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Detection of virulence indicator of E. coli O157 causing diarrhea

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Abstract

This study was planned to determine sources of contamination of E. coli O157 that are mainly discovered in animal, children's feces, milk, and water samples and serve as reservoirs for contamination by E. coli O157. About 500 samples (40 cattle fecal swabs, 150 sheep fecal swabs, 120 children fecal swabs, 100 milk samples, and 90 drinking water) were collected for bacteriological examination and identified by the Vitek2 system beside molecular detection of 16 isolates of E.coli O157 by PCR as well as detection of virulence genes (*stx1*, *stx2,* and *eae*) for STEC and EPEC isolates. Out of 500 examined samples 222 (44.4%) were positive for E. coli, by theVitek2 system 16 (7.2%) isolates were positive for E. coli O157 (7.2%) with high incidence in cattle samples (20%), water samples (12.5%), children fecal samples (12.5% & 10%) but low percent (7.9%) in raw milk and (1%) in sheep. Molecular identification of 16 isolates ofE. coli O157 by PCR detected 9 *stx1*, 6 *stx2*, and 16 *eae-positive* strains. It could be concluded that its important to put a control strategic plan for contamination by E. coli O157 on farms and hospitals to minimize the incidence of different infections in animals and humans.

Keywords: E. coli O157; fecal swabs; milk; children; sheep; cattle; Vitek2 system; and PCR

1. Introduction

Enterohemorrhagic Escherichia coli (EHEC) is an important emerging zoonotic foodborne pathogen that can cause watery and/or bloody diarrhea, hemorrhagic colitis (HC), hemolyticuremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (**Barnes et al 2008)**. In humans, EHEC O157 is recognized as a major etiological agent of these diseases, especially in infants and the elderly (**Xiong et al., 2012**). Diarrhea in calves is caused by a variety of aetiological agents including Escherichia coli (**Abd-Ellha 2004).** E. coli strains belonging to enterobacteriaceae family are G-ve, rod-shaped, flagellated, motile, oxidase negative, facultative anaerobic organisms which found as normal habitants of the digestive tract in humans and warm-blooded animals **(Bazeley, 2003)**. Various strains of this species have been classified into different pathogenic types on basis of pathogenesis and virulence factors (**Croxen et al., 2013**). Biochemical tests (IMVIC and TSI) were performed on the non-sorbitol fermenting colonies for conformity identification of E. coli O157 (**Mohmmed et al. 2012**). Escherichia coli colonies on eosin methylene blue agar showed a green metallic sheen (**Yan, et al. 2011)**. E. coli O157 colonies appear smooth and colorless on SMAC agar at 24-48 hrs (**Adamu et al., 2014**).

The pathogenesis of E. coli O157 is associated with several virulence factors, such as Shiga toxin 1 and 2 (encoded by *stx1* and *stx2* genes) and intimin (encoded by *eae* gene). Intimin is a type III secretion system effector protein that facilitates the intimate adherence of E.coli O157 cells to the intestinal epithelium (**Gyles et al., 2007)**. **El-Jakee et al., (2012)** isolated 28 E. coli strains from 250 samples [chicken (100), buffaloes (50), cattle (30) fecal swabs, (30) mastitic cow milk, (50) raw meat, and (30) milk samples] obtained from the same geographical area in Egypt. One E. coli O157 strain of chicken origin contained *stx2* and *eae* genes; 1 E. coli O157 from mastitic milk which had *stx2* gene, and 3 O157 from buffaloes mastitic milk which had *stx1* (100%), *stx2* (33.34%) and *eae* (33.34%) genes and serve as reservoirs for contamination by E.coli O157. The objective of the present work was to determine several sources of contamination by E. coli O157 in animal, children's feces, milk, and water samples.

2. Materials and methods

Numbers and types of samples

A total of 500 samples (40 diarrheic cattle fecal swabs, 150 diarrheic sheep fecal swabs, 120 diarrheic children fecal swabs, 100 milk samples - 90 drinking water) were collected from different sources in Egypt

Isolation of E. coli O157

Twenty five ml of each sample of water and milk was enriched with

225 ml of modified tryptic soy broth (mTSB- DifcoLa Jolla, CA/USA), incubated for 24 h at 37°C. Each fecal swab was directly transferred to 10 ml enrichment broth as described by **(Karuniawati, 2001).** After enrichment; aliquots of 100 µl were plated onto MacConkeys agar (MAC-Difco) and Eosin Methylene Blue (EMB-Difco) agar and onto Sorbitol MacConkey s agar (SMAC-Difco) to test for sorbitol non-fermenting bacteria (colorless colonies). After 18 to 24 h at 37°C, characteristic colonies from SMAC agar were transferred onto Tryptic Soy agar (TSA, Difco) and used for biochemical identification tests.

Phenotypic characterization of E. coli isolates

Purified suspected E. coli-like colonies (n=230) were identified by examining the morphology and biochemical properties of growing colonies. Gram staining was evaluated following the procedure described by (**Cruickshank et al., 1975**) and E. coli-like colonies were subjected to different biochemical tests, including sugar fermentation tests, indole production test, Methyl-Red, and Voges-Proskauer (IMIVC) tests, following the standard methods described by **(Holt et al., 1994**) and (**Quinn et al,. 2002**).

Identification of E. coli O157 by Vitek2 compact system

From the isolated colonies grown on the Mueller Hinton agar 24 h at 37°C, a bacterial suspension was prepared in 3 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12x75 mm clear plastic (polystyrene) test tube. The turbidity of the suspension was adjusted to a McFarland standard of 0.5 with the help of a VITEK-2 Densi Check instrument. The time between the preparation of the inoculum and filling of the card was always less than 30 min. Identification with the VITEK-2 system was performed using a Gram Negative (GN) card according to the Manufacturer's instructions **(Biomeriux, 2006)**. The culture suspension was inoculated into the GN Card with the help of a vacuum device inside the filling chamber. The cards were later transferred into the loading chamber where the cards were sealed and incubated in a rotating carousel at 37°C. Each loaded card was removed from the carousel for every 15 minutes, transported to the optical system for reaction readings and returned to the carousel incubator until the next read time. Data were collected at 15-minute intervals during the entire incubation period.

Serotyping of E. coliO157 isolates

All E. coli isolates were serotyped by slide agglutination test according to (Edwards and Ewings., 1972) using standard monovalent E. coli O157antisera.

Congo red (CR) binding activity

The individual E. coli O157isolates were tested for their binding activity with Congo red dye, which is an indicator of intestinal invasion (Berkhoff and Vinal, 1986). Individual E. coli O157colonies were cultured onto Congo red medium and incubated at 37°C for 24 h. Culture plates were then transferred at room temperature for an additional 24- 48h. of incubation. The growth of red colonies indicates a Congo red positive (CR+).

Detection of virulence genes

DNA extraction. DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. Primers used were supplied from Metabion (Germany, table 1). Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. However in *stx1* and *stx2* PCR, primers were utilized in a 50- µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 9 µl of water, and 12 µl of DNA template. The reactions were performed in an Applied biosystem 2720 thermocycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Aleichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot **(Sambrook, et al 1989)**. Gelpilot 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences		Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Stx1	F	ACACTGGATGATCTCAGTGG	614	94˚C 10 min.	94˚C 1 min.	58˚C 1 min.	72˚C 1 min.	72˚C 10 min.	Dipinetoet al., 2006
	R	CTGAATCCCCCTCCATTATG							
Stx2	F	CCATGACAACGGACAGCAGTT	779						
	R	CCTGTCAACTGAGCAGCACTTTG							
eaeA	F	ATGCTTAGTGCTGGTTTAGG	248	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Bisi-Johnson et al., 2011
	R	GCCTTCATCATTTCGCTTTC							

Results and discussion

Table (2): prevalence, virulence factors and genes among *E. coli* O157 isolates:

Source	Type of Samples	Isolated E.coli	Positive E.coli O157		Invasion of E.coli O157 on Congo Red Agar	Virulence genes		
			+VE	%		Stx1	Stx2	eaeA
Cattle	Fecal swabs	30	6	20	+++	5	4	6
Sheep	Fecal swabs	100	1	1	+++	1	0	1
Children	Fecal swabs	30	3	10	+++	2	1	3
Milk	Raw milk	38	3	7.9	+++	0	0	3
Water	Animal drinking water	24	3	12.5	+++	1	1	3

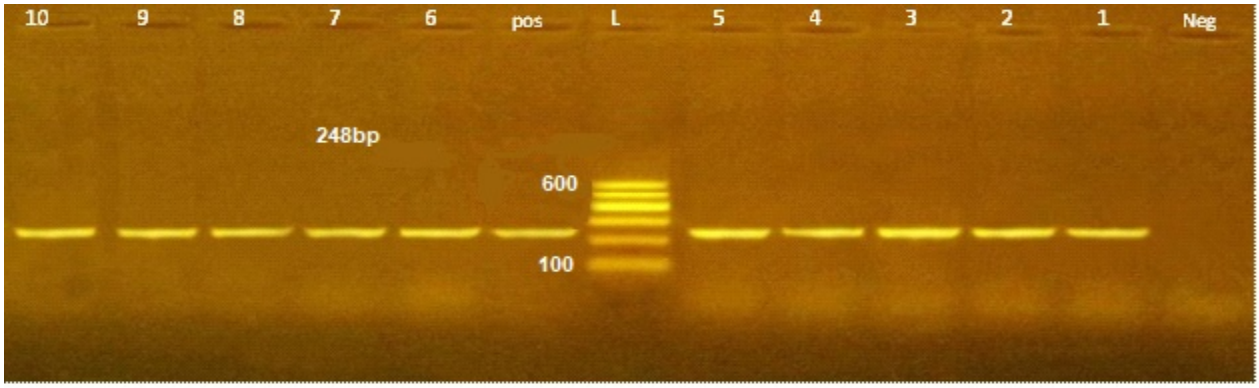

Fig.1. Agarose gel electrophoresis showing results of uniplex PCR for detection of eaeA gene (248 bp) from samples No. (1 to 10) of E.coli O157. Lanes 1-6): Positive eaeA gene from cattle samples. Lane 7: Positive of eaeA gene from sheep samples. Lanes 8 - 10: Positive of eaeA gene from children samples. Neg: Negative control. L: molecular marker (100bp). Pos: Positive control of eaeA gene.

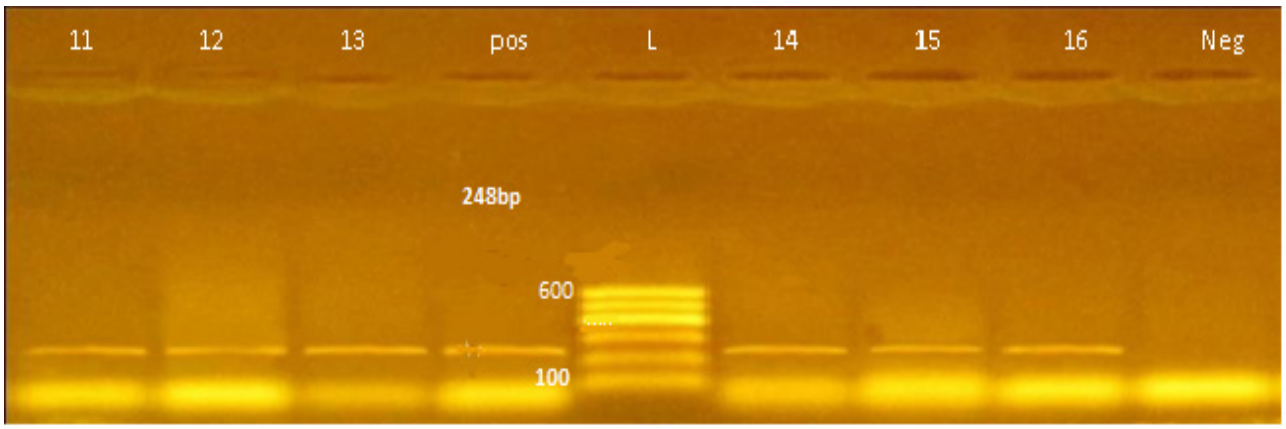

Fig. 2. Agarose gel electrophoresis showing the continuous results of uniplex PCR for detection of eaeA gene (248 bp) fin E. coli O157 isolates. Lanes 11-13: Positive eaeA gene from milk samples. Lanes 14-16: Positive eaeA gene from water samples. L: molecular marker (100bp). Neg: Negative control. Pos: Positive control of eaeA gene (248 bp).

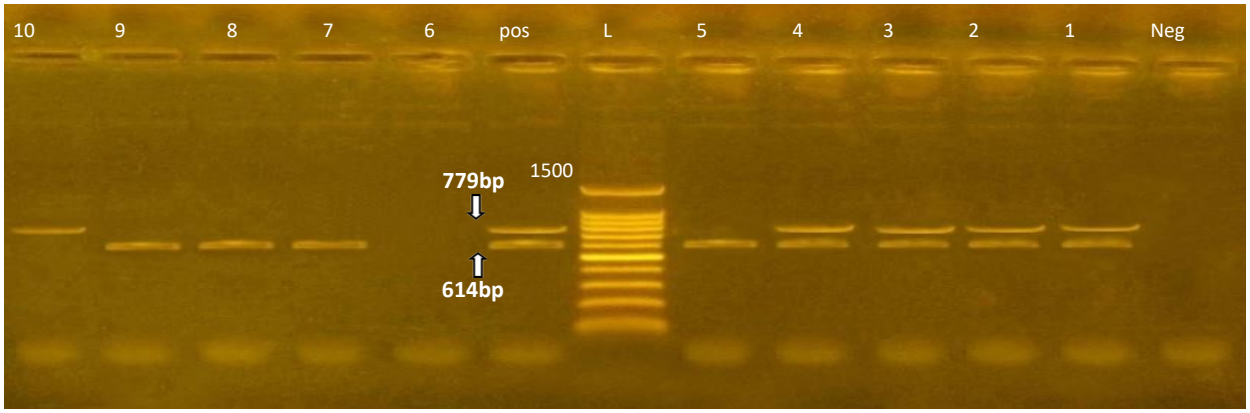

Fig.3. Agarose gel electrophoresis showing results of multiplex PCR for detection of stx1 (614 bp) and stx2 (779 bp) genes from E.coli O157 isolates. Lanes 1-6: Positive stx1 and stx2 gene from cattle samples. Lane 7: Positive of stx1 and stx2 gene from sheep samples. Lanes 8-10: Positive of stx1 and stx2 gene from children samples. Neg: Negative control. L: molecular marker (100bp). Pos: Positive control of stx1 and stx2 gene.

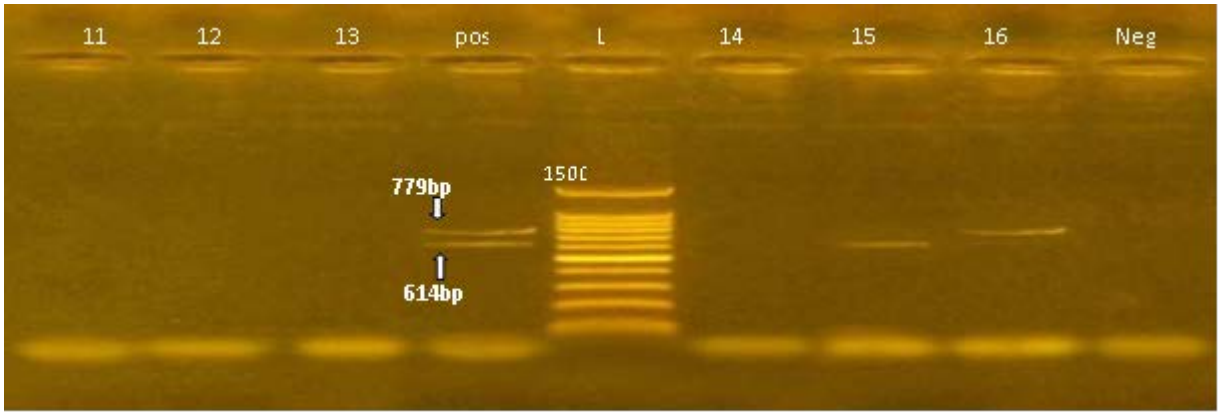

Fig.4. Agarose gel electrophoresis showing the continuous results of multiplex PCR for detection of *stx1* (614 bp) and *stx2* (779 bp) genes from *E. coli* O157 isolates. Lanes 11-14: negative stx1 and stx2 gene from milk samples. Lanes 15 and 16: Positive stx1 and stx2 genes from water samples. L: molecular marker (100bp). Neg: Negative control. Pos: Positive control.

Escherichia coli O157 is a serotype of Shiga toxin E. coli, causing a range of foodborne illnesses (from hemorrhagic diarrhea to acute renal failure) through consumption of contaminated raw food and milk **(Karch et al 2005; Tamparo and Carol 2011**). Although, acute sudden deaths of children less than five years of age, elderly patients, and immunologic patients had been recorded. The fecal-oral route is the main way of transmission and most illness has been through the distribution of contaminated raw vegetables, undercooked meat, and raw milk **(CDC, 2016)**. Escherichia coli O157 causes severe bloody or mucoid diarrhea in calves and death occurs in severe complicated cases fatal meningoencephalitis and septicemia in one-month-old goats were reported by verotoxogenic E. coli O157 (VTEC O157**) (CFSPH, 2016)**. Moreover, Egypt during the period from August 2017 to February 2019. Diarrhea was demarcated as three or more discharges within 12 hours or just one liquid or semiliquid stool with mucus, pus, or blood. **(Shimaa and Gamal, 2020).**

The technique of the vitek2 system has improved the field of bacterial screening by providing a more reliable, faster, cheaper, and highly sensitive technique for bacterial identification. In addition, this can apply as a routine method for laboratory microbiology **(Wallet et al., 2005)**. The results in Table 2 showed that 16 isolates of E. coli O157 were recovered from 500 samples collected from different sources and identified by the vitek2 system. The prevalence of E. coli O157 from cattle fecal samples was (20%). Nearly similar results of E. coli O157 (19%) were reported by **Cernicchiaro, et al., (2012)** from bovine fecal samples. A study by **Hussein and Bollinger, (2005)** indicated the prevalence of VTEC O157 from cattle feces ranged from (0.2%) to (27.8%). The prevalence of E. coli O157 from sheep fecal samples was (1%). The higher percentage (1.4%) and (6.5%) of E. coli O157 in sheep were detected by **Novotna et al (2005) and Ogden et al. (2005),** respectively while a lower % of E. coli O157 (0.7%) in sheep in Great Britain **(Milnes, 2008)**. The E. coli O157 isolated from milk samples were (7.8%). Similar findings were recorded by **Adamu et al., (2014)** who reported that about10 (5%) out of (198) human stool samples were E. coli O157 positive and E. coli O157 isolates from the patients who lived in rural areas were (7) (70%) isolates especially raw milk consumers. These percentages were near to those obtained by**, Mohamed et al., (2003) (**7.1%). while **Abdul-Raouf et al. (1996)** found that, E. coli O157 in raw milk samples was (6%). In this study, the prevalence of E. coli O157 in the water was (12.5%). The same result (12.5%) for E. coli O157 from Nile river samples was achieved by **Mohamed et al., (2003).** However, **Mohmmed et al., (2012)** reported a higher incidence (23%) for E. coli O157 isolates from drinking water in Basrah Province.

All E. coli O157 isolates exhibited an invasive phenotype on Congo Red agar (Table 2). The description of E. coli O157 isolates characters on Congo Red agar was previously described by **Samy et al., (2013) and Shome et al.,(2005)**. Moreover, **Verónica et al (2017)** analyzed 388 samples from milk, air, water, feed, and feces of 10 dairy farms by culture methods and PCR. A total of (47) isolates of Shiga toxin-producing E. coli were obtained, (4) (8.5%) of them belonging to serotype E. coli O157 (3) (6.3%) from milk samples and (1) (2.1%) from water samples. Our results were also similar belonging to serotype E. coli O157 (3) (6.3%) from milk samples and (1) (2.1%) from water samples. Our results were also similar to that reported by **Hiko et al. (2008)** who mentioned that E. coli O157was highly sensitive to amikacin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, norfloxacin, polymyxin B and trimethoprim-sulfamethoxazole and highly resistant to streptomycin, cephalothin, tetracycline, ampicillin and trimethoprim. The antimicrobial susceptibility testing by Vitek-2 on most common Gr-ve bacilli isolated from intensive care unit patients**.**

The pathogenicity of E. coli O157 isolates are associated with different virulence factors, including Shiga toxin that is encoded by (stx1andstx2 gene), intimin (encoded by the eaeA gene), and enterohemolysin (encoded by the Ehly gene) (**Kang et al., 2004).** Intimin is encoded by eaeA gene which is essential for Attaching/Effacing (A/E) lesions resulting in the destruction of the microvilli and helping the colonization of

pathogens in the gastrointestinal tract of the host **(Woodward et al., 2003).** The Shiga toxins that encoded by stx1 and sxt2 genes are consists of five identical B subunits that are responsible for binding the holotoxin to the glycolipid globotriaosylceramide (Gb3) on the target cell surface, and a single A subunit that cleaves ribosomal RNA, causing cessation of protein synthesis leading to cell death **(Bellmeyer et al. 2009).** The result in fig (1,2, 3, and 4) revealed that Out of the 16 isolates of E. coli O157 isolated from different sources (cattle, sheep, milk, children feces, and water) have virulence genes 9 (56.25%), 7(43.75%) and 16(100%) for stx1, stx2, and eaeA, respectively. These results agree with **Alam and Zurek, (2006)** tested all isolates of E coli O157:H7 in beef cattle and showed positive for stx2 (Shiga toxin 2) and eaeA (Intimin) genes, and only (12.8%) were also carried stx1. There are several studies that proved significantly higher frequency of eaeA genes in strains from diarrhoeic calves (60.3%) than in non-diarrheic calves (18.6%; $P < 0.001$) **(Herrera, et al.,2009).** Moreover, they reported a high prevalence of sxt1 gene in the diarrheic strains (41.3%) as well as in non-diarrheic (44.2%). Another study by **Güler et al., (2008)** in Turkey, demonstrated that the frequency of stx1 and sxt2 genes of E coli strains from diarrheic calves were 13.5% and 5.4%, respectively. However, in healthy calves, no E. coli isolates were found positive for these genes (stx1 and stx2). Concerning results obtained by **Khanjar and Alwan, (2014)** concluded that detection of stx1 and stx2 genes in most isolates from diarrheic and non-diarrheic calves indicated that these isolates have the ability to be virulent and possess the pathogenic effect on humans similar results were reported by **Yousif and Mohammed (2015)** isolates E. coli O157 (19) (59.4%) from the calves were possessed stx1 gene and (10) (31.25%) isolates were possessing stx2.

It could be concluded that its important to put a control strategic plan for contamination by E. coli O157on farms and hospitals to minimize the incidence of different infections in animals and humans.

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