Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 12, 2015

Original Article

PREVALENCE AND MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM β-LACTAMASES IN *KLEBSIELLA PNEUMONIAE* ISOLATES FROM CANCER PATIENTS AND OTHERS

TAGHRID S. EL-MAHDY[1,2]*, MOHAMMED O. ABDELAZIZ[2], RAMADAN A. EL-DOMANY[3]

[1]Dept. of Biomedical Sciences, College of Clinical Pharmacy, King-Faisal University, Al Ahsa, Saudi Arabia, [2]Dept. of Microbiology and Immunology, Faculty of Pharmacy, Helwan University, Cairo, Egypt, [3]Dept. of Microbiology and Immunology, Faculty of Pharmacy, Kafr El-Sheikh University, Egypt

Email: telmahdy@kfu.edu.sa, & sata186@hotmail.com

Received: 27 Aug 2015 Revised and Accepted: 27 Oct 2015

ABSTRACT

Objective: *Klebsiella pneumoniae* is highly prevalent in hospitals and causes many nosocomial infections. The study sought to determine prevalence rates of extended spectrum β-lactamases (ESBLs) in clinical isolates of *K. pneumoniae* from Cairo, Egypt and to detect the ESBL-encoding genes in the isolates.

Methods: *K. pneumoniae* isolates were collected through two-year period (2011-2012). Identification of *K. pneumoniae* was carried out using automated Microscan and standard biochemical tests. ESBL pattern and minimum inhibitory concentrations (MICs) were detected using Clinical and Laboratory Standards Institute guidelines and confirmatory tests. Multiplex polymerase chain reaction for ESBL-encoding genes and plasmid profiling were performed.

Results: In the present work; 112 isolates, 75 of them from cancer patients, were characterized. High proportion (52 of 112, "46 %") of ESBLs among the isolates were detected. Highest prevalence of ESBLs was seen among cancer patients, 39 isolates of 75 (52%). Plasmid profile for ESBL-producing *K. pneumoniae* isolates showed different sizes and numbers of plasmids in all isolates. MICs for all ESBL-producing isolates revealed high resistance rates with tetracycline (100%), cefepime (96%), gentamycin (90%) and ciprofloxacin (79%). Whereas, only two isolates (4%) were resistant to both carbapenem drugs tested, imipenem and meropenem. bla_{TEM}, bla_{SHV}, and bla_{CTX-M} were performed for all ESBL-producing isolates. Five patterns of ESBL-encoding genes were detected. The most prevalent ESBL-encoding gene was bla_{TEM}; alone in 40% and with other ESBL-encoding gene(s) in 48% of the isolates.

Conclusion: High prevalence of ESBL (46%) in our isolates suggesting the need for continuous monitoring of emergence of this pattern in our region.

Keywords: Antimicrobial resistance, Egypt, ESBL, *Klebsiella pneumoniae*.

INTRODUCTION

Among the most clinically important β-lactamases are the extended-spectrum β-lactamases (ESBLs). Infections due to ESBL-producing organisms are associated with increased mortality among adults and children and are increasing in frequency [1]. Considerable proportions of Enterobacteriaceae, including *Klebsiella pneumoniae*, have been observed to harbor ESBLs worldwide [2-4]. *K. pneumoniae* has become an important pathogen in nosocomial infections, and its clinical isolates are the major hosts for ESBLs [5, 6]. ESBL-producing organisms mostly carry self-transferable plasmids, which co-transfer enzymes conferring resistance to fluoroquinolones, aminoglycosides and tetracyclines [7, 8]. Antimicrobial resistance problems in African countries, including Egypt, have not been illustrated adequately yet, due to low financial resources. Knowledge of the prevalence rates of antimicrobial resistance in common nosocomial pathogens in these countries is an urgent need to prevent healthcare-associated infections and to improve guidelines of empirical antibiotic therapy [9]. TEM and SHV ESBLs spread during the 1980s and 1990s, largely in *Klebsiella* spp. and particularly in intensive care units. A massive shift in the distribution of ESBLs has occurred since 2000 with the spread of CTX-M types [10]. The current study aims to determine the prevalence rates of ESBLs in *K. pneumonia* clinical isolates, mainly from cancer patients, from multi-centers in Cairo; Egypt and to characterize the ESBL-encoding genes in our isolates.

MATERIALS AND METHODS

Selective isolation of microorganisms

Clinical isolates of *K. pneumoniae* were collected during a two-year period (2011-2012) from four geographically distributed hospitals in Cairo, namely, National Cancer Institute (NCI), Naser Medical Institute (NMI), National Heart Institute (NHI), and Helwan Hospital (HH). Isolates were limited to one per patient, were accepted regardless of specimen source and were collected from inpatients and outpatients. Upon isolates receipt, they were sub-cultured on MacConkey's agar and incubated at 37 °C/24 h. Their taxonomic identities were confirmed using standard biochemical tests, as follow: pink colonies on MacConkey's agar, black mucoid colonies on EMB agar, colorless ether layer in indole test, yellowish color in methyl red test, red color in Voges-Proskauer test and blue color with growth on citrate medium. Further confirmation of identification was performed by Microscan in the faculty of medicine laboratories at Cairo University. Pure cultures were emulsified in 100 µl sterile nutrient broth and 100 µl sterile glycerol using sterile cotton swabs and stored in cryovials at -80 °C for long preservation.

Agar dilution antimicrobial susceptibility testing for ESBL screening and for different antimicrobials

The minimum inhibition concentrations (MICs) of different antibiotics were determined by the agar diffusion method according to Clinical and Laboratory Standards Institute (CLSI) [11] on Muller-Hinton agar. Experiments were repeated at least twice for results confirmation. Plates were incubated at 37 °C for 18 to 24 h and the MICs were calculated. Spots with the lowest concentrations of antibiotic showing no growth were defined as the MIC. For ESBL screening, a more than 3 two-fold concentration decrease in MIC for ceftazidime+clavulanic acid in combination versus ceftazidime alone is considered to be positive result for ESBL phenotype [11].

Iodometric method for detection of β-lactamases

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolorizes starch-iodine complex within 5 min [12]. This reaction can be exploited to detect β-lactamase activity in tubes. Positive and

negative controls are vital, as extraneous protein reduces iodine, and over-heavily inoculated tests may give false-positive results.

Confirmatory tests for detection of ESBL in *K. pneumoniae*

Escherichia coli ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as a negative and positive control for ESBL test, respectively. The double-disk synergy test was performed by a standard disk diffusion assay on Muller-Hinton agar following CLSI guidelines. Disks containing ceftazidime, ceftriaxone, or cefotaxime (30µg each) were placed at distances 30 mm from center to center around a disk containing amoxicillin (20µg) plus clavulanic acid (10µg). Enhancement of the inhibition zone toward the amoxicillin/ clavulanic acid disk was considered suggestive of ESBL production [13]. The combination disks (cefotaxime 30µg/clavulanic acid 10µg or ceftazidime 30µg/clavulanic acid 10µg) were placed on each plate. An organism was interpreted as the ESBL producer if there was an increase of ≥5 mm in inhibition zone of the combination disk when compared to the corresponding cephalosporin disk alone [11].

Plasmid extraction and profiling for ESBL-producing *K. pneumoniae*

Examination of plasmid DNA was performed by plasmid DNA extraction (Gen Elute plasmid miniprep kit, Sigma) following the manufacturer recommendations followed by direct agarose gel electrophoresis of extracted DNA [14].

Table 1: Ceftazidime and ceftazidime/clavulanic acid MICs, source of specimen and hospital of isolation for ESBL-producing *K. pneumoniae* isolates

Isolate code	MIC (µg/ml)		Source of specimen	Hospital
	Ceftazidime	Ceftazidime+clavulanic acid		
KP1	64	4	Sputum	NCI
KP2	512	16	Stool	NCI
KP3	128	4	Blood	NCI
KP4	64	2	Blood	NCI
KP5	512	32	Urine	NHI
KP6	512	16	Sputum	NCI
KP7	512	64	Sputum	NHI
KP10	256	8	Sputum	NCI
KP13	128	4	Urine	NMI
KP16	128	2	Blood	NCI
KP17	64	2	Sputum	NMI
KP18	256	16	Sputum	NCI
KP19	128	8	Blood	NCI
KP20	64	4	Urine	NMI
KP21	128	4	Urine	NHI
KP25	256	8	Sputum	NMI
KP28	128	8	Urine	HH
KP29	32	2	Blood	NCI
KP31	128	4	Pus	NCI
KP34	512	16	Urine	NCI
KP37	128	8	Pus	NCI
KP39	128	4	Sputum	NCI
KP40	32	2	Urine	NCI
KP41	512	16	Urine	NCI
KP42	128	8	Urine	HH
KP48	64	4	Sputum	NHI
KP50	128	8	Sputum	NCI
KP51	256	32	Urine	NCI
KP53	518	8	Sputum	NCI
KP54	64	2	Pus	NCI
KP57	32	2	Blood	NCI
KP59	128	8	Blood	NCI
KP60	1024	32	Pus	NCI
KP64	512	32	Stool	NCI
KP65	128	16	Urine	NMI
KP70	64	2	Urine	NCI
KP72	256	8	Sputum	NCI
KP73	128	8	Sputum	NCI
KP74	256	32	Sputum	NCI
KP75	256	16	Urine	NCI
KP76	128	4	Sputum	NCI
KP78	256	16	Sputum	NCI
KP79	256	8	Sputum	NCI
KP80	64	2	Sputum	NMI
KP81	128	4	Urine	NHI
KP86	256	8	Blood	NCI
KP87	256	8	Blood	NCI
KP90	256	4	Pus	NCI
KP91	1026	24	Sputum	NCI
KP92	512	8	Blood	NCI
KP93	128	4	Blood	NCI
KP94	1024	8	Blood	NCI

NCI: National Cancer Institute, NMI: Naser Medical Institute, NHI: National Heart Institute, HH: Helwan Hospital

DNA template preparation and PCR-based screening for ESBL genes

Rapid DNA preparation was performed using boiling technique for 10 min. After centrifugation at 5000 rpm for 5 min, the supernatant was used as a template for amplification or stored at -20 °C for later use. Screening for the presence of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes in the ESBL-producing *K. pneumoniae* was done according to described multiplex PCR protocol [15]. The amplification was done using Ready Mix™ PCR Reaction Mix (Sigma-Aldrich LP, USA). PCR reactions were carried out using 1µl DNA solution, Ready Mix™ PCR Reaction Mix (Sigma-Aldrich LP, USA) and 10 p mol of each gene-specific primer in a final volume of 25µl. PCR amplification conditions were as follows: initial denaturation step at 95 °C for 15 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min.

RESULTS

Isolation and identification of *K. pneumoniae*

A total of 112 *K.pneumoniae* isolates were collected from different inpatient and outpatient cases in four geographically distributed hospitals in Cairo, Egypt. Isolates were collected during a two-year period (2011-2012) and identified as *K. pneumoniae* by Microscan and standard biochemical tests. Out of 112 *K. pneumoniae* isolates, 75 isolates were collected from NCI "cancer patients", 15 isolates from NHI, 12 isolates from NMI, and 10 isolates from HH.

Table 2: MICs determination of antimicrobial agents against ESBL-producing *K. pneumoniae* by agar dilution technique

Isolate code	MICs (µg/ml) of different antimicrobials against tested *K. Pneumonia*							
	FEP	SXT	GEN	AMK	MER	IPM	TET	CIP
KP1	32	4/76	512	4	1	1	512	2
KP2	128	1/19	1024	32	1	1	1024	16
KP3	512	4/76	8	8	1	1	128	64
KP4	64	2/38	8	8	1	2	512	64
KP5	1024	2/38	256	4	1	1	256	128
KP6	1024	2/38	1024	128	1	1	128	128
KP7	1024	2/38	1024	16	1	1	256	128
KP10	32	4/76	64	16	1	1	1024	128
KP13	32	4/76	8	8	1	1	512	16
KP16	256	4/76	16	2	1	1	256	128
KP17	512	4/76	1024	16	1	1	128	128
KP18	64	4/76	1024	16	8	8	256	32
KP19	32	4/76	128	8	1	1	256	2
KP20	32	4/76	1024	8	1	1	128	128
KP21	64	4/76	1024	128	1	1	1024	32
KP25	32	4/76	512	64	1	1	256	128
KP28	64	4/76	256	4	1	1	128	128
KP29	16	4/76	128	64	1	1	256	128
KP31	512	4/76	512	32	1	1	128	32
KP34	1024	1/19	128	8	1	1	256	2
KP37	256	1/19	1024	128	1	1	1024	64
KP39	32	1/19	32	2	1	1	1024	128
KP40	64	4/76	1024	32	1	1	1024	16
KP41	512	4/76	1024	16	1	1	1024	2
KP42	16	4/76	1024	16	1	1	128	2
KP48	32	4/76	256	8	1	1	256	2
KP50	64	4/76	64	4	1	1	128	2
KP51	64	4/76	64	4	1	1	128	2
KP53	256	4/76	128	32	1	1	256	128
KP54	1024	2/38	1024	8	1	1	1024	32
KP57	32	4/76	128	4	1	1	1024	64
KP59	256	4/76	256	4	1	1	256	128
KP60	64	4/76	1024	8	1	1	128	2
KP64	256	2/38	1024	64	1	1	512	128
KP65	64	2/38	1024	32	1	1	1024	64
KP70	32	2/38	512	64	1	1	128	128
KP72	512	4/76	1024	32	1	1	256	16
KP73	256	4/76	256	8	1	1	256	128
KP74	256	4/76	256	8	1	1	128	128
KP75	32	4/76	1024	64	1	1	1024	64
KP76	256	4/76	1024	64	1	1	256	128
KP78	64	4/76	1024	64	8	8	1024	32
KP79	512	2/38	8	1	1	1	128	128
KP80	512	2/38	1024	64	1	1	1024	128
KP81	1024	2/38	1024	32	1	1	512	32
KP86	256	4/76	1024	64	1	1	128	64
KP87	256	4/76	16	2	1	1	1024	32
KP90	32	4/76	512	16	1	1	128	128
KP91	512	4/76	512	32	1	1	256	64
KP92	32	4/76	8	1	1	1	1024	2
KP93	32	4/76	1024	128	1	1	128	4
KP94	512	4/76	256	8	1	1	256	2

FEP: cefepime, SXT: trimethoprim/sulfamethoxazole, GEN: gentamycin, AMK: amikacin, MER: meropenem, IPM: imipenem, TET: tetracycline, CIP: ciprofloxacin.

Table 3: Number and percentages of resistant isolates in the 52 ESBL-producing *K. pneumoniae* to tested antimicrobial agents

Antimicrobial agent	Resistant *K. Pneumonia*	
	No.	%
FEP	50	96
SXT	37	71
GEN	47	90
AMK	13	25
MER	2	4
IPM	2	4
CIP	41	79
TET	100	100

FEP: cefepime, SXT: trimethoprim/sulfamethoxazole, GEN: gentamycin, AMK: amikacin, MER: meropenem, IPM: imipenem, TET: tetracycline, CIP: ciprofloxacin

Screening of ESBLs

A more than 3 two-fold concentration decrease in MIC for ceftazidime in combination with clavulanic acid versus ceftazidime MIC alone, when tested by agar dilution method, is considered to be positive result for ESBL phenotype according to CLSI recommendations. Out of the 112 *K. pneumoniae* isolates, 52 isolates (46%) were identified as ESBL-producing isolates (table 1).

Of the 52 ESBL-producing *K. pneumoniae* isolates, 39 isolates (75%) were collected from NCI "cancer patients", 6 isolates (11%) from NMI, 5 isolates (10%) from NHI, and 2 isolates (4%) from HH. The results also show that the prevalence of ESBL-producing *K. pneumoniae* was highest in the NCI hospital, 52 % (39 isolates of 75 isolated from the hospital) and was lowest in the HH, 20 % (2 isolates out of 10). Whereas, the prevalence of ESBL-producing *K. pneumoniae* was 50 % (6 of 12) and 33% (5 of 15) in NMI and NHI, respectively. Regarding the prevalence of ESBL-producing *K. pneumoniae* among different clinical samples, sputum was the most prevalent source (37%) and stool was the least prevalent one (4%) (table 1).

Antimicrobial susceptibility testing for ESBL-producing *K. pneumonia*

Table 2 shows MICs estimated by agar dilution technique for ESBL-producing *K. pneumoniae* for different antimicrobials.

Interpretation of MICs was performed according to CLSI guidelines. The numbers and percentages of resistant isolates in ESBL-producing *K. pneumoniae* to the eight tested antimicrobial agents were summarized in table 3. All ESBL-producing isolates were resistant to tetracycline, whereas two isolates (KP18 and KP78) were resistant to carbapenems tested, imipenem and meropenem. The two isolates were also resistant to the remaining antimicrobial agent tested except isolate KP18 with amikacin. Additionally, most of our ESBL-producing *K. pneumoniae* (96%) were resistant to the fourth generation cephalosporin, cefepime.

Iodometric method for detection of β-lactamases

The presence of blue color using iodometric method was indicative of the β-lactamase production. All ESBL-producing *K. pneumoniae* were positive in the screening of β-lactamases using the iodometric method.

Confirmatory tests for detection of ESBL in *K. pneumoniae*

Enhancement of the inhibition zone toward the amoxicillin+ clavulanic acid disk was considered suggestive of ESBL production. All our 52 ESBL-producing *K. pneumoniae* isolates were positive for this test. Moreover, the combined disk method confirmed ESBL production in the 52 *K. pneumoniae* clinical isolates as evidenced by an increase in zone of inhibition of cephalosporin (cefotaxime or ceftazidime) plus β-lactamase inhibitor (clavulanic acid) ≥ 5 mm than any single cephalosporin.

Plasmid extraction and profiling for ESBL-producing *K. pneumoniae*

The plasmid profiles of all ESBL-producing isolates were analyzed by agarose gel electrophoresis. All 52 ESBL-producing *K. pneumoniae* isolates harboured plasmids with different sizes. The number of plasmids in each isolate varied from one plasmid to eight plasmids (isolate KP28).

PCR-based screening for ESBL genes

All 52 positive ESBL-producing *K. pneumoniae* were examined for the prevalence of ESBL-encoding genes by multiplex PCR. The results of amplification showed the presence of three bands of different sizes, 445bp equivalent to bla_{TEM}, 593bp equivalent to bla_{CTX-M} and 747bp equivalent to bla_{SHV} as shown in fig. 1.

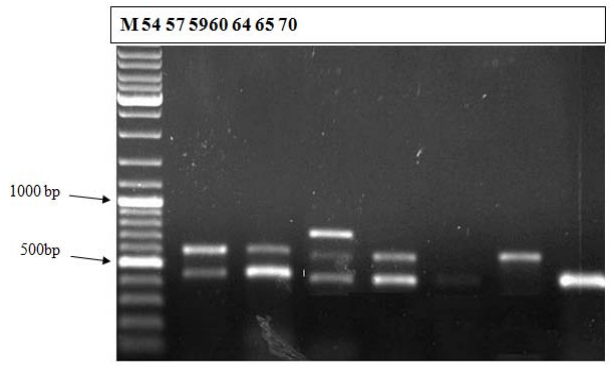

Fig 1: PCR of the amplified genes with a relative size of 445, 593, 747 bp for bla_{TEM}, bla_{CTX-M}, and bla_{SHV}, respectively. M: is GeneRuler DNA ladder mix (Thermo Scientific, USA).

Five patterns of ESBL-encoding genes were detected in our isolates. Among the tested isolates, the most prevalent ESBL-encoding gene was bla_{TEM} alone (40%) while the least prevalent pattern of ESBL-encoding genes was bla_{CTX-M}+bla_{SHV} (2%) (table 4). The bla_{TEM} was evident in 46 of 52 isolates (88%) either alone or with other ESBL-encoding gene(s).

Table 4: Prevalence and pattern of ESBL-encoding genes among ESBL-producing *K. pneumoniae*

	ESBL-encoding genes pattern of				
	bla_{TEM}	bla_{TEM}+bla_{CTX-M}	bla_{TEM}+bla_{CTX-M}+bla_{SHV}	bla_{CTX-M}	bla_{CTX-M}+bla_{SHV}
Number of isolates harboring ESBL-encoding genes (%)	21 (40%)	15 (29%)	10 (19%)	5 (10%)	1 (2%)

DISCUSSION

The emergence of ESBLs in *K. pneumonia* continues to be of critical concern for the choice of treatment options against infections caused by this bacterium. ESBLs represent the second and a greater plasmid-mediated threat to oxyimino-cephalosporins, affecting fourth as well as third-generation analogues [10]. The prevalence of ESBLs differs among patient groups, harboring bacteria and clinical and geographic settings. A recent systematic review about ESBL-producing Enterobacteriaceae in African studies published 2005 onwards reported that the proportion of ESBL-producing isolates was < 15% in 16 out of 26 studies included in their systematic search [9]. They also noticed that ESBLs were more commonly identified among *Klebsiella* spp. than *E. coli* isolates, which is consistent with data from Europe [2].

Moreover, a study from Egypt detected that 79% of their *K. pneumoniae* isolated from bloodstream infections in intensive care units between 2006 and 2007 carry ESBLs [16]. However, it should be noted that ESBL acquisition from blood cultures of intensive care unit patients with nosocomial infections may occur more commonly. In our study, 46 % of *K. pneumoniae* isolates were ESBL producers. This is higher than reported by others from Algeria (19.9%) [17], Germany (3%) [18], Italy (30%) [19] and Lebanon (20%) [20]. On the other hand, a global surveillance database collected from Europe, North and South America, and Asia, showed that the detection frequencies for ESBL-producing *K. pneumoniae* isolates were 7.5–44% [21]. In a recent study from a cancer center in Japan [22], the ESBL producing *K. pneumoniae* isolated between 2009-2013 represented only 0.01 per 100 admissions throughout the study period. The situation in our study is much worse, where the prevalence of ESBL among our *K. pneumoniae* isolates from cancer patients were 52%.

Our ESBL-producing isolates were also resistant to fluoroquinolones, aminoglycosides, tetracycline and trimethoprim/ sulfamethoxazole suggesting that ESBL-encoding genes are carried on self-transferable plasmid(s) which often carry other genes encoding resistance to different drug classes making treatment options extremely limited. Among aminoglycosides, amikacin is likely to show the greatest percentage of susceptible strains, particularly in the United States with the resistance of approximately 10%. Amikacin is a likely alternative for empirical therapy when other agents cannot be used [23]. In the current study, 75% of ESBL-producing *K. pneumoniae* were susceptible to amikacin allowing its use as alternative therapy for ESBL-producing bacterium.

The current results show high dissemination of bla_{TEM} gene in our isolates (88%) either alone or with other ESBL-encoding gene(s). This finding is in contrast to previous reports where bla_{SHV} and $bla_{\text{CTX-M}}$ was the most prevalent ESBLs [24-26].

The present study showed that only two isolates (4%) exhibited resistance to the carbapenems tested, imipenem and meropenem with MIC value of 8µg/ml. In accordance to our finding, Abdallah *et al.* (2015) [27] noticed that 10 of 15 (66.7%) *K. pneumoniae* isolated from Egypt in 2013 were ESBL producer but none of them was resistant to imipenem or meropenem. In contrast, Metwally *et al.* (2013) [28] reported a high prevalence of non-susceptibility to imipenem (40%) and meropenem (37.8%) in 45 clinical isolates of *K. pneumoniae* from Egypt during 2011 using CLSI breakpoints of ≥ 2 µg/ml for both drugs.

CONCLUSION

The present study described the prevalence of ESBL-producing *K. pneumoniae* and their encoding genes in multi-centers in Cairo. The high carriage rate (46%) of ESBL in our isolates, especially in cancer patients, extended our knowledge the worrisome situation of high prevalence rates of ESBL-producing Gram-negative organisms isolated from hospitals in Egypt [16, 29, 30]. To our knowledge, this is the first study reported the incidence of ESBL in *K. pneumoniae* isolated mainly from cancer patients in Egypt. Surveillance of emergence and dissemination of ESBL-producing *K. pneumoniae* is an urgent public health need in our region.

ACKNOWLEDGMENT

The authors greatly thank the Deanship of Scientific Research at King Faisal University (Hofuf, Saudi Arabia) for funding the publication through the research project no. 140049.

CONFLICT OF INTERESTS

Declared None

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