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The objective of this study is to estimate the most common G genotypes of human rotaviruses in addition to the most common sequence of partial VP7 gene in clinical specimens, raw sewage, and Nile water samples in Egypt. A total of five hundred and fifty-four stool specimens were collected from December 2020 to April 2022 from children ≤ 3 years old. Of 554 specimens, 182 specimens were positive for rotavirus VP6 using RT-PCR (32.85%). Using multiplex nested and semi-nested RT-PCR, a higher prevalence of common P genotypes than that of common G genotypes was observed in clinical specimens, raw sewage, and Nile water samples. Only common P genotypes could be detected, while P[9] genotype (uncommon P genotype) could not be detected in clinical specimens, raw sewage, and Nile water samples. Of the common G genotypes, G1 was the most prevalent in clinical specimens, raw sewage, and Nile water samples followed by G3. The uncommon G genotypes detected were G9 and G8 in clinical specimens, raw sewage, and Nile water samples. Sequence analysis of 1062 bp of VP7 gene for G1 genotype (the most common G in clinical specimens, raw sewage, and Nile water samples) showed high similarity with human rotavirus Wa strain. In conclusion**,** the highest frequency of G1 genotype among common G genotypes in clinical specimens, sewage, and Nile water samples may make it a suitable G genotype to be added to the common P genotypes in the candidate recombinant subunit vaccine in Egypt and similar patterns in other countries.

Keywords: Human rotaviruses, G genotypes and P genotypes, Clinical specimens and water samples, Silent and non-silent mutations, Vaccine development, Recombinant subunit vaccine

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INTRODUCTION

Human rotaviruses represent a challenge in both developed and developing countries (Du et al., 2022; Gbebangi-Manzemu et al., 2023). Severe gastroenteritis causes 30% to 50% of diarrheal hospitalization cases with a high mortality rate (128000 cases) and 258 million morbidity cases, especially in children less than 5 years old (Mohy et al., 2023). Higher infection rates and higher mortality rates were observed in developing countries than in developed countries. This may be due to better hygienic conditions in developed countries and may be due to the higher percentage of vaccination against human rotaviruses for children in developed countries (Madhi et al., 2010; Parashar et al., 2016). Although two commercial live-attenuated oral rotavirus vaccines, the monovalent Rotarix and the pentavalent Rotateq, are available against human rotaviruses even in developing countries, lower percentages of vaccination were recorded in developing countries. This may be due to the high cost of these vaccines; however, they are out of the list of pathogens in the mandatory vaccination program for children. An additional problem may be that these commercial vaccines do not cover all circulating human rotavirus G and P genotypes (36 G genotypes and 51 P genotypes). VP7 and VP4 are the two outer capsid proteins in the rotaviruses responsible for serotyping and genotyping designations based on the independent neutralization determinants and molecular characterizations of these two outer capsid proteins (Estes, 2001; Matthijnssens et al., 2011). So, this may cause less efficiency of these vaccines in developing countries such as the case with African children (Tate et al., 2016; Harris et al., 2017). Rotavirus G1P[8] strain was the most frequent genotype in a lot of studies worldwide. This may be the reason for using the genotype G1P[8] in the Rotarix vaccine. After that, a high frequency of other G and P genotypes was observed. So, the need for another vaccine emerged. Although Rotateq vaccine contains all common G genotypes (G1, G2, G3, and G4), it may cause some problems in patterns of rotavirus frequency in some countries which have a relatively higher frequency rate of common P genotypes (P[8], P[4], and P[6]) and in the same time a relatively lower frequency rate of common G genotypes (Villena et al., 2003; El-Senousy et al., 2004; Harris et al., 2017).

A previous study with raw sewage, treated effluents, and Nile and drinking water samples collected from El-Berka, Balaks, and Zenin Wastewater Treatment Plants (WWTPs) and El-Giza Water Treatment Plant (WTP) in Cairo, Egypt, in 1998-1999 indicated that common P genotypes are highly frequent in relation to common G genotypes (Villena et al., 2003; El-Senousy et al., 2004). Also, the same pattern of P and

G types was observed in wastewater (raw and treated) and water (raw and treated) from El-Gabal El-Asfar and Zenin WWTPs and El-Giza WTP in 2015-2017 from Cairo, Egypt, with increasing the frequency of common P genotypes and decreasing the frequency of common G genotypes (El-Senousy et al., 2020). The same pattern of frequency was observed for the frequency of common P genotypes and common G genotypes of rotavirus which was observed in other countries (Ouermi et al., 2017; Lartey et al., 2018; Tagbo et al., 2019). On the other hand, sequence analysis of partial rotavirus VP8 gene in the rotavirus isolates in the study of El-Senousy and coworkers (2020) indicated a high similarity to the human rotaviruses Wa strain and A strain with a low percentage of non-silent mutations in clinical specimens and environmental samples in Egypt. These results suggested common P genotypes of human rotaviruses as a good target for recombinant subunit human rotavirus vaccine in Egypt and other countries with the same pattern of prevalence of common P and G genotypes (El-Senousy et al., 2020). The efficiency of the suggested vaccine may be increased if it contains the most frequent G genotypes in addition to the common P genotypes. Also, it is necessary to study the silent and non-silent mutations in the sequences of the most prevalent G genotypes. The objectives of this present study are to determine the most frequent human rotaviruses P and G genotypes in clinical specimens in Egypt and, on the other hand, to determine the most frequent sequences of the highly prevalent G genotypes in the specimens with estimation of silent and non-silent mutations.

MATERIALS AND METHODS

Sewage and Water Samples

A total of 33 sewage samples (4-5-liter volume for each sample) were previously collected from El-Gabal El-Asfar and Zenin WWTPs from January 2021 to April 2022 and from March 2021 to April 2022, respectively. They included 19 raw sewage samples from El-Gabal El-Asfar WWTP and 14 raw sewage samples from Zenin WWTP (14 raw sewage and 14 treated effluents). Also, 14 raw Nile water samples (10-liter volume for each sample) were collected from El-Giza WTP from March 2021 to April 2022. They were previously concentrated and examined for rotavirus VP6 (El-Senousy et al., in press). Positive VP6 samples (27 samples: 13 raw sewage samples from El-Gabal El-Asfar WWTP, 8 raw sewage samples from Zenin WWTP, and 6 Nile water samples) will be further examined for G and P genotypes as explained later. El-Gabal El-Asfar WWTP receives most of the sewage of Cairo Governorate (1,700,000 cubic meters per day (m^3/day)), while Zenin WWTP receives most of the sewage of El-Giza Governorate (330,000 cubic meters per day (m^3/day)).

Collection of Clinical Specimens

A total of five hundred and fifty-four stool specimens were collected from Abo El-Reesh Children Hospital in Cairo, Egypt. Specimen details are shown in Table 1. Abo El-Reesh Children Hospital is the largest children´s hospital in Egypt which receives patients from all Egyptian governorates, so, it may represent a suitable model for rotavirus frequency in children suffering from diarrhea due to viral causes in Egypt.

Concentration of Viruses from Stool Specimens

It was performed according to El-Senousy and coworkers (El-Senousy et al., 2020), whereas 0.1 g of stool specimens was concentrated to a final volume of 50 µl.

Viral Nucleic Acid Extraction

It was performed for concentrated stool specimens using BIOZOL (Bioflux) according to the manufacturer's instructions.

RT-PCR of a Fragment of the VP6-Coding Gene of Rotavirus Group A

According to Iturriza-Gómara et al. (2002), RT-PCR was performed using both the forward VP6-F and the reverse VP6-R primers (1 µm for each) and using 200 U of M-MLV reverse transcriptase enzyme (Promega, USA) and 1.5 U of Taq DNA polymerase (Biobasic, Canada) in a Thermal Cycler machine (Applied Biosystem) to amplify 379 bp. So, nested PCR was performed according to Gallimore et al. (2006) to amplify the 155 bp fragment.

Genotyping for Positive Human Rotavirus Group A VP6 Sewage and Water Samples and Clinical Specimens

Positive rotavirus VP6 sewage and water samples and stool specimens were analyzed for common G and P genotypes according to Gouvea et al. (1990) and Gentsch et al. (1992).

Sequencing of the 1062 bp of VP7 Gene

Sequence analysis was performed for the 1062 bp of VP7 gene using Beg 9 and End 9 primers (Gouvea et al., 1990) for 17 of the genotyped specimens which contain G1 genotype.

Table 1. Number of clinical specimens collected from December 2020 to April 2022 (554 specimens)

Month	Age group																						Total
	0-3		3-6		6-9		9-12		12-15		15-18		18-21		21-24		24-27		27-30		30-33		33-36		
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	
Dec. 2020	3	0	14	14	18	8	9	6	1	0	3	4	0	0	1	2	0	0	1	0	0	0	1	0	85
Jan. 2021	1	0	8	6	23	7	8	5	3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	63
Feb. 2021	1	1	4	3	8	3	10	5	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	38
Mar. 2021	2	0	5	9	10	4	6	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	39
Apr. 2021	3	2	2	3	4	2	10	2	0	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	32
May 2021	2	0	2	4	6	2	4	2	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	24
June 2021	0	1	1	4	2	0	6	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17
July 2021	5	4	1	1	0	2	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	17
Aug. 2021	2	0	2	4	1	0	4	0	0	0	2	0	0	0	3	0	0	0	0	0	0	0	0	0	18
Sep. 2021	3	1	1	0	1	0	7	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	16
Oct. 2021	1	0	2	0	2	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	7
Nov. 2021	0	0	3	0	4	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Dec. 2021	2	0	12	8	16	4	10	3	5	0	2	3	0	0	2	2	0	0	1	0	0	0	1	0	71
Jan. 2022	3	0	11	8	25	10	6	7	5	2	0	2	0	0	1	1	0	0	0	0	0	0	0	0	81
Feb. 2022	0	0	7	2	6	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19
Mar. 2022	0	0	5	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Apr. 2022	0	0	2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Total	28	9	82	67	133	45	84	36	15	5	10	13	0	0	13	7	0	0	3	0	0	0	3	1	554
Total	37		149		178		120		20		23		0		20		0		3		0		4		
Total females	183																								
Total males	371																								
Total	554																								

Statistical Analysis

Pearson's Chi-squared (χ^2) test was used for gender rotavirus infection; rotavirus-positive cases were compared with rotavirus-negative cases. McNemar's test was used for comparison between percentages of rotavirus common P and G genotypes.

RESULTS

Detection of Rotaviruses in Clinical Specimens

One hundred and eighty-two specimens were positive for rotavirus VP6 (182/554, 32.85%). One hundred and twenty-six specimens were positive for rotaviruses in the male group (126/371, 33.96%) and fifty-six specimens were positive for rotaviruses (56/183, 30.6%) in the female group. The highest peak for rotavirus detection was in January 2021 and January 2022 (35/63, 55.56% and 44/81, 54.32%), followed by December 2020 and December 2021 (42/85, 49.41% and 36/71, 50.7%) and then November 2021 (4/11, 36.36%). Rotavirus incidence was low in children ≤ 6 months of age (31/186, 16.67%). The incidence increased in the age group > 6 and ≤ 12 months old (126/298, 42.28%) followed by a slight decrease in rotavirus infection in children aged > 12 and ≤ 18 months old (16/43, 37.21%). Then a rapid decrease in the rotavirus incidence was noted with increasing age to 36 months old (9/27, 33.33%) (Table 2).

Table 2. Percentage of rotaviruses in different age groups in clinical specimens.

Age group	Percentage of frequency
≤ 6 months old	16.67%
6-12 months old	42.28%
12-18 months old	37.21%
18-36 months old	33.33%

Number of Rotavirus Genome Copies in Positive Clinical Specimens

Results of real-time PCR to quantify rotavirus genome copies in positive clinical specimens indicated that, of the 182 positive specimens, 43 specimens contain more than 1×10^7 genome copies/ml stool specimen, with Ct value of 26 or less. Also, 52 specimens contain more than 1×10^6 genome copies/ml stool specimen with Ct value of 30 or less, 35 specimens contain more than 1×10^5 genome copies/ml stool specimen with Ct value 34 or less, 38 specimens contain more than 1×10^4 genome copies/ml stool specimen with Ct value 38 or less and 9 specimens contain more than 1×10^3 genome copies/1ml stool specimen with Ct value 42 or less. Five positive samples could not be quantified. The highest number of genome copies was observed in the peak of rotavirus (autumn and winter months).

Results of Rotavirus G and P Genotypes in Positive Clinical Specimens

Ninety VP6-positive clinical specimens were analyzed for rotavirus G and P types. Seventy-nine clinical specimens could be genotyped for P types (79/90, 87.78%) and fifty-five specimens could be genotyped for G types (55/90, 61.11%). The most frequent P genotypes were P[4] (37 specimens (37/90), 41.11%), P[8] (32 specimens (32/90), 35.56%), and P[6] (10 specimens (10/90), 11.11%), whereas P[9] was the only uncommon P examined (0%), and eleven specimens were untypeable (11/90, 12.22%). The most frequent G genotypes were G1 (22 specimens (22/90), 24.44%), G3 (11 specimens (11/90), 12.22%), G2 (3 specimens (3/90), 3.33%), G9 (13 specimens (13/90), 14.44%), and G8 (6 specimens (6/90), 6.67%), and thirty-five specimens were untypeable (35/90, 38.89%) (Table 3). Common P genotypes were detected in 79/90 (87.78%) of total positive VP6 specimens, while P[9] (the only uncommon P examined) could not be detected in all positive VP6 specimens. Common G genotypes (G1, G2, G3, and G4) were detected in 36/90 (40%) of total positive VP6 specimens, while uncommon G genotypes (G9 and G8) were detected in 19/90 (21.11%) of total positive VP6 specimens.

Results of Rotavirus G and P Genotypes in Positive Sewage and Water Samples

Twenty-five (12 influents of El-Gabal El-Asfar WWTP, 8 influents of Zenin WWTP, and 5 Nile water samples) of the twenty-seven sewage and water samples (13 influents of El-Gabal El-Asfar WWTP, 8 influents of Zenin WWTP, and 6 Nile water samples) could be genotyped for common P genotypes (25/27, 92.59%), while only twelve (12/27, 44.44%) could be genotyped for common G genotypes (6 influents of El-Gabal El-Asfar WWTP, 4 influents of Zenin WWTP, and 2 Nile water samples). Eleven samples had P[4] genotype (11/27, 40.74%), while ten samples had P[8] genotype (10/27, 37.04%). Four samples had P[6] genotype (4/27, 14.81%) and two were untypeable samples (7.41%). Uncommon P genotype P[9] could not be detected in all samples. The genotyped samples for G genotypes had G1 genotype in nine samples (9/27, 33.33%), which were 5 influents of El-Gabal El-Asfar WWTP, 3 influents of Zenin WWTP, and 1 Nile water sample. Genotype G3 could be detected in three samples (3/27 (11.11%), 1 influent of El-Gabal El-Asfar WWTP, 1 influent of Zenin WWTP, and 1 Nile water sample). Uncommon G genotypes could be detected in 8 samples (3 influents of El-Gabal El-Asfar WWTP, 3 influents of Zenin WWTP, and 2 Nile water samples). Genotype G9 could be detected in five samples (5/27, 18.52%, 2 influents of El-Gabal El-Asfar WWTP, 2 influents of Zenin WWTP, and 1 Nile water sample) and genotype G8 could be detected in three samples (3/27, 11.11%, 1 influent of El-Gabal El-Asfar WWTP, 1 influent of Zenin WWTP, and 1 Nile water sample). There were seven (7/27, 25.93%) untypeable samples (Table 4).

Sequencing of Human Rotavirus VP7 Gene in Sewage and Water Samples and Clinical Specimens

Of the 22 genotyped samples with G1 genotype, sequence analysis for the 1062 bp showed two groups of results. Sequence analysis of 16 specimens showed similar sequences and the highest relation to the Wa strain (GenBank nucleotide accessions: FJ423153.1, GenBank protein accessions: ACR22823.1) with 98.31% nucleotide identity and 97.55% amino acid identity. They showed 18 nucleotide substitutions in the coding sequence with 8 amino acid changes (non-silent mutations) (Pro to Arg, Leu to Pro, Leu to Pro, Pro to Arg, Leu to Pro, Leu to Pro, Pro to Arg, and Leu to Pro at positions 58, 102, 107, 131, 148, 171, 196, and 223, respectively). The other 6 specimens had similar sequences and 100% nucleotide identity and amino acid identity with Wa strain (GenBank nucleotide accessions: FJ423153.1, GenBank protein accessions: ACR22823.1). The same results were observed in the sewage and water samples. The twelve genotyped samples with G1 genotype showed similar results to clinical specimens. Sequence analysis of 8 samples (4 raw sewage samples from El-Gabal El-Asfar WWTP, 2 raw sewage samples from Zenin WWTP, and 2 Nile water samples) showed similar sequences and the highest relation to the Wa strain (GenBank nucleotide accessions: FJ423153.1, GenBank protein accessions: ACR22823.1) with 98.31% nucleotide identity and 97.55% amino acid identity. They showed 18 nucleotide substitutions in the coding sequence with 8 amino acid changes (non-silent mutations) (Pro to Arg, Leu to Pro, Leu to Pro, Pro to Arg, Leu to Pro, Leu to Pro, Pro to Arg, and Leu to Pro at positions 58, 102, 107, 131, 148, 171, 196, and 223, respectively). The other 4 samples (2 raw sewage samples from El-Gabal El-Asfar WWTP and 2 raw sewage samples from Zenin WWTP) had similar sequences and 100% nucleotide identity and amino acid identity with Wa strain (GenBank nucleotide accessions: FJ423153.1, GenBank protein accessions: ACR22823.1).

Table 3. Percentage of frequency of rotavirus P and G genotypes in clinical specimens.

P genotypes	% of frequency	G genotypes	% of frequency
P[8]	35.56%	G1	24.44%
P[4]	41.11%	G2	3.33%
P[6]	11.11%	G3	12.22%
Uncommon P types	0%	G4	0%
Untypeable P specimens	12.22%	Uncommon G types (G8 and G9)	21.11%
		Untypeable G specimens	38.9%

Table 4. Percentage of frequency of rotavirus P and G genotypes in sewage and water samples

P genotypes	% of frequency	G genotypes	% of frequency
P[8]	37.04%	G1	33.33%
P[4]	40.74%	G2	0%
P[6]	14.81%	G3	11.11%
Uncommon P types P[9]	0%	G4	0%
Untypeable P samples	7.41%	Uncommon G types (G8 and G9)	29.63%
		Untypeable G samples	25.93%

Alignment of the nucleotide sequences and amino acid sequences of the sequenced clinical and environmental isolates with the vaccine strain Rotarix® showed 95.84%-97.77% nucleotide identity and 94.11%-98.42% amino acid identity.

DISCUSSION

In the present study molecular methods were used to estimate the prevalence of rotavirus as well as to compare the prevalence of rotavirus common and uncommon G and P genotypes among children ≤ 3 years of age in Egypt. Rotavirus VP6 positivity was 32.85%. Significantly higher percentage of positivity was observed in males (33.96%) than females (30.6%). These percentages are in accordance with the results reported by El-Senousy and coworkers (El-Senousy et al., 2020) with a slightly higher percent of rotavirus frequency in our present study. The highest peak for rotavirus detection in our present study was in January 2021 and January 2022 (55.56% and 54.32%, respectively), followed by December 2020 and December 2021 (49.41% and 50.7%, respectively), and then November 2021 (36.36%). In addition to qualitative results, quantitative results in our present study also confirmed the highest prevalence of rotavirus in its peak months (autumn and winter). The highest number of genome copies was observed in the peak of rotavirus (autumn and winter months). Most of the positive specimens in peak months had a high number of genome copies ≥ 10^5 genome copies/ml stool. This may confirm the highest incidence of rotavirus infection in Egypt for children less than 5 years of age in autumn and winter months. Samples were collected from Abo El-Reesh Children Hospital which is the largest children hospital in Egypt and receives patients from all Egyptian governorates, so, it may represent a suitable model for rotavirus frequency in children suffering from diarrhea due to viral causes in Egypt. Also, 17 months of collection including the peak of rotavirus prevalence (autumn and winter) in two years may confirm our results about the prevalence of rotaviruses.

On the other hand, in accordance with the study of El-Senousy and coworkers (El-Senousy et al., 2020), in the present study, rotavirus incidence was low in children ≤ 6 months of age (16.67%). The incidence increased in the age group > 6 and ≤ 12 months old (42.28%) followed by a slight decrease in rotavirus infection in children aged > 12 and ≤ 18 months old (37.21%). Then a rapid decrease in the rotavirus incidence was noted by increasing the age to 36 months old (33.33%). These results are supported by Junaid and coworkers (Junaid et al., 2011). In the present study, the rotavirus positivity rate was lower among children ≤ 6 months old (16.67%). Passive immunity acquired by the infants from their mothers in the first six months may be the reason for this low percentage. This was also confirmed by other studies (Rodrigues et al., 2007; Ouermi et al., 2017). Increasing the rotavirus infection rate between 6 and 18 months may be because not only breastfeeding but also other types of food and drinks may be available for children at this age. Another reason is the disappearance of immunity acquired from mothers after 6 months of age. Collection of samples from El-Gabal El-Asfar WWTP, which receives the majority of

sewage from Cairo Governorate (1,700,000 cubic meters per day (m^3/day)), and Zenin WWTP, which receives the majority of sewage from El-Giza Governorate (330,000 cubic meters per day (m^3/day)), in addition to the one-year survey including the peak of the rotavirus (autumn and winter) may make the sampling pattern a good model that expresses the prevalence of rotaviruses.

Our results of studying rotavirus G and P genotyping in positive rotavirus VP6 clinical specimens and sewage and water samples confirmed the higher prevalence of common P genotypes than that of common G genotypes in clinical specimens, raw sewage, and Nile water samples in Egypt. Also, a higher prevalence of uncommon G genotypes than uncommon P genotypes was confirmed by our results in children less than 5 years of age and results in raw sewage and Nile water samples in Egypt. This pattern of rotavirus genotype distribution was supported by other reports (Ouermi et al., 2017; Lartey et al., 2018; Tagbo et al., 2019).

The most frequent G genotype in clinical specimens, raw sewage, and Nile water samples was G1 with a percentage of 24.44% in clinical specimens and 33.33% in raw sewage and Nile water samples. This high frequency of human rotavirus G1 genotype in the Egyptian community was supported by other reports (Villena et al., 2003; Ouermi et al., 2017). Sequence analysis of clinical specimens, raw sewage, and Nile water samples which had G1 genotypes showed similar results between the clinical isolates and the environmental samples. The high similarity of the frequent G1 in clinical specimens, raw sewage, and Nile water samples in Egypt with the human rotavirus Wa strain and the vaccine strain Rotarix® with a low percentage of non-silent mutations may put it as a strong candidate to be added to common P genotypes suggested by El-Senousy and coworkers (2020) in a candidate recombinant subunit vaccine in Egypt and similar patterns in other countries.

CONCLUSIONS

High prevalence of common P genotypes and, in comparison, low prevalence of common G genotypes in clinical specimens, raw sewage, and Nile water samples in Egypt with higher frequency of G1 genotype among common G genotypes and high similarity with the human rotavirus Wa strain and the vaccine strain Rotarix® and also with the low percentage of non-silent mutations in the partial VP7 gene may make G1 as a suitable G genotype to be added to the common P genotypes in the candidate recombinant subunit vaccine in Egypt and in similar patterns in other countries.

CONFLICT OF INTERESTS

All authors declare that there is no conflict of interests.

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