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Original Article

Antibiotic resistance assessment and multi-drug efflux pumps of *Enterococcus faecium* isolated from clinical specimens

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Abstract

Introduction: *Enterococcus faecium* is a major cause of community and hospital-acquired infections. Due to limited options for infection with fluoroquinolones-resistant Enterococci, novel therapeutics are urgently needed. Efflux pumps are contributed to fluoroquinolones resistance phenotype in this bacterium and novel inhibitors that target these efflux pumps could be effective in patients. In this research, the possible synergistic effect of an efflux pump inhibitor (EPI), thioridazine, with ciprofloxacin was investigated against clinical isolates of *E. faecium*.

Methodology: A total of 88 isolates of *E. faecium* from clinical specimens were studied from August 2017 to September 2018. Conventional phenotypic and molecular methods characterized all the isolates. Standard susceptibility tests and molecular assays determined the antibiotic resistance profiles and the frequency of efflux pump genes. Minimum inhibitory concentrations (MICs) to ciprofloxacin (CIP) in the presence and absence of thioridazine were measured by the micro-broth dilution method.

Results: The highest antibiotic resistance rate among *E. faecium* isolates was related to ciprofloxacin (96.8%), levofloxacin (94.3%), and imipenem (90.9%), respectively. The highest frequency of efflux pump determinants was related to *efmA* (60, 68%), followed by *emeA* (48, 54.5%), and *efrA* and/or *efrB* genes (45, 51%). The efflux pump inhibitor showed ≥ 2-fold decrease in the MIC value of ciprofloxacin in 48.2% of the isolates.

Conclusions: Efflux pump inhibitor genes *efrA*B, *efmA,* and *emeA* are common among the *E. faecium* clinical isolates. Our results supported the administration of thioridazine, as an efflux pump inhibitor, in fluoroquinolone-resistant *E. faecium* infections due to its synergistic effect with CIP.

Keywords: *Enterococcus faecium*; efflux pumps; antibiotic resistance; thioridazine.

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Introduction

Enterococcus faecium is Gram-positive common commensal bacteria that have emerged as an important nosocomial pathogen in the last decade [1]. The microorganism is responsible for urinary tract infections (UTI), bacteremia, wound infections, surgical site infections, and endocarditis [2]. Concerns about this bacterium have been linked to the increasing prevalence of infections in humans, the intrinsic and acquired resistance to antibiotics (e.g., β-lactams, aminoglycosides, quinolones, and a few other antibiotics), the ability to acquire virulence gene determinants, multiple-drug resistance traits rapidly, and the reduced effectiveness of therapeutic agents. This acquired resistance can occur through mutations or horizontal gene transfer by plasmids and transposons with other bacteria [1]. Various mechanisms are involved in the development of resistant strains [3]. Several studies have shown that efflux pumps have become a serious concern for the treatment of infections with multidrug-resistant *E. faecium* strains [3-6]. Efflux pumps can not only repel a wide range of antibiotics due to their poly-substance properties but also achieve greater resistance mechanisms by reducing the concentration of intracellular antibiotics and increasing the accumulation of mutations [7]. Furthermore, it involves bacterial pathogenicity, iron metabolism, and exporting a toxic compound during anaerobic respiration [8].

Ciprofloxacin (CIP) is a fluoroquinolone antibiotic that is used to treat a wide range of bacterial infections

[9]. There have been many studies on antibiotic resistance against beta-lactams, aminoglycosides, fluoroquinolones, and vancomycin in *E. faecium* [10,11], but little is known about drug efflux pumps in this bacterium. Various studies have shown multidrug efflux pumps in *E. faecium*, including EfmA (*E. faecium* multidrug resistance) [12] belonging to the major facilitator superfamily (MFS), EmeA (Enterococcal multidrug resistance efflux) homolog of NorA and belonging to the MFS and ABC-type MDR transporters [4], and *efr*AB belonging to the ATP-binding cassette (ABC) [13] superfamily of multidrug efflux transporters that are involved in resistance to fluoroquinolones (FQ).

Several studies were shown the inhibition of efflux pumps by thioridazine, the efflux inhibitor (EPI), against multidrug-resistant *Mycobacterium tuberculosis, Staphylococcus aureus*, *Escherichia coli*, multidrug-resistant *Acinetobacter baumannii,* and *Burkholderia pseudomalle* strains [14-16]. Currently, is it possible to use these types of inhibitors in other treatment-resistant infections, especially MDR *E. faecium* infections? Few studies support efflux pumps' role and their involvement in resistance to fluoroquinolones in *E. faecium* isolates from clinical specimens. Inhibition of FQ efflux transporters by EPIs could increase the sensitivity of FQ-resistant strains, which seems to be a promising strategy to restore antibacterial potency [17]. Therefore, in this work, we investigated the overall effect of thioridazine on the induction of sensitivity to ciprofloxacin at the MIC level in the resistant *E. faecium* clinical isolates presenting different genetic patterns of efflux pump inhibitors.

Methodology

Patients and bacterial strains

A total of 88 isolates of *E. faecium* were collected from different kinds of clinical specimens including blood, urine, wound, tracheal, fluids, sonde urinaire, sputum, and fistula of hospitalized patients who attended Milad Hospital between August 2017 and September 2018 in Tehran, Iran. This study was approved by the ethics committee of Shahroud University of Medical Sciences (Code IR.SHMU.REC.1397.075). All the patients provided written informed consent, similar to the Declaration of Helsinki before entry into the study. Bacterial isolates were inoculated on Sheep Blood agar and identified by the bacteriological conventional methods, including catalase, bile esculin test, sugar fermentation test, and growth in 6.5% NaCl solution. PCR was performed with species-specific primers (see the *Molecular examinations* section) to confirm the results of the biochemical tests. All the strains were stored at -70 °C in brain–heart infusion (BHI) broth medium supplemented with 20% glycerol. *E. faecalis* ATCC 29212 was used as the control strain for both biochemical and molecular identification methods.

Antimicrobial susceptibility testing

Antibiotic susceptibility patterns of *E. faecium* isolates to 12 antibiotics were determined by the Kirby-Bauer disk diffusion technique on Mueller-Hinton Agar medium based on the Clinical and Laboratory Standard Institute guidelines (CLSI) [18]. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. The following antibiotics were used: Ampicillin (10 µg), vancomycin (30 µg), teicoplanin (30 µg), linezolid (30 µg), tetracycline (30 µg), minocycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nitrofurantoin (300 µg), gentamicin (120 µg), imipenem (10µg), and tigecycline (15 µg) (Himedia, Mumbai, India).

Minimum Inhibitory Concentrations Test

MIC of CIP was determined using the broth microdilution method in 96-well microtiter plates. Pump phenotypic evaluation of ciprofloxacin-resistant *E. faecium* was assessed by measuring the minimum inhibitory concentrations (MICs) for CIP before and after exposure to the efflux pump inhibitor (EPI), thioridazine (Sigma-Aldrich, Shanghai, China). In this study, a set of different concentrations for both conditions was prepared using serial dilution, and similar amounts of bacterial suspension were added to each sample. The final concentration of thioridazine was 15 µg/mL and after incubation for 24 hours, MICs were recorded as the lowest concentration of the experimental compound that was able to inhibit visual growth. A plate containing (15 µg/mL) of thioridazine without antibiotics was used as a control. A 4-fold or greater reduction in MIC following the addition of thioridazine indicated that an efflux pump could extrude antibiotics [19]. All assays were performed three times. The test results were interpreted according to CLSI guidelines.

DNA Preparation

PGA DNA extraction kit (Pouya Gene Azma Company, Iran) was used to extract genomic DNA from all the Enterococci isolates according to the manufacturer's protocol. Suspected Enterococci

colonies were cultured on the Blood agar medium. The grown colonies were centrifuged at 13,000 rpm and the pellets were resuspended in 100 µL of buffer I. Then buffer II and buffer X were added, respectively. After centrifugation and transfer of supernatant to the new tube, 96 to 100% cold ethanol and then 70% were added for washing. Finally, 20 to 50 µL of solvent buffer was added to the lysates in the tube, and the DNA samples were kept at -20 °C for polymerase chain reaction (PCR).

Molecular characterization and Efflux Pump Genes

Molecular characterization of *E. faecium* was performed by species-specific primers for $ddl_{E.\ faecium}$ gene (Table 1). Amplification was performed in a 25 µL reaction mixture containing 12.5 µL of Master Mix (Amplicon, Denmark), 10.5 µL of distilled water, 0.5 µL of each of the primers (F and R), and 1 µL of template DNA. Sequence-specific primer sets were used to amplify four efflux pump determinants (*efmA, emeA, efrA*, and *efrB*) in clinical isolates of *E. faecium.* PCR was performed on a thermal cycler (Eppendorf, Germany) under the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles including denaturation at 95 °C for 1 minute, annealing ranging from 56 °C to 57.5 °C (Table 1) for 1 minute, 72 °C for 1 minute, and final extension at 72 °C for 10 minutes.

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (IBM SPSS Statistic). The Chi-Square test was used and the *p*-value < 0.05 was considered statistically significant.

Ethical standards

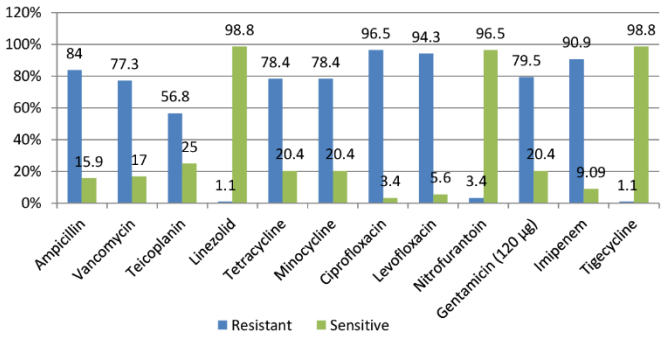
This study has an ethics code number (IR.SHMU.REC.1397.075) from the research deputy of Shahroud University of Medical Sciences.

Results

Bacterial isolates and epidemiological characteristics

A total of 88 isolates of *E. faecium* were collected from different kinds of clinical specimens, including blood (n = 16, 18.2%), urine (n = 43, 48.9%), fluids (n = 9, 10.2%), wound (n = 8, 9.1%), sonde urinaire (n = 3, 3.4%), tracheal (n = 3, 3.4%), CSF (n = 2, 2.3%), sputum, fistula, catheter, and ascetic fluid (n = 1, 1.1%). The highest number of *E. faecium* isolates was obtained from urine (48.9%) and the lowest amount was obtained from sputum, fistula, catheter, and ascetic fluid samples (1.1%). The age range of the patients was 1 to 93 years, with 43 (48.9%) female, and 45 (51%) male. The isolates showed positive results for biochemical tests and their identity was confirmed by the species-specific PCR assay.

Figure 1. Susceptibility of *E. faecium* isolates to different antibiotics in different clinical specimens.



Antimicrobial resistance patterns among E. faecium strains

The susceptibility of *E. faecium* isolates to different antibiotics in different clinical specimens is summarized in Figure 1. The highest susceptibility of antibiotics to linezolid, tigecycline, and nitrofurantoin were 98.8%, and 96.5%, respectively, and the highest antibiotic resistance was related to ciprofloxacin (96.8%), levofloxacin (94.3%), and imipenem (90.9%), respectively. The MIC-resistant breakpoint for CIP was ≥ 4 µg/mL according to the CLSI guidelines [18]. In this study, CIP MIC values of tested clinical isolates ranged from 2 to 1024 µg/mL, indicating high resistance of the isolates to CIP. A majority of the *E. faecium* isolates (85, 96.6%) showed MIC of CIP ≥ 4 µg/mL and were considered resistant.

Table 1. Oligonucleotide primers and conditions used to amplify different efflux pump determinants in *E. faecium* strains by PCR.

Genes	Primer sequence (5′- 3′)	Annealing temperature	PCR product size (bp)	Reference
ddl (E. faecium)	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	57	658	[20]
efmA	TCCAAATGCCAACGATCCA TAGAGCTGGCGCAATGATGA	56.5	119	[12]
emeA	GTGACAGCCTTTGTGGCAGAT TAGTCCGTTGATGGTTCCTTG	57.5	687	[11]
efrA	ACGCCAGTGATGTTTATTGC ACGAATAGCTGGTTCCATGT	57	543	[21]

A majority of the *E. faecium* isolates (68, 77.2%) showed MIC levels ≥ 32 µg/mL and resistant phenotype to vancomycin. The intermediate phenotype was only detected in five isolates (5.6%, MIC of 8-16 µg/mL) and 17% of the isolates showed MIC ≤ 4 µg/mL and were sensitive to vancomycin.

The multi-drug resistance (MDR) phenotype was detected in 76.1% (67/88) of *E. faecium* isolates from the clinical specimens. The most common MDR phenotype was tetracycline^R/ minocycline^R/ gentamicin120µg^R/ ciprofloxacin^R/ levofloxacin^R/ ampicllin^R/ teicoplanin^R/ imipenem^R phenotype (Table 2).

Prevalence of efflux pump determinants in the studied isolates

PCR amplification results showed that the highest frequency of efflux pump determinants was related to *efmA*, 60 (68%), followed by *emeA* and *efrA* and/or *efrB* genes (48, 54.5%, and 45, 51%, respectively). Frequency of efflux pump determinants in CIP-resistant *E. faecium* isolates revealed that 70.5% (*60*/88), 52.9% (45/88), and 49.4% (42/88) of the isolates carried *efmA, emeA, and efrA and/or efrB* genes, respectively. There was a significant correlation between the presence of the *efmA* gene and CIP-resistant *E. faecium* (*p*-value = 0.03). The co-carriage of efflux pump determinants was detected in 28.2% (24) of the CIP-resistant *E. faecium* isolates.

Effects of efflux pump inhibitor MIC levels of CIP

MIC results of ciprofloxacin were observed in 85 *E. faecium* isolates in the presence and absence of thioridazine (as a chemical inhibitor of efflux pumps) in different patterns. The results showed that most of the isolates, 48.2% (41/88), presented at least a 2-fold or greater reduction in MIC value of ciprofloxacin in the presence of the studied efflux pump inhibitor (Table 3).

Table 2. Antibiotic resistance patterns in *E. faecium* isolates from clinical specimens.

Antibiotic resistance patterns	N (%)
TET,MIN,GEN120,CIP,LEV,AMP,LIN,TEC,TGC,IMI,VAN	1 (1.1%)
TET,MIN,GEN120,CIP, AMP,LIN,TEC,TGC,IMI,VAN	1 (1.1%)
TET,MIN,GEN120,CIP,LEV,AMP,NIT, IMI,VAN	3 (3.4%)
TET,MIN,GEN120,CIP,LEV,AMP,TEC, IMI,VAN	**24 (27.2%)**
TET,MIN,GEN120,CIP,LEV, TEC, IMI	5 (5.6%)
TET,MIN,GEN120,CIP,LEV, TEC, IMI,VAN	1 (1.1%)
TET,MIN,GEN120,CIP,LEV,AMP, IMI,VAN	2 (2.2%)
TET,MIN,GEN120,CIP,LEV,AMP, IMI	2 (2.2%)
TET,MIN,GEN120,CIP,LEV,IMI	4 (4.5%)
TET,MIN, CIP,LEV,AMP,TEC, IMI,VAN	3 (3.4%)
TET,MIN, CIP,LEV,AMP, IMI,VAN	3 (3.4%)
TET,MIN, CIP,LEV,AMP, IMI	1 (1.1%)
TET,MIN, CIP,LEV, IMI	1 (1.1%)
TET,MIN, IMI	1 (1.1%)
TET,MIN,GEN120,CIP,LEV,AMP, TEC,VAN	3 (3.4%)
GEN120,CIP,LEV,AMP, TEC, IMI,VAN	3 (3.4%)
TET, GEN120,CIP,LEV, TEC, AMP,IMI,VAN	1 (1.1%)
GEN120,CIP,LEV, AMP,IMI,VAN	5 (5.6%)
GEN120,CIP,LEV, AMP,IMI	1 (1.1%)
CIP,LEV, TEC, AMP,IMI,VAN	1 (1.1%)
GEN120,CIP,LEV, TEC, AMP,VAN	1 (1.1%)
Total	**67 (76.1%)**

TET: tetracycline; MIN: minocycline; GM120: gentamicin 120 µg; CP: ciprofloxacin; LEV: levofloxacin; AMP: ampicillin; VAN: vancomycin; IMI: imipenem; TEC: teicoplanin; TGC: tigecycline; LIN: linezolid; NIT: nitrofurantoin. Resistance phenotypes were determined for all antibiotics by disk diffusion (Kirby–Bauer) method according to CLSI 2019 guidelines (Himedia, Mumbai, India).

Discussion

Treatment of multidrug-resistant Enterococci has created a serious problem in hospitals [1]. High-level resistance to fluoroquinolones requires the accumulation of multiple mutations in target genes, such as *parC* and *gyrA*, and genes regulating drug efflux. These mutations could evolve either through the overuse of antimicrobial agents or natural selection to improve the growth and fitness of the bacteria [20]. Treatment of infections associated with multidrug-

Table 3. Effects of thioridazine on the ciprofloxacin MIC in *E. faecium* isolates.

Isolates, No. (%)	MIC (µg/mL)	MIC (µg/mL) + Thioridazine	Fold reduction in MIC + Thioridazine
14 (16.4%)	64	4-32	1-4
4 (4.7%)	64	64	0
10 (11.7%)	128	8-64	1-4
3 (3.5%)	128	128	0
4 (4.7%)	256	16-32	3-4
1 (1.7%)	256	256	0
9 (10.5%)	512	16-64	3-5
4 (4.7%)	512	512	0
15 (17.6%)	1024	32-512	1-5
5 (5.8%)	1024	1024	0

MIC: minimum inhibitory concentration.

resistant Enterococci is very difficult. At present, the efflux pumps are recognized as one of the most important complexes involved in resistance to most classes of antibiotics. Furthermore, recent studies show that these efflux pumps, in addition to pumping out antibiotics, are also associated with the sustained formation and increased spontaneous mutation rates, both of which can contribute to persistence at the site of infection [21]. Survival at the site of infection provides an opportunity for the low-resistance population to evolve by obtaining secondary mutations in the target genes of the antibiotic, resulting in clinical resistance to the therapeutic antibiotic [22].

In our study, we found a high prevalence of resistance to ciprofloxacin and levofloxacin in *E. faecium* isolates. This resistance rate was in agreement with the reports published by Arshadi *et al.* [23], Sattari-Maraji *et al.* [24], and Jia *et al*. [22], in Iran and China, but higher than those reported by Kateete *et al.* [25], Karna *et al.* [26], Tian *et al.* [27], and Arbabi *et al.* [28] in Uganda, India, China, and Iran, respectively. Extensive or irrational use of these antibiotics to treat a wide range of bacterial infections may explain the higher resistance to this antibiotic compared to other antimicrobials.

The frequency of resistance to imipenem in these isolates was 90%, which is consistence with the reports published by Boccella *et al.* [29], Said and Abdelmegeed [30], Asadollahi *et al.* [31], and Sharifi *et al.* [32] in Italy, Egypt, and Iran, respectively. *E. faecium* isolates are intrinsically resistant to imipenem, which may be the reason for the high resistance of this antibiotic in our study.

In the current study, 84% of the isolates were resistant to ampicillin. This resistance rate was in agreement with the reports published by Jia *et al.* [11], Arshadi *et al.* [23], and Boccella *et al.* [29] in China, Iran, and Italy, respectively, but higher than those reported by Karna *et al.* [26] in India and Asadollahi *et al.* [31] in Iran. The frequency of resistance to tetracycline, minocycline, and teicoplanin was 78.4%, 78.4%, and 56.8%, which was relatively similar to those reported by Asadollahi *et al.* [31] and Arabestani *et al.* [33] in Iran, but higher than those reported by Jia *et al.* [11] in China, Boccella *et al.* [29] in Italy, and Karna et al. [26] inIndia and lower than the results obtained by other researchers from Egypt, India, and Iran [23,26,30]. The frequency of resistance to gentamicin 120 µg was 79.5%, which is comparable to the rate reported in previous studies from Egypt and Iran [30,31], but higher than those reported by Jia *et al*. [11], Boccella *et al.* [29], and Karna *et al.* [26] in China, Italy, and India, respectively. Differences in antibiotic resistance in our region and other countries may be related to the regional variation in antibiotic practice patterns. The frequency of resistance to vancomycin was 77.3%, which is comparable to the rate reported in previous studies from Iran [28,33], and India [34], but higher than those reported by Jia *et al*. [11], Boccella *et al.* [29], and Karna *et al.* [26]. Different rates of antibiotic resistance in different countries can be due to the geographical distribution of resistance, differences in antibiotic use patterns, and environmental factors.

The increase of vancomycin-resistance *E. faecium* (VRE) in hospitals is a serious threat to patient care systems because, in addition to vancomycin resistance, these bacteria inherently acquired resistance to a wide range of antibiotics. Furthermore, VREs may serve as reservoirs and sources for other antimicrobial resistance genes and transfer to other gram-positive cocci such as methicillin-resistance *Staphylococcus aureus* (MRSA) [35].

Most of the isolates in our study showed high sensitivity to linezolid, nitrofurantoin, and tigecycline, which is consistent with previous data reported by Boccella *et al*. [29] in Italy and Arshadi *et al.* [23] in Iran.

Isolation of MDR *E. faecium* isolates has become a worldwide concern in hospitals and multi-drug efflux pumps serve as one of the important mechanisms of MDR. In our study, the high frequency of the MDR phenotype (76%) was detected in the strains isolated from clinical samples. This frequency was consistent with the reports published by Jia *et al*. [11], and Rana and Sande [34] in China and India, but fewer than those reported by Nasaj *et al.* [36], and Said *et al.* [30] in Iran and Egypt. Several studies have shown various multidrug efflux pumps in *E. faecium*, such as *efmA*, *emeA*, and *efrAB*, which are involved in resistance to fluoroquinolones. In this study, the frequency of *emeA* was 54.5%, which is in agreement with the report published by Jia *et al.* [11] in China, but higher than Kang *et al.* [6] and Sobhanipoor *et al.* [37] in Korea and Iran, respectively. Also, the frequency of *efrA/efrB* was 51%, which is higher than those reported by Kang *et al.* [6], Sobhanipoor *et al.* [37], and Valenzuela *et al.* [38] in Korea, Iran, and Spain, respectively and lower than the report published by Jabbari *et al.* [39] in Iran. There are no data about the frequency of *efmA* in *E. faecium* isolates in clinical specimens. It seems that the source and types of samples are effective in the distribution of these genes. For ciprofloxacin-resistant isolates that do not have efflux pumps, the

resistance mechanism may be due to mutations in *parC* and *gyrA*.

Efflux pump inhibitors could reduce resistance to fluoroquinolones in *E. faecium* isolates. The role of efflux pump inhibitors, such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has been studied by some researchers, but so far, no study has been done on thioridazine as an efflux pump inhibitor in *E. faecium* isolates. The results of our study revealed that thioridazine, as CCCP, was able to decrease CIP MIC from one- to fivefold among the clinical isolates. Regarding the increasing prevalence of resistant *E. faecium* strains to fluoroquinolones in the clinical samples of hospitalized patients and the observed synergistic effect of this CCCP with CIP, its usage in combination therapy should be considered in the future.

Conclusions

Different factors, such as the transfer of resistance genes between bacteria, mutations in functional genes, increase in selective pressure, and natural selection for improving growth and fitness, play roles in spreading resistance and increasing the occurrence of MDR-*E. faecium* strains in hospital settings and the community. Our results showed a high prevalence of vancomycin- and carbapenem-resistant *E. faecium* strains in the clinical samples. The efflux pumps genes *efrAB*, *efmA*, and *emeA* were common among the clinical isolates, which were significantly associated with resistance phenotype to CIP. Our results supported the synergistic effect of thioridazine, a known inhibitor for efflux pumps, with ciprofloxacin in these isolates, leading to two folds or greater levels of reduction in its MIC value. Further studies are needed to show the effectiveness of thioridazine in the treatment of fluoroquinolone-resistant and MDR *E. faecium* infections.

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