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Efficacy of Combinations of Piperacilline/Tazobactam, Ceftazidime, Amikacin and Bacteriophage against *Enterobacteriaceae* Sepsis in Neonates: I*n Vitro Study*

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ABSTRACT

Background neonatal sepsis is considered as one of the main causes of morbidity and mortality in preterm and full-term neonates. It is a clinical disorder caused by bacterial infections of blood stream. *Enterobacteriaceae* group (including *Klebsiella Pneumonia* and *Escherichia coli*) is a gram-negative (G-ve) bacteria which is one of the most serious causative organisms of neonatal sepsis. *Klebsiella Pneumonia* and *Escherichia coli* phages are bacterial viruses that infect bacteria and pursue in two possible ways: lytic and lysogenic life cycle. The risk of neonatal sepsis increase with multi drug resistant organisms which make the treatment of their infections difficult. Therefore, antibiotics and phage combinations are possible choices to solve this problem.

Aims of the study: this study aims to evaluate the antibacterial activity of bacteriophages alone and in combination with certain antibiotics against most common *Enterobacteriaceae* that cause neonatal sepsis and to obtain desired antibacterial combinations for the treatment of most common *Enterobacteriaceae* in neonatal sepsis with synergistic effects.

Materials and methods: thirty isolated bacterial cultures from blood samples of neonates with sepsis were collected from the laboratories of Al-Kadimia pediatric hospital and Central teaching hospital of pediatric from December 2019 to March 2020. They were identified using morphological characteristics, Api20E test and by Vitek2 compact system. Fourteen isolates among them (46.67%) were *Klebsiella Pneumonia* and eleven isolates (36.67%) were *Esherichia Coli.* **Results:** The bacterial profile of the collected isolates was found as follows: *Klebsiella Pneumonia* (46.67%), *E.Coli*(36.67%), *Enterobacter*(13.33%) and *Serratia* (3.33%). The percentages of resistance to the tested antibiotics were as follows: of *Klebsiella Pneumonia* isolates amikacin14.28%, aztreonam ,cefepime and ceftazidime 71.43% for each, ciprofloxacin 7.14%, piperacillin/tazobactam, gentamicin, tobramycin, imipenem and meropenem 21.43% for each, minocycline 0%, piperacillin 78.57%, ticarcillin 100%, ticarcillin/clavulanic acid 50%, and trimethoprim-sulfamethoxazole 28.57%. For *Escherichia Coli* isolates amikacin 0%, aztreonam , ticarcillin, piperacillin, cefepime and ceftazidime 72.73% for each, piperacillin/tazobactam, ticarcillin/clavulanic acid, ciprofloxacin and trimethoprim-sulfamethoxazole 27.27% for each, gentamicin, tobramycin and minocycline 9.09% for each, imipenem and meropenem 18.18%. (64.29%) of *Klebsiella Pneumonia* isolates and (45.45%) of *E.Coli* were MDR. Antibiotic-antibiotic combinations of piperacillin/tazobactam or ceftazidime with amikacin for isolate 11, piperacillin/tazobactam or amikacin with ceftazidime for isolate 12 were resulted in synergistic effects. Also, antibiotic-antibiotic combinations of piperacillin/tazobactam or ceftazidime with amikacin for isolates 4 and 6 resulted in synergistic effects. Combinations of phage with each of 1/4and 1/2 MIC piperacillin/tazobactam or ceftazidime for tested isolates (4and6 which were *E.Coli* and *Klebsiella Pneumoia* respectively) and phage with 1/4 and 1/2 MIC amikacin for *E.Coli* isolate 4 showed synergistic effects to the antibiotics.

Conclusions: all combinations of piperacillin/tazobactam, ceftazidime with amikacin and phage- antibiotic combinations with piperacillin/tazobactam, ceftazidime or amikacin showed highly synergistic effects against resistant and MDR *Klebsiella Pneumonia* and *E.Coli* strains that cause neonatal sepsis.

Keywords: Neonatal sepsis, *Klebsiella pneumonia, Escherichia coli,* lytic, lysogenic life cycle, antibiotic resistance

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INTRODUCTION

Sepsis is one of the main causes of morbidity and mortality in preterm newborn and infants. The risk increases with multi drug resistant organisms**.** (Folgori L. & Bielicki J.,2019). The most common cause of EONS is group *B Streptococcus*, while its main cause in developing countries are *Enterobacteriaceae*. *Staphylococcus* species or *Enterobacteriaceae* are the most commonly isolated bacteria in nosocomial LONS. (Lona Reyes JC. *et al.*,2015). The incidence of neonatal sepsis is higher in very –low birth weight infants with increase in mortality rate (Simonsen KA. *et al.*,2014). The successful treatment and improvements in the outcome depend largely on early initiation of appropriate antibiotic therapy; the etiological bacteria and their antibiotic susceptibility which differ from country to country. (Silva NBS. *et al.*,2017). *Enterobacteriaceae* are big and different family of Gram-negative rods, its members are both free-living and present as indigenous flora of humans and animals (Ryan KJ.*et al.*,2014). (*Escherichia Coli, Klebsiella pneumonia*, *Enterobacter cloacae*, *Serratia marscenes* and *Proteus mirabilis*) are *Enterobacteriaceae* species that causing sepsis (Warren L., 2016). Antimicrobial resistant bacteria exert a great problem on healthcare systems, management of the infectious diseases becoming progressively complicated. (Spicer WJ., 2008). The important factor that enhances the spread of antibiotic resistance is excessive use of antibiotic especially without

treatment indication. (Exner M.*et al.*,2017). Bacteriophage can be defined as viruses that have the ability to infect and kill bacteria without making any negative effect on the human or animal cells. (Principi N.*et al.*,2019). Two different cycles that bacteriophages pursue when infect their bacterial hosts: Lytic cycle in which the infecting phage ultimately kills the host cell to produce many of their own progeny (Domingo-calap P. and Delgado-martinez J.,2018) and Lysogenic cycle in which bacterial cells with phage genetic material are created, as temperate phages (Whittbole X.*et al.*,2014). Lytic phages are those phages that are only involved in phage therapy to treat bacterial infection, with special use against antibiotic resistant bacteria. (Sarhan SR.*et al.*,2016). Combination therapy is widely used in patients especially those who are critically ill due to widespread emergence of multi drug resistant organism, and is mostly practiced to Broadening the antibacterial spectrum, used for their synergistic action to improve the efficacy and reduce emergence of resistance (Tyers M. & Wright GD.,2019). Phages combination with antibiotics can be beneficial in terms of minimizing the chances of bacterial resistance to either antibiotic or to therapeutic phages. Moreover, when combining phages with antibiotics, it is possible to use half or quarter of the killing dose of antibiotic, the approach useful to lower side effects and collateral toxicity of antibiotics. (Nilsson AS.,2014). phage- antibiotic therapy less likely to fail because the mode of action of phage is different from that of antibiotic so the bacterial strains that are resistant to one agent are susceptible to another one (Burrowes B.*et al.*,2011).

MATERIALS AND METHOD

Collection of samples

Thirty isolated bacterial cultures were collected from laboratories of al-kadimia pediatric hospital and Central teaching hospital of pediatric and these cultures belong to blood samples of neonate's patients with sepsis infection who attended to the hospitals.

Identification of *Klebsiella Pneumonia* and *Escherichia Coli:*

Microscopic examination and API20 E system used for identification of bacteria, also The BioMérieux VITEK2 compact system was used for identification and antibiotics susceptibility test.

Bacteriophage isolation:

The first enrichment step was carried out by using 20–30 ml of sewage water filled up to 30-40 ml with nutrient broth according to the volume of collected sewage samples, then the target bacterial strains added to enrich phages in the sample. These enrichments were incubated over night at 37 ºC. (Mattila S.*et al.*,2015). The bacteria removed from this enrichment culture by filtration through 0.22 µm syringe filter and about 10 µl of this filtrate was put on agar plate lawn with tested bacteria and incubate the plate over night at 37 ºC. The observed plaque on the bacterial lawn indicates presence of phage. (Weber-DabrowskaB.*et al.*,2016)

Top Layer Plaque assay: Plaque assay is the most common technique that used for determination of the concentration of infectious phage particles, in which dilutions of the phage preparation are mixed with a permissive host bacterium and dispersed evenly onto solid medium. This technique is based on mixing of dilutions of phage suspension with host bacteria in a dilute, molten agar or agarose matrix (the "top agar" or "overlay"), which is distributed evenly to solidify on a standard agar plate (the "bottom agar" or "underlay"). After incubation, usually overnight, plaques are visualized as zones of clearing (or diminished growth) in the bacterial lawn, which grows in the overlay. (Kropinski A.*et al.*,2009). Ten-fold serial dilutions to 10^{-9} of phage filtrate made with the buffer for the phage stock solution by taking 100ul of phage solution into 900ul of buffer. 3 ml of hot, melted top agar in test tubes held in 47°C water bath. To each tube of top agar, 0.1 ml of target bacteria added and 0.1 ml of the phage dilution quickly piped into a tube of top agar containing host bacteria, well but gently mix to avoid bubbles and quickly pour the mixture onto the surface of bottom agar, the plates tilted rapidly and distributed over the surface of the bottom layer (nutrient agar), this procedure was repeated with each phage dilutions. The top agar allows solidifying, all the plates inverted and incubated for 24 hrs at 37°C. The plates that had between 30-300 plaques are counted Then we determined the phage titer by counting the number of plaque forming units (PFU) for each dilution according to the following formula :- (Sarhan SR.*et al.*,2016, Sarhan SR.,2017). Phage titer = Number of plaques per plate×100 × dilution factor. The buffer solution that used in our work is Saline – magnesium plus gelatin (SMG) (Kropinski A.*et al.*,2009).

Determination of Multiplicity of Infection (MOI)

The multiplicity of infection represents the ratio of the numbers of phages to the numbers of bacterial cells. (The MOI is determined by dividing the number of phage added (ml added x PFU/ml) by the number of bacteria added (ml added x cells/ml). The average number of phages per bacterium in the population could be 0.1, 1, 2, 10, etc., depending upon how the experiment did is set up. (Http://www.sci.sdsu.edu/~smaloy).

Determination of Fractional Inhibitory Concentration (FIC) value for antibiotics combinations

The FIC used to determine the effect of antibiotics in combination. FIC used to evaluate the effect between each of piperacillin/tazobactam, ceftazedime and amikacin when two of these drugs used in combination. FIC can be calculated according to the following equation:

$$FIC = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

The combinations effect result depending on FIC was determined as follow: (≤0.5) synergy, (0.5-<1) additive, (1-<4) indifference and (≥4) antagonism. (Killic S.*et al.*,2008). The procedure is done by preparation of 5ml of sterile nutrient broth contains 1/4 and 1/2 MIC of antibiotics for antibiotic- antibiotic combinations, then each tube inoculated with a loop full of previously prepared overnight bacterial culture and incubated at 37°C for 24hr. the results determined according to the turbidity of the tube and for antibiotic- bacteriophage combinations 1/4 and 1/2 MIC of antibiotics inoculated with phages and bacterial volumes that produce MOI equal to 1 for the *klebsiella Pneumonia* and *E.coli* phage. (American society for microbiology.,2002).

RESULTS

The isolates were grown on MacConkey agar media which is selective media for gram negative bacteria. Among the 30 isolates, 14 (46.67%) were *Klebsiella Pneumonia*, 11 (36.67%) were *Escherichia Coli,* 4(13.33%) were

Enterobacter and 1 (3.33%) was *Serratia*. The percentage of resistance of *Klebsiella Pneumonia* isolates to antibiotics included: amikacin14.28%, aztreonam, cefepime and ceftazidime 71.43%, ciprofloxacin 7.14%, piperacillin/tazobactam, gentamicin, tobramycin, imipenem and meropenem 21.43%, minocycline 0%, piperacillin 78.57%, ticarcillin 100%, ticarcillin/clavulanic acid 50%, and trimethoprim-sulfamethoxazole 28.57%. The percentage of resistance of *Escherichia Coli* isolates to antibiotics included: amikacin 0%, aztreonam, cefepime , ceftazidime, piperacillin and ticarcillin 72.73%, piperacillin/tazobactam, ticarcillin/clavulanic acid, ciprofloxacin and trimethoprim-sulfamethoxazole 27.27%, imipenem and meropenem 18.18%, gentamicin, tobramycin and minocycline 9.09%. The percentage of resistance of *Klebsiella Pneumonia* isolates and *Escherichia Coli* isolates to antibiotics are shown in figure (1-1)

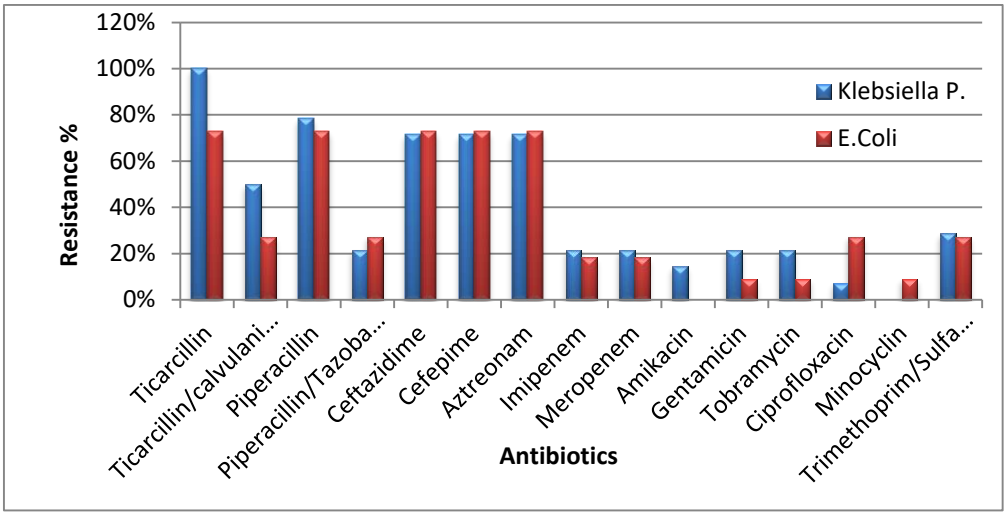

Figure 1-1. percentages of resistance of *Klebsiella Pneumonia* and *Escherichia Coli* isolates to antibiotics.

Among fourteen *Klebsiella Pneumonia* isolates, eleven isolates (78.57%) were obtained from male and three isolates (21.43%) were obtained from female while among eleven *E.Coli* isolates, three isolates (27.27%) were obtained from male and eight isolates (72.73%) were obtained from female. Consideration of *Klebsiella Pneumonia* and *E.Coli* as MDR was according to the criterion of non- susceptibility to at least one agent in three or more antimicrobial categories. (Cilloniz C.*et al.,*2019). Depending on this criterion the percentage of MDR isolates for *Klebsiella Pneumonia* isolates was 64.29% and for *E.Coli* isolates was 45.45%.

Antibiotic- antibiotic combinations:

Combinations of each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Ceftazidime with 1/4 and 1/2 MIC of Amikacin for isolate no. 11(*E.Coli*)and combination of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Amikacin with 1/4 and 1/2 MIC of Ceftazidime for isolate no. 12(*Kleb.P.*).Both tested isolates were sensitive to Amikacin and resistant to Ceftazidime, isolate 11 was resistant to Piperacillin/Tazobactam, while isolate 12 was sensitive to it, the combinations showed synergestic effects in which the fractional inhibitory concentration value was 0.5. Combinations of each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Ceftazidime with 1/4 and 1/2 MIC of Amikacin for isolate no. 4 (*E.Coli*) and combination of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Ceftazidime with 1/4 and 1/2 MIC of Amikacin for isolate no.6 (*Kleb. P.*). Both tested isolates were sensitive to Amikacin and resistant to each of Piperacillin/Tazobactam and Ceftazidime, also these combinations showed synergistic effects with fractional inhibitory concentration value equal to 0.5.

Antibiotic- Phage combinations:

Combinations of 1 MOI of *E.Coli* phages with each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam , Ceftazidime or Amikacin were done for isolate no.4(*E.Coli*) and combinations of 1 MOI of *Klebsiella Pneumonia* phages with each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Ceftazidime were done for isolate no.6 (*Kleb. P.*) As shown in tables (1-1 and 1-2).

Table 1-1. the effects of combinations of 1 MOI of *E.Coli* phages with each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam, Ceftazidime or Amikacin for isolate no.4(*E.Coli*).

		***FIC values	
Antibiotic	**1MOI *Φ**	**FIC**	**Interpretation**
1/4 MIC *TZP	**-ve	**0.5**	**synergism**
1/2 MIC TZP	**-ve**	**0.5**	**synergism**
1/4 MIC *CAZ	**-ve**	**0.5**	**synergism**
1/2 MIC CAZ	**-ve**	**0.5**	**synergism**
1/4 MIC *AN	**-ve**	**0.5**	**synergism**
1/2 MIC AN	**-ve**	**0.5**	**synergism**

*: **Φ**=phage, TZP= Piperacillin/Tazobactam, CAZ=Ceftazidime, AN=Amikacin. **: -ve mean no growth. ***: FIC which determined as follow: (≤0.5) synergism, (0.5–<1) additive, (1-<4) indifference, (≥4) antagonism. (Killic S.*et al.,*2008).

Table 1-2. the effects of combinations of 1 MOI of *Klebsiella Pneumonia* phages with each of 1/4 and 1/2 MIC of Piperacillin/Tazobactam or Ceftazidime for isolate no.6(*Kleb. Pneumonia)*.

Antibiotic	1 MOI *Φ	***FIC values	
		FIC	Interpretation
1/4 MIC* TZP	**-ve	0.5	synergism
1/2 MIC TZP	-ve	0.5	Synergism
1/4 MIC *CAZ	-ve	0.5	Synergism
1/2 MIC CAZ	-ve	0.5	Synergism

*: **Φ**=phage, TZP= Piperacillin/Tazobactam, CAZ=Ceftazidime. **: -ve mean no growth. ***: FIC which determined as follow: (≤0.5) synergism, (0.5–<1) additive, (1-<4) indifference, (≥4) antagonism. (Killic S.*et al.,*2008).

DISCUSSION

There is wide spectrum of different organisms have been described that cause neonatal sepsis. (Ibrahim AH., 2005). In the current study, 30 isolates of *Enterobacteriaceae* that belong to neonates with sepsis were taken and the bacterial profile of these isolates found that most common bacteria were *Klebsiella Pneumonia* which constitute fourteen isolates (46.67%) and *Escherichia Coli* were eleven (36.67%). The results of the present study are in agreement with many studies which found that *Klebsiella* and *E.Coli* which belong to *Enterobacteriaceae* group, among all other types of gram negative bacteria, were the most common pathogens responsible for high percentage of neonatal sepsis infection including many studies that done in Iraq (Al-Hamadani AH.*et al.,*2008, Ibrahim MF.,2011, Sadiq ZM.& Al-Anee AH.,2010, Al-Bayaa YJ.*et al.,*2014). Other studies done in Egypt and India showed similar bacterial profile (Fahmey SS.,2013, Muley VA.*et al.,*2015). The difference in the percentages or number of *Klebsiella Pneumonia* and *E.Coli* isolates from one study to another might be due to the difference in the numbers of isolates collected and investigated in different studies. The type of microorganisms that causes neonatal sepsis was changing overtimes and varying from region to region this may be due to changing in pattern of antibiotic use and lifestyle changes. (Jyothi P.*et al.,*2013). In the current study, the MDR isolates for *Klebsiella* were 9(64.29%) and non MDR isolates were 5(35.71%), for *E.Coli* the MDR isolates were 5(45.45%) and non MDR isolates were 6(54.55%);this was classified according to the criterion of non-susceptibility to at least one agent in three or more antimicrobial categories considered as MDR..(Cilloniz C.*et al.,*2019). Concerning MDR isolates, the current study agrees with a study done in Egypt in which most of *Klebsiella Pneumonia* isolates were MDR (69.7%). (Almohammady MN.*et al.,*2020)

The increase in the bacterial resistance to antibiotics with development of life threatening infections is due to the emergence of multi drug resistant bacterial strains, therefore it is an important issue to use a combination of antibiotics to improve patient's outcomes and minimize their side effects and toxicity. (Dudeja S.,2020). The advantage of using antibiotics combinations to prevent the emergence of bacterial resistance in which bacterial resistance to two drugs would be less probable than that against single antibiotic. (Ahmed A.*et al.,*2014).

In this study, the combinations of piperacillin /tazobactam or ceftazidime with amikacin and combinations of piperacillin/tazobactam or amikacin with ceftazidime were done for two of most resistant isolates of *E.Coli* isolate no.11 and *Klebsiella Pneumonia* isolate no.12 respectively which were resistant to twelve and six different antibiotics respectively.

This disposition was done in order to evaluate the benefits of antibiotics combinations used in the treatment of MDR *Klebsiella* and *E.Coli* infections. The combinations of pieracillin/tazobactam or ceftazidime with amikacin were used for *E.Coli* and *Klebsiella* isolates (no.4and no.6) which were resistant to twelve and eleven different antibiotics respectively including piperacillin/tazobactam and ceftzidime also done to evaluate the antibacterial activity of *Klebsiella Pneumonia* and *E.Coli* bacteriophage and benefit of usage them to help in the treatment of MDR strains.

The results of antibiotics combinations in this study agree with a study done in South Korean (Cha MK.*et al.,*2015) which found that the combinations of beta-lactam antibiotics (piperacillin/tazobactam, cefepime and ceftazidime) with amikacin increased susceptibility of extended spectrum beta-lactamase producing *E.Coli* isolates to antibiotic combinations and antibacterial synergism was shown with β-lactam and aminoglycoside combinations. The synergism effect that was found may be attributed to the use of different antibiotics with different mechanisms of action as the combinations used in this study would help in reducing the resistance of the selected isolates. In addition, the synergism found may be occurred due to the strain's susceptibility to one of the two antibiotics used in the combination. (Rahal JJ.,2008).

In this study, all antibiotics combinations showed synergism. This enhance the activity of the agents used in the combinations against sensitive, resistant and MDR isolates of *Klebsiella* and *E.Coli* and may cause improvement in therapeutic outcomes with prevention or decrease in the emergence of resistance; furthermore, the lower MIC values obtained from these combinations would help in reducing the occurrence of side effects and toxicity which are dose dependent.

The emergence of antibacterial resistance in bacterial strains and the antibacterial activity of phages give great importance of using phages alone or in combination with antibiotic(s) as antibacterial agents. In addition, the rapid entry of phages into the circulation when delivered by I.V, I.M and I.P injection, achieve rapid distribution of phage systemically and would be found in different tissues and organs like kidney, bladder, skeletal muscles, salivary gland and brain and this gives arise for medical use of phages in systemic infections. (Dabrowska K.,2019). In this study, the results showed that the phages had potent antibacterial activity and when combined with 1/4 or1/2 MIC of piperacillin/tazobactam, ceftazidime and amikacin, they gave synergestic effect against MDR *E.Coli* and *Klebsiella Pneumonia* isolates tested.

Similar results were found in a study done in Iran (Moradpour Z.*et al.,*2020) in which phage- antibiotic combination therapy increased the sensitivity of *E.Coli* isolates to antibiotic (pencillins group used) to which

they were resistant as well as to the antibiotic to which they were moderate sensitive by increasing the diameter of inhibition zones using antibiotic disks and the susceptibility status was converted from resistant and intermediate to sensitive. This synergistic effect may be due to many mechanisms include: the combination may result in increasing the lytic phage growth (Comeau AM.*et al.,*2007), binding of phage to one or more surface receptors on the host bacteria including efflux pump would change the efflux pump function when occupied by the phage and this will restrict the resistance to antibiotic and increase the susceptibility. (Summers WC.,2001, Chan BK.*et al.,*2016) The phage effect related to the direct bacteriolysis or associated with immune activation by spreading bacterial components. The synergism effect of phage-antibiotic treatment is dependent on the specific combinations of antibiotic, phage, bacterial isolates and mechanism of interaction. (Morrisette T.*et al.,*2020).

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