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Molecular study of *bla$_{VEB-1}$* gene in *Proteus mirabilis* isolated from clinicalSamples from Baghdad City's hospitals

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Abstract : From different hospitals in Baghdad city, 25 clinical isolates of *Proteus spp.* were collected from different clinical samples, all isolates were identified as *Proteus* mirabilis by using bacteriological and biochemical assays in addition to Vitek-2 identification system. 15 (60%) isolates were identifying as *Proteus mirabilis*. The susceptibility of *P. mirabilis* isolates towards cefotaxime and ceftazidime was (66.6 %), (20%) consecutively; while extended spectrum β-lactamases producing *P. mirabilis* percentage was (30.7 %).
Because *bla$_{VEB-1}$* was documented as an important indicator for increasing risk of extended spectrum beta ß-lactamases producing *P. mirabilis* isolates that began to spread from many geographic area to Far east which increase the possibility of its occurrence in the middle east; *bla$_{VEB-1}$* gene was detected by using multiplex polymerase chain reaction technique (PCR) that followed extracted DNA from 5 isolates of *P. mirabilis*. Results showed the presence of this gene in all tested isolates. Sequencing of DNA nucleotides was preformed by automated sequencer (Macrogen/ Korea) which showed that 3 isolates of *P. mirabilis* have *bla$_{VEB-1}$* gene. In order to indicate the *bla$_{VEB-1}$* gene was harbor on plasmid or chromosomal DNA, curing of plasmid DNA was carried out by using Ultraviolet rays (230 A°) for 20 min. the results confirmed that the *bla$_{VEB-1}$* gene in local isolates of *P. mirabilis* was found on plasmid DNA.

Keywords – Cephlosporine resistant, beta-lactamases, bla$_{VEB-1}$ gene, EXBLs enzymes, *Proteus mirabilis*

I. Introduction:

Enterobacteriaceae are Gram-negative, rod shaped 1-3 μm large bacteria. They are facultative anaerobic bacteria and can be found in nature, in the human and animal intestine, where they belong to the commensal microbial flora, such as: *Escherichia coli, Klebsiella* spp., *Proteus* spp., *Morganella spp., Providentia spp., Enterobacter spp., Serratia spp.* These bacteria can be pathogens of urinary tract, respiratory tract, bloodstream and wounds. *Proteus* spp. are also widely distributed in the natural environment, including polluted water, soil. Their important was noticed in their rule in the decomposing of the organic matter of the animal origin due to their proteolytic activity, the ability to hydrolyze urea to ammonia and carbon dioxide, as well as the oxidative deamination of amino acids. *Proteus mirabilis* is a common opportunistic pathogen in human, it is considering as the second most common cause of urinary tract infections and an important cause of nosocomial infections, wound and burns infections; their pathogenicity is due to several virulence factor including: enzymes, such as: urease and protease; fimbriae; endotoxin (lipopolysaccharide); hemolysin and swarming phenomena. *Proteus* spp. are distinguishable from most other genera by their ability to swarm across agar surfaces of solid media. [1, 2, 3, 4].While most *P. mirabilis* are susceptible to ß-lactam antimicrobial agents, some isolates produce ESBLs. Recently, a progressive increase of ß-lactam antimicrobial agents resistance mediated by the production of acquired ß-lactamases enzymes has occurred in this species. Extended-spectrum ß-lactamases (ESBLs) active on expanded-spectrum cephalosporins have also started spreading in *P. mirabilis*, including most frequently plasmid mediated ß-lactamases, such as: CTX-M-2, SHV- derivatives, TEM- derivatives and plasmid mediated cephalosporinase. [3, 5], these informations become an indicator of increasing occurrence of this gene in the meddle east, for this reason this study was preformed to indicate the presence of *bla* $_{VEB-1}$ gene in local isolates of *P. mirabilis.*

ESBLs are a group of plasmid-mediated, diverse, complex and rapidly evolving enzymes which are capable of conferring bacterial resistance by hydrolyzing penicillins, broad-spectrum cephalosporins, aztreonam (but not cephamycins or carbapenems) and monobactams and are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. While ESBLs are generally derived from TEM and SHV-type enzymes, CTX –M type enzyme are isolated from ESBL producing microorganism had been an important subtype leading to multi drug resistance. SHVs are more prevalent in Europe, TEMs are dominantly present in the USA while the CTX-Ms are being increasingly detected worldwide. The plasmids encoding ESBLs also frequently carry genes that encode resistance to other antimicrobial agents, such as amino- glycosides and quinolones. Therefore, the selection of antimicrobial agents against ESBL producing microorganisms in clinical practice is often difficult and complicated [1, 6]. Resistance to extended spectrum ß-lactams antimicrobial agents are important clinical problem. Infections caused by ESBL-producing microorganisms are increased in

epidemic pro- portions and cause a serious threat and a challenge to health care efforts around the World. The most prevalent ESBLs enzymes that responsible of resistant to antimicrobial agents among *Enterobacteriaceae* are TEM-type; SHV, CTX-M and PER-1. In addition to the large TEM, SHV, and CTX families, several minor ESBLs have been identified, recent reports of presence VEB type were documented. VEB-1 has been found in *Enterobacteriaceae* from southeast Asia and France for the first time before the emergence of VEB-1 is reported in Far East which caused nosocomial outbreak in Korea. VEB (Vietnam extended-spectrum beta-lactamase) has 38% homology with PER. The gene encoding VEB-1 was found to be plasmid mediated and such plasmids frequently carry non-beta-lactam resistance determinants. Up to date all VEB enzymes which identified are (VEB 1, 1a, 1b, 2, 3, 4, 5, 6); all these derivatives are minor variants of VEB-1, which confers a high level of resistance to ceftazidime, cefotaxime, and aztreonam while inhibited by clavulanic acid. *bla*$_{\text{VEB}}$ genes have been identified in a different species of *Enterobacteriaceae* from Asia, Europe, the Middle East, Africa, and North and South America on both plasmids and the chromosome [1, 6, 7, 8, 9].

Some ß-lactamases genes may be encoded in gene cassettes that are detached mobile elements comprising with a gene, and a recombination site that is recognized by an integrase which the cassete associated with. Class 1 integron, which are isolated predominantly from antibiotic- resistant clinical isolates, commonly possess two conserved region located on either side of the integrated gene cassette. *bla*$_{\text{VEB-1}}$ was first described in a gene cassette in a class 1 integron, and most other examples of *bla*$_{\text{VEB}}$ genes are also found in cassette arrays in class 1 integrons that are mostly related, containing different combinations from a limited set of cassettes in different configurations, suggesting rearrangements mediated by both homologous and IntI-catalyzed recombination [9, 5].

II. Material and methods

a. Isolation and identification of bacterial isolates:

Different clinical samples (urine, wounds and burns) were collected from patients attending to different hospitals in Baghdad city, Iraq through the period from October 2013 to march 2014.

All samples were cultured on some bacteriological media, such as: Blood agar, MacConkey agar, Xylose lysine deoxychochlate agar (XLD) for primary identification of microorganisms.

After aerobically incubation at 37C° of cultured plates for 24 hrs.; the yield isolates were identified by bacteriological and biochemical assay [10,11].

By morphological features (such as: swarming on blood agar, non-lactose fermenter isolates on MacConkey agar and colored colonies on XLD agar) and morphological features of bacteria by using Gram stain. Vietk 2 identification system (Biomerieux- France) was used to confirm identification of bacterial isolates. According to all above identification assays that used, 25 isolates of *Proteus mirabilis* were identified.

b. Antibiotic susceptibility test:

All identified *P. mirabilis* isolates were tested for antibiotic susceptibility towards cefotaxime and cftazidime antimicrobial agents by using Kiraby-Bauer method [10,11]. According to the clinical laboratories standard institute (CLSI, 2011), the susceptibility of tested isolates was detected depending on the size of inhibition zone formed by bacterial isolates.

c. Detecting of Extended Spectrum Beta lactamases:

The ability of *Proteus* isolates to produce Extended Spectrum Beta lacatamases enzymes (ESBLs) were tested by using double disk – synergy method [3, 5, 12].

Bacterial suspension standardized to $1.5x10^8$ CFU/ml by matching the suspension to the turbidity of the 0.5 MacFarland turbidity standard, then spread it on the surface on Muller Hinton agar plates by sterile cotton swab; all plates were lifted to dry for about 10 min.

Antibiotic disk of cefotaxime, ceftazidime and aztronam were placed on Muller Hinton agar plates around amoxicillin/ clavulanic acid (30 µg) antibiotic disk within 2-3 cm distances. The plates are inverted and incubated for 18-24 hrs. at 37C°.

Synergism activity were detected after incubation period between amoxicillin/ clavulanic acid (30 µg) disk and anyone of surrounding antimicrobial agent's disks.

d. Extraction of DNA:

Commentarial purification kit (Presto mini Genomic DNA kit, Geneaid, Thialand) was used for extraction of genomic DNA form 5 isolates of *P. mirabilis* isolates by using Gram negative extraction protocol according to manufacturing instructions.

Nano-drop system was used to estimate the concentration and purity of extracted genomic DNA. Confirmation of extracted DNA was performed by using gel electrophoresis technique in 0.8% agarose gel concentration, then

it is illuminated by UV transilluniator documentation system after staining the gel with ethidium bromide and the results were documented and photographed by digital camera [13].

e.Amplification of *bla$_{VEB-1}$* by monoplex Polymerase Reaction Chain (PCR):
In order to detect the *bla$_{VEB-1}$* gene from 47- 946 bp of whole 1081 bp gene in 5 isolates of *P. mirabilis* by using specific primers (table1) according to [8] which experimented by [14]; monplex PCR was carried out.

Table (1): specific primers of *bla$_{VEB-1}$* gene according to (Kim *et. al.*, 2004) experimented by (Al-Hamando *et. al.*, 2017)

gene	sequences	Product /pb
bla$_{VEB-1}$	F- 5` CGACTTCCATTTCCCGATGC- 3` R - 5` GGACTCTGCAACAAATACGC- 3`	636

PCR reactants & amplification program were prepared in final concentration 50 µl , template genomic DNA < 250 ng (5 µl), forward and reverse primer 10 pmol/ µl (5 µl), Go Taq green Master mix 2X (25 µl), de-ionized distilled water (10 µl), while the PCR reaction was performed; Initial denaturation at 94 °C for 10 min., denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, extension at 72 °C for 3 min. and final extension for 72 °C for 7 min. repeated for 35 cycles [15].
The product of this process was visualized by using gel electrophoresis technique in 1.5% agarose gel after staining the gel with ethidium bromide and UV illuminator documentation system equipped with digital camera.

f. Sequencing of monoplex PCR product:
The product of monoplex PCR amplification of *bla$_{VEB-1}$* gene in 5 isolates of *P.mirabilis* was confirmed by gel electrophoresis was purified by gel/ PCR DNA fragment extraction kit (Geneaid- Thialand). The sequencing of nucleotides was preformed according to Macrogen Company, Korea by an automatic sequencer, DNA sequences were analyzed and similarity searches were carried out with the Basic Local Alignment Search tool (BLAST) in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

g. Detection of bacterial plasmid profile:
Bacterial plasmid DNA curing was preformed to examine if the *bla$_{VEB-1}$* gene is harbor on chromosomal or plasmid DNA.
Commercial kit (pureyeild™ plasmid miniprep system; Promega, USA) was used to extract and purify plasmid DNA, by follow the manufacturing instructions, for 3 isolates of *P. mirabilis* which confirmed by gel electrophoresis in 0.8% agarose gel to visualize the patterns of plasmid in the tested isolates.
Curing was carried out by exposing the extracted plasmid DNA of tested isolated to Ultra violet with wave length of (230 A°) for 20 min.; the results recorded after performing gel electrophoresis in 0.8% agarose gel to investigate the ability of UV to cure plasmid DNA of *P. mirabilis* isolates [13].

III. Results and Discussion:

Identification of 25 isolates was performed by microscopic examination of Gram stain and morphological feature that developed on blood agar medium, MacConkey agar medium and XLD agar medium [10, 11]; to confirm the identification of tested isolates, Vitek 2 identification system (Biomerieux, France) was carried out. The results of identification show that 15 isolates (60%) were *P. mirabilis* and 10 isolates (40%) were *P. vulgaris.*
Disk diffusion method was used for testing the susceptibility of *P. mirabilis* isolates towards cefotaxime and ceftazidime [10, 11] which are the drugs of choice for treatment the infections that caused by *Proteus*, that the recent researches show the risk of increasing resistant of *Proteus* towards these antimicrobial agents [6].
The results show that 10 isolates (66.6%) out of 15 isolates of *P. mirabilis* showed resistant to cefotaxime while 3 isolates (20%) were resistant to ceftazidime, [14], Figure (1).

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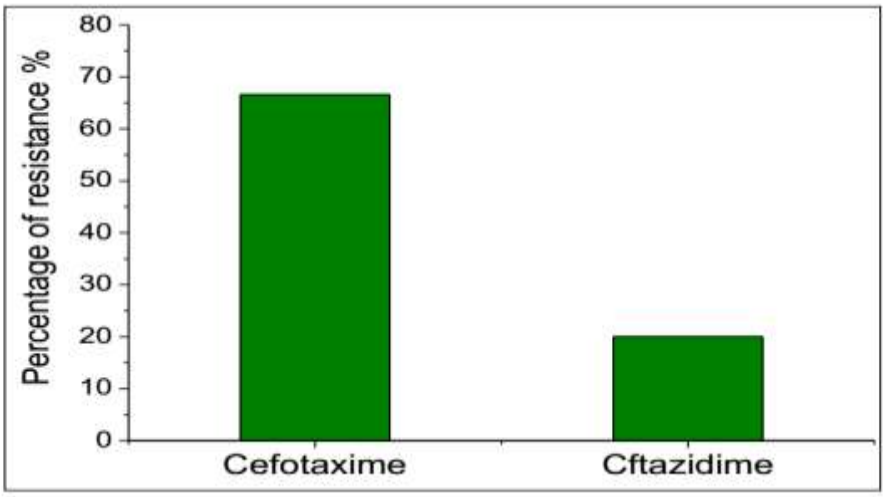

Fig. (1): susceptibility of *P. mirabilis* isolates towards cefotaxime and ceftazidime antimicrobial agents (Al-Hamando *et. al.*, 2017).

To examine the ability of these bacterial isolates to produce ESBLs enzymes, double disk synergy method was performed [3, 5, 12].
Out of 15 isolates of *P. mirabilis*, 6 isolates (40%) were able to produce ESBLs enzymes, Figure (2). The results were detected by increasing of inhibition zones against several antimicrobial agents, such as: cefotaxime, ceftazidime, aztronam and amoxicillin/ clavulanic acid in comparison with the inhibition zones formed by the same antimicrobial agents lacking clavulanic acid.

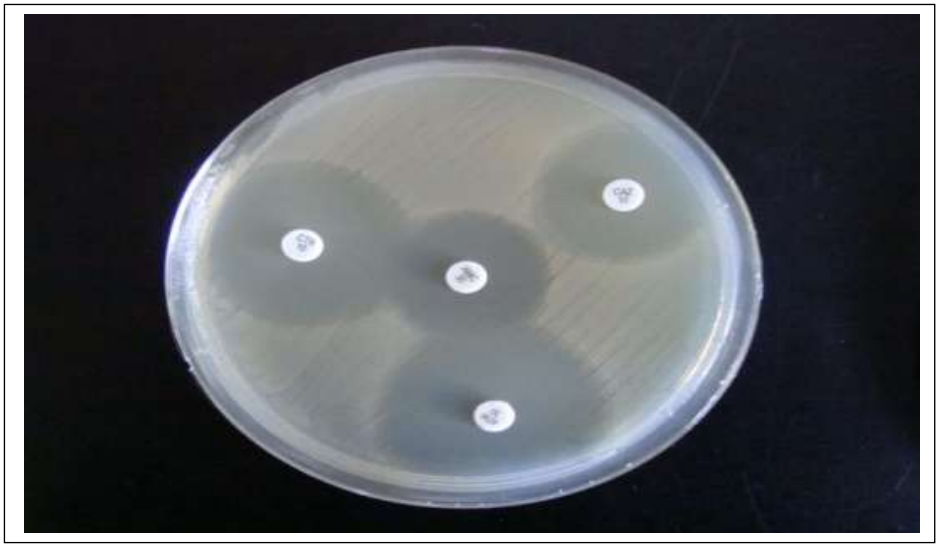

Fig. (2): *Production of ESBLs by* P. mirabilis *isolate using double disk synergy method (Cefotaxime, Ceftazidime, Aztronam, Amoxicillin/ Clavulanic acid)* (Al-Hamando *et. al.*, 2017).

P. mirabilis producing enzymes, such as: ESBLs is considered as an urgent medical concern that reported worldwide because of the ability of *P. mirabilis* to resist antimicrobial agents, such as: cefotaxime and ceftazidime by producing this kind of enzyme with a relatively high prevalence of 40% in some areas which is a matter of concern as resistance to third generation of cephalosporins that represented the drug of choice for *Proteus* infections treatment [7].
Increasing the percentage of *Proteus* producing ESBLs recently is due to the random usage of antimicrobial agents, ability of these bacteria of transferring gene harbor these enzymes in addition of bad health and nutrition conditions of some populations, especially the populations of third world countries.
Because of increasing resistant of *P. mirabilis* towards cefotaxime by producing ESBLs and the concern of *bla$_{VEB-1}$* gene as a one of the genes that harbor ESBLs enzymes was develop; detection of *bla$_{VEB-1}$* gene was

performed as one of genes that harbor ESBLs, due to its importance as a new factor effecting the susceptibility to cefotaxime by using Monoplex PCR technique that used for *bla$_{VEB-1}$* gene detection in 5 isolates of *P. mirabilis* which it was found in all tested isolates with 636 bp fragment size of gene with molecular weight of *bla$_{VEB-1}$* according to (Kim *et. al.,* 2004) that experimented by (Al-Hamndo *et. al.,* 2017) by using 100 bp DNA as ladder (figure 3), since it was found for the first time in Far East in Korea according to [8] and following researches that showed its increasing spread in Asia especially in middle and far east, while it was bounded in the beginning to Europe, Africa and America [8].

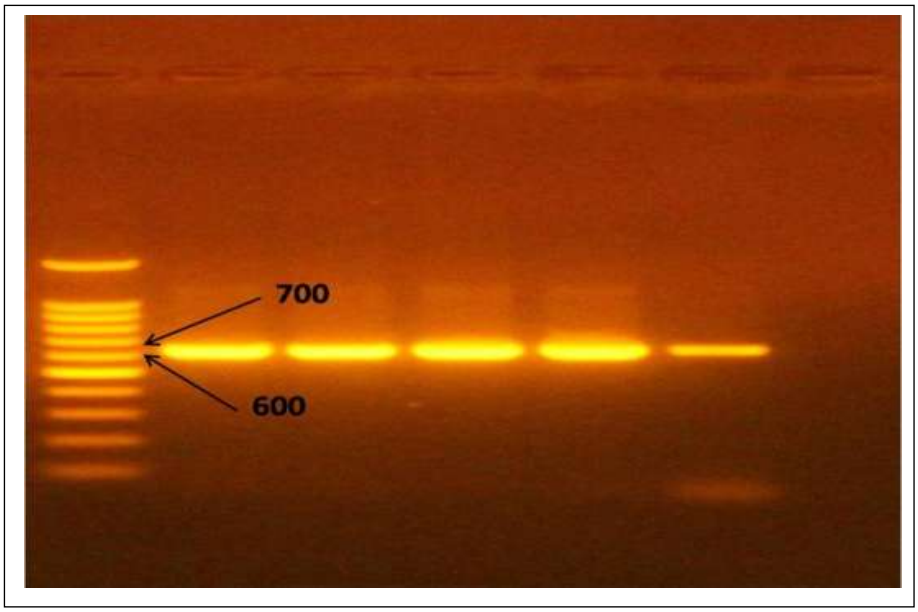

Fig. (3): ***PCR reaction with monoplex set of primers for* P. mirabilis *isolates that contain* bla$_{VEB-1}$ *gene with DNA marker 1000 bp ladder (M), in 1.5 % agarose gel at 5 volt/cm for 1-2 h, visualized by photograph gel documentation equipped by UV light source* (Al-Hamando *et.al.*,2017).**

According to Macron company/ Korea; sequencing of *bla$_{VEB-1}$* gene in 5 isolates of *P. mirabilis* was performed by automated sequencer. The results showed that out of 5 isolates of *P. mirabilis*, 3 isolates harbor *bla$_{VEB-1}$* gene while one isolate have *bla$_{VEB-3}$* gene [14]. These finding were referred to the presence of *bla$_{VEB-1}$* genes in new geographic area which give evidences for increasing *P. mirabilis* resistance towards antimicrobial agents and mark as a risk factor that faced the possibility of treating infections caused by these bacteria, and the ability of spreading these genes that harbor enzymes encoding antimicrobial agents resistant by transmission of plasmids or transposons or other genetic material may be the major cause of increasing antimicrobial agents resistant.
In order to examine the locus of gene of concern (*bla$_{VEB-1}$*), if it is could be found on either plasmid or chromosomal DNA; plasmid curing experiment was carried out by extracting plasmid from 3 isolates of *P. mirabilis* containing *bla$_{VEB-1}$* by using DNA-spin ^{TM} plasmid DNA purification kit (Intron – Korea) according to the manufacturing instructions followed by confirming the bands of plasmid DNA by gel electrophoresis technique and exposure the extracted plasmid from bacterial isolates to UV rays (230Aº) for 20 min., then the experimented plasmid electrophorizied in gel agarose. The results showed that the tested isolates of *P. mirabilis* have mega plasmid band that cured by espousing to the UV rays and disappeared when gel electrophoresis was done to clarify the results (Figure 4).

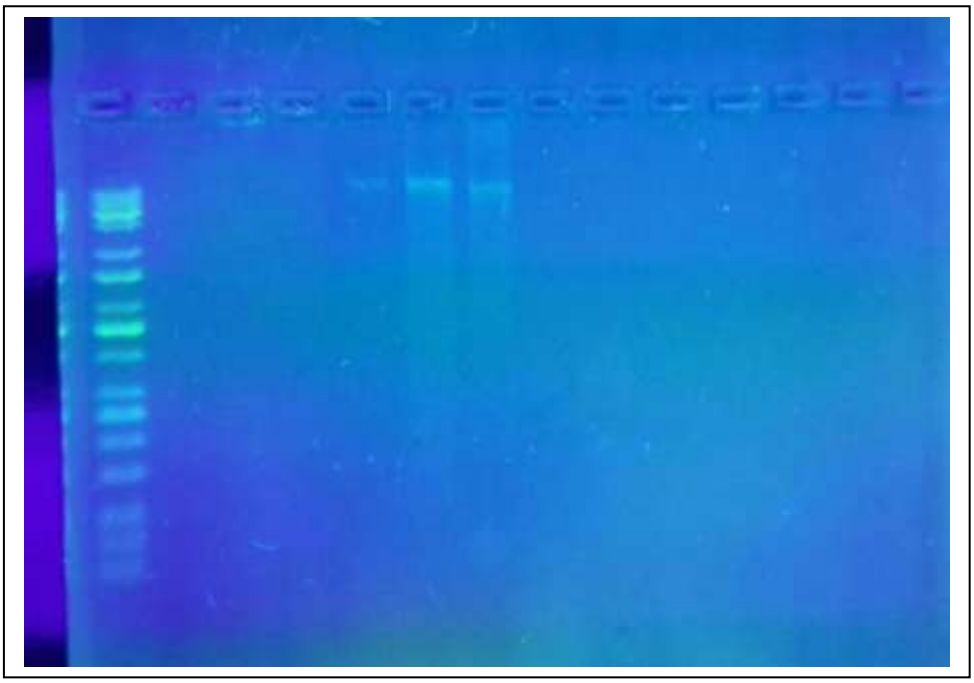

Fig. (4): plasmid DNA curing experiment by exposing the *P. mirabilis* isolates to UV rays (230A°)

IV. CONCLUSION

This study showed the presence of *bla$_{VEB-1}$* gene in local isolates of certain species of bacteria in a country in the middle east which it gives an indicator of spread this gene from certain geographic area to another by its ability to be transferred by genetic elements leading to increase the resistant of bacteria towards antimicrobial agents that constitute a carious medical concern in these areas.

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