

## The characterization of ESBL genes in *Escherichia coli* and *Klebsiella pneumoniae* causing nosocomial infections in Vietnam

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### Abstract

**Background:** Extended-spectrum  $\beta$ -lactamases (ESBLs) are enzymes capable of hydrolyzing oxyimino- $\beta$ -lactams and inducing resistance to third generation cephalosporins. The genes encoding ESBLs are widespread and generally located on highly transmissible resistance plasmids. We aimed to investigate the complement of ESBL genes in *E. coli* and *Klebsiella pneumoniae* causing nosocomial infections in hospitals in Ho Chi Minh City, Vietnam.

**Methodology:** Thirty-two non-duplicate isolates of *E. coli* and *Klebsiella pneumoniae* causing nosocomial infections, isolated between March and June 2010, were subjected to antimicrobial susceptibility testing. All isolates were PCR-amplified to detect the *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> ESBL genes and subjected to plasmid analysis.

**Results:** We found that co-resistance to multiple antimicrobials was highly prevalent, and we report the predominance of the *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub> genes, located on highly transmissible plasmids ranging from 50 to 170 kb in size.

**Conclusions:** Our study represents a snap shot of ESBL-producing enteric bacteria causing nosocomial infections in this setting. We suggest that antimicrobial resistance in nosocomial *E. coli* and *Klebsiella pneumoniae* is rampant in Vietnam and ESBL organisms are widespread. In view of these data and the dramatic levels of antimicrobial resistance reported in Vietnam we advocate an urgent review of antimicrobial use in the Vietnamese healthcare system.

**Key words:** Enterobacteriaceae; Extended-spectrum beta-lactamases; ESBL-encoding genes; Plasmid-mediated resistance; antimicrobials

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### Introduction

The production of  $\beta$ -lactamases is the most common mechanism of bacterial resistance to the  $\beta$ -lactam antimicrobials.  $\beta$ -lactamase genes are widespread and mutate in response to continuous antimicrobial exposure. This prolonged exposure has led to the emergence of extended-spectrum  $\beta$ -lactamases (ESBLs) [1]. ESBLs are enzymes capable of hydrolyzing oxyimino- $\beta$ -lactams, such as third generation cephalosporins, which include the commonly used antimicrobials, ceftriaxone and cefixime. The dissemination of ESBLs is a global problem, particularly in sentinel members of the *Enterobacteriaceae* [2]. Among the known ESBL enzymes, the CTX-M-type  $\beta$ -lactamases, which preferentially hydrolyze cefotaxime, were first reported in the late 1980s and have undergone a rapid,

global spread. The spread of CTX-M-type  $\beta$ -lactamases has been dramatic and greater than the impact of the TEM- and SHV-type ESBLs [3-6].

In Vietnam, the presence of pathogens expressing ESBLs has been increasingly reported over the past ten years. A study conducted in 2001 in seven hospitals across Ho Chi Minh City in the south of Vietnam, found that 5.6 % of all Gram negative bacterial isolates were ESBL positive, with the rate of positivity in *Escherichia coli* and *Klebsiella pneumoniae* being 58 % and 23.6 %, respectively [7]. A further study, also conducted in Ho Chi Minh City, between February 2002 and May 2005, found that 33 % of all Gram-negative bacterial isolates were ESBL positive. From these ESBL positive isolates, *E. coli* and *K. pneumoniae* accounted for 74.1 % of all the organisms isolated [8]. A pan Asia-Pacific study

regarding Gram-negative bacilli from intra-abdominal infections in 2007, found that the ESBL positivity rate in Vietnam was 35.6 % (34.4 % and 39.1 % of the ESBL positive strains were *E. coli* and *K. pneumoniae*, respectively) [9]. As such, the prevalence of ESBL producing strains in Vietnam and the Asia-Pacific region is now higher than those observed in Europe, suggesting differing geographical pressures and exposures to antimicrobials [9, 10].

ESBL producers can often transfer resistance to multiple bacterial species through plasmid-mediated conjugation [11]. The widespread use of antimicrobials, coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and integrons, contribute to increasing the prevalence of antimicrobial resistance in pathogenic members of the *Enterobacteriaceae* [12]. These elements pose serious problems in hospital settings worldwide. Therefore, surveillance of ESBLs producing *Enterobacteriaceae* is necessary to add insight into ESBL transmission, the emergence of predominant ESBL groups and the mobile elements inducing the dissemination of ESBL determinants. In Vietnam, limited studies have been performed investigating the molecular characteristics of ESBL genes and their corresponding mobile elements. Here, we aimed to define the characteristics of common ESBL genes and their encoding plasmid profiles in members of the *Enterobacteriaceae* causing nosocomial infections in hospitalized patients in Ho Chi Minh City, Vietnam.

## Methodology

### *Ethics statement*

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the institutional ethical review boards of the participating hospitals. Samples were collected as part of “standard of care” for treatment and diagnosis; therefore, the institutional ethical review boards did not require us to collect informed consent.

### *Clinical isolates, antimicrobial susceptibility testing and ESBL phenotyping*

The microbiology laboratories at Cho Ray and Thong Nhat hospitals in Ho Chi Minh City isolated 72 bacterial isolates (*E. coli* or *K. pneumoniae*) causing nosocomial infections demonstrating resistance to ceftriazone and ceftazidime between March and June, 2010. Thirty-two of these isolates, comprising 23 *E. coli* and 9 *K. pneumoniae*, were latterly confirmed to be

ESBL producing at the laboratories of Oxford University Clinical Research Unit by the double-disc synergy test (the remainder were ESBL negative) [13]. The double-disc synergy method utilizes discs containing cefotaxime (CTX) (30 µg) and ceftazidime (CAZ) only (30 µg) and both antimicrobials in combination with clavulanic acid (CLA) (10 µg). ESBL producing strains were identified as those with a greater than 5 mm increase in zone with the single antimicrobial compared to the combined antimicrobials. All 32 bacterial isolates were additionally subjected to susceptibility testing by assessing the minimum inhibitory concentrations (MICs) against amoxicillin/ clavulanic acid (AMC), cefepime (FEP), ceftriaxone (CRO), imipenem (IPM), ciprofloxacin (CIP), nalidixic acid (NAL), trimethoprim/ sulfamethoxazole (SXT) and chloramphenicol (CHL) by E-test (AB Biodisk, Solna, Sweden). All antimicrobial susceptibility tests were performed on Mueller-Hinton agar and the resulting data were interpreted according to the Clinical and Laboratory Standards Institute guidelines [13].

### *Nucleic acid amplification and sequencing*

Genomic DNA was isolated from all bacterial isolates from 1 mL of an overnight bacterial culture using the Wizard Genomic DNA Extraction Kit (Promega, Fitchburg, USA), according to manufacturer’s recommendations. All isolates were screened for the presence of *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub> ESBL genes using previously published primers [14, 15]. Further characterization of the *bla*<sub>CTX-M</sub> was performed using the primers specific for CTX-M-1, CTX-M-2, CTX-M-9 subgroups [11]. PCR amplifications were performed using 30 cycles, of 30s at 95°C, 30s at 57°C, and 90s at 72°C. All PCR amplifications were performed using Taq DNA polymerase and appropriate recommended concentrations of reagents (Bioline, London, UK). All PCR amplicons were sequenced using big dye terminators in a forward and reverse orientation on an ABI3130XL sequencing machine (ABI, Advanced Biotechnology Inc, Columbia, USA), according to the manufacturer’s recommendations. Resulting DNA sequences were verified and aligned using BioEdit and Vector NTI Suite 7 software. BLASTn at NCBI was used to compare all resulting ESBL gene sequences against additional ESBL sequences.

**Table 1** The antimicrobial resistance patterns of ESBL producing organisms

Bacterial isolates	Number of antimicrobials resistant <sup>a</sup>	Number of isolates	Antimicrobial resistance phenotypes <sup>b</sup>
<i>E. coli</i> (n = 23)			
	6	3	FEP-CHL-CRO-CIP-NAL-SXT
	5	1	FEP-CRO-CIP-NAL-SXT
	5	5	CHL-CRO-CIP-NAL-SXT
	4	10	CRO-CIP-NAL-SXT
	4	1	FEP-CRO-CIP-NAL
	3	1	CHL-CRO-SXT
	3	1	CRO-CIP-NAL
	2	1	CHL-CRO
<i>K. pneumoniae</i> (n=9)			
	6	1	FEP-CHL-CRO-CIP-NAL-SXT
	5	5	CHL-CRO-CIP-NAL-SXT
	3	1	CHL-CRO-SXT
	2	1	CRO-SXT
	1	1	CRO

<sup>a</sup> From 8 tested, see methods<sup>b</sup> AMC; amoxicillin/ clavulanic acid, FEP; cefepime, CRO; ceftriaxone, IPM; imipenem, CIP; ciprofloxacin, NAL; nalidixic acid, SXT; trimethoprim/ sulfamethoxazole and CHL; chloramphenicol.

The DNA sequences for *bla*<sub>TEM-1</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-3</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-27</sub>, *bla*<sub>CTX-M-55</sub> genes the accession numbers J01749, X92506.1, Y10278, AF174129.3, AF252622.2, AY044436.1, AY156923.1 and DQ885477.1 were downloaded from NCBI and aligned with the resulting sequences.

#### Plasmid extraction and visualization

Plasmid DNA was isolated from all ESBL bacterial isolates using an adapted methodology originally described by Kado and Liu [11]. Briefly, plasmid DNA was separated by agarose gel electrophoresis in 0.7 % agarose gels with 1X TBE buffer. Agarose gels were subjected to 100V for 4 hours, stained with ethidium bromide and photographed. *E. coli* 39R861 containing plasmids of 7, 36, 63 and 147 kb was used for sizing plasmid extractions on agarose gels [16]. Plasmid DNA was size-separated and analyzed using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

#### Southern blotting and hybridization

Plasmid DNA was electrophoresed and transferred to a Hybond N<sup>+</sup> membrane (Amersham Biosciences, Little Chalfont, UK). The PCR amplicons of *bla*<sub>TEM</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-9</sub> were labeled with horseradish peroxidase using the ECL direct nucleic acid labeling

and detection systems kit (Amersham Biosciences, Little Chalfont, UK), and were used as hybridization probes. Hybridization and detection were performed according to the manufacturer's instructions.

#### Bacterial conjugation

Conjugation was performed by combining equal volumes (500 µL) of overnight cultures grown in Luria-Bertani (LB) media of donor and recipient strains in 4 mL of sterile LB media. The donor strains were ESBL-producing isolates (*E. coli* and *K. pneumoniae*) and the recipient was a sodium azide resistant *E. coli* (strain J53 resistant). Bacteria were mixed in a 1:1 ratio at 37°C and incubated without agitation overnight. Transconjugants were selected on LB media containing sodium azide (100 µg/mL) and ceftriaxone (6 µg/mL) and were verified by plasmid extraction and visualization, as before. Conjugation frequency was calculated by dividing the mean number of transconjugants by the mean number of recipient cells.

## Results

#### Antimicrobial susceptibility

All ESBL-producing isolates were resistant to ceftriaxone, of which 27/32 (84.3 %) isolates exhibited an MIC of greater than 256 µg/ml. Resistance to additional antimicrobials was common with 27/32 (84.3 %) resistant to ciprofloxacin, 28/32 (87.5 %) resistant to

**Table 2.** The characterization of ESBL genes and their corresponding plasmids

Bacterial isolates	CAZ zone size (mm)	ESBL gene(s) detected	Size of ESBL Plasmid (Kb) <sup>a</sup>		Number of transferable plasmids	Maximum conjugation frequency <sup>b</sup>
			CTX-M <sup>a</sup>	TEM		
<i>E. coli</i>						
EC2	15	CTX-M-15, CTX-M-14	144	ND	2	1.17 x 10 <sup>-4</sup>
EC7	14	CTX-M-15, TEM-1	125	125	0	0
EC8	12	CTX-M-15, TEM-1	89	89	3	2.86 x 10 <sup>-4</sup>
EC9	14	CTX-M-15	156	ND	2	2.86 x 10 <sup>-7</sup>
EC10	17	CTX-M-15, TEM-1	ND	155	0	0
EC13	14	CTX-M-15, TEM-1	ND	ND	0	0
EC14	6	CTX-M-15, TEM-1	118	118	2	2.73 x 10 <sup>-5</sup>
EC15	15	CTX-M-15, TEM-1	116	116	0	0
EC19	12	CTX-M-15	134	ND	3	1.25 x 10 <sup>-4</sup>
EC21	9	CTX-M-15, TEM-1	146	146	2	6.25 x 10 <sup>-6</sup>
EC12	15	CTX-M-55, TEM-1	87	87	2	1.17 x 10 <sup>-3</sup>
EC16	14	CTX-M-55, TEM-1	143	144	6	4.28 x 10 <sup>-5</sup>
EC4	6	CTX-M-14, TEM-1	157	157	0	0
ET1	21	CTX-M-14, TEM-1	124	124	0	0
ET2	23	CTX-M-14, TEM-1	111	111	4	1.43 x 10 <sup>-7</sup>
EC1	19	CTX-M-27	132	ND	5	3.75 x 10 <sup>-7</sup>
EC3	18	CTX-M-27, TEM-1	70	94	3	1.25 x 10 <sup>-3</sup>
EC5	16	CTX-M-27, TEM-1	98	98	2	1.00 x 10 <sup>-3</sup>
EC6	16	CTX-M-27, TEM-1	93	93	2	1.67 x 10 <sup>-3</sup>
EC11	23	CTX-M-27, TEM-1	139	113	2	1.00 x 10 <sup>-3</sup>
EC17	20	CTX-M-27	140	ND	6	6.25 x 10 <sup>-8</sup>
EC20	19	CTX-M-27, TEM-1	100	73	3	4.00 x 10 <sup>-6</sup>
EC22	17	CTX-M-27, TEM-1	99	99	2	2.50 x 10 <sup>-4</sup>
<i>K. pneumoniae</i>						
KT7	21	CTX-M-3	57	ND	1	7.50 x 10 <sup>-5</sup>
KC3	6	CTX-M-15, TEM-1	155	155	2	1.00 x 10 <sup>-3</sup>
KC4	7	CTX-M-15, TEM-1	152	152	0	0
KT2	7	CTX-M-15, TEM-1	146	146	0	0
KT3	7	CTX-M-15, TEM-1	104	104	0	0
KT5	6	CTX-M-15	141	ND	0	0
KC1	15	CTX-M-27	100 and 76	ND	2	3.33 x 10 <sup>-4</sup>
KT1	23	CTX-M-27, TEM-1	113	157	3	3.33 x 10 <sup>-5</sup>
KT4	23	CTX-M-27, TEM-1	54	54	1	1.20 x 10 <sup>-5</sup>

<sup>a</sup> Estimated plasmid size by agarose electrophoresis with markers of known sizes

<sup>b</sup> Conjugation frequency per recipient cell

ND Not detected

resistant to trimethoprim-sulfamethoxazole, 27/32 (84.3 %) resistant to nalidixic acid and 17/32 (53.1 %) resistant to chloramphenicol (Table 1). More than 80 % of the isolates were resistant to between four and six of the antimicrobials tested (Table 1).

Six out of 32 isolates (18.8 %) were resistant to the fourth generation cephalosporin, cefepime, with an additional 11/32 (34.4 %) demonstrating intermediate resistance. All ESBL-producing strains were sensitive to the carbapenem, imipenem.

#### Characterization of *bla* genes

PCR amplifications were performed to detect the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes. The resulting amplifications demonstrated that all 32 of the ESBL-producing isolates carried a *bla*<sub>CTX-M</sub> gene, 24/32 isolates harbored an additional *bla*<sub>TEM</sub> gene and no

isolates carried a *bla*<sub>SHV</sub> gene. All strains were additionally amplified with primers specific for the three major CTX-M clusters, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub> and *bla*<sub>CTX-M-9</sub>. With these specific CTX-M cluster primers, one *E. coli* isolate, produced an amplicon with both *bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-9</sub> primers, the remaining strains produced single amplicons with either the *bla*<sub>CTX-M-1</sub> primers or the *bla*<sub>CTX-M-9</sub> primers and none tested positive with the *bla*<sub>CTX-M-2</sub> primers (Table 2). All 33 PCR amplicons were DNA-sequenced to identify the specific *bla*<sub>CTX-M</sub> variants. DNA sequence analysis showed that multiple *bla*<sub>CTX-M</sub> loci were circulating in the *E. coli* and *K. pneumoniae* isolates. We identified one *bla*<sub>CTX-M-3</sub>, 15 *bla*<sub>CTX-M-15</sub>, two *bla*<sub>CTX-M-55</sub>, four *bla*<sub>CTX-M-14</sub> and 11 *bla*<sub>CTX-M-27</sub> genes (Table 2). The *bla*<sub>CTX-M-15</sub> gene was the most predominant variant (15/18 strains) among the *bla*<sub>CTX-M-1</sub> cluster, and

*bla*<sub>CTX-M-27</sub> (11/15 strains) was the most predominant variant within the *bla*<sub>CTX-M-9</sub> cluster. Among the 15 *bla*<sub>CTX-M-15</sub> carrying isolates, five were additionally resistant to cefepime (MIC > 32 µg/ml) and seven exhibited intermediate cefepime resistance (median MIC = 16µg/ml).

#### Characterization of ESBL encoding plasmids

Plasmid profiling of 32 ESBL-positive isolates demonstrated that all strains harbored at least one large plasmid, ranging from 50 to 171 kb in size (Table 2). Furthermore, the majority of the strains also harbored multiple other plasmids, ranging from 1 to 50 kb in size. The number of plasmids in these isolates ranged from one to nine and after gel sizing analysis, using a binary scoring system with a Pearson correlation, we found that plasmid profiles were not specific to *E. coli* or *K. pneumoniae* (data not shown).

Plasmid DNA hybridization demonstrated that the majority of the ESBL-producing strains (31/32 strains) carried the *bla* genes on a large plasmid (ranging from 53.8 to 157 kb in size) (Table 2). These large plasmids encoded a *bla*<sub>CTX-M</sub> only or both a *bla*<sub>CTX-M</sub> and a *bla*<sub>TEM</sub> gene. Among the 24 strains carrying both a *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> genes, 18 strains carried these genes on the same plasmid and four strains carried these genes on different plasmids; we were unable to confirm the PCR result for two strains as a presumed consequence of plasmid instability after in vitro passage (this was, however, latterly confirmed by PCR) (Table 2). ESBL-producing strains containing genes belonging to the *bla*<sub>CTX-M-9</sub> gene cluster exhibited less activity against ceftazidime in comparison to strains carrying gene belonging to the *bla*<sub>CTX-M-1</sub> gene cluster. Susceptibility testing against ceftazidime with ESBL strains showed two distinct zone clearance areas with the *bla*<sub>CTX-M-9</sub> cluster (median; 18.3 mm) and the *bla*<sub>CTX-M-1</sub> cluster (median; 12.2 mm) (Table 2).

We performed bacterial conjugation experiments on all 32 ESBL-producing strains (donors) using *E. coli* J53 as a recipient. Results demonstrated that plasmids harboring ESBL genes of twenty-two isolates (69%) were transmissible via conjugation, with conjugation frequencies ranging from  $6.25 \times 10^{-8}$  to  $1 \times 10^{-3}$  per recipient cell (Table 2). Of the 10 isolates carrying non-transmissible plasmids, eight carried ESBL plasmids with sizes greater than 100 kb and we were unable to confirm the presence of ESBL genes on plasmids by Southern Blotting in two (Table 2). We further confirmed the transmission of ESBL plasmids

between both species (*E. coli* to *E. coli*) and genus (*K. pneumoniae* to *E. coli*).

## Discussion

Our work shows that the CTX-M-type ESBLs are the most common ESBL found amongst *E. coli* and *K. pneumoniae* causing nosocomial infections in hospitals in Ho Chi Minh City. Among the *bla*<sub>CTX-M</sub> variants, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-27</sub> were the most common in *E. coli* and in *K. pneumoniae*, and *bla*<sub>CTX-M-15</sub> accounted for 45 % of all *bla*<sub>CTX-M</sub> variants. A study regarding resistance gene characterization of ESBL positive *Shigella sonnei* isolated at the Hospital for Tropical Diseases in Ho Chi Minh City in 2009 found that *bla*<sub>CTX-M-15</sub> was the most dominant *bla*<sub>CTX-M</sub> variant, found in all but one ESBL positive *Shigella sonnei* [11]. Our observations reflect the current rapid and successful dissemination of CTX-M-type ESBLs and the emergence of *bla*<sub>CTX-M-15</sub> in Asia and globally. *Bla*<sub>CTX-M-15</sub> first arose in India in 2000 and has become predominant globally within a decade [3, 10, 17, 18]. This particular *bla*<sub>CTX-M</sub> gene is generally found on large conjugative plasmids and is located downstream of an *ISEcp1* insertion sequence which explains its remarkable transmission success [7, 11]. The CTX-M-15 type enzyme differs from that of CTX-M-3 type by an asparagine to glycine substitution at codon 240, which leads to increased activity against ceftazidime. These CTX-M-15 type enzymes may have been selected by the increasing use of ceftazidime in clinical practice [19-21].

We can additionally show that the ESBL-producing organisms additionally exhibited co-resistance against multiple antimicrobials from other classes. Many studies have also reported co-resistance to tetracycline, aminoglycosides, and fluoroquinolones in ESBL producers [7, 8, 11]. It has also been demonstrated that CTX-M-15 ESBL hydrolyzes cefepime with higher efficiency than other ESBL variation [5]. Here, ESBL producers also demonstrated a high level of resistance against ciprofloxacin, trimethoprim-sulfamethoxazole, nalidixic acid and chloramphenicol. Our work shows that ESBL producing strains carrying *bla*<sub>CTX-M-15</sub> exhibit complete resistance and intermediate resistance to cefepime with significantly higher MICs than other *bla*<sub>CTX-M</sub> alleles. This complexity in antimicrobials resistance combinations limits suitable drug of choice for antimicrobial therapy, leaving carbapenems and aminoglycosides the last options for treatment in some cases. The emergence of NDM-1 clearly compounds

potential treatment options, and more recent data additionally suggests an association between resistance to beta-lactams and aminoglycosides in ESBL-producing bacteria [22]. Furthermore, organisms carrying ESBLs are highly efficient at transferring their resistance to other organisms within the same or different species through conjugation, increasing the rate of antimicrobial resistance transmission. Selective pressure, from heavy use of extended-spectrum beta lactam will presumably maintain the presence of these ESBL-producing pathogens resulting in the persistence and transmission of ESBL resistance determinants among Gram-negative bacteria. Therefore, further characterization of other antimicrobial resistance mechanisms will be important to understand the co-transmission of a range of antimicrobial determinants.

Our study represents a snap shot of ESBL producing enteric bacteria causing nosocomial infections in our setting. We report that antimicrobial resistance in hospital isolates is common in Vietnam and ESBL organisms are widespread. CTX-M-type ESBLs were the most common enzymes found in both *E. coli* and *K. pneumoniae*. Furthermore, the ESBL genes were consistently located on highly transmissible plasmids ranging from 50 to 170 kb in size. We suggest that the rampant use of extended-spectrum cephalosporins in the hospital is driving the on-going selection, maintenance and dissemination of these ESBL genes across a spectrum of Gram-negative organisms and recommend a stringent review of antimicrobial use in the Vietnamese healthcare system.

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