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EFFECTS OF BACTEROCIN FROM MRSA AND *PSEUDOMONAS AERUGINOSA* AGAINST BIOFILM OF FOOD BORN PATHOGEN

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Abstract

A major challenge in settings hospital are multi- antibiotic-resistant bacteria. When ability bacteria strain establish surface-attached bio-film census. Many recent studies have puriûed and identiûed there is a need for create new antimicrobials that are operant contra multi-drug-resistant and surface-close fitting bacteria bacteriocins for application in food technology, both crude bacterocin are antibacterial proteins produced by MRSA and *P. seudomonas aeruginosa*, one hundred clinical samples (urin, ears and nasals) swaps were collected from patients of Al-Yarmouk Hospital and Teaching Baghdad Hospital during the period from November / 2015 to January / 2016. Cultural and morphological characteristice examination, biochemical tests were conducted and confirmed the diagnosis by antibiotics sensitivity test and Vitek-2 system. *In vitro* experiment was carried out on the antibacterial activity of bacterocin crude extract against different isolates cusing food born included (*Listeria monocytogenes*, *Salmonella typhimurium, E. coli* O157:H7 and *Staphylococcus aureus*) by using well assay method for planktonoc cells and microtiter plate was also used against biofilm embedded cells. The results showed that first bacterocn more actine then second extract was active against these isolates when found as planktonic and biofilm state. The higher effect of crude extract from MRSA at concentration (32.5 µg/ml) was observed against *Staphylococcus aureus* biofilm while other bacterocin from *Pseudomonas aeruginosa* at concentration (30.5 µg/ml) less effect. Significant effect of both bacterocin extract killing food-borne pathogens cells in a planktonic and biofilm state at concentration 32.5 µg/ml. This study proposse that bacteriocins can be an potently way to adjudge surface-attached pathogenic bacteria.

Key words : Biofilm, *Pseudomonas aeruginosa*, MRSA, *K. pneumoniae.*

Introduction

Biofilms-adherent communally of bacteria bounding by a matrix of (extracellular polymeric substance) (EPS)-are the dominion microbial life pattern in the environment (Batoni *et al*., 2011). The biofilm is turn the attach to surfaces abiotic, the epithelia of polycellular organisms and facades such as that between air and water. Surface adhesion of bacteria is an step essentialy and is required for the bacteria to arrangement themselves positively in their environment (Regev-Shoshani *et al*., 2010). The biofilm now is a major target for the develop of drugs pharmacological, a biofilm ministering to bolstering bacteria persistence by resisting antibiotic treatment and immune replies in host (Carneiro, 2011). The use of medicinal plants, which forms the backbone of blandness medicine, has grown with an appraises 80% of the populations, especial in developing countries, relying on traditional medicines for their primary health care (Dashiff *et al*., 2011). Anti biofilm effect of biocompatible polymer encapsulated aerial fragments extracts of anti- clinical isolate of *E.coli* biofilm was convassed in last years there is an excess demand of minimal processed foods with fresh like specific; moreover, modernized allot. In Gram-negative bacteria, an substitute strategy for antibiotic detecting is to exploit the lower -spectrum antibiotics against bacteria for intra species compete. The high-molecular-weight protein bacteriocins antibiotics as known pyocin S2 (*in vitro*) that displays effective against bacterial growing the biofilm state of *P. aeruginosa*. This pyocin S2 is greatly active in an invertebrate model of *P. aeruginosa* infection (Smith *et al*., 2011). *Listeria monocytogenes* is the causality organism of the serious

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food-borne illness listeriosis and food-processing equipments may grow as biofilms on food and that conservation it against environmental stress. *Salmonella typhimurium* as a typical food-borne pathogen, which can form biofilms on food-processing expedient surfaces According to the scholarly report of European Food Safety Authority (EFSA) (Liu *et al*., 2015). Several kind of packaging food useful in preservation food techniques in order to extending the influence of the (food preservation chain). One of the key needed during storage the food from spoilage microbial and contamination of pathogenic micro-organisms, preservation Food methods such as addendum of preservatives (organic compounds, antibiotics such as, benzoate, propionate acetate and lactate) decrease of pH and activity water (dehydration, acidification) and treatment (pasteurization, heating, sterilization) used to prevent food poisoning.

Although, these methods have been inhibits to be successful, however, have been consumers abidingly concerned the possible about health effects due to the presence of chemical in curing foods (Chopra *et al*., 2015). Grows *P. aeruginosa* predominantly as a bioûlm infected in lung, and this state is associated with high levels of resistance to small-molecule antibiotics to determine if display pyocins potent activity against *P.aeruginosa* growing in the state bioûlm. The pyocin S2 complex was isolated by nickel afûnity chromatography by virtue, the purified pyocin S2-ImS2 complex was highly active anti *P. aeruginosa* growing on LB (Smith *et al*., 2016).

Materials and Methods

Specimen's collections of *P. aeruginosa* and MRSA, thirty three specimens included: (Ear and Nasal swab) were collected from Al-Yarmouk Hospital. All Swab on MacConkey agar plates and on Mannitol Salt Agar plates, the specimens were transferred at present to the microbiology laboratory isolation of bacterial pathogens were incubated at 37^{0}C for 24 hrs later incubation the isolated colonies were identified on the basis of morphological, cultural and biochemical characteristics (McCaughey *et al*., 2016).

Antibiotic susceptibility test

Antimicrobial susceptibility test (AST) was performed according to the guidelines of Clinical and Laboratory Standards Institute (Wright, 2014). The *S. aureus* strain was prepared to control the quality of the antibiotic susceptibility test. Different disks were used in AST, including Oxacillin (1 mg), Erythromycin (15 mg), Clindamycin (2 mg), Vancomycin (2 mg) and Tetracycline (30 mg) (all purchased from MAST, UK) and four antibiotics for *P. aeruginosa* Gentamycine CN(01 mg), Amoxiline AX(52mg), Erythromycine E(15 mg) and Tetracycline TE (30).

Detection of bacterocin isolates by Cup assay method

Cup assay method reported by Al-Qassab and AlKhafaji (1992). Method was followed for screening both bacteocin extraction. From loopful an overnight (LB) broth culture. Suspension of indicator isolate were spread on the surface of Muller hinton agar then left to dry at 37^{0}C for 10 min and for each isolate plates were used (Diplicate). Removed discs from medium agar were gently sticked on the surface of the medium spread by the sensitive isolate then incubated at 37^{0}C for overnight. Sensitivity was detected by measuring the zone of inhibition. The indicator strain (*Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7 and *Staphylococcus aureus*) isolation and identification was obtain from Laboratory of Microbiolog in College of Science for Women, University of Baghdad.

Detection of MRSAcin and Pyocin activity by Well Diffusion assay

The quantitative determination of Both in bacterial culture was performed by using the Wells Assay method that described by Vimalin and Sudha (2011), two fold dilution of crude extract was prepared. The indicator strain was cultured on Muller Hinton gar after comparsion with Macferland tube (0.5×10^8), wells were made in the plate by cork borer, dilution from the crude both bacterocin was added in these wells.

In vitro inhibitory activity of Bacterocin extract on planktonic

Two fold dilutions for crude extract of bacterocin was used. Were cultured on (Brain heart infusion agar) in order to confirm the absence of MRSA and *Pseudomonas aeruginosa* cells. They were stored at 4°C until the assay. Well diffusion method described by Pozzi *et al*. (2012) was followed to detect both bacterocin extract inhibitory effect.

Biofilm assay

Method described by Atshan *et al*. (2012) was followed to achieve biofilm formation:

To study the ability of adherence all four indicator strain were growing in (trypticase soy broth) includes (1% glucose) in 96-well polystyrene tissue culture plates at 37^{0}C incubated for 24 hrs. The planktonic cells after incubation were washed many times with deionized water and the adhering bacterial cells in every well were attested with 200 µl of absolute methanol for 20 mints. The adhering cells were stained (200 µl of 0.1% crystal

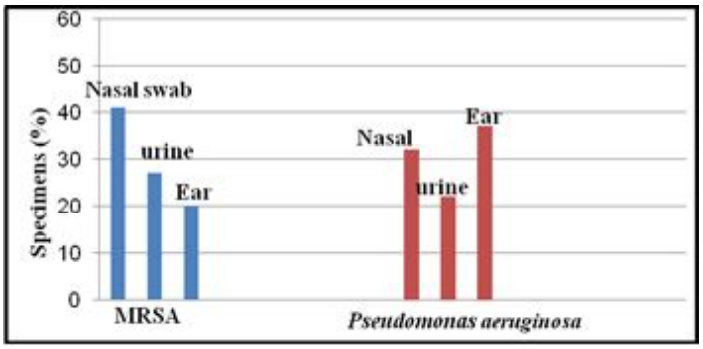

Fig. 1 : Different sampling sites for collection of the isolates.

Table 1 : Classification of bacterial adherence by tissue culture plate method (Atshan *et al*., 2012).

OD values	Adherence	Biofilm formation
< OD c	Non	Non
OD < OD ≤ 2*ODc	Weakly	Weakly
2*ODc < ODt ≤ 2*ODc	Moderately	High
4ODc<OD t	Strong	High

***** *Listeria monocytogenes*, *Salmonella typhimurium, E .coli* O157:H7 and *Staphylococcus aureus*.

violet) for 15min and excessive stain was beveled. The plates with distilled water were douched and overnight airdried. The crystal violate dye fixed to the adherent cells was deiced with 1ml of 95% ethanol per well, and the plates were read at 490nm using a spectrophotometer. The assay was performed in triplicates and the absorbance of wells includes sterile TSB was wield as the negative control the outcome calculate as in table (1).

Inhibitory effect of crude extract of bacterocin on biofilm as treatment

To detect the inhibitory effect of crude Bacterocin on biofilm, method described by Nathan and Pei (2012) was followed, the isolates of *Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7 and *Staphylococcus aureus* were selected to be assayed according to inhibition activity of both using bacterocin against planktonic cells of it on plate agar. Followed to produce a biofilm same protocol described earlier. Then, before the staining step the previously prepared crude containing media were added to the biofilm containing wells Subsequently the tray was incubated for another 24 hours at 37ºC after incubation period all wells were washed and stained as the same procedure described above.

Estimation of protein by Lowry's method (Lowry *et al*., 1957)

The Lowry's method were analyzed for protein using the samples.

Biofilm formation on a polystyrene plate

The study of strain to form biofilms following treatment with (Crude Bacterocin from *P.aeruginosa* and MRSA) according to the methodology proposed by Lindsay and von Holy (2006). Briefly, MRSA and *P. aeruginosa* was cultured with 1% Glc in 5 mL TSB for 18 h at 37°C and treated with Bacterocin and control medium. The cultures were first diluted (1:100) in the same medium, 200 µL was inoculated in a 96-well plate and plates were incubated at (37°C for 24 h). The plates were washed twice with (phosphate-buffered saline), dried for 1 h at 65°C, 1% crystal violet was added and the plates were incubated for a further (30 min at 25°C). Each well was washed twice with PBS and 200 µL PBS obtained using following formula :

Biofilm degree = Mean OD 630 of tested bacteria – Mean OD 630 of control.

Results and Discussion

Antibiogram profile results for MRSA isolated from clinical samples showed that 86 isolates from 100 samples of *S. aureus* were diagnosed as Methicillin resistance *S. aureus* (MRSA). The results of the present study showed that highest percentage isolation (42%) were isolation MRSA and *P.aeruginosa* from nasal swap and less from ear (20%) and urin (22%), respectively. The results of the present study showed that (60%) were wound infaction (15%) from urine (25%) frome ear and eye infaction (fig. 1). The result not agree with Ali *et al*. (2015). Clinical isolates of *Pseudomonas aeruginosa* were collected highest percentage from urine.

Vitek 2 system and antibiotic susceptibility test

Gave confirmation of positive results for MRSA and *Pseudomonas aeruginosa* as a selected organism with a probability 98-99%. MRSA isolates were resistant to all tested antibiotic especially Methicillin, Oxacillin, Clindamycin, Penicillin G, Tetracycline and while it is sensitive to vancomycin (fig. 2). In addition, MRSA was resistant to most tested antibiotic except vancomycin and

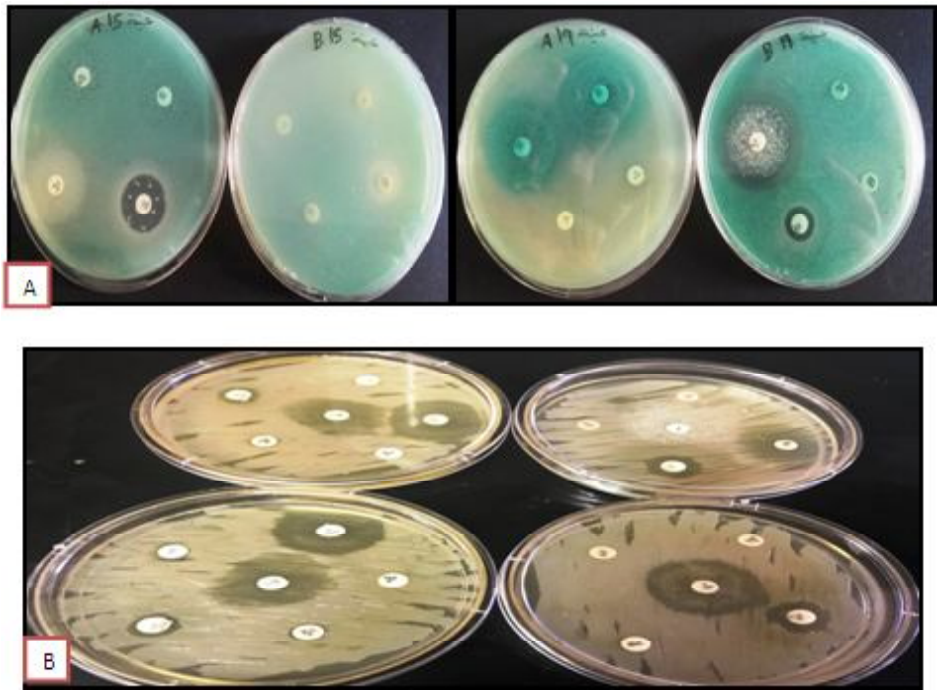

Fig. 2 : (A)Antibiotic susceptibility test of *P. aeruginosa* isolates (B) Antibiotic susceptibility test of MRSA isolates.

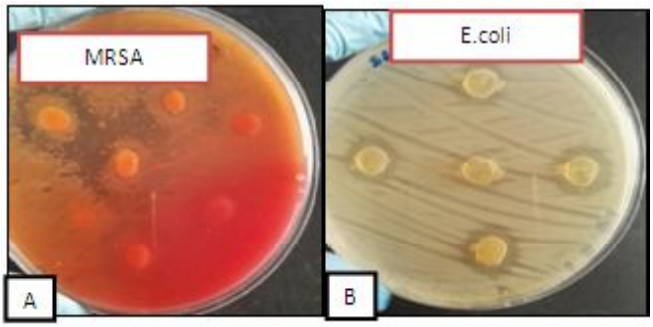

Fig. 3 : Cup assay method for detection crude *bacteriocins* from A) MRSA B) *Pseudomonas aeruginosa* at 37°C for 24 hr on Muller-Hinton agar and Manitol salt agar.

Table 2 : Types of Isolates that used for Biofilm Formation.

Biofilm formation	***Staphylococcus aureus***	***E.coli***	***L. monocytogenes***	***S.typhimurium***
O.D	0.4	0.3	0.226	0.2
Classification	**Strong**	**Strong**	**Moderate**	**Moderate**

this contraindicated the results of Alwan *et al*. (2011), whom mention *S. aureus* was less resistance to chloramphicol, while *P. aeruginosa* there resistant to most tested antibiotic except levofloxacin agree result with Sous and Pereira (2014) the spective potency of either Ciprofloxacin, Levofloxacin and Ormoxifloxacin against *P. aeruginosa*.

Comparison between antimicrobial activity for crude bacteriocin using agar methods

Locally isolates MRSA and *Pseudomonas aeruginosa* were screened in system to select the aptitude isolates in both production. The capability of these isolates in two bacterocin production was probation after culturing at 37^{0}C in Brain heart infusion agar, then wells were made on this agar and put on Muller hinton agar that contained the sensitive strain. So the antagonistic effect against the sensitive strain was detected by measuring inhibition zone according to cup assay and well diffusion assay method. Diameters of inhibition zone ranged in well diffusion rang (10 to 15) mm bast then cup assay from (5-7 mm) (figs 3 and 4).

In vitro inhibitory activity of Bacterocin extract on planktonic cells

To confirm the antibacterial activity of extracted from both bacteriocin isolates contain, their MIC were determined against 4 species of Enterobacteriaceae

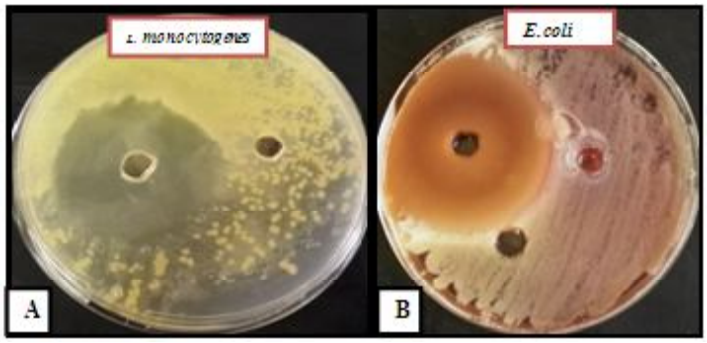

Fig. 4 : Well diffusion assay method for detection crude *bacteriocins* from A) MRSA B) *Pseudomonas aeruginosa* at 37°C for 24 hrs.

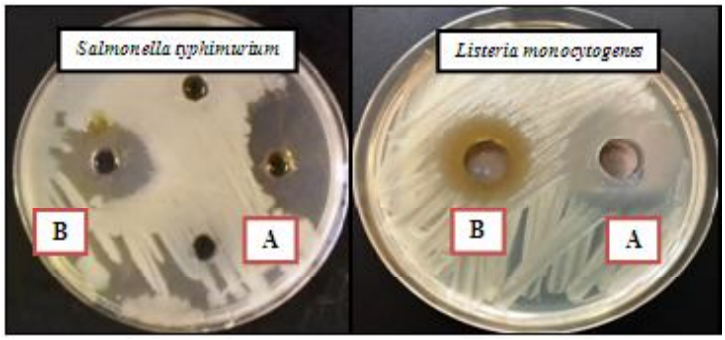

Fig. 5 : Well diffusion assay method for detection crude *bacteriocins* from 1) MRSA 2) *Pseudomonas aeruginosa* on concentration A: 30 µg/ml, B: 15 µg/ml at 37°C for 24 hr on Muller-Hinton agar.

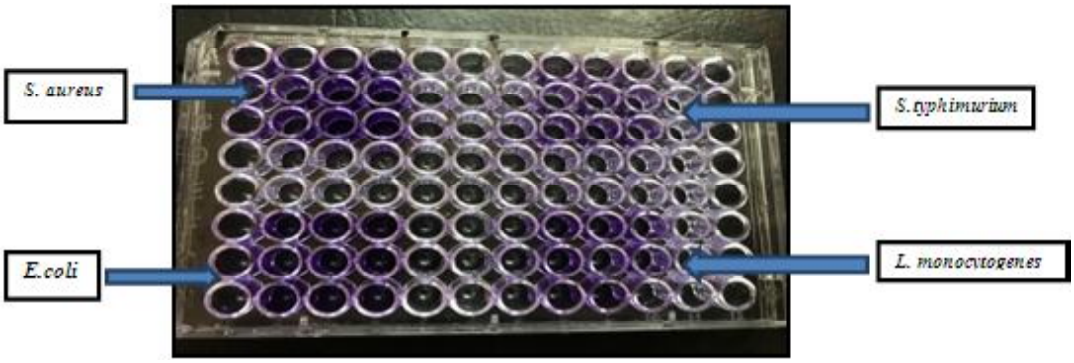

Fig. 6 : Screening of Biofilm producer by TCP method :high and moderate Slime producer differentiation with crystal violet staining in 96 well tissue culture plate.

(*Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* O157:H7 and *Staphylococcus aureus*) was detected MIC by two fold dilution preparation. Results were showed that concentration (30 µg/ml) more effected than 15 µg/ml on planktonic cell in Petri dish by well assay (fig. 5).

According to these results, the MIC value against these isolates was selected to study the effect of crude *bacteriocins* from MRSA more then another extract on biofilm formed by different isolates of enterobacteriaceae. The MIC on plate agar was determined and measured depending on the activities of the antimicrobial proteins against planktonic bacteria (Penfold, 2000). *Staphylococcus* species specially *Staphylococcus aureus* reason mastitis, wound and Bourn contagion in livestock and food poisoning in humans through ingestion of contaminated foods connotation meat and dairy, meet products (Hennekinne *et al*., 2012). Salmonella is a major cause of food poisoning outbreaks especially in the third world countries against *Salmonella enterica* biofilm was examined connotation foodborne pathogens grow predominantly as biofilms in most of their inbred habitats, rather than in planktonic mode, adhere to a surface and generative extracellular polymers that facilitate the adhesion and evidenced a structural matrix which resided the biofilm (Giaouris *et al*., 2012).

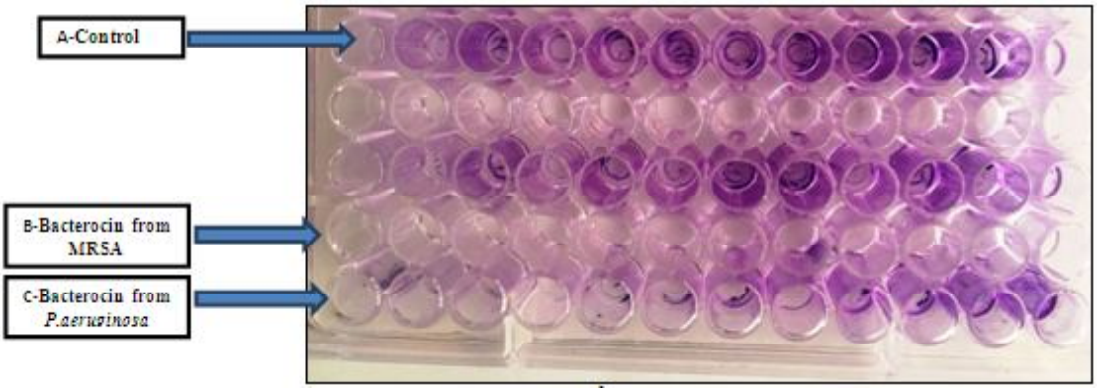

Fig. 7 : Culture wells were inoculated with 10^8 CFU of disrupted biofilms of (*Staphylococcus aureus*) A- Control, B-Bacterocin from MRSA, C-Bacterocin from *P. aeruginosa*.

Biofilm formation

The result of the study showed *Staphylococcus aureus* was high ability to biofilm formation then followed by *E.coli*, *L. monocytogenes* and *S. typhimurium*. According to Lewis (2010) calculation of the mechanisms by which bacteria in biofilms with stands killing by antibiotics are not completely articulate. Suggested biofilm antibiotic resistance mechanisms consist the attend of persister cells, micro environments within the biofilm that debars the antibiotic adequacy, and reduced antibiotic entrance to bacteria within a biofilm (Lewis, 2010) (fig. 6 and table 2).

In inhred environments, bacteria obtain form biofilms, microbial communally in which adhere bacteria to an (abiotic or biotic) roof via surface charges as well as produce of (fimbriae, pili and exopolysaccharides). Cells of Microbial in biofilms show discriminate properties, resiste to antibiotics particularly, antiseptics (Harvey *et al*., 2006).

By the micro-liquid dilution method at first, the MICs of Bacterocin against *Staphylococcus aureus* were determined. Incubation times for complete bactericidal action for Bacterocin from MRSA more active then another . The ability of Bacteriocin to depend the bioûlms of the *S. aureus* multiplicate strains was tailored by ocularly comparing the variation of safranin stain or Gram stain density between Bacterocin from MRSA more active then Bacterocin from *P. aeruginosa* treated bioûlms (fig. 7).

Conclusion

It can be concluded from the results of this study that the changes induced in the surface of materials influence bacterial adhesion, so the modification of food contact materials, including their treatment with both bacterocin is a promising method for the reduction of bacterial attachment and biofilm formation.

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