Insertional inactivation of oprD in carbapenem-resistant *Pseudomonas aeruginosa* strains isolated from burn patients in Tehran, Iran

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Abstract

In this study, we report the insertion sequence IS*Ppu*21 in the *opr*D porin gene of carbapenem-resistant *Pseudomonas aeruginosa* isolates from burn patients in Tehran, Iran. Antibiotic susceptibility tests for *P. aeruginosa* isolates were determined. Production of metallo-β-lactamases (MBLs) and carbapenemase was evaluated and the β-lactamase-encoding and aminoglycoside-modifying enzyme genes were investigated by PCR and sequencing methods. The mRNA transcription level of *oprD* and *mex* efflux pump genes were evaluated by real-time PCR. The outer membrane protein profile was determined by SDS−PAGE. The genetic relationship between the *P. aeruginosa* isolates was assessed by random amplified polymorphic DNA PCR. In all, 10.52% (10/95) of clinical isolates of *P. aeruginosa* harboured the IS*Ppu*21 insertion element in the *opr*D gene. The extended-spectrum β-lactamase-encoding gene in IS*Ppu*21-carrying isolates was *bla*TEM. PCR assays targeting MBL and carbapenemase-encoding genes were also negative in all ten isolates. The *rmt*A, *aad*A, *aad*B and *arm*A genes were positive in all IS*Ppu*21 harbouring isolates. The relative expression levels of the *mex*X, *mex*B, *mex*T and *mex*D genes in ten isolates ranged from 0.1- to 1.4-fold, 1.1- to 3.68-fold, 0.3- to 8.22-fold and 1.7- to 35.17-fold, respectively. The relative expression levels of the *oprD* in ten isolates ranged from 0.57- to 35.01-fold, which was much higher than those in the control strain *P. aeruginosa* PAO1. Evaluation of the outer membrane protein by SDS−PAGE suggested that *opr*D was produced at very low levels by all isolates. Using random amplified polymorphic DNA PCR genotyping, eight of the ten isolates containing IS*Ppu*21 were shown to be clonally related. The present study describes a novel molecular mechanism, IS*Ppu*21 insertion of the *opr*D gene, associated with carbapenem resistance in clinical *P. aeruginosa* isolates.
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Introduction

Carbapenems, such as imipenem and meropenem are often used as last resort antibiotics for the treatment of multidrug-resistant (resistant to at least three different classes of antibiotics) *Pseudomonas aeruginosa* infections [1]. *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen causing a variety of infections such as endocarditis, pneumonia, urinary tract infection, septicaemia, and skin, eye and ear infections. This organism is also a leading cause of morbidity and mortality among hospitalized burn patients worldwide [2]. Selecting the appropriate drug to initiate antibiotic therapy is important to control the clinical outcome. Unfortunately, treatment with the most appropriate antibiotics is complicated because of the possible resistance of this bacterium to multiple classes of antibacterial agents, such as carbapenems (imipenem and meropenem) [3]. *Pseudomonas aeruginosa* can use

a combination of chromosomally encoded and/or plasmid-encoded mechanisms to evade carbapenem therapy. However, in non-carbapenemase-producing *P. aeruginosa*, resistance to doripenem and meropenem is associated with other mechanisms [4]. The main reported mechanism of resistance to carbapenems involves the loss of OprD porin from the outer membrane through deletions, mutations or insertions in the *opr*D gene. One important mechanism of carbapenem resistance is insertional inactivation of the *opr*D gene by insertion sequence (IS) elements of various sizes [5]. In this study we define a novel insertion sequence in the *opr*D porin gene of carbapenem-resistant *P. aeruginosa* isolates from burn patients in Tehran, Iran.

Materials and methods

Ethics statement
This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences "IR.SBMU.R-ETECH.REC.1396.85" and financially supported by the Infectious Diseases and Tropical Medicine Research Centre, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant No 9369).

Clinical samples and bacterial identification
A total of 95 clinical isolates of *P. aeruginosa* were obtained from patients hospitalized in Shahid Motahari Burn Care Centre, Tehran, Iran from March 2014 to March 2015. Wound swabs were collected from patients and immediately transported to the microbiology laboratory of the Department of Microbiology of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Blood culture was also carried out in patients with a suspicion of sepsis. Strains were identified as *P. aeruginosa* using the standard biochemical tests, including OF, SIM and MR-VP tests, and pigment production on Mueller−Hinton agar (Merck, Darmstadt, Germany). Isolates identified as *P. aeruginosa* were preserved at −70°C in trypticase soy broth (Merck) supplemented with 20% glycerol until further processing [6].

Susceptibility testing
Susceptibility of the *P. aeruginosa* isolates to imipenem (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), meropenem (10 μg), doripenem (10 μg), ticarcillin (75 μg), piperacillin (100 μg), piperacillin/tazobactam (100/10 μg), ciprofloxacin (5 μg), cefepime (30 μg), aztreonam (30 μg), amikacin (30 μg) and gentamicin (10 μg) were determined using a disc diffusion method according to the CLSI [7]. The MIC values of seven selected antibiotics—amikacin, gentamicin, meropenem, imipenem, ceftazidime, ciprofloxacin and colistin—were also determined using the microbroth dilution method at a final concentration

for each antimicrobial agent from 0.5 mg/L to 256 mg/L [7]. *Pseudomonas aeruginosa* ATCC 27853 was used as the quality control strain.

Carbapenemase and metallo-β-lactamase determination
Phenotypic determination of metallo-β-lactamase (MBL) producers and carbapenemase producers was performed by the combined disc diffusion test [8] and Modified Hodge test, respectively [1].

Conventional PCR and sequencing analysis of resistance determinants
Responsible resistance genes encoding extended spectrum β-lactamases, MBLs, carbapenemases and aminoglycoside-modifying enzymes are listed in Table 1. DNA was extracted using the DNA extraction kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000) according to the manufacturer's guidelines. PCR was conducted in a final volume of 25 μL with 12.5 μL of 2× Master Mix (SinaClon, Tehran, Iran; Cat. no. PR901638), including 1× PCR buffer, 0.4 mmol/L dNTPs, 3 mmol/L MgCl$_2$ and 0.08 IU *Taq* DNA polymerase, 1 μL of 10 pmol of each primer and 7.5 μL of sterile distilled water. Amplification reactions were performed on a thermal cycler (Eppendorf, Mastercycler Gradient; Eppendorf, Hamburg, Germany) followed by 36 cycles of denaturation at 94°C for 45 s, annealing at 50°C to 59°C, according to the primers for each gene, for 45 s and extension at 72°C for 45 s. PCR products were electrophoresed on a 1%−1.5% agarose gel, visualized by ethidium bromide staining and photographed under UV light. Amplicons representing each studied gene were confirmed by sequencing analysis (Macrogen Korea). The obtained sequence results were examined by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Insertion sequences were characterized by the online tool ISfinder (https://www-is.biotoul.fr/).

Real-time RT-PCR
The expression levels of *opr*D, *mex*B, *mex*D, *mex*T and *mex*X genes were assessed by RT-PCR. After an overnight culture of bacteria on Luria−Bertani broth [9], RNAs were extracted using the RNX-Plus kit (Cat. No., RN7713C; SinaClon) according to the manufacturer's instructions. The contaminating DNA was removed by DNase I (Fermentas, Waltham, MA, USA). The total RNA concentration was determined using the Nanodrop (DS-11 Spectrophotometer, USA). DNase-treated RNA was reverse-transcribed into cDNA using the Takara kit (Shiga, Japan). The primers used for real-time RT-PCR are shown in Table 1. Real-time RT-PCR assay was performed using the Power SYBR Green PCR Master Mix (Bioneer, Daejeon,

TABLE 1. DNA sequences used in PCR and real-time RT-PCR

Primer	Sequence (5′→3′)	Expected size of amplicon (bp)	Reference
*opr*D for PCR	ATGAAAGTGATGAAGTGGAG CAGGATCGACAGCGGATAGT	1329	[13]
*opr*D for RT-PCR	CGCCGACATCAGCAACACC GGGCCGTTGAAGTCGGAGTA	195	[14]
*mex*B	CAAGGGCGTCGGTGACTTCCAG ACCTGGGAACCGTCGGGATTGA	297	[15]
*mex*D	CTCGAGCTATACGTGCCTAAC GTCCCTCTTCCCATTTCACG	79	[16]
*mex*T	TGTCAGTGATCCTATGCCCC ACACGATCAGCAGGTTCAGA	165	This study
*mex*X	GGCTTGGTGGAAGACGTG GGCTGATGATCCAGTCGC	64	[9]
*rps*L	GGCGTGCGTTACCACACCGT GGACGCTTGGCGCCGTACTT	92	[16]
TEM	TCGGGGAAATGTGCGCG TGCTTAATCAGTGAGGCACC	972	[17]
SHV	TTAGCGTTGCCAGTGCTC GGTTATGCGTTATATTCGCC	861	[17]
CTX-M	TTTGCGATGTGCAGTACCAGTAA CGCTATCGTTGGTGGTGCCATA	544	[17]
VEB	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	643	[18]
GES	TTGCAATGTGCTCAACGTTC TATCACAACCAATATTGTCG	351	This study
KPC	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798	[19]
IMP	GAAGGCGTTTATGTTCATAC GTAAGTTTCAAGAGTGATGC	587	[19]
VIM	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	[20]
NDM	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	[20]
GIM	TCGACACACCTTGGTCTGAA AACTTCCAACTTTGCCATGC	477	[20]
SPM	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	271	[20]
*rmt*A	CTAGCGTCCATCCTTTCCTC TTTGCTTCCATGCCCTTGCC	635	[21]
*aad*A	ATGAGGGAAGCGGTGATCG TTATTTGCCGACTACCTTGGTG	320	[21]
*aad*B	ATGGACACAACGCAGGTCGC TTAGGCCGCATATCGCGACC	120	[21]
*arm*A	TGGGAAGTTAAAGACGACGA CCATTCCCTTCTCCTTTCCA	212	[21]

Korea) on a Corbett Rotor-Gene 6000 Real-Time rotary analyser (Corbett Life Science, Sydney, Australia). Samples were run in triplicate and contained 2 μL of cDNA per reaction. Controls run without reverse transcriptase confirmed the absence of contaminating cDNA in any of the samples. The relative expression of the investigated genes was normalized against the *rps*L housekeeping gene, and was calculated based on a $2^{-\Delta\Delta CT}$ method. Results were obtained as the relative expression of the mRNA compared with that of *P. aeruginosa* PAO1. MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-oprM efflux systems and OprD porin were considered as overexpressed when *mex*B, *mex*C, *mex*E, *mex*X and *opr*D transcriptional levels were at least 3-, 100-, 100-, 4- and 2-fold higher than those in *P. aeruginosa* PAO1 strain, respectively [10–12].

Outer membrane protein analysis

Bacterial outer membrane proteins were examined using previously described methods [9]. Following sonication, membranes were collected by ultracentrifugation at 100 000 *g* for 45 min. Outer membrane proteins were separated by SDS–PAGE, and gels were stained with Coomassie blue.

DNA fingerprinting analysis

Random amplified polymorphic DNA (RAPD)-PCR was performed as described previously [6]. The RAPD fingerprints were analysed and genotypes were assigned on the basis of weight and number of band differences.

Nucleotide sequence accession numbers

The DNA sequences of IS*Ppu*21/*opr*D and IS*Ppu*21 have been deposited in GenBank under accession numbers KT736319 and KT728193, respectively.

Results

In this study, 95 *P. aeruginosa* isolates were identified as imipenem-resistant with MICs ranging from 4 to 256 mg/L. Antibiotic susceptibility tests in this study showed that *P. aeruginosa* isolates were resistant to all currently used antibiotics, including β-lactams, fluoroquinolones and aminoglycosides, but remained susceptible to colistin. In all, 10.52% (10/95) clinical isolates of *P. aeruginosa* harboured an insertion element in the *opr*D gene (Fig. 1).

Phenotypic and genotypic determination of carbapenemase and MBLs in 10.52% (10/95) of *P. aeruginosa* isolates revealed absence of any carbapenemase activity and production of MBL in isolates. In contrast, these isolates were positive for the ESBL gene (*bla*TEM). In this study, the *rmt*A, *aad*A, *aad*B and *arm*A genes were positive in all IS*Ppu*21-harbouring isolates. The relative expression levels of the *mex*X, *mex*B, *mex*T and *mex*D genes in ten isolates ranged from 0.1-fold to 1.4 fold, 1.1-fold to 3.68-fold, 0.3-fold to 8.22-fold and 1.7-fold to 35.17-fold, respectively and relative expression levels of the *opr*D in ten
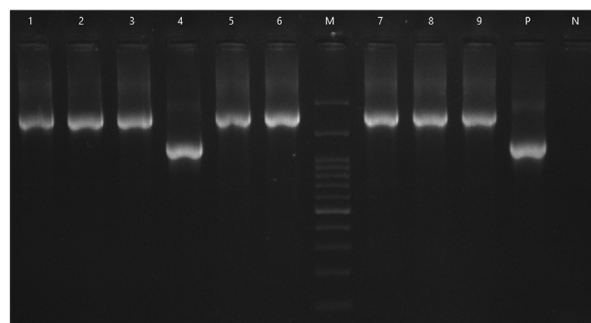
FIG. 1. PCR amplification of *opr*D gene from carbapenem-resistant *Pseudomonas aeruginosa*. M, DNA Ladder 100 bp; N, negative control; P, *P. aeruginosa* PAO1 strain; lane 4, *opr*D gene; lanes 2,3,5–9, IS*Ppu*21/*opr*D combination.

isolates ranged from 0.57-fold to 35.01-fold, which was higher than those in the control strain *P. aeruginosa* PAO1 (Table 2).

Outer membrane profiles obtained by SDS−PAGE indicated that except for one isolate, nine remaining isolates had a corresponding band for *opr*D in comparison to that of PAO1. Using RAPD-PCR genotyping, eight of the ten isolates containing IS*Ppu*21 were shown to be clonally related (Fig. 2, Table 2).

Discussion

Carbapenems, such as imipenem and meropenem have been widely used as active antimicrobials to treat infections associated with *P. aeruginosa*. Producing carbapenemases has been reported as a major mechanism of carbapenem resistance in Iran [22]. Loss of OprD has also contributed to carbapenem resistance, especially imipenem resistance, in Iran [10]. The present study describes isolates of *P. aeruginosa* that exhibit reduced susceptibility to carbapenems other than carbapenemase production [1]. Antibiotic susceptibility tests in this study showed that *P. aeruginosa* isolates were resistant to all antibiotics, but remained susceptible to colistin. PCR assays targeting MBL and carbapenemase-encoding genes were also negative in all ten isolates. It seems that imipenem resistance in such bacteria is not mediated by carbapenemase enzymes. It has been shown that one of the most important mechanisms for imipenem resistance in *P. aeruginosa* is the absence of OprD production due to insertions, mutations and/or deletions in the *opr*D gene, as well as the down-regulation of *opr*D transcription [23]. In the present study, PCR assay using the *opr*D-specific primers demonstrated that 10.52% (10/95) of imipenem-resistant *P. aeruginosa* isolates harbored an IS element in the *opr*D gene. PCR of *opr*D gene using the corresponding primer is expected to obtain an amplicon with size 1323 bp or 1329 bp, while a fragment with a size about 2511 bp was produced. After purification and sequencing, it was found that the *opr*D gene was disrupted at nucleotide position 728 by the insertion of a 1179-bp sequence (GenBank accession no. KT736319) containing features of an IS*Ppu*21 element. This insertional inactivation of the *opr*D gene mediated by IS*Ppu*21 was found to be associated with ten of 95 isolates. These findings are in agreement with Fowler and Hanson, in which insertion of an IS element has resulted in a reduction of carbapenem susceptibility and also loss of OprD production [4]. Different IS elements have been identified worldwide that may inactivate the *opr*D gene and confer resistance to imipenem in clinical isolates of *P. aeruginosa*. To date, the presence of IS elements that disrupt the *opr*D gene has been reported in South Africa (IS*Pa*26), Croatia (IS*RP*10) [1], Iran (IS*Pa*1328) [2], Spain

TABLE 2. Antibiotic susceptibility, PCR results for genes and gene expression data for ISPpu21-carrying *Pseudomonas aeruginosa* isolates

Isolate	MIC (μg/mL) CO	GEN	AK	IMP	MEM	CAZ	CIP	ESBL genes	MBL genes	Aminoglycoside-Modifying Enzyme Genes	Relative expression levels mexX	mexB	mexD	mexT	oprD	Inserted into oprD	ISPpu21/oprD	RAPD-PCR
PA11	<2	256	64	8	64	128	16	*bla*TEM	–	aadA- aadB- rmtA-armA	1.2	1.1	21.11	ND	12.22	+	+	A
PA17	<2	128	32	4	128	128	32	*bla*TEM	–	aadA- aadB-rmtA	2.1	1.2	35.17	0.97	10.25	+	+	A
PA22	<2	32	32	256	64	128	16	*bla*TEM	–	aadA- aadB- rmtA-armA	1.3	1.1	1.7	0.89	0.57	+	+	A
PA31	<2	32	32	256	128	128	128	*bla*TEM	–	aadA- aadB-armA	1.2	1.3	4.56	0.35	1.37	+	+	A
PA42	<2	256	256	256	128	64	32	*bla*TEM	–	aadA- aadB- rmtA-armA	1.2	1.1	19.2	8.22	30.36	+	+	A
PA43	<2	256	256	256	128	128	32	*bla*TEM	–	aadA- aadB- rmtA-armA	1.3	1.1	4.22	2.07	16.14	+	+	C
PA46	<2	256	256	256	8	64	4	*bla*TEM	–	aadA- aadB- rmtA-armA	0.18	1.2	15.24	2.31	25.56	+	+	A
PA48	<2	128	256	4	8	256	8	*bla*TEM	–	aadA- aadB- rmtA-armA	0.53	1.2	23.57	1.06	9.01	+	+	A
PA49	<2	32	256	4	4	16	16	*bla*TEM	–	aadA- rmtA	1.4	ND	ND	ND	4.07	+	+	A
Psa6	<2	256	256	4	4	4	16	*bla*TEM	–	aadA- rmtA-	1.10	3.68	2.21	2.36	35.01	+	+	B

Abbreviations: GEN, gentamicin; AK, amikacin; IMP, imipenem; MEM, meropenem; CAZ, cefotaxime; CIP, ciprofloxacin; ESBL, extended-spectrum β-lactamases; MBL, metallo-β-lactamases; RAPD-PCR, random amplified polymorphic DNA polymerase chain reaction.
–, Negative; +, Positive; ND, not determined.
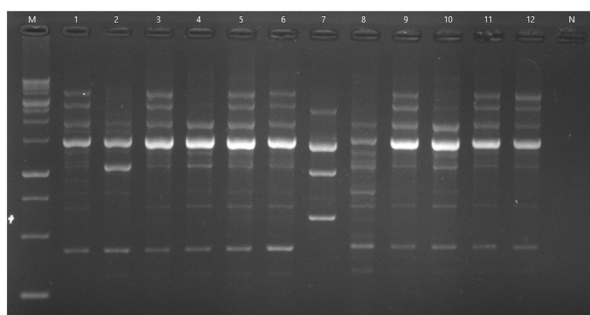
FIG. 2. Random amplified polymorphic DNA-PCR fingerprints of *Pseudomonas aeruginosa* isolates from burn patients using primer 272. M, DNA molecular weight marker (1 kb); N, negative control; 1–12, clinical samples.

(IS*Pa*133), China (IS*Pa*1328, IS*Pre*2), the USA (IS*Pa*8, IS*Pa*1635 and IS*Pa*1328) and France (IS*Pa*46 and IS*Pa*1328) [1]. Mobile elements, such as IS can help Gram-negative bacteria to promote their survival and adaptation to altered environmental niches through the interruption of genes and genomic modifications. This type of adaptation has been well reported in clinical isolates of *P. aeruginosa* that are resistant to β-lactams [4]. IS*Ppu*21, identified in the present study, is a 1179-bp insertion sequence with a couple of 6-bp inverted repeats (TCTGAA) at its extremes. Interestingly, IS*Ppu*21 elements introduced at insertion site 1 and insertion site 2 generate a pair of 7-bp and a pair of 4-bp target site duplication direct repeats, respectively. Just a single open reading frame was discovered in IS*Ppu*21, which encodes a protein with 326 amino acids. The IS*Ppu*21 nucleotide sequence is 100% identical to a region of O antigen biosynthesis gene cluster (GenBank accession no. AF498406) of *P. aeruginosa* serotype O15-ATCC with the open reading frame in this IS*Ppu*21 orthologue transcribed in the opposite direction from the *P. aeruginosa* chromosomal gene cluster [24]. The putative product of the open reading frame in IS*Ppu*21 has a homology (identities from 61% to 93%) to transposase proteins belonging to the IS*5* family of transposases [24]. A previous study has demonstrated that resistance to carbapenems, such as meropenem and doripenem in *P. aeruginosa* isolates that do not produce metallo-β-lactamase, could be developed through both down-regulation and loss of OprD in the outer membrane and up-regulation of MexAB-OprM in these strains [4]. In this study, the relative expression levels of the *mex*X, *mex*B, *mex*T and, *mex*D genes in the isolates ranged from 0.1-fold to 1.4-fold, 1.1-fold to 3.68-fold, 0.3-fold to 8.22-fold, and 1.7-fold to 35.17-fold, respectively, and the *opr*D expression level in IS*Ppu*21-carrying isolates ranged from 0.57-fold to 35.01-fold, which was much higher than in the control strain *P. aeruginosa* PAO1 [4]. However, the susceptibility analysis results for nine isolates were contrary to others, so imipenem resistance could not be attributed to the increased expression of the *opr*D gene. These results were similar to those by Sun and Dennis, in which the relative expression levels of the *opr*D gene in IS-positive isolates were higher than the control strain *P. aeruginosa* ATCC 27853 [24]. In addition, we found one isolate that showed decreased expression of the *opr*D gene. These results were also similar to those of Wolter *et al.*, who reported how an IS element with 97% similarity to IS*Pa*16 inserted in the promoter of the *opr*D gene in two isolates, *P. aeruginosa* PA323 and *P. aeruginosa* PA415, reduced the *opr*D expression and increased imipenem MIC value [25]. Outer membrane profiles obtained by SDS–PAGE indicated that, with the exception of one isolate, the nine remaining isolates had a corresponding band for *opr*D in comparison to that of PAO1. Although these results signify the presence of a functional OprD in our clinical isolates, they strongly indicate that the OprD is absent or produced at very low levels. These results were similar to those of Shen *et al.*, who reported a reduction in the size of OprD through the insertion of an IS element in the *opr*D gene of six isolates. In contrast, both imipenem-susceptible strains and the PAO1 control strain showed the expected protein band for OprD at 46 000 MW [26]. In the other study, Fournier *et al.*, showed that an IS insertion in the *opr*D gene of one isolate may be associated with down-regulated OprD production [27]. RAPD-PCR was performed for typing all the isolates harbouring IS*Ppu*21 in order to survey whether the IS-carrying strains collected in this study were associated with nosocomial infections. On observing DNA fingerprinting results, three patterns of these isolates were obtained. In the other word, eight of the ten isolates were clonally related. The results showed that the high prevalence of IS*Ppu*21-carrying isolates was related to nosocomial infections.

Conclusion

Carbapenem resistance mediated by OprD deficiency in *P. aeruginosa* clinical isolates may be more frequent in Iranian hospitals, which could in turn be derived from the insertional inactivation. In this study, we report the emergence of IS*Ppu*21 in carbapenem-resistant *P. aeruginosa* strains isolated from burn patients in Iran.

Transparency declaration

The authors declare that they have no competing interests.

Acknowledgements

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