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ORIGINAL PAPER

Prevalence and Molecular Differentiation of *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, and *Entamoeba hartmanni* in Egypt

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Abstract

Introduction *Entamoeba histolytica*-caused amoebiasis is a major cause of mortality worldwide. *E. histolytica* is morphologically indistinguishable from nonpathogenic species like *E. dispar*, *E. moshkovskii*, and *E. hartmanni*. Polymerase chain reaction (PCR) is the approved method by World Health Organization for diagnosis and differentiation of amoebiasis. This study aims to molecularly differentiate the four *Entamoeba* spp. using conventional PCR and correlate their prevalence with the patients' sociodemographic data.

Methods We collected fecal samples of 175 patients with gastrointestinal diseases at Damanhour General Hospital (El-Behira, Egypt). All microscopically positive samples were subjected to conventional PCR.

Results The overall prevalence of *Entamoeba* infection was 65.7% (115/175). The differentiation by PCR was successfully attained in 102 samples. The species distribution was as follows: *E. histolytica* (14.7%), *E. dispar* (61.8%), *E. moshkovskii* (11.8%); besides, 11.8% of samples revealed mixed infection. Of note, the infection rate was higher in men, patients from rural areas and patients who did not have sanitation facilities for sewage disposal.

Conclusion This study demonstrates a high prevalence of infections caused by the nonpathogenic *Entamoeba* spp. *E. dispar*, *E. moshkovskii*, and *E. hartmanni* along with the pathogenic *E. histolytica*. Hence, we recommend PCR assay as an accurate, rapid, and effective diagnostic method for the detection and differentiation of the four morphologically indistinguishable *Entamoeba* spp. in both routine diagnosis of amoebiasis and epidemiological surveys.

Keywords Amoebiasis · Egypt · PCR · Epidemiology · Microscopy

Introduction

Protozoal gastrointestinal infections are mainly associated with the lack of suitable sanitation and hygiene measures, as well as environmental contamination with fecal matter from infected individuals; thus, these infections are most prevalent in developing countries [1]. Amoebiasis is one of the leading parasitic intestinal infections worldwide, accounting for thousands of deaths annually [2]. While some *Entamoeba* infections are mild, the protozoan can also cause severe infections like infectious diarrhea, abscesses and extraintestinal invasion, and damage of other host organs, including the liver, lungs, and brain [3, 4].

To date, several epidemiological and molecular studies have demonstrated that the organism previously known as *Entamoeba histolytica* (*E. histolytica*) now comprises two genetically distinct but morphologically identical species: The first species is *E. histolytica*, which is highly pathogenic and causes invasive, severe diseases; *E. dispar*, a nonpathogenic commensal [3]. Besides these two *Entamoeba* spp., the third species, *E. moshkovskii*, is primarily considered as a ubiquitous free-living amoeba found in anoxic sediments [2]. However, sporadic cases of human infections due to *E. moshkovskii* have been reported; *E. moshkovskii* has been isolated in pediatric cases from

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Bangladesh that had mixed infections involving *E. histolytica* and *E. dispar* [4]. The fourth species, *E. hartmanni*, is a nonpathogenic commensal organism residing in the large intestines of humans, and it is also worth considering [1]. Besides, a new *Entamoeba* spp. was identified in Bangladesh, *E. Bangladeshi*, is morphologically identical to *E. histolytica* and cannot be differentiated from it by light microscopy. A study investigating the phylogenetic relationship between *E. bangladeshi* and other *Entamoeba* spp. revealed that *E. bangladeshi* was related to *E. histolytica* more than *E. moshkovskii* and less than *E. dispar* [5].

Lately, the nonpathogenic species, *E. dispar* and *E. moshkovskii*, have been associated with amoebic dysentery and extraintestinal amoebiasis. Thus, further research is warranted in this field to comprehend the pathogenic behavior and the public health contribution of the indistinguishable *E. histolytica/E. dispar/E. moshkovskii* complex and *E. hartmanni* protozoa [1].

In most countries, amoebiasis is diagnosed through microscopic examination of wet or stained smears of patients' fecal samples to detect amoebic cysts and trophozoites. Although microscopy as a diagnostic method is cost-effective and straightforward, it cannot identify the exact *Entamoeba* spp. present specimens as it cannot distinguish between cysts and trophozoites of the four *Entamoeba* spp. Hence, the differentiation of the *Entamoeba* species in clinical samples by other means is imperative to achieve an accurate diagnosis of infections to the species level for valuable epidemiological studies and selection of treatment strategies [4].

Accordingly, several methods and approaches have been tested to overcome the limitations of the microscopic diagnostic method. For example, culturing trophozoites and determining isoenzyme patterns by gel electrophoresis can be used; however, these methods are expensive, time-consuming, and unsuitable for regular diagnosis [2]. Moreover, antibody-based methods have been developed to differentiate between *E. histolytica* and *E. dispar* in stool or serum samples; however, serological testing could pose challenges in distinguishing past from current infections [4]. The World Health Organization has approved the polymerase chain reaction (PCR) as a diagnostic method for differentiating the four *Entamoeba* spp. to attain accurate diagnosis and valuable epidemiological studies [2]. Besides, the identification of *Entamoeba* to the species level is crucial for patient care management and selection of treatment strategies as patients infected by *E. moshkovskii*, *E. hartmanni*, and *E. dispar* could be unnecessarily treated with antiamoebic chemotherapy [1]. Furthermore, PCR has enhanced sensitivity and specificity over enzyme-linked immunosorbent assay (ELISA)-based kits in the precise diagnosis of amoebiasis [2].

This study aims to detect and differentiate the four morphologically similar *Entamoeba* spp. found in humans *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. hartmanni*, as well as estimate the prevalence of their infections in El Behira governorate in Egypt using conventional PCR. In addition, this study aims to identify and investigate the possible environmental and socioeconomic factors related to these infections.

Materials and Methods

Sample Collection

We collected fecal samples were collected from 175 patients (145 men and 30 women) with gastrointestinal diseases at Damanhour General Hospital (El-Behira, Egypt) between September and December 2018. The patients' age ranged from 4 to 65 years. The samples were examined by light microscopy, both unstained and iodine stained, to detect *Entamoeba* uninuclear, binuclear, trinuclear, or tetranuclear cysts and trophozoites. In addition, we collected the patients' sociodemographic characteristics, including age, gender, community, education level, gastrointestinal symptoms, sanitation facilities, and dealing with cattle. Of note, patients were diagnosed with diarrhea if they reported experiencing loose, liquid, or watery bowel movements, at least, three times a day for a few days. In this study, post-secondary education was considered high education; while, any other lower education level was considered low education.

DNA Extraction and PCR for the Identification of the Four *Entamoeba* Species

Using a FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corp., Vienna, Austria.), DNA extraction from stool samples was performed directly according to the manufacturer's instructions. The extracted DNA was stored at − 20 °C. All DNA extracts were amplified by PCR to detect *E. histolytica*, *E. dispar, E. moshkovskii*, and *E. hartmanni*, as described by Lau et al. and Gomes et al. with slight modifications using the following species-specific primers. *Eh-L* (5-ACATTTTGAAGACTTTATGTAAGTA-3) and *Eh-R* (5-CAGATCTAGAAACAATG CTTCTCT-3) were used to detect *E. histolytica* with a product size of 427 bp. *Ed-L* (5-GTTAGTTATCTAATTTCGATTAGAA-3) and *Ed-R* (5-ACA CCACTTACTATCCCTA CC-3) were used to detect *E. dispar* with a product size of 195 bp. *Mos* 1 (5-GAAACC AAGAGTTTCACAAC-3) and *Mos* 2 (5-CAATATAAGGCT TGGA TGAT-3) were used to detect *E. moshkovskii* with a product size of 553 bp. *EhartR1* (5-ATTGTCTTCACTATT CCATGCC-3) and *EhartF* (5-CCAGCTTTCCAAACATGA TG-3) were used to detect *E. hartmanni* with a product size

of 186 bp. All PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination [1].

In this study, the PCR conditions were as follows: 95 °C for 3 min for initial denaturation, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min.

Ethics

This research adhered to the accepted principles of ethical conduct according to the approval reference number (1116PO1) by the Research Ethics Committee of the Faculty of Pharmacy, Damanhour University. We obtained Informed consents from adult patients and parents/guardians of young patients before performing molecular studies on their stool specimens.

Statistical Analysis

The correlation between the four *Entamoeba* spp. and different sociodemographic characteristics of patients was determined statistically using the χ^2 test and Monte-Carlo method. All statistical analyses were performed using IPM SPSS version 20.0 (IBM Corp., Armonk, NY). In addition, $P < 0.05$ was considered statistically significant.

Results

Out 175 clinical stool samples, 115 (65.7%) were identified as *Entamoeba* spp. by light microscopy; the four *Entamoeba* spp. could not be differentiated microscopically. Out of these 115 patients, 30 had diarrhea and 85 had no diarrhea.

DNA extraction was performed for 115 stool samples (fresh samples: 102; frozen samples: 13). Of these microscopically positive 115 samples, 102 (88.7%) tested positive by PCR. All 102 positive samples were the fresh samples; however, all 13 frozen samples displayed negative results by PCR. Table 1 presents the sociodemographic characteristics of 102 patients positively diagnosed by PCR.

The detection rate of the four *Entamoeba* spp. using the species-specific primers was as follows: *E. histolytica*: 14.7% ($n = 15$); *E. dispar*: 61.8% ($n = 63$); *E. moshkovskii*: 11.8% ($n = 12$); and mixed infections: 11.8% ($n = 12$). Of mixed infection samples, 6 (5.9%) contained *E. histolytica, E. dispar*, and *E. hartmanni*, which were obtained from members of the same family, while the other 6 (5.9%) samples contained *E. histolytica and E. dispar*, which were also obtained from members of the same family. Furthermore, PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination (Figs. 1 and 2).

Table 1 The sociodemographic characteristics of the 102 patients positively diagnosed by PCR

Characteristics	*N* (%)
Gender	
Male	87 (85.3%)
Female	15 (14.7%)
Age group	
4–12 years	21 (20.6%)
13–30 years	42 (41.2%)
31–65 years	39 (38.2%)
Community	
Rural area	78 (76.5%)
City	24 (23.5%)
Education level	
Low	57(55.9%)
High	45(44.1%)
Sanitation facilities	
Latrine	51 (50%)
Open defecation	51 (50%)
Dealing with cattle	
Dealing	51 (50%)
Not dealing	51 (50%)

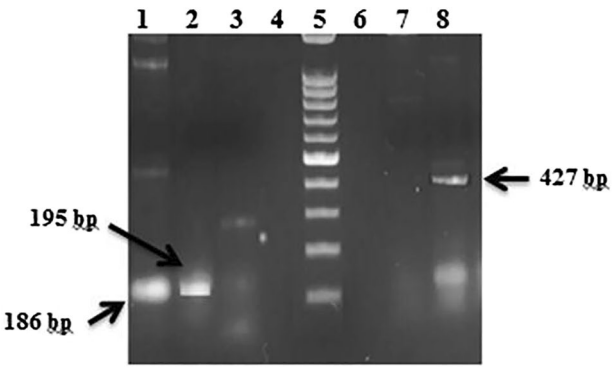

Fig. 1 Detection and differentiation of *Entamoeba histolytica*, *E. dispar* and *E. hartmanni* by polymerase chain reaction (PCR). PCR products were visualized in 1.5% agarose gel with ethidium bromide staining. Lane 1, fecal sample positive for *E. hartmanni*; lane 2, fecal sample positive for *E. dispar*; lanes 3 and 7, negative fecal samples; lane 5, a 100-bp DNA ladder; lanes 4 and 6, empty wells; and lane 8, fecal sample positive control for *E. histolytica*

The four *Entamoeba* spp. were prevalent in patients from rural areas more than urban patients; we found a statistically strong positive correlation between *E. dispar* and mixed infections and the community ($P < 0.05$). In addition, the prevalence of *E. histolytica* and mixed infections was higher in patients lacking sanitation facilities for sewage disposal; we found a statistically significant correlation between *E. histolytica, E. dispar*, and mixed infections

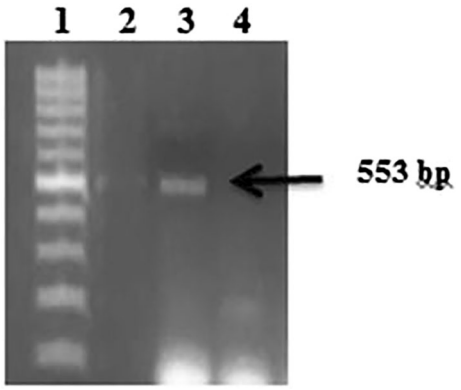

Fig. 2 Detection of *Entamoeba moshkovskii* by polymerase chain reaction (PCR). PCR products were visualized in 1.5% agarose gel with ethidium bromide staining. Lane 1, a 100-bp DNA ladder; lane 2, empty well; lane 3, fecal sample positive control for *E. moshkovskii*; and lane 4, negative fecal sample

and the presence of sanitation facilities ($P < 0.05$). The prevalence of *E. dispar* and mixed infections was higher in patients dealing with cattle, and a statistically significant correlation was observed ($P < 0.05$). Besides, the prevalence of the four *Entamoeba* spp. was higher in men than in women, and the prevalence of *E. histolytica*, *E. dispar*, and *E. moshkovskii* was higher among patients aged from 13 to 30 years. Surprisingly, the prevalence of *E. histolytica* and *E. dispar* was marginally higher in patients with high education levels (Table 2). Statistically, we observed no significant correlation between the four *Entamoeba* spp. and sex, age, or education levels ($P < 0.05$).

Discussion

Although *Entamoeba* infections are often mild in most cases, some strains could invade the intestinal wall, resulting in amoebic colitis and serious extraintestinal diseases. Clinically, amoebiasis is always diagnosed by microscopically examining patients' fecal samples; however, microscopy cannot distinguish and differentiate *E. histolytica* from the morphologically similar nonpathogenic species *E. dispar, E. moshkovskii,* and *E. hartmanni* [6]. This limitation could lead to the treatment of patients who are not actually infected with *E. histolytica* with antiamoebic drugs, such as metronidazole, unnecessarily [1]. The unnecessary treatment of numerous individuals with antiamoebic drugs has already resulted in the development of resistant *Entamoeba* strains and reporting of incorrect epidemiological data regarding both the organism and the disease [6].

In this study, 85% of amebic infections were not caused by *E. histolytica*; this observation is, especially, significant because increasing attention is currently given to traditionally nonpathogenic species as their relationship with invasive amoebiasis has been reported. Compared with *E. dispar* and *E. moshkovskii*, the cysts and trophozoites of *E. hartmanni* can be distinguished from *E. histolytica* by light microscopy; however, this distinction warrants detailed observation of nuclear structures, permanent smear staining, and a highly

Table 2 The distribution of the four *Entamoeba* species according to the sociodemographic characteristics of the 102 patients

	E. histolytica	*E. dispar*	*E. moshkovskii*	Mixed infections
Sex				
Male	15 (100%)	51 (81%)	12 (100%)	9 (75%)
Female	0 (0%)	12 (19%)	0 (0%)	3 (25%)
Age groups				
4–12 years	3 (20%)	9 (14%)	3 (25%)	6 (50%)
13–30 years	9 (60%)	21 (33%)	6 (50%)	6 (50%)
31–65 years	3 (20%)	33 (53%)	3 (25%)	0 (0%)
Community				
Rural area	12 (80%)	45 (71%)	9 (75%)	12 (100%)
City	3 (20%)	18 (29%)	3 (25%)	0 (0%)
Education level				
Low	6 (40%)	30 (48%)	9 (75%)	12 (100%)
High	9 (60%)	33 (52%)	3 (25%)	0 (0%)
Sanitation facilities				
Latrine	6 (40%)	39 (62%)	6 (50%)	0 (0%)
Open defecation	9 (60%)	24 (38%)	6 (50%)	12 (100%)
Dealing with cattle				
Dealing	3 (20%)	33 (52%)	3 (25%)	12 (100%)
Not dealing	12 (80%)	30 (48%)	9 (75%)	0 (0%)

skilled parasitologist, which is often hard to fulfill in many laboratories [1].

In our research, we used the conventional PCR described by Lau et al. [2] and Gomes et al. [7], with slight modifications to detect and differentiate the four species of *Entamoeba* (*E. histolytica*, *E. dispar, E. moshkovskii*, and *E. hartmanni*) directly from fecal samples. Of 115 microscopically positive fecal samples, 102 (88.7%) displayed positive results by PCR; our percentage was higher than that reported by Parija et al. [8] in India, as only 38.8% of their microscopically positive samples revealed positive results by PCR. DNA extraction and PCR amplification failed for the 13 (11.3%) frozen samples. This may be due to the delay in their freezing in the hospital laboratory and the DNA stool extraction kit instructions ensured that stool samples must be fresh for valid DNA extraction. In addition, the failure of DNA amplifications of these samples may be due to inhibition of the reaction by impurities present in the stool samples that contaminated the target DNA and/or reasons related to the extraction procedure, such as inefficient nucleic acid isolation.

In this study, the most abundant *Entamoeba* spp. was *E. dispar*, which consistent with Gomes et al. in Brazil [7], Verweij et al. in Ghana [9], Kebede et al. in Ethiopia [10], Calderaro et al. in Italy [11], Lebbad and Svard, in Sweden [12], Ramos et al. in Mexico [13], Samie et al. in Zimbabwe and Cameroon [14], and Fotedar et al. in Sydney [15]. Besides, several studies reported the predominant prevalence of *E. histolytica* in Malaysia, the Philippines, Gaza and Thailand [16].

In our study, *E. histolytica* was detected in 14.7% of fecal samples, which was lower than those reported by Blessmann et al. (11.2%) and Calegar et al. (23.8%) but higher than those reported by Khairnar et al. (7.4%) [1, 17, 18]. In addition, *E. dispar* was detected in 61.8% of our fecal samples, which was lower than those reported by Parija et al. (57.9%) and Gomes et al. (71%); however, a lower percentage was also reported by El Bakri et al. (2.5%) [6–8]. Using ELISA, Abd-Alla and Ravdin detected *E. histolytica* and *E. dispar* antigens in 43% and 14% of their fecal samples, respectively, in Egypt, which were in contrast to our results [19]. In our study, *E. moshkovskii* was detected in 11.8% of fecal samples, which was higher than those reported by Parija et al. (5.26%) and El Bakri et al. (2.5%) [6, 8]. Moreover, Calegar et al. reported that none of their samples was positive for *E. moshkovskii* [1]. Regarding *E. hartmanni*, 5.9% of our samples were positive and all these samples were mixed infections containing *E. histolytica* and *E. dispar.* Of note, our findings were marginally higher than those obtained by Calegar et al. (4.8%), and their samples were also mixed infections containing both *E. hartmanni* and *E. dispar* [1].

Regarding mixed infections of *E. histolytica* and *E. dispar*, 5.9% of our samples were positive, which was lower than those reported by Calegar et al. (14.3%) and higher than those reported by El Bakri et al. (3.3%) [6, 17].

After comparing the findings in several countries, we inferred that the economic differences and social and environmental risk factors should be cautiously considered as they markedly affect the epidemiology and prevalence of amoebiasis [20]. Of note, the distribution of the four *Entamoeba* spp. among different age groups was variable in our study. Regarding *E. dispar*, its prevalence rate was directly proportional to age and its peak was reported in patients aged< 30 years. Our findings countered Anuar et al. in Malaysia as their highest prevalence rate was observed in children aged> 15 years [16]. In our study, the prevalence of *E. histolytica* was at its peak among patients aged 13–30 years; however, several studies reported the highest infection rates in young children [16, 21–24]. In our study, the prevalence of *E. moshkovskii* was at its peak among patients aged 13–30 years, which corroborated with Anuar et al. as the infection was observed more frequently in older individuals [16].

Regarding gender as a risk factor, the prevalence of the four *Entamoeba* spp. was markedly higher in males in our study, which could be because men are more exposed to the sources of infections than women, such as: more frequent consumption of contaminated outdoor food and water and contact with infected individuals [20]. In Yemen, Al-Areeqi et al. [20] reported similar results for *E. moshkovskii*; however, the prevalence rate was similar in males and females for both *E. histolytica* and *E. dispar*. Conversely, higher prevalence rates of *E. histolytica* infection in females were reported by Ozgumus and Efe in Turkey [25].

Surprisingly, in our study, the prevalence of *E. histolytica* and *E. dispar* was marginally higher in patients with high education level; however, the prevalence of *E. moshkovskii* and mixed infections was higher in patients with low education level. Al-Areeqi et al. reported that the prevalence of their infection correlated with patients with low education level; this could be because non-educated or low educated individuals are supposed to have less health-related knowledge and awareness about parasitic intestinal infections and the ways of their transmission compared with highly educated individuals [20].

Lack of toilets and proper sanitation facilities for sewage disposal is a major risk factor for the dissemination of amoebic infections. In our study, about 50% of patients did not have toilets or proper sanitary facilities at home. In addition, we found a significant correlation between this risk factor and *E. dispar* and *E. histolytica* infections. Likewise, Anuar et al. reported that individuals with no access to adequate sanitary infrastructure demonstrated 1.2 times higher risk of infection, compared with those having access to toilets [16].

Dealing with domestic animals is another crucial risk factor worth considering; the impact of this risk factor on

exacerbating the risk of amoebic infections is of utmost concern to public health, as amoebiasis is not a zoonotic disease. The *Entamoeba* cysts are found in infected 'animals' stool, which could contaminate the 'animals' surface and, thus, be easily transmitted to persons dealing with these animals closely. In our study, nearly 50% of patients dealt with cattle, and we found a significant correlation between this risk factor and *E. dispar* infection. Indeed, all mixed amoebic infections in our study were collected from patients dealing with cattle. Alikewise, Anuar et al. [16], Alyousefi et al. [26], and Pham Duc et al. [27] reported a significant correlation between dealing with cattle and amoebic infections.

In addition, we detected a higher infection rate in patients living in rural areas than those living in the city for the four *Entamoeba* spp., which could be attributed to the lack of personal hygiene and sanitation measures in this community that facilitates the transmission of infection. Statistically, a significant correlation was found between *E. dispar* and community.

Indeed, this study also highlighted the risk for transmission of amoebic infection among the same family members as we found that mixed infections were detected among members of the same family. Similar findings have been reported in Yemen, Malaysia, and Mexico. Notably, the infection could be easily transmitted among family members by consuming cyst-contaminated food and drinks prepared by infected family members who have inadequate personal hygiene. The cysts are the infective stages of *Entamoeba* spp. as they can survive for extended periods on the hands, clothes, bed linens, and toilet seats, withstanding unfavorable environmental conditions, which implies that amoebic infection could also be transmitted by sharing patients' contaminated objects [16, 20].

In addition, amoebiasis can be transmitted orally by drinking water contaminated by *Entamoeba* cysts. In our study, the water source of all patients was the tap water, and none of them depended on mineral water or filtered water as its only water source. Thus, the likelihood of water supply pollution cannot be eliminated as a potential source of infection, as El Behira governorate relies on water derived from the river as its water source. Furthermore, amoebiasis can be transmitted by person-to-person contact, especially among children, crowding, and lack of personal hygiene [28].

Conclusion

This study provides crucial and representative data regarding the public healthcare system in Egypt, as this is the first study to detect and distinguish the four *Entamoeba* spp. molecularly *E. histolytica, E. dispar, E. moshkovskii*, and *E. hartmanni*, by conventional PCR. In addition, the findings revealed the prevalence of amoebic infections caused by the nonpathogenic *Entamoeba* spp., *E. dispar/E. moshkovskii* and *E. hartmanni*, along with the pathogenic *E. histolytica*. Hence, this study recommends that amoebiasis should be diagnosed by PCR to avoid redundant treatment of numerous individuals with antiamoebic drugs which would increase the development of resistant parasitic strains, and obtain proper and accurate epidemiological data concerning the organism.

Compliance with Ethical Standards

Conflict of Interest We declare that we do not have any conflict of interest.

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