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Detection of Plasmid-Mediated Quinolone Resistance Determinants in ESBLs and Non-ESBLs Producing *Enterobacteriaceae* from Urinary Tract Infections

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Abstract

Background: Plasmid-mediated quinolone resistance determinants have been identified in *Enterobacteriaceae*, with varying prevalence rates worldwide. In addition, the emergence of PMQR and its association in ESBL producing *Enterobacteriaceae* are raising public health concerns.

Aim: To assess the prevalence of PMQR genes among ESBL and ESBL non-producing *Enterobacteriaceae* isolated from patients with UTIs.

Methods: All the ESBL and non-ESBL *Enterobacteriaceae* that were phenotypically resistant to nalidixic acid, ciprofloxacin and levofloxacin were tested by PCR for the presence of five PMQR determinants.

Results: The study showed that 28.2% of the *Enterobacteriaceae* isolates were confirmed to be ESBL producers while 71.8% were ESBL non-producers with significant difference (P<0.05). PMQR genes were detected in 93.1% of the ESBL producers and in 76% of the ESBL non-producers with no significant difference. *qnr*A gene showed the highest association with ESBL (58.6%) followed by *qnr*B gene (51.7%), while *qnr*B and aac(6')-Ib-cr genes showed the highest association with non-ESBL (32% each) followed by *qnr*A and *qnr*S (28% each). There were no statistically significant differences regarding the association between ESBL production and the presence of the different PMQR genes except for *qnr*A. Coexistence of more than one PMQR gene in each of the 54 phenotypic quinolone resistance isolates was detected; 4 genes in 2 isolates (3.7%), 3 genes in 7 (12.9%) and 2 genes in 27 (50%) isolates.

Conclusion: Our results suggest high frequencies of PMQR genes among both ESBL producing and ESBL non-producing *Enterobacteriaceae* isolates.

Keywords: Quinolones; ESBL; *Enterobacteriaceae*; PMQR gene

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Background

The *Enterobacteriaceae* is a large diverse family of bacteria that are found primarily in the colon of human and other animals as part of the normal flora but can cause a variety of community and health care associated infections such as Urinary Tract Infections (UTIs), wound infections, and bacteremia [1]. Beta-lactam antimicrobial agents are frequently used for treatment of infections caused by members of this family. Production of beta-lactamases is the most common mechanism of bacterial resistance to these antimicrobial agents. These enzymes mutate continuously leading to the development of Extended Spectrum Beta Lactamases (ESBLs) which induce resistance to penicillins, narrow-and extended-spectrum cephalosporins, and aztreonam [2].

Quinolones are synthetic antimicrobial agents that are used widely in routine clinical practice. The new quinolones compounds (6-fluoroquinolones) have broad spectrum activity against Gram-negative pathogens, mycobacteria, and anaerobes [3]. Quinolones prevent bacterial DNA replication by inhibiting DNA gyrase (topoisomerase II) and topoisomerase IV enzymes of the bacteria [4]. For more than 30 years, the only known resistance mechanisms to quinolones were chromosome borne [5]. Later on, several studies revealed that quinolone resistance can also be mediated by plasmid-carried genes, so called Plasmid-Mediated Quinolone Resistance (PMQR) determinants [6]. In addition, the emergence of PMQR and its association in ESBL-

producing *Enterobacteriaceae* is raising public health concerns since the inappropriate use of antimicrobial agents can transfer PMQR genes on the same plasmid carrying β-lactamase genes inducing therapy failure [7]. Therefore, this study was conducted to assess the prevalence and genotypes of PMQR genes among ESBLs and ESBLs non-producing *Enterobacteriaceae* isolated from patients with UTIs at Suez Canal University Hospitals (SCUH), Ismailia.

Objectives

To assess the prevalence of PMQR genes among ESBL and ESBL non-producing *Enterobacteriaceae* isolated from patients with UTIs.

Methods

A cross-sectional descriptive study was carried out during the period from May 2016 to February 2018 on 205 patients with clinical presentations suggestive of health care associated UTIs. Patients were admitted to different wards in SCUH. All age groups were included. Informed consent was taken from each patient to use their data in the current research work. Urine specimens were collected from all patients and processed to identify ESBL producing and ESBL non-producing *Enterobacteriaceae.* PMQR genes were detected in these *Enterobacteriaceae* isolates by PCR. The ethics committee of Faculty of Medicine, Suez Canal University had reviewed and approved the study.

Identification of *Enterobacteriaceae*

Urine specimens were cultured on blood and MacConkey's agar plates and incubated for 24 hours at 37°C. After incubation, Gram staining was done and smears were examined for Gram-negative bacilli. *Enterobacteriaceae* were identified by oxidase test, tube indole test, citrate test, MRVP test, MIO test, LIA test, TSI test, urease test and carbohydrate fermentation tests according to [8].

Antimicrobial susceptibility testing and ESBL screening method

Antimicrobial susceptibility testing and phenotypic ESBL screening tests were performed by Kirby-Bauer disc diffusion method. The antibiotics tested were ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), cefepime (30 µg), cefoxitin (30 µg), amikacin (30 µg), gentamicin (10 µg), meropenem (10 µg) and imipenem (10 µg) (Oxoid, Basingstoke, UK). Interpretations of the test results were done according to the guidelines of the CLSI (2017) [9]. ESBL production was suspected if the inhibition zone diameter was ≤ 22 mm for ceftazidime, ≤ 27 mm cefotaxime, or ≤ 27 mm for aztreonam. Strains suspected to be ESBL producers by screening tests were further examined by confirmatory tests to confirm ESBL production.

Phenotypic ESBL confirmatory test

Phenotypic confirmation of ESBLs production was performed using Combination Disk Test (CDT) between ceftazidime alone (30 µg) *vs.* ceftazidime/clavulanic acid (30/10 µg) and cefotaxime alone (30 µg) versus cefotaxime/clavulanic acid (30/10 µg) (Oxoid, Basingstoke, UK) placed on Muller-Hinton agar plate lawned with the test organism and incubated at 35°C ± 2°C for 16 h to 18 h. Regardless of the zone diameters, a ≥ 5 mm increase in the inhibition zone diameter for either antimicrobial agent tested in combination with clavulanic acid *vs.* its zone size when tested alone confirmed ESBL production [9].

Phenotypic detection of fluoroquinolones resistance

Quinolone Resistance (QR) was detected phenotypically by disk diffusion method as recommended by the CLSI using the following antibiotic disks: nalidixic acid (30 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg) disks (Oxoid, Basingstoke, UK) [9]. Isolates with phenotypic quinolone resistance were suspected to harbor PMQR genes and examined by PCR for detection of these genes.

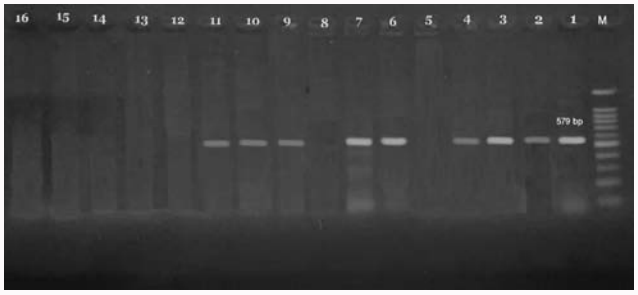

Figure 1: Agarose gel electrophoresis of *qnr*A gene amplicons (579 bp). Lane M shows 100 bp molecular size standard DNA ladder. Lane 1 was positive control and lane 16 was negative control for the gene. Lanes from 2 to 4, 6, 7 and 9 to 11 show amplicons of *qnr*A gene from different isolates.

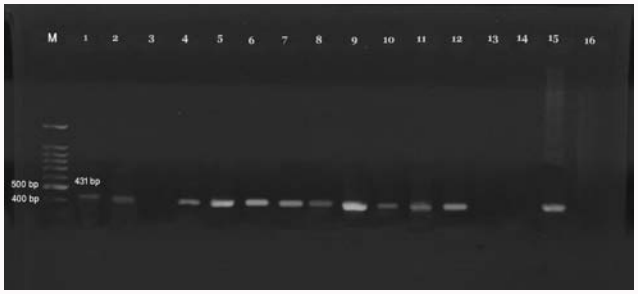

Figure 2: Agarose gel electrophoresis of *qnr*B gene amplicons (431 bp). Lane M shows 100 bp molecular size standard DNA ladder. Lane 1 was positive control and lane 16 was negative control for the gene. Lanes 2, 4 to 12 and 15 show amplicons of *qnr*B gene from different isolates.

Genotypic detection of PMQR genes by PCR

All the ESBL producing and ESBL non-producing *Enterobacteriaceae* strains that showed phenotypic QR were examined by conventional PCR for the presence of five PMQR determinants, namely *qnr*A, *qnr*B, *qnr*S, *qep*A and aac(6')-Ib-cr genes with a set of primers as described in Table 1. Plasmid DNA was extracted from the test isolates using plasmid DNA extraction kit according to the manufacturer's instructions (Genetix biotech, Asia). The reaction mixture was prepared in a total volume of 25 µl including 2 µl of template DNA, 2.0 U of Taq DNA polymerase, 10 mM Deoxynucleotide Triphosphates (dNTP) mix at a final concentration of 0.2 mM, 50 mM MgCl2 at a final concentration of 1.5 mM, 1 µM of each primer and 1X PCR buffer [10]. Reaction mixtures without a DNA template served as negative controls.

For *qnr*A, *qnr*B and *qnr*S genes, amplifications were carried out in a thermal cycler (Eppendorf AG, Hamburg, Germany) with the following thermal cycling conditions: 10 min at 95°C as the primary denaturation step and 35 cycles of amplification consisting of 1 min of denaturation at 95°C, 1 min of hybridization using temperature specific for each primer as shown in Table 1, 1 min of extension at 72°C and 10 minutes at 72°C for the final extension [11]. For *qep*A and aac(6')-Ib-cr genes, amplifications were carried out with the following thermal cycling conditions: 10 min at 95°C as the primary denaturation step and 34 cycles of amplification consisting of 45

Table 1: Primers for the detection of PMQR genes in *Enterobacteriaceae.*

Target	Sequence (5′ → 3')	Annealing temp.	Amplicon size (bp)	Reference
*qnr*A1-*qnr*A6	F:5'- AGAGGATTTCTCACGCCAGG -3'	54°C	579	[11,25]
	R: 5'- TGCCAGGCACAGATCTTGAC -3'			
*qnr*B1-3, 5, 6,8	F: 5'- GGCACTGAATTTATCGGC -3'	49°C	431	[10]
	R: 5'- TCCGAATTGGTCAGATCG -3'			
*qnr*S1- *qnr*S2	F:5'- GCAAGTTCATTGAACAGGGT -3'	54°C	427	[11,25]
	R:5'- TCTAAACCGTCGAGTTCGGCG-3'			
*qep*A	F:5'- AACTGCTTGAGCCCGTAGAT-3'	55°C	596	[12]
	R:5'- GTCTACGCCATGGACCTCAC-3'			
aac (6')-Ib-cr	F:5'-TTGCGATGCTCTATGAGTGGCTA-3'	55°C	482	[28]
	R:5'- CTCGAATGCCTGGCGTGTTT-3'			

Table 2: Prevalence of phenotypic ESBL production in the *Enterobacteriaceae* isolates.

	No.	(%)	p-value
ESBL production	48	28.20%	0.035*
ESBL non-production	122	71.80%	
Total	170	100%	

sec of denaturation at 94°C, 45 sec of annealing at 55°C and 45 seconds of extension at 72°C [12]. Amplicons were analyzed by gel electrophoresis in 1.5% agarose gel in 1X Tris-Borate-EDTA (TBE) buffer containing 0.1 µl/mL ethidium bromide at 120 volts for 45 min and finally visualized with ultraviolet light [10]. Amplicon size (bp) of the tested gene was identified and compared to a 100 bp molecular size standard DNA ladder (Cleaver scientific, UK).

Phylogeny

The nucleotide sequences of the five PMQR genes were obtained from GenBank, with accession numbers as follows: AY070235. 1 for *qnr*A; DQ351241. 1 for *qnr*B; AB187515. 1 for *qnr*S; AB263754.2 for *qep*A and HQ166949. 1 for *aac(6')-Ib-cr*.

Statistical analysis

Collected data were entered into a database film. All statistical analyses were performed using Statistical Package for Social Science program (SPSS version 22 for windows). Descriptive data were managed according to its type; mean, standard deviation and range summarized continuous data; while qualitative data were summarized by frequencies. In analytical data, chi square test was used to detect the difference between qualitative data. Statistical significance was considered at p value ≤ 0.05.

Results

One hundred and seventy *Enterobacteriaceae* isolates were isolated from 205 urine specimens collected from patients with health care associated UTIs with a prevalence rate of 82.9%. *Escherichia coli* was the most commonly isolated species (61.2%), followed by *Klebsiella pneumoniae* (21.8%) and Proteus mirabilis (10%), while *Klebsiella oxytoca* showed the lowest rate of isolation (1.2%). No pathological growth was detected in 5.9% of the cases. The highest rate of UTIs was in the urology department and ICU (31.8% for each) while the lowest rate was in the pediatrics and burn units (4.7% for each).

One hundred and three of the tested *Enterobacteriaceae* isolates (60.6%) were proved to be resistant to ceftazidime and cefotaxime,

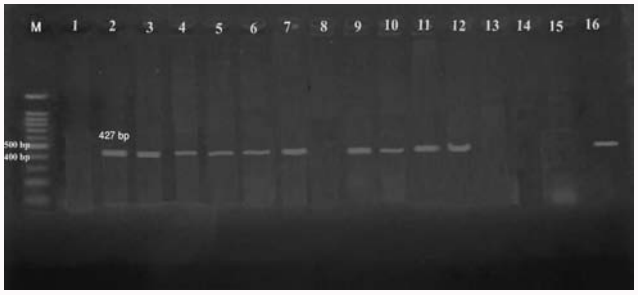

Figure 3: Agarose gel electrophoresis of *qnr*S gene amplicons (427 bp). Lane M shows 100 bp molecular size standard DNA ladder. Lane 1 was negative control and Lane 2 was positive control for the gene. Lanes 3 to 7, 9 to 12 and 16 show amplicons of *qnr*S gene from different isolates.

which required phenotypic confirmation for ESBL production by the Combination Disk Test (CDT); 48 (28.2%) of the *Enterobacteriaceae* isolates were confirmed to be ESBL producers with significant difference between the prevalence of ESBL production and ESBL non-production among isolated *Enterobacteriaceae species* (P<0.05) in favorite of the ESBL non-producers (Table 2). Out of 48 ESBL producers, 73% were *Escherichia coli*, 20.8% were *Klebsiella pneumoniae* and 6.2% were *Proteus mirabilis*.

Fifty four isolates (31.8%) were proved to be resistant to nalidixic acid (quinolones), ciprofloxacin and levofloxacin (fluoroquinolones), which were tested for harboring PMQR genes by conventional PCR. Among the 48 ESBL producers, 29 isolates (60.4%) were QR, while among the 122 ESBL non-producers, 25 isolates (20.5%) were QR with statistical significant difference (P<0.05). On testing the phenotypic QR isolates by PCR (Figures 1 to 5), PMQR genes were detected in 27 (93.1%) of the ESBL producers and in 19 (76%) of the ESBL non-producers with no significant difference. *qnr*A gene showed the highest frequency (52.2%), followed by *qnr*B gene (50%), each of *qnr*S gene and aac(6')-Ib-cr gene (37%) and then *qep*A gene (26%).

Regarding the distribution of the different 5 PMQR genes among the ESBL producers and ESBL non producers with phenotypic QR, *qnr*A gene showed the highest association with ESBL (58.6%), followed by *qnr*B gene (51.7%), then *qnr*S (34.4%), while *qnr*B and aac(6')-Ib-cr genes showed the highest association with ESBL non producers (32% for each) followed by *qnr*A and *qnr*S (28% for each) then *qep*A (12%). There were no statistically significant differences regarding the association between ESBL production and the presence of the type of PMQR genes except for *qnr*A. Regarding the presence of

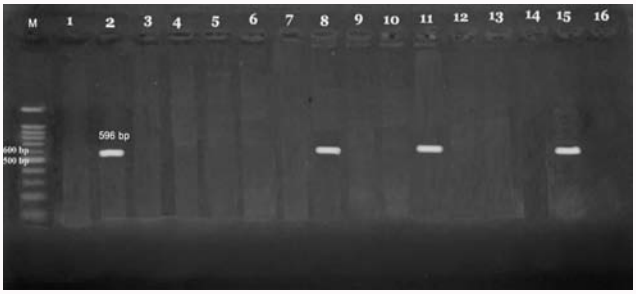

Figure 4: Agarose gel electrophoresis of *qep*A gene amplicons (596 bp). Lane M shows 100 bp molecular size standard DNA ladder. Lane 1 was negative control and Lane 2 was positive control for the gene. Lanes 8, 11 and 15 show amplicons of *qep*A gene from different isolates.

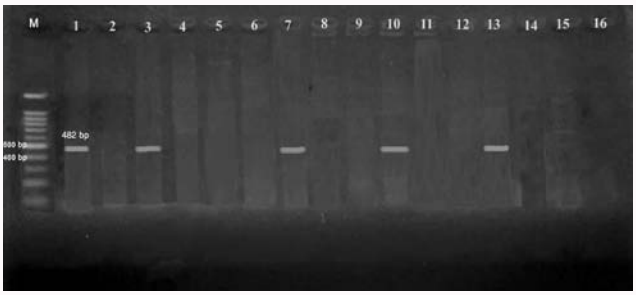

Figure 5: Agarose gel electrophoresis of *aac (6')-Ib-cr* gene amplicons (482 bp). Lane M shows 100 bp molecular size standard DNA ladder. Lane 1 was positive control and Lane 2 was negative control for the gene. Lanes 3,7,10 and 13 show amplicons of *aac (6')-Ib-cr* gene from different isolates.

more than one PMQR gene in each of the 54 phenotypic QR isolates, it was found that 2 isolates (3.7%) harbored 4 genes, 7 (12.9%) harbored 3 genes and 27 (50%) harbored 2 genes.

Discussion

Quinolones are among the most common antimicrobials routinely used for the treatment of serious infections caused by members of the family *Enterobacteriaceae*. The development of resistance to these antibiotics makes the treatment decision difficult, leading to treatment failures [7]. This study aimed to assess the prevalence and genotypes of PMQR genes among ESBL producing and ESBL non-producing *Enterobacteriaceae* isolated from patients with UTIs in SCUH to detect the magnitude of QR among ESBL *Enterobacteriaceae* aiming to help to restrict the use of antibiotics through applying a proper antibiotic policy to avoid emergence and spread of antibiotic resistant bacteria.

In this study, *Escherichia coli* were the most commonly isolated species (61.2%), followed by *klebsiella pneumonia* (21.8%). The study of El-Mahdy on nosocomial infections at Mansoura University Hospitals, Egypt, reported that *Klebsiella pneumonia* showed the highest isolation rate (41.5%), followed by *Escherichia coli* (32.5%) [13]. In another study at Ain Shams University Hospital, *Klebsiella pneumonia* was found to be the most commonly isolated species (57.4%), followed by *Escherichia coli* (28.4%) then *P. mirabilis* (14.2%) [14]. These differences from our results may be explained by the fact that these studies were conducted on various clinical specimens, but our study was conducted on patients infected with UTIs in which *Escherichia coli* is the most common causative agent.

The current study revealed that 60.6% of all the isolated *Enterobacteriaceae* were resistant to ceftazidime and cefotaxime, 51.2% were resistant to aztreonam and 31.8% were phenotypically resistant to nalidixic acid, ciprofloxacin and levofloxacin. Other studies in Egypt also revealed the high rates of antibiotic resistance to beta lactamse and fluoroquinolones drugs among Enterobacteriaceae species. A study conducted at Menoufia University Hospitals revealed that 82.5%, 84.2%, 94.2% and 62.5% of the isolated *Enterobacteriaceae* strains from cases of UTIs were resistant to ceftazidime, cefotaxime, ciprofloxacin and levofloxacin, respectively [15]. At Al-Azhar University, it was found that 55.88%, 61.76%, 73.53% and 58.82% of hospital-acquired Uropathogenic *Escherichia coli* (UPEC) were resistant to cefotaxime, ceftazidime, ciprofloxacin and levofloxacin, respectively [16]. In the USA, a study showed that 21% and 12% of UPEC were resistant to quinolones and fluoroquinolones, respectively [17]. In Iran, it was reported that 69.3%, 39.3%, 28%, 50.6% and 28% of *Escherichia coli* isolated from UTIs patients were resistant to ceftazidime, cefotaxime, cefpodoxime, cefteriaxone and aztreonam, respectively, while only 19.3% of all isolates were resistance to ciprofloxacin [18].

We also found that 28.2% of all isolated *Enterobacteriaceae* were ESBL producers, among them 73% were *Escherichia coli*, 20.8% were *Klebsiella pneumoniae* and only 6.2% were *P. mirabilis*. Out of ESBL producers, 60.4% were proved to be phenotypically resistant to quinolones and fluoroquinolones. Hassan et al. found that 42.9% of their *Escherichia coli* isolates were ESBL producers and among the ESBL producers, 60% were ciprofloxacin resistant [19]. In Iran, it was demonstrated that 51.7% of nosocomial UPEC were ESBL producers, among them, 78%, 80% and 70% were resistant to nalidixic acid, ciprofloxacin and norfloxacin, respectively [20]. Other studies reported lower frequencies of ESBL production; Cruz et al. [21] and Abreu et al. [22] reported ESBL production among *Enterobacteriaceae* at rates of 3.2% and 7.6% respectively [21,22]. The low rates of ESBL production in these studies may be due to the policy of restriction of the use of beta lactam antibiotics in these sites.

The present study demonstrated that 93.1% of phenotypic QR ESBL and 76% of phenotypic QR non-ESBL were proved to harbor at least one of the PMQR genes. Although the difference was not statistically significant, still the percentage in QR strains is higher among phenotypic QR ESBL. *qnrA* gene showed the highest association with ESBL producers (58.6%), followed by *qnr*B gene (51.7%), *qnr*S gene (34.4%) and *qep*A and *aac(6')-Ib-cr* genes (31% for each), while *qnr*B and aac(6')-Ib-cr genes showed the highest association with non-ESBL (32% for each) followed by *qnr*A and *qnr*S (28% for each) then *qep*A (12 %). El-Mahdy reported that 68.8% of his nosocomial *Enterobacteriaceae* isolates were positive for PMQR genes; 77.3% and 66% were positive for the *qnr* genes and *aac(6′)-Ibcr* gene, respectively [13]. Cruz et al. [21] also found that 66% of ESBL-producing *Enterobacteriaceae* had at least one PMQR determinant [21]. Lavilla et al. [23] reported that *qnr*A gene had the highest frequency (93.3%) among *qnr* positive isolates [23]. Other study also reported higher association of *qnr*A with QR *Enterobacteriaceae* [24]. On the other hand, some studies reported that *qnr*B gene showed the highest frequency; Kim et al. [12] found that *qnr*B was the predominant gene (85.7%) among both *Escherichia coli* and *Klebsiella pneumonia* isolates [25]. Peymani et al. [10] reported that *qnr*B1 was the most common gene (30.6%) followed by *qnr*B 4 (9.7%) and *qnr*S1 (1.6%) genes, while their isolates were negative for *qnr*A gene [10]. We reported the coexistence of more than one PMQR gene among the 54 phenotypic QR isolates; 2 isolates (3.7%) harbored 4 genes, 7

(12.9%) harbored 3 genes and 27 (50%) harbored 2 genes. Stephenson et al. [26] reported that 36% of their *Enterobacteriaceae* isolates were positive for *qnr*A and *qnr*S, and two isolates had all the three *qnr* determinants [26]. Banadkouki et al. also reported that 15.2% of *Klebsiella pneumonia* isolated from urine samples were positive for both *qnr*B and *qnr*S genes [27].

The absence of PMQR genes among isolates with phenotypic QR is possibly caused by another resistance mechanisms such as chromosomal mutations (changes of DNA gyrase (gyrA) and/or topoisomerase IV (parC) genes), decreased intracellular concentration due to impermeability of the membrane and/or over expression of efflux pumps which were not evaluated in the present study.

Conclusion

There are high frequencies of QR and PMQR genes among ESBL positive *Enterobacteriaceae* isolates and as these genes are carried on transmissible plasmid, controlled use of these antibiotics and effective infection control measures may limit the spread of resistant genes.

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