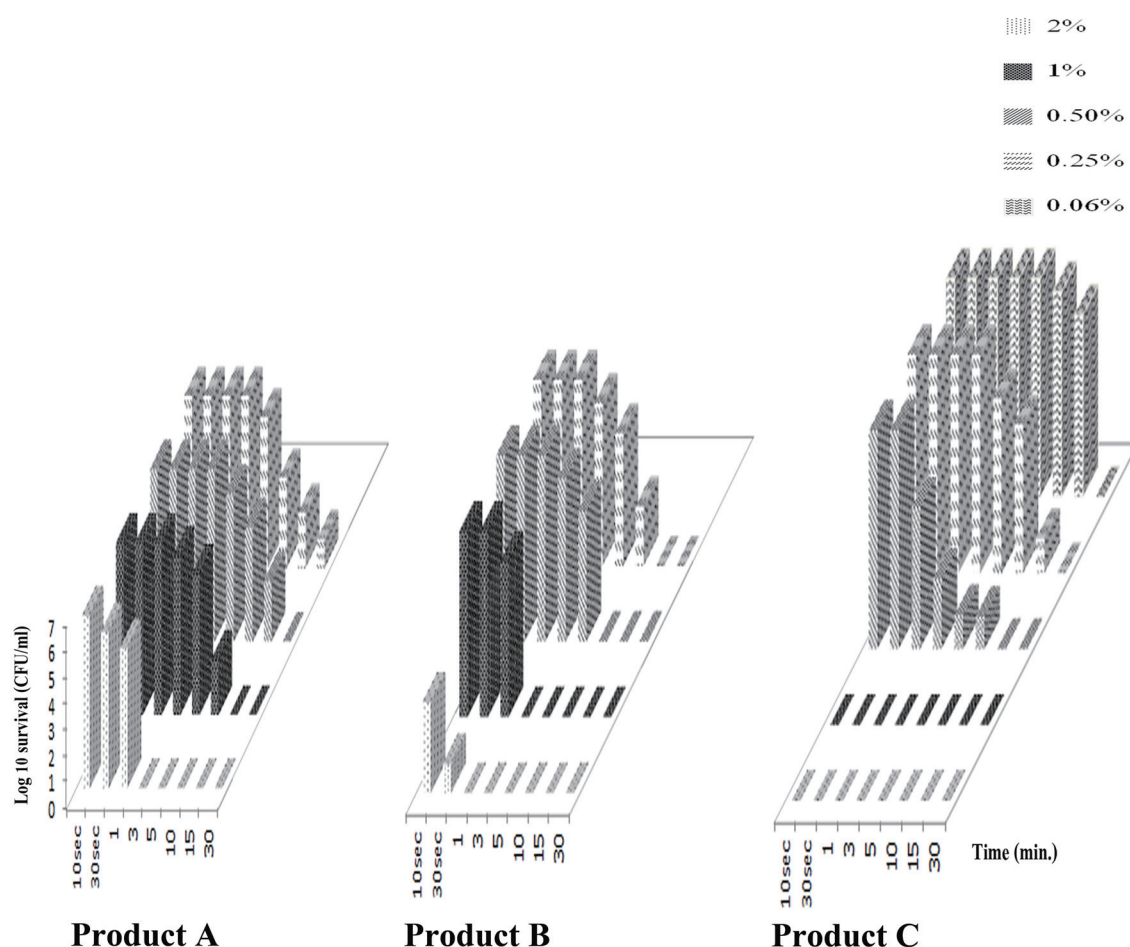


Fig. 3. Comparison of survival rate of *C. neoformans* mixed in sterile pigeon droppings and exposed to different concentrations of the three disinfectants for different times.

mendations for suitable concentrations and time exposure to achieve fungicidal effect against *C. neoformans* in pigeon droppings. The efficacies of Products A and B, both chloride releasing agents, are reduced by organic matter, such as soil, feces and blood [12, 15], as well as by chlorhexidine [23]. An increase in duration of exposure can increase their efficacies in practical use. Product C is a cationic detergent that is a quaternary ammonium compound used for a variety of clinical purposes [15]. This derivative was the most outstanding agent and did not show reduced efficacy in organic matter with any time-kill effect (at 2% to 0.06%). In conclusion, *C. neoformans* var. *grubii* (serotype A) was found at a variable but sometimes very high prevalence (13–56%) in pigeon droppings in only three of 11 Bangkok districts (Nong Kham, Khlong Toei and Lat Phrao). To complete eradication of *C. neoformans* in organic matters, increase of time-kill and concentration-kill may be an option in the guidance of disinfectant preparation. The quaternary ammonium compound was the most effective against *C. neoformans* from all sources in this study and may be recommended for decontamination in an endemic area.

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