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УСТОЙЧИВОСТЬ И РЕЗИСТЕНТНОСТЬ К АНТИБИОТИКАМ ДИАРЕЕГЕННЫХ *ESCHERICHIA COLI* У ФЕКАЛЬНЫХ НОСИТЕЛЕЙ РАННЕГО ДЕТСКОГО ВОЗРАСТА

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РЕЗЮМЕ. Значимость носительства диареегенных *E. coli* у детей обусловлена связью с уровнем распространенности резистентности к антибиотикам и качеством оказания медицинской помощи в педиатрических отделениях. В статье мы представляем данные о различных вариантах диарегенных *E. coli*, выделенных от фекальных носителей раннего детского возраста, и о чувствительности выделенных микроорганизмов к антибиотикам. Исследовались образцы кала детей, поступающих в детский сад. Изоляты *E. coli* исследовались на наличие генов вирулентности путем культивирования и ПЦР-анализа в реальном времени. Все изоляты кишечных возбудителей тестировали на антимикробную чувствительность диско-диффузионным методом в соответствии с протоколами EUCAST (Научный комитет по определению руководящих принципов для интерпретации устойчивости к противомикробным препаратам). В общей сложности было исследовано 680 образцов фекалий, 165 из которых содержали искомые диареегенные изоляты *E. coli*. Только 11,51% фекальных изолятов *E. coli* имели гены вирулентности различных патотипов. Антибиотикорезистентность диареегенных *E. coli*, выделенных от детей раннего возраста без диареи, показала самую высокую фенотипическую устойчивость к AK (амикацин) — 12,3% и SXT (триметоприм / сульфаметоксазол) — 9,7%, CIP (ципрофлоксацин) — 4,3%, FOX (цефокситин) — 2,83 и LEV (левофлоксацин) — 1,58. Одновременная устойчивость более чем к двум антибактериальным препаратам не наблюдалась. Несмотря на низкую распространенность диареегенных *E. coli* среди детей раннего возраста, нельзя недооценивать факты возникновения и распространения эпидемий в детских коллективах. Группы риска должны находиться под регулярным и пристальным наблюдением. Мониторинг антибиотикорезистентности социально значимых инфекционных агентов должен стать частью долгосрочной стратегии в каждой стране.

КЛЮЧЕВЫЕ СЛОВА: диареегенные *E. coli*; чувствительность к антибиотикам; фекальные носители; ранний детский возраст.

IMPACT AND ANTIBIOTIC RESISTANCE OF DIARRHOEAGENIC *ESCHERICHIA COLI* FROM FAECAL CARRIERS IN EARLY CHILDHOOD

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SUMMARY. The importance of the children's faecal diarrhoeagenic E.coli carriers determines the prevalence of antibiotic resistance and the quality of medical care in paediatrics departments. We report the incidence of different categories of diarrhoeagenic E.coli isolated from faecal carriers in early childhood and antibiotic susceptibility. The exams were conducted through routine faecal examinations needed to start kindergarten. *E. coli* isolates were examined by culturing and real-time PCR analyses for *E. coli* virulence genes. All isolates of intestinal pathogens were tested for antimicrobial susceptibility by the disk diffusion method and according to EUCAST protocols. A total of 680 faecal samples were obtained, 165 with suspected diarrheagenic *E. coli* isolates. Only 11.51% of faecal *E. coli* isolates had virulence genes of various pathotypes. Antimicrobial resistance of diarrheagenic *E. coli* isolated from young children without diarrhoea showed the highest phenotypic resistance to AK — 12.3% and SXT — 9.7%, CIP — 4.3%, FOX — 2.83 and LEV — 1.58. Simultaneous resistance to more than two antibacterial drugs was not observed. Despite the low rate of diarrheagenic *E. coli* among young children, the factor for the emergence and spread of epidemics in children's groups should not be underestimated. Risk groups should be closely and regularly monitored. Monitoring the antibiotic resistance of socially significant infectious agents should be a sustainable strategy for each nation**.**

KEY WORDS: diarrheagenic *E. coli*; antibiotic susceptibility; faecal carriers; young children.

INTRODUCTION

Diarrheagenic *Escherichia (E.) coli* (DEC) are being recognized as an important pediatric enteropathogen worldwide. Currently, these organisms are classifiedinto six categories: enteropathogenic *E. coli* — (EPEC), enterotoxigenic *E. coli* — (ETEC), enteroinvasive *E. coli* — (EIEC), diffusely adhering *E. coli* — (DAEC), enteroaggregative *E. coli* — (EAEC), enterohemorrhagic *E. coli* — (EHEC/STEC) [12]. DEC are one of the main causes of sporadic and epidemic diarrhoea in children, with the most commonly affected age group being less than 5 years [9]. In this age group, diarrhoea can quickly become complicated, especially if the etiological agent is phenotypically resistant to antimicrobials commonly used in therapy.

AIM OF STUDY

To assess the severity of early faecal carriers of diarrheagenic *E. coli* as a reservoir for enterocolitis in at-risk pediatric groups (2–6 years), in which the spread of intestinal pathogens is facilitated by close person-to-person contact, and still poorly developed personal hygiene habits in young children.

MATERIALS AND METHODS

For a period of 4 months (August 2021 — November 2021) in the National Reference Laboratory for Intestinal Diseases, NCIPD, Sofia, a total of 680 faecal samples from children without diarrhoea aged 2–6 years were tested. Preventive tests for intestinal pathogens (*Salmonella, Shigella*, DEC) are mandatory before the children

proceed to kindergartens in Bulgaria or after the absence of children from kindergartens for more than a month. The collected faecal samples are from three major cities in the country — Sofia (capital), Varna and Burgas. All samples were aerobically cultured, serologically tested and molecularly examed for virulent *E. coli* genes. The faecal samples for viruses and parasites have not been tested, as this is not the focus of our study. All isolates of DEC, *Salmonella sp.* and *Shigella sp.* have been tested for antibiotic susceptibility.

All parents were informed in writing about the purpose of our study. In addition, according to the normative documents of the Ministry of Health of the Republic of Bulgaria, a child, which is laboratory-confirmed as positive for *Salmonella sp., Shigella sp.*, DEC, has no right to proceed to kindergarten until the presentation of three consecutive negative microbiological tests on faecal samples.

Parents of laboratory-confirmed children positive for *Salmonella, Shigella* or DEC were also examed.

Faecal samples. Faecal samples were collected in sterile containers for this purpose and stored at 4–8 °C for no more than 12 hours until delivery to the microbiological laboratory.

Isolation. Sample faeces were cultured aerobically at 37 °C for 16–18 hours to become differentiating, selective and enriching nutrient media: McConkey agar, Deoxycholate agar, Levin agar and Selenite broth for the enrichment of *Salmonella sp.* and *Shigella sp*. After overnight enrichment, it proceeded with secondary cultures of Selenite broth on a solid Deoxycholate agar medium for detection of *Salmonella sp.* and *Shigella sp.*

Phenotypic identification. All suspected isolates for *Salmonella, Shigella* and diarrhoeagenic *E. coli* were tested with KIA and API20E. Subsequently, they were serotyped with commercial sera (Sifin — Germany, BioRad — USA, SSI — Denmark and BB — NCIPD, Ltd., Bulgaria). DNA was isolated from serotyped DEC isolates with certain OK groups to detect virulent genes. Commensalbacteria were identified with MALDI TOF.

Antimicrobial susceptibility. All isolated *Salmonella enterica, Shigellas p.* and DEC were tested for antimicrobial susceptibility by the disk diffusion method according to the EUCAST protocols to the following antibiotics: Amikacin — 30 μg (AK); Ampicillin — 10 μg (AMP); Levofloxacin — 5 μg (LEV); Ciprofloxacin — 5 μg (CIP); Cefoxitin — 30 μg (FOX); Amoxicillin-Clavulanic acid — 20–10 μg (AMC) and Trimethoprim-Sulfamethoxazole — 1.25–23.75 μg (SXT), (Oxoid ™) on Mueller-Hinton agar, at 37 °C for 18–24 hours. For control, we used *Escherichia coli* ATCC 25922.

DNA extraction. Of all the OK-typed *E. coli* isolates, we extracted bacterial DNA with QIAamp DNA Kits, Germany according to the manufacturer's instructions. The extracted DNA was stored at 80 °C for genetic testing for virulence genes.

Real-time PCR analysis. DEC isolates were examined by real-time PCR to detect virulence genes: *eae* for atypical EPEC; *pEAF* for typical EPEC; *aatA* for EAEC; *daaC* for DAEC; *elt* (lable tox) for ETEC; *est* (stable tox) for ETEC, *ipaH* for EIEC; *stx1* and *stx2* for EHEC [11]. Exemplary pairs and reaction conditions are presented in Table 1. *E. coli* ATCC 25922 and dH_2O were used as negative controls. Positive controls were ready for use DNA od ETEC, EPEC, EAEC and VTEC provided by Satum Serum Institute, Denmark.

RESULTS

Of all the 680 collected and microbiological tested faecal samples from children 2–6 years of age without diarrhoea, nine strains of *Salmonella enterica subsp. Enterica — S. typhimurium* (5/9) and *S. enteritidis* (4/9), 3 strains of *Shigella flexneri* 2b and 165 strains of *Escherichia coli*, belonging to different OK groups, with leading ones O111 and O6 were obtained. And no co-infections were detected.

The results of molecular genetic studies of DEC isolates confirmed 4.24% (7/165) as tEPEC positive for the *pEAF* gene and 1.21% (2/165) as aEPEC, positive for *eae* gene, belonging to OK — group O111; 1.81% (3/165) as lt-ETEC and 0.6% (1/165) as st-ETEC belong to OK group O6; 1.21% (2/165) were EIEC, positive for *ipaH* gene (O112ac and O112ab), 2.24% (4/16) were EAEC, positive for *aatA* gene (O44, O15: H15, O126 — 2 isolates) presented in Table 2, Fig. 1 and 2. Of the 165 *E. coli* isolates that tested positive for agglutination with commercial sera for DEC typing, only 11.51% had virulence genes. *Citrobacter sp., Shigella sp.* and *Enterobacter sp*. show well-observed co-agglutination with polyvalent sera for groups I, II and III of *E. coli* (BB — NCIPD, Ltd., Bulgaria).

Antimicrobial resistance of DEC isolated from young children without diarrhoea. The studied isolates show the highest phenotypic resistance to AK — 12.3% and SXT — 9.7%. In

Table 1

Primers and conditions for Real-time PCR analisis of DEC

Target gene / primername		Concn (μM)	Tm/ bp size	Each 20-μl reaction mixture contained: 10 μl qMAXSen™ Green qPCR Master Mix (2x) Forward and Reverse Praimers (0.35x) DNA (2 μl)+ NF water to final volume	The reaction mixture was subjected to 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. After 45 cycles, a melting curve with a ramp speed of 2.0 °C/s between 70 °C and 95 °C was determined with a reading every 0.2 °C
eae / EAE-S for	ACT GGA CTT CTT ATT RCC GTT CTA TG	0.35	82 °C		
EAE-B2 rev	CCT AAA CGG GTA TTA TCA CCA GA		189 bp		
pEAF / EP-1 for	GTT CTT GGC GAA CAG GCT TGT C	0.35	84 °C		
EP-2 rev	TTA AGC CAG CTA CCA TCC ACC C		107bp		
aatA / EA-1 for	AGG TTT GAT ATT GAT GTC CTT GAG GA	0.35	75 °C		
EA-2 rev	TCA GCT AAT AAT GTA TAG AAA TCC GCT GTT		52bp		
daaC / DAA-F	ATT ACG TCA TCC GGG AAG CAC ACA	0.35	87 °C		
DAA-R	GCT TGC TCA TAA AGC CGC AGA CAA		146bp		
Elt/LTf	GGC GAC AGA TTA TAC CGT GC	0.35	78 °C		
LTr	CGG TCT CTA TAT TCC CTG TT		450bp		
Est / STa-F	ATT TTT MTT TCT GTA TTR TCT T	0.35	73 °C		
STa-R	CAC CCG GTA CAR GCA GGA TT		190bp		
ipaH / IpaH1	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	0.35	85 °C		
IpaH2	GCC GGT CAG CCA CCC TCT GAG AGT AC		603bp		
***stx*1** / stxA1 598	AGT CGT ACG GGG ATG CAG ATA AAT	0.35	80 °C		
stxA1 1015	CCG GAC ACA TAG AAG GAA ACT CAT		418bp		
***stx*2** / Stx2f	GGC ACT GTC TGA AAC TGC CC	0.35	89 °C		
Stx2r	TCG CCA GTT ATC TGA CAT TCT G		246bp		

Table 2

Prevalence of the different diarrheagenic *Escherichia coli* isolated from 2–6 year ages children with no diarrhoea

DEC	No isolates	%	OK-groupes	Genes
tEPEC	7	4.24	O111, O44, O15: H15; O126	*pEAF*
aEPEC	2	1.21	O111	*eae*
lt-ETEC	3	1.81	O6	*elt*
st-ETEC	1	0.6	O6	*est*
EIEC	2	1.21	O112ac, O112ab	*ipaH*
EAEC	4	2.24	O44, O15:H15, O126	*aatA*

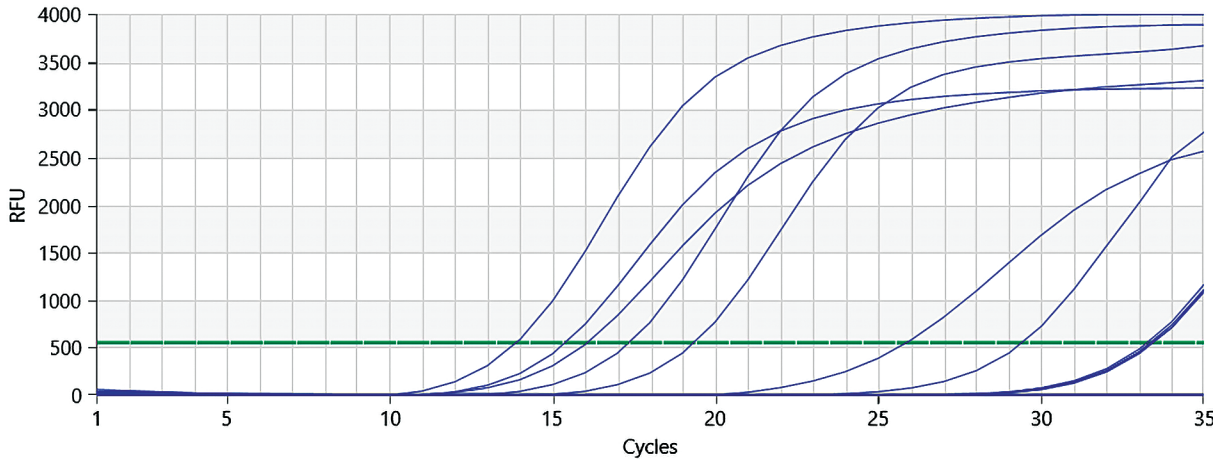

Fig. 1. Amplification analysis of DEC positive control strains — tEPEC, aEPEC, EIEC, ETEC, EAEC, VTEC, DAEC. Negative control dH_2O and *E. coli* ATCC 25922 amplifications are afer 30 cycles

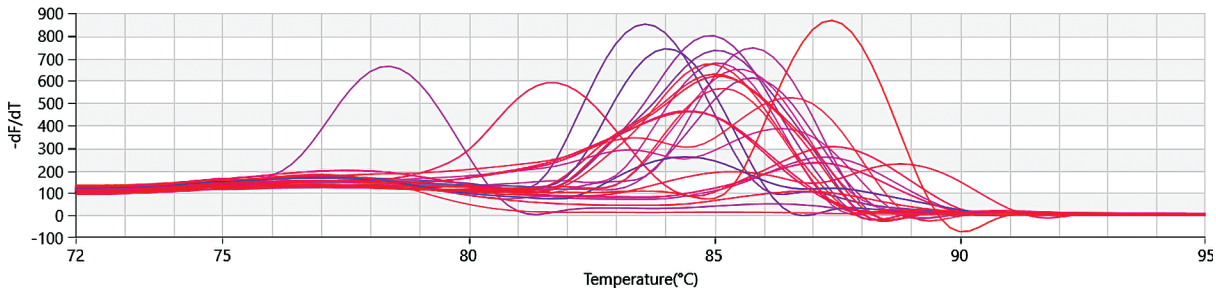

Fig. 2. Melting analisis showed peaks for target genesin DEC starins

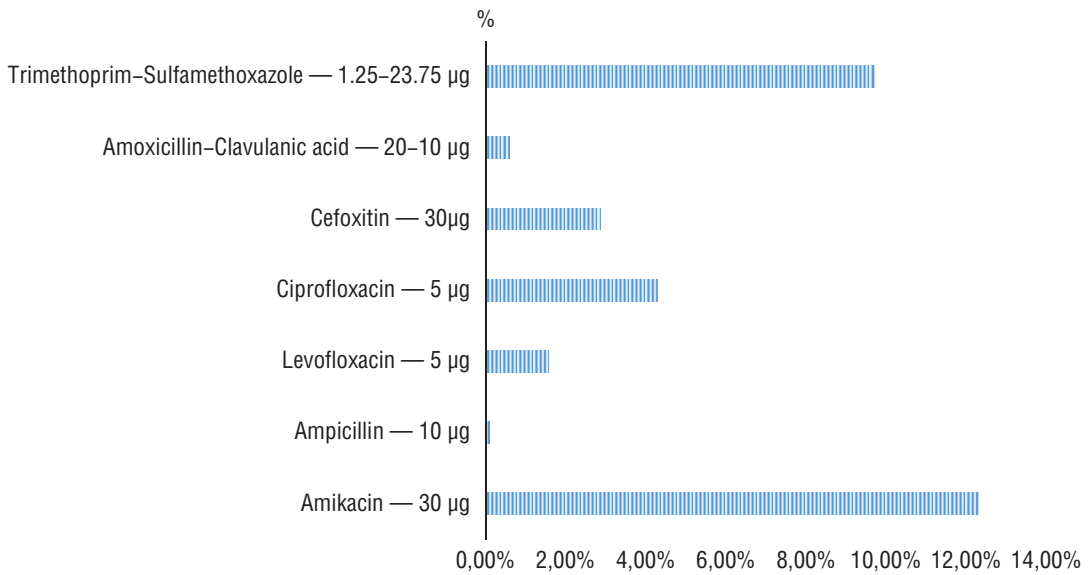

Fig. 3. Antimicrobial resistance of tested DEC isolates from faecal carriers 2–6 year of ages

second place is the resistance to CIP — 4.3% and FOX — 2.83. In particular, we must point out the expressed resistance to LEV — 1.58% (Fig. 3).

From the antimicrobial susceptibility testing of *Shigella flexneri* and *Salmonella enterica* isolates, the results are as follows: (1/4) *S. enteritidis* demonstrates resistance to Trimethoprim-sulfamethoxazole, (1/4) *S. enteritidis* is resistant to Ampicillin, the other two isolates, as well as the three isolates of *Shigella flexneri* 2b, are sensi-

tive to all tested antimicrobial agents. Of all 5 *S. typhimurium* isolates, only one was resistant to Amikacin.

It should be noted that in a large number (53/680) of the studied faecal samples, growth in pure culture of *K. pneumoniae, Enterobacter sp., C. braakii, C. freindii* and *M. morgani.* In addition, we reported *sterile* (11/680) faecal samples, in which primary (24 hours) and secondary (48 hours) cultures after 37 °C cultivation were deprivedof bacterial growth.

No pathogens were isolated from examed parents as contact persons of laboratory-confirmed *Salmonella, Shigella* ordiarrhoeagenic *E. coli*-positive children.

DISCUSSION

In recent years, the NRL for Enteric Diseases has been receiving DEC isolates toconfirm bacterial diagnosis, mainly from hospitalized patients. The Covid epidemic has minimized laboratory-confirmed cases of enterocolitis and the reporting of etiologically confirmed bacterial diarrhoea. Mostly for these reasons, there are no data on the carrier of the OK-serogroups *E. coli* in the country. DEC is being recognized as important pediatric enteropathogens worldwide. Enterotoxigenic *E. coli* is the most common enteropathogen in developing countries, accounting for approximately 210 million episodes of diarrhoea and approximately 380,000 deaths [4, 14]. In Bulgaria, the data on the prevalence of ETEC, as the most common cause of infectious diarrhoea, especially in pediatric patients, do not differ. In our study, ETEC take first place in isolation among children aged 2–6 years without diarrhoea.These are the most common OK-serological groups, O6, which so far have the greatest etiological significance in *E. coli* enteritis, manifested as sporadic or epidemically related diseases. Regardless of this established pattern in our country, it is necessary to take into account the other OK-groups in the course of microbiological research. EPEC and EAEC take second place after ETEC in our study with O111, O44, O15: H15, and O126. Many studies have found a significant association of EPEC with infant diarrhoea. Healthy transmission of intestinal pathogens, in general, is very common in developing countries. Colonization, not disease, can be the result of the interaction of many factors, including host sensitivity (related to the child's age, breastfeeding, nutritional and immunological status), bacterial factors (various virulence genes) and environmental factors. hygiene and high faecal contamination [3, 5, 8]. And since children attending kindergartens are a vulnerable group to the epidemic spread of ETEC, we recommend the strict application of methods for early clinical and etiological diagnosis of carriers and timely and correct collection of materials for microbiological studies of faeces.

Impressive is the large number of *E. coli* isolates serologically identified with commercial *E. coli* sera, and yet only 11.51% of these isolates have virulence genes. This fact raises a significant problem for our country, as the diagnosis of DEC is made solely based on serology and typing, which is a factor in misdiagnosis and misreported positive DEC cases, especially in the absence of clinical manifestations.

Another significant problem is one company's widespread use of *E. coli* serums due to low prices. However, these sera show co-agglutination with other bacterial species, as we found in our study, which provides data showing the importance of a complete biochemical evaluation of other enteropathogenic bacteria before proceeding with typing.

Of all 680 faecal samples of young children without diarrhoea, 53 of them reported growth in pure culture of various commensals such as *K. pneumoniae, Enterobacter sp., C. braakii, C. Freindii* and *M. morgani* important for intestinal microbiota and general immunity [2, 13]. As we do not have data on whether these children took antibiotics shortly before the study or were on a special diet, we can only assume that commensals in the mass are associated with an unhealthy diet or drug therapy.

In recent years, several researchers have reported increased resistance to antibacterial drugs of DEC isolated from sporadic and especially epidemic-related infectious diarrhoea. This fact is essential for the practice, given on the one hand the increasing use of antibiotics and on the other hand the epidemic spread of R-factors, which most often cause this resistance [1, 7]. One of the factors in this problem is excessive consumption and irresponsible prescribing of antibiotics. Also, the use of antibiotics in animal husbandry favours the spread and persistence of resistant bacteria in humans through two different mechanisms: consumption of antibiotic-contaminated meat, in which antibiotics cause selective pressure on the host microbiota, and/or consumption of meat contaminated with antibiotic bacteria.Various studies around the world have shown that ready-to-eat animal products are contaminated with *E. coli* strains resistant to various types of antibiotics, mainly to β-lactams through

the production of broad-spectrum β-lactamases (ESBL) [6, 10]. This is also confirmed by the tested Bulgarian isolates of DES from healthy children. The results obtained from the phenotypic testing of isolates still show low values, but clearly expressed resistance to AK — 12.3% and SXT — 9.7% followed by CIP — 4.3% and FOX — 2.83. In particular, we must point out the expressed resistance to LEV — 1.58%. This could be explained by the widespread use of these antibacterial agents in recent years and the rapid development and spread of resistance to them. Analyzing the obtained results, we hypothesize that the increasing values of resistant DEC isolates among young children are most likely of origin to sick adults and/or asymptomatic faecal carriers, which are often proven over the years as a source of epidemics transmitted by food.

CONCLUSION

Due to the ever-increasing number of infections caused by resistant *E. coli* and their ease of faecal-oral transmission among humans and environmental sources, understanding the epidemiology of these strains and their mechanisms of resistance is crucial in combating these infections. Furthermore, timely detection, isolation and treatment of enteric infectious agents among children will prevent the development of outbreaks, and reduce the cost of hospital care and the risk of developing severe infectious diarrhoea with the unwanted lethal exit.

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Conflicts of interest. The corresponding author states that there is no conflict of interest.

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