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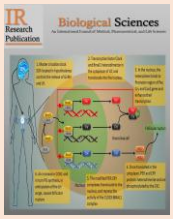

Molecular Identification of *bla*$_{CTX-M}$ producing clinical isolates of *E. coli*

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ABSTRACT

Extended spectrum-β-lactamases (ESBLs) producing *E. coli* is an emerging and serious threat to the public health sector around the globe. These pathogens are responsible for high rates of morbidity and mortality and cause severe infections. blaCTX-M is one of the potent ESBLs that produce resistance against cephalosporin antibiotics. Clinical isolates (n=100) of *E. coli* from various clinical samples (blood, urine, and pus) were collected from tertiary care hospitals, Lahore. Isolates were subcultured on MacConkey and blood agar and incubated at 37 ºC aerobically overnight. *E. coli* was initially identified based on their colony morphology and cultural characteristics and confirmed by API 20E. Antimicrobial susceptibility testing was performed as per CLSI 2015 guidelines. Phenotypic detection of ESBLs was done by double disk synergy test. DNA extraction was performed by heat method. Molecular identification of blaCTX-M-1, blaCTX-M-10 and blaCTX-M-14 was performed using specific primers by PCR. Out of 100 isolates, 64 were ESBL producers. Most of the *E. coli* displayed 100 % resistance to first generation cephalosporin, 94% to nalidixic acid and 56% to co-trimoxazole while most effective drugs were carbapenems. Molecular characterization revealed predominantly CTX-M-1 (44 %) followed by CTX-M-10 (23%) and CTX-M-14 (14%). Most the ESBL variants were found in urine samples (n=35) followed by blood (n=15) and pus (n=9). This study concluded a higher prevalence of ESBLs producing *E. coli* with predominant CTX-M-1. Therefore, it is the need of the hour to conduct a surveillance study at national level.

Introduction

Escherichia coli is a Gram-negative rod and member of Enterobacteriaceae family which are capable to thrive in the facultative environment (1). They are considered as opportunistic pathogens causing various infections like bacteremia, septicemia, meningitis and hemolytic uremic syndrome (2). Most of the *E. coli* strains are not deleterious however some of the strains contribute to various severe human diseases (3). ExPEC cause infections at extra intestinal sites viz, peritonitis, wound infections, pneumonia gastroenteritis, meningitis, septicemia and urinary tract infections (UTIs) (2). Diarrhoea is the major cause of children death (4). E. coli is contributing its role in mortality by causing diarrheal diseases globally. This problem is still

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present and significant reasons for death and disease cases in less developed countries in those children who are under five years of age (5). Advancements in antibiotics have been proven to decrease the cost burden and play a vital role in stopping *E. coli* and other bacterial infections (6). β-lactams are frequently used in clinical settings against pathogens especially enterobacterial members globally (Shaikh et al., 2015).

Cephalosporin antibiotics are a broad class of β-lactam antibiotics including ceftazidime, cefotaxime, ceftriaxone, and cephaloridine (7). β-lactam antibiotics show various mechanisms of action against bacterial infections but the most effective one is inhibiting bacterial cell wall synthesis (8).To escape from the effects of β-lactams most bacteria, especially *E. coli* have developed a variety of different resistance mechanisms such as mutation, efflux pumps, target alterations, and synthesis of β-lactamase enzymes but the enzyme production has a major role in drug resistance in *E. coli* bacteria (9).

The synthesis of β-lactamase enzymes in Gram-negative bacteria has led to the proliferation of bacterial genetic subspecies (10). β-lactamases having broad range effectivity against β-lactam drugs are designated as Extended Spectrum β-lactamases (ESBLs). Mostly β-lactamases are frequently found in *E. coli, K. pneumoniae P. aeruginosa* and Acinetobacter species (11). ESBLs grouping was proposed by Ambler and Bush and grouped into various groups such as TEM, SHV, and CTX-M ESBLs, the latter one being the widespread globally among all CTX-M ESBLs originated in the beginning of 21st century and the capability of hydrolyzing cefotaxime β-lactam antibiotic and its initial discovery in Munich, Germany is the reason to assign 'CTX-M' terminology to these β-lactamases (12).

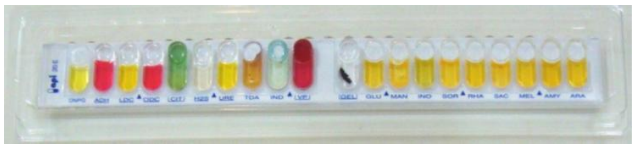
They have been found frequently located in *E. coli* and *K. pneumoniae* isolates and in Western countries geographically (13). CTX-M ESBLs have appeared as predominant ESBLs and disseminated globally as 99% dominance has been recorded among clinical isolates of Enterobacter species collected from various tertiary care hospitals in China (14). A genetically altered subtype of *E. coli* has been recorded one of the causes of the spread of these ESBLs globally and in hospitals as well (15). The CTX-M-14 has been recorded as the second most commonly found enzyme globally while CTX-M-15 with the most common distribution in the world and in USA, Asian and European countries with higher concentrations (16). CTX-M family has been divided into different families on the basis of amino acid sequence similarity viz CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (17). In Asia, especially in Pakistan the incidence of CTX-M β-lactamases is still rapidly growing which is much quicker as compared to SHV and TEM type β-lactamases (18). There are about 190 allelic variants of CTX-M have discovered recently (19).

Materials and methods

A total of 100 clinical isolates of *E. coli* were collected randomly from different tertiary care hospitals, Lahore, Pakistan from different clinical sources such as blood, urine and pus using aseptic sampling technique. Specimen source for all *E. coli* samples was recorded. Samples were incubated aerobically. The number of ESBL producing isolates along with their antibiotic susceptibility profiles was noted.

Identification/Purification of isolates

Positive specimens were subcultured on MacConkey agar to identify *E. coli* and their cultural characteristics using different biochemical tests. Gram staining procedure was done to designate *E. coli* as Gram-negative rod. MRVP battery of tests was performed for its biochemical characteristics. Then further reconfirmation was observed using analytical profile index (API) with the help of API 20 E multi step system.



Antimicrobial susceptibility testing

Antimicrobial susceptibility of isolates was determined by Kirby-Baur disk diffusion method using Mueller-Hinton agar (Oxoid UK), according to the Clinical laboratory Standards institute (CLSI) 2015 guidelines. The plates were prepared and incubated at 37 º C for 18-20 h. The implanted antibiotics were Ampicillin (10µg), Amoxicillin/clavulanate (20/10µg), Ampicillin/sulbactam (10/10µg), Piperacillin/tazobactam (100/10µg), Cefepime (30µg), Cefotaxime (30µg), Cefuroxime (30µg), Ceftazidime (30µg), Aztreonam (30µg), Imipenem (10µg), Meropenem (10µg), Gentamicin (10µg), Doxycycline (30µg), ciprofloxacin (5µg), Trimethoprim/sulfamethoxazole (1.25/23.75µg). The interpretation of susceptibility tests was done according to CLSI guidelines. Statistical analysis was done using SPSS 22.0.

Phenotypic detection of ESBLs

Phenotypic detection of ESBLs was confirmed by performing double disk synergy test using antibiotic disks having a combination of amoxicillin/clavulanate (20/10µg) along with respective cephalosporin disk alone. The antibiotic susceptibility pattern of ESBL producing isolates against a panel of different antibiotics including amikacin, amoxicillin plus clavulanic acid, meropenem, gentamycin and cefotaxime was recorded.

PCR amplification

Preparation of DNA template and PCR amplification of CTX-M beta-lactamase genes were carried out on a thermal Cycler Optimum 96G (Quality Lab System, England). The primers' sizes of amplification product along with annealing temperature used in PCR amplification is given in Table 1.

Table 1. The primers' sizes of amplification product.

Primers	Sequences (5'-3')	Product Size (bp)	Annealing Temp
$bla_{CTX\text{-}M\text{-}1}{}^{-F}$	CCGTTTCCGCTATTACAAACCG	944	56^0C
$bla_{CTX\text{-}M\text{-}1}{}^{-R}$	GGCCCATGGTTAAAAAATCACTGC		
$bla_{CTX\text{-}M\text{-}10}{}^{-F}$	GCAGCACCAGTAAAGTGATGG	524	56^0C
$bla_{CTX\text{-}M\text{-}10}{}^{-R}$	GCGATATCGTTGGTGGTACC		
$bla_{CTX\text{-}M\text{-}14}{}^{-F}$	GAGAGTGCAACGGATGATG	941	56^0C
$bla_{CTX\text{-}M\text{-}14}{}^{-R}$	TGCGGCTGGGTAAAATAG		

Results

Out of 100 isolates 64 were positive for ESBL production. The majority of ESBLs producing isolates (56.9%) were recovered from urine samples, 23.4% from pus cultures, 14.06% from blood cultures and 7.81% from various other sources (e.g., high vaginal swabs, tracheal section and tissue specimens). Out of 100 *E. coli* isolates 61 were isolated from female patients and 39from male patients. All isolates showed 100% resistance to cephalexin and cephradine (first generation cephalosporin antibiotics), (94%) to nalidixic acid, 56% to co-trimoxazole, 20% to cefoperazone / sulbactum and 17% to piperacillin / tazobactum. However, most effective drugs were imipenem (100%) and meropenem (100%).

Molecular Identification of blaCTX-M

Among ESBLs producing isolates of *E. coli* (64%), different CTX-M variants were identified.CTX-M-1 producing *E. coli* isolate was more prevalent ESBL (44%) followed by CTX-M-10 (23%) and CTX-M-14 (14%). Furthermore, co-existence of CTX-M-1 and CTX-M-10 was detected in 8 (12.5%) *E. coli* isolates while only 1 (1.5%) *E. coli* isolate was carrying three CTX-M variants (CTX-M-1, CTX-M-10 and CTX-M-14).

blaCTX-M-1

blaCTX-M-1 producing isolates were mainly isolated from urine (20%) followed by pus (13%) and blood (8%) (Table 4.2). The antimicrobial resistance of CTX-M-1 producing *E. coli* is given in Figure 4.7. *E. coli* harbouring CTX-M-1 enzyme were completely resistant to cephalexin and cefradine antibiotics that belong to first generation cephalosporin β-lactams. They showed 100% sensitivity to carbapenem drugs (imipenem and meropenem). They were also resistant (78%) to cefotaxime (3rd generation cephalosporin). All isolates harbouring CTX-M-1 enzyme showed high resistance than sensitivity to all the drugs.

blaCTX-M-10

blaCTX-M-10 producing isolates were mainly isolated from urine (13%) followed by pus (6%) and blood (5%) (Table 4.2). The antimicrobial resistance of CTX-M-10 producing *E. coli* is given in Figure 4.8. *E. coli* possessing CTX-M-10 enzyme showed strong hydrolytic activity and complete resistance to cefalexin and cefradine drugs while nitrofurantoin was the most effective drug. In fact, most of the *E. coli* producing CTX-M-10 enzyme was more resistant and less susceptible to the antibiotics.

blaCTX-M-14

blaCTX-M-14 producing isolates were mainly isolated from urine (6%) followed by pus (5%) and blood (2%) (Table 4.2). The antimicrobial resistance of CTX-M-14 producing *E. coli* is given in Figure 4.9. Interestingly, isolates harbouring CTX-M-14 enzyme showed 100% resistance to most of the drugs and very few were effective. CTX-M-14 are the enzymes that display strong hydrolytic activity against many antibiotics of different classes.

Discussion

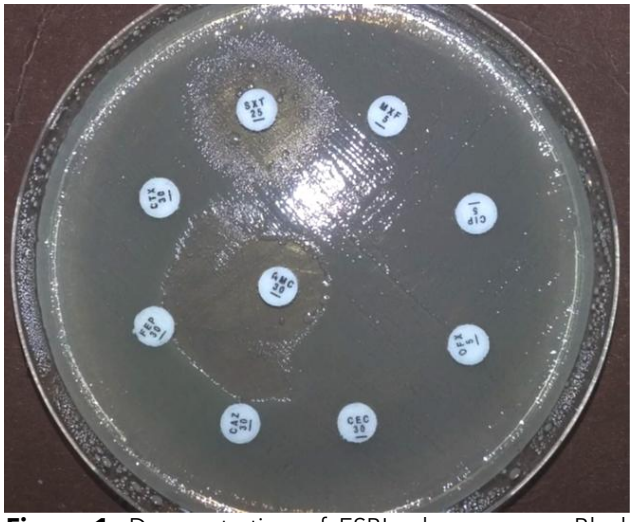
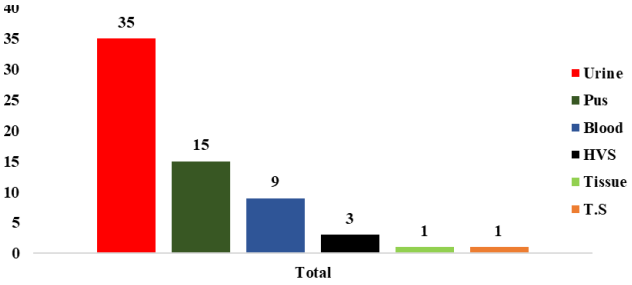


Figure 1. Demonstration of ESBL phenomenon. Black arrows indicate the enlarged zone of inhibition of each of the CTX, CAZ, FEP disks which placed 15 mm distance from AMC disk. AMC; Co-amoxiclave, CTX; Cefotaxime, CAZ; Ceftazidime, FEP; Cefipime.



showed the emergence of clonally related CTX-M producing isolates of *E. coli* and alarming threat in our community hospitals. In our study, 100 clinical isolates of *E. coli* were collected from different specimen sources from different tertiary care hospitals Lahore, Pakistan. Among these specimens, urine was the major source of specimen followed by blood and pus. These specimens were collected from both male and female patients. Most of isolates of *E. coli* were collected from urine specimens (n=55) followed by blood (n=23) and pus (n=14) as given in Table 4.1. Female urine was the main reservoir of E. coli. Similar findings were also testified in a study conducted in Karachi, Pakistan by (20). Most of the *E. coli* isolates were ESBLs producers (64%). ESBLs are β-lactamases produced by certain bacteria which degrade β-lactam antibiotics and serious threat to health issues. Frequency of the *E. coli* isolates responsible for ESBLs production were mainly of urine specimens (n=35) followed by blood (n=15) and pus (n=9). A similar study was conducted in America and Canada which showed 6% ESBL positive *E. coli* from urine specimens (21). The main reason of such difference is the hygienic conditions and uncontaminated edible items in these countries as compared to our region. In another study in Taiwan, sixty five percent of *E. coli* isolated from urine specimens collected from children less than one year were responsible for ESBLs production (22). Taiwan is also suffering from water pollution as water is not normally usable but after boiling, so ESBL producing *E. coli* is highly

Table 1. Percentage of CTX-M ESBLs from clinical specimens

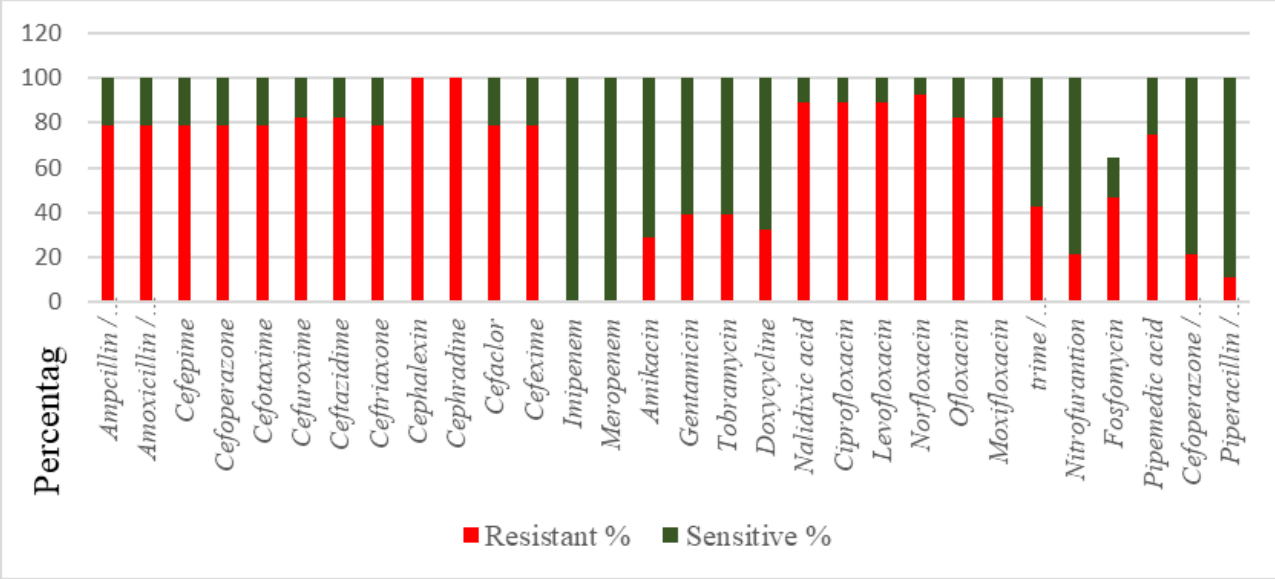
Specimens	CTX-M-1	CTX-M-10	CTX-M-14
Urine	20%	13%	6%
Pus	13%	6%	5%
Blood	8%	5%	2%
HVS	2%	0%	2%
Tissue	0%	0%	0%
Tracheal Section	2%	0%	0%

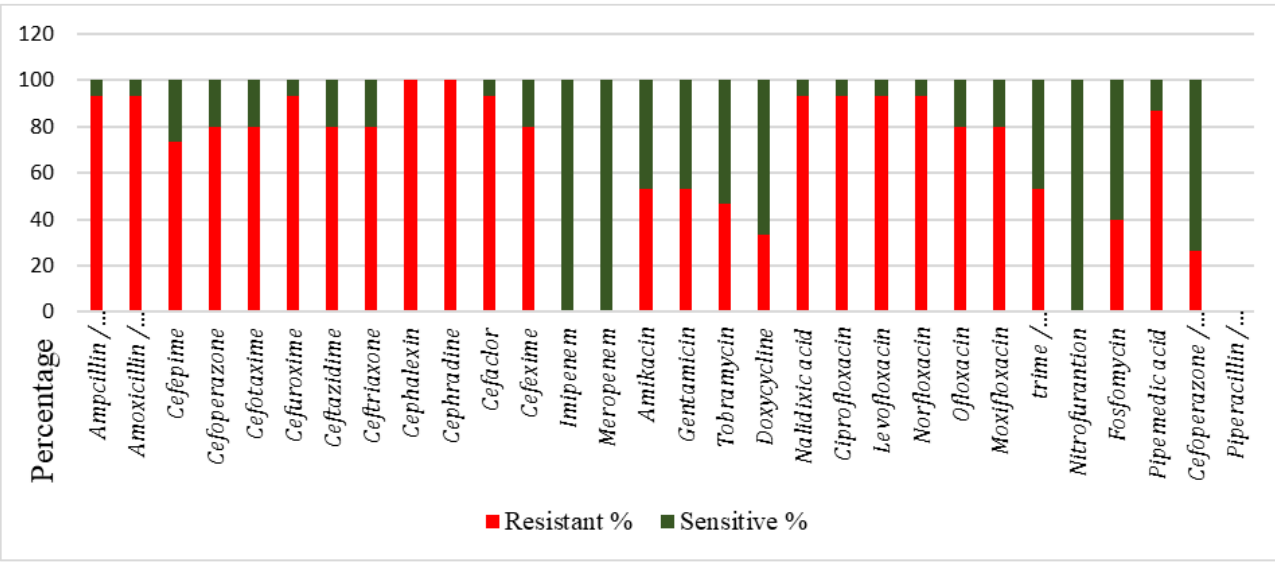
Table 1. Percentage of ESBLs positive E. coli isolates

bla$_{CTX-M}$ Type	Positive ESBLs	Total ESBLs Positive isolates	Percentage
bla$_{CTX-M-1}$	28	64	44%
bla$_{CTX-M-10}$	15	64	23%
bla$_{CTX-M-14}$	9	64	14%

It is interesting to note that organisms producing CTX-M β-lactamases have been isolated in the regions of the world mostly in America, Europe and Japan. In short, our study demonstrates that molecular epidemiology of CTX-M producing *E. coli* strains in Lahore region is vibrant with a change in CTX-M variants with the passage in time. In conclusion this study highlights a significant role of molecular surveillance for tracking of CTX-M producing isolates of *E. coli* in the community and examining their invasion into hospitals. Our study

prevalent. In another study conducted in India 92% of clinically isolated *E. coli* detected as positive for ESBLs production. Among the 50 ESBLs synthesizing *E. coli* 20% found to be positive for CTX-M type ESBLs (23). It is a big difference between this and our study. Comparatively, population explosion, poverty, lack of health and sanitation facilities are the major reasons for such a huge ratio of ESBLs production in this country. In another study conducted in Spanish region to know





prevalence of ESBLs carrying *E. coli* during a five year duration, only six percent of *E. coli* for ESBLs production were detected (24). As living standard and health facilities in this region are too well so less

prevalence of ESBLs as compared to our locality. Antimicrobial resistance is increasing rapidly causing economic burden and serious issues to health agencies. In present study, all isolates of *E. coli* showed highest resistance to different cephalosporin antibiotics while 100% resistance to first generation cephalosporins. Carbapenems were most effective drugs Figure 4.1. The similar resistance to cephalosporins and ciprofloxacin was shown by ESBL *E. coli* and higher susceptibility to carbapenems in a study conducted in Karachi, Pakistan (25). In contrast a study with similar results conducted in Asian countries, *E. coli* showed higher resistance against cephalosporin antibiotic on average (26). Sensitivity to carbapenem drugs was also same. Similarity in results is based on poor hygienic and other health facilities along with unawareness to germs spread. A study conducted in Tanzania revealed higher resistance against cephalosporin antibiotics in ESBLs harbouring *E. coli* (27). This similarity is due to the weak health conditions and poverty in this country. In contrast, *E. coli* displayed prominent effectivity against cephalosporin antibiotics while distinct susceptibility to carbapenems that were effective drugs in comparison according to a study conducted in Rwanda (28). The resistance ability in *E. coli* to advanced cephalosporins in this region is due to the contamination of food, water and lack of health facilities. According to study conducted in Arabian countries a different antibiotic resistance pattern has reported with higher resistance to cephalosporin but no against amikacin and mean susceptibility to carbapenems (29). Better health and hygienic facilities and no self-medication are the reasons for this antibiotic pattern.

In present study, 81% of ESBL producing *E. coli* carried three CTX-M genes. Among the clinical isolates, 28 out of 64 (44%) isolates were positive for blaCTX-M-1 while 15 out of 64 (23%) isolates were positive for blaCTX-M-10 and 9 out of 64 (14%) were positive for blaCTX-M-14. The clinical specimens of urine positive for CTX-M-1 gene were 20% followed by pus (13%) and blood (8%) as given in table 4.2.*E. coli* isolates producingblaCTX-M-1 showed 100% and relatively high resistance against cephalexin, cefradine and other drugs respectively. In contrast, in a study CTX-M-1 showed higher effectivity against cephalosporins (30). The clinical specimens of urine positive for CTX-M-10 gene were 13% preceding pus specimen (6%) and blood (5%). *E. coli* isolates harbouring this gene showed 100% hydrolytic activity to cephalexin and cefradine, strong effectivity against other cephalosporin and quinolone antibiotics. They showed 100% susceptibility to carbapenems. The isolates were completely susceptible to carbapenems. They were 100% susceptible against carbapenems and nitrofurantoin. No data was found relative to antibiotic resistance pattern in *E. coli* isolates. The clinical specimens of urine positive for CTX-M-14 gene were (6%) followed by pus specimens (5%) and of blood (2%). *E. coli* isolates positive for this gene were completely resistant to ampicillin/sulbactam, ampicillin/clavulanic acid, cephalosporin antibiotics and nalidixic acid. In a study conducted in Korea demonstrated that *E. coli* isolates producing CTX-M-14 genes were moderately resistant to aminoglycosides and 3rd generation cephalosporin (31).

Conclusion

This study is of significant importance as emergence of multi-drug resistant ESBLs producing *E. coli* is increasing day by day. This study showed the increasing percentage of different CTX-M producing *E. coli* in clinical samples of patients especially in females of Lahore region of Pakistan.

Contribution of authors

Not applicable

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Conflict of interest

The authors declare no conflict of interest.

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