Ministry of Higher Education
And Scientific Research
University of Al-Qadisiyah
College of Biotechnology
Department of Medical
Biotechnology

Isolation and Characterization of Bacteriophages Infecting. Klebsiella pneumoniae and 0xytoka

A Researche

Submitted to the Council of the dept. Of Biotechnology College/ University of Al-Qadisiyah in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Medical Biotechnology

By

Alaq Abd Alqadir Hussein

Zahraa Fares Abd Ali

SUPERVISOR

Prof. Dr.Ziad M.F.AL-Khozai

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﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

صدق الله العلي العظيم

[سورة المجادلة: 11]

Dedication

To my mother and my father

To the light that enlightens me the path of success...To the great heart...

To the professors

To whom it was credited for the completion of this research Professor Ziad M.F.AL-Khozai

To all who taught me the characters in this world

To those who save this country from loss and exile

Dedicate this work, asking the Lord to be with us.

Alaq &Zahraa

Acknowledgments

Praise be to God who made thanks for the key to mention and peace and blessings on the best creation Aba al-Qasim Muhammad and on the Tahirin

Either after

We would like to thank Dr. Ziad M.F.AL-Khozai, the supervisor of this research, who has had the greatest role in preparing and evaluating it, and because of our time and efforts, we have many thanks and appreciation.

We also extend our thanks and gratitude to all faculty members

Biotechnologies have provided us with a scientific experience in our university life

And do not forget all those who helped us from near or far, even by word or call it good

Thanks God first and last

Alaq &Zahraa

Contents

Summary

This study was carried out during the period from November 2017 to March 2018, to isolate and characterization the phages that cause infection in an attempt to find alternative therapeutic phage.It included the isolation of 10 sample , isolates from Diwaniyah Teaching Hospital of different patients . Samples were subjected to laboratory culturing to identification.

Obtained result included the isolation and diagnostic procedures we have obtained 5 types of phages (A,B,C,D,E) which have been categorized into virus classification criteria which include (phage diameter mm),number of plaques, turbidity , halo) and four from isolated phages (A ,B ,D ,E) are malignant phages which generated round , clear , no halo and medium sized (1.0-4.0 mm in diameter) plaques but isolated phage C produce irregular shaped , turbidity , halo and large sized(8.0-11.0 mm).A,B,C phages were capable to lyse *K. oxytoca* and D,E phages had lysis effect against *K. pneumoniae.* The isolated phage particles recorded highly significant ability to cause lysis of the bacterial cells and these effects reached to the higher rate after one hours of mixing of phage particles with bacterial cells. Also results showed that there is a dramatic decrease in number of bacterial cells with time. Complete lysis time of Phage particles was 5.5 hours. The overall findings of this work indicate the crucial role of phages that control & drive bacterial populations in the environment.

Chapter One

Introduction
Review of references

Introduction

Klebsiella are opportunistic pathogens, bar- formed microbes, generally measures 0.3 to 1.5 µm wide by 0.5 to 5.0 µm long with a noticeable (distinguished) polysaccharide-based capsule. It is belong to family of Enterobacteriaceae, gram-negative, oxidase-negative and non-motile. [1]. *Klebsiela* include species; K. *granulomatis , K. oxytoca, K. michiganensis,K. pneumoniae,K. quasipneumoniae, K. grimontii, K. variicola.* Klebsiella spp are found omnipresent in nature, where they are found in surface water, soil, sewage and on plants [2,3] and other and the other being colonized the mucosal surfaces of mammals such as mares, humans and pig[4].In predominantly, the medically most important Klebsiella species, Klebsiella pneumoniae found in animals, human(K. pneumoniae is present in the nasopharynx and in the intestinal tract), sewage, and dirtied waters and soils, ,Although found in the normal flora of the mouth, skin, and intestines but represents a huge part of hospital-acquired urinary tract infections, pneumonia, septicemia, meningitis, diarrhea, and soft tissue infections [5] .For get a K. pneumonia infection , occurs after enter the body through the respiratory system to cause pneumonia , blood to cause infection of the bloodstream. In general bacteria are spread among people, for example health care personnel (contaminated hands, use of bacteria-contaminated instruments, etc.) or may be transferred to patients by ventilators or who have an intravenous catheter and wounds [6,7]. *Klebsiella oxytoca* is one of several Klebsiella bacteria. These bacteria are naturally found in the intestinal tract, mouth, and nose. They're considered healthy gut bacteria inside your intestines. Outside the gut, however, these bacteria can cause

Serious infections. They're is commonly spread in healthcare environments. These environments include nursing homes and intensive care units [8]. They're can cause a serious infection. One type of infection causes pneumonia-like symptoms. *Klebsiella oxytoca* can also lead to urinary tract infections (UTIs), wound infections, and more [9]. *Klebsiella* follows many of the mechanisms to become resistant to many drugs and that through produce enzyme to degrade antibiotic, prevent drug from reach to target, and modify drag target and drug injection pumps [12]. For example *K. pneumonia* is an important cause of multidrug-resistant infections worldwide, which produce an enzyme called Carbapenemases which actually degrade the carbapenem drug that would normally used to treat it thus its resistance and that reduce the number of antibiotics that we have to fight any infection [10]. Infection with microorganisms is resistant to many drugs, which leads to long-term hospital stay, high health care costs and increased deaths rates [11]. Due to emergence the resistance of pathogens for many chemical drugs therefore, the study aims to resort to the use of another type of treatment, which is phage therapy indicate to use bacteriophages to treat pathogenic bacterial infections [13]. For the medical importance of this genus, this work was aimed to isolate and characterization the phages that cause infection in an attempt to find alternative therapeutic phage.

Chapter Two

Material and Methods

2 -1 Materials

2-1-1 Equipment

Laboratory equipment and tools

Table (2-1): The equipment and laboratory tools used in the study and the manufacturers

No.	Equipments	Manufacturing
1	Autoclave	Delama, USA
2	Burner	Amal, Turkey
3	Centrifuge –universal 16 A	Hittch Germany
4	Fine and adjustable micropipettes	Gilson, France
5	Hot plate	Gallenkamp (England)
6	Incubator	Memmert(Germany)
7	Laminar air flow	Prettl , Germany
8	Light microscope	Olympus(Japan)
9	Magnetic stirrer with hot plat	Vision , Korea
10	Micro pipettes	Eppendorff\Germany
11	Microplate shaker	Heidolph, Germany
12	Oven	Olympus, Japan
13	PH-meter	Thermo Electron , USA
14	Sensitive balance	Sartorius (U.S.A)
15	Vortex	Griffin, Germany
16	water bath	Tafesa , Germany

2-1-2 Plastic and Glass Wares:

Table (2-2): Plastic and Glass Wares

No.	***Plastic and Glass Ware***	***Manufacturing***
1	Automatic pipette	Birhit, Finland
2	Disposable Petri-dishes	EAPIF (Germany)
3	Gloves	Hungary
4	Pasteur's pipettes	Volac (England)
5	Pipette Tips (10 µl , 20 µl , 100 µl , 200 µl and 1	Star Lab,UK
6	Plain tubes	AFMH, England
7	Screw caps tubes	HBG, Germany
8	Whitman filter papers	MEHE, China

2-1-3 Biological Materials:

Table (2-3): Biological Materials

	Biological Materials	**Manufacturing**
1	Nutrient agar	Oxoid (England
2	Nutrient broth	Oxoid (England)
3	chloroform	BDH Chemical, England
4	Peptone water	Oxoid (England)

2 Methods

2-1 Samples Collection:

2-1-1 Bacteria strains

Klebsiella were isolated at 10 samples from Diwaniyah Teaching Hospital. Samples were subjected to laboratory culturing, Nutrient agar container of Peptone water broth for 18 hours at 37 ° C. After that, labeling was applied to each plate which contain of bacteria. Growth and propagation of the culturing was observed on all petri dish. Tests were included Biochemical and serological tests. (19, 18, 17).

2-2-1-2 Water samples

Phage samples were collected from the sewage water and from different environmental sources within the closed laboratory bottle with covered at the size of about 500 ml of these samples.(16)

2-2-2 Sterilization Methods

Equipment's, Biological Materials were Sterilization during the experiment was performed using an Autoclave at 121 ° C and 1 psi for 15 minutes.

Plastic and Glass Ware were sterilized using sterilization ovens and at a temperature of 168 and for an hour and a half.(16)

liquids were sterilized which used in the experiment by filtered using filters with diameters ranging from 0.27 microns to 0.4 microns to ensure the elimination of contaminant

2-2-3 Preparation Culture media

Media used in this study were prepared according to the manufacturer's instructions fixed on containers.

2-2-3-1 Preparation Nutrient agar

Agar powder were weighted at 28 g of nutrient in 1 liter of distilled water, then putting on the hotplate to dissolve completely. Suspension were sterilized by using Autoclave as required.

Suspension were Cooled to 55°C.(16)

Cotton plug and flame were taken the mouth of the flask over a Bunsen burner, and then suspension was poured into sterile empty of Petri dishes (10-15 mL into each Petri dish) and the Petri dishes were kept horizontally until the medium completely solidifies

2-2-3-2 Preparation Broth agar

Agar powder were weighted at 28 g of nutrient in 1 liter of distilled water, then putting on the hotplate to dissolve completely. Suspension were sterilized by using Autoclave as required

2-2-3-3 Preparation Nutrient Soft agar

Agar powder were weighted at 0.7 g of nutrient in 100 mL of distilled water, then putting on the hotplate to dissolve completely. Suspension were sterilized by using Autoclave as required

2-2-2 Phage Isolation

Phage samples were collected previously from the sewage water and from different environmental sources. Then were centrifuged

(10,000 rpm, 10 minutes) and passed through super-sterilization filters (0.45 µm or 0.22 µm Millipore filter). Filtered sewage were mixed at 1 ml to 1 ml sterile nutrient broth which contain bacteria in the test tube

Mixture from phage and bacteria were incubated for 18-24 hours in the incubator at 37 ° C. than we take 0.2 ml from chloroform organic solvent and will be added to the bacteria, so that all the bacteria were eliminated except phage in the test tube for 1 minutes.(16)

2-2-3 Plaque Assay and Spot Test:

Phages were verified presence in the test tube by Plaque Assay was performed an as previously known. (18). for the isolation specific phage and determination through a double layer agar technique, Where each of tube diluted were mixed at 1 ml of phages in 4 ml soft agar that contain host bacteria as *Klebsiella* into other tube , then mixture that contain of phages and bacteria were added to plate (19). Plates were incubated for 24 hour at 37oC.

2-2-4 Phage Propagation and Purification:

Phages were isolated by one Petri Dishes and sequentially until the dishes are obtained homogenously container on Plaque (19). During this period, the Plaque were taken one at a time in a well-sequenced mode, under ideal conditions in terms of providing a sterile atmosphere. until sacrament so as not to contaminate them with fungus ,bactericidal and bacteria , one well separated phage was picked with sterile pasture pipette and inject them into the 5.0 ml nutrient broth, mixture which contain specific phage and *Klebsiella* were shaken by vortex and were left 24 hour of the host strain and then incubated at 37^{o}C. For complete decomposition. Until ensure the replicated of phage (16).

2-2-5 Lysis Time:

sample of cells were taken at 10-ml to mixed with soft agar at 10^7 pfu/ml ,then left for grown in test tube at five to six minutes, then do series of diluted to sample , 10^7 , 10^8 to 10^3 fold. Suspension were separate into flasks until to stop adsorption. For calculate time, untreated free phage plus infected cells were mixed of infective centers at various time points to determine changes in title (16).

Chapter Three

Results

Results

3-1- Isolation of *Klebseilla* Phages:

Five types of *Klebseilla* phages were isolated from samples of sewage water primarily named Phage A, Phage B, Phage C, Phage D and Phage E, using two *Klebsiella ssp (K. pneumoniae and K. oxytoca) that* were used as hosts for primary isolation of phages, the phages effectively were tested against *Klebseilla* isolates by using plaque assay method as in the Figure (3-1), Figure (3-1). Characteristics of these phages were determined by the plaques appearance, diameter, turbidity and the presence of a halo in the culture media as in table **(3-1), (3-2).**

Table (3-1): Characteristics of *K. pneumoniae* phages

Phage name	Plaque diameter (mm)	Number of plaques	Turbidity	Halo
Phage A	1-2	8	C	-
Phage **B**	3-4	6	C	-

C**:** clear; **+:** presence of halo; **-:** no visible halo

Table (3-2): Characteristics of *K. oxytoca* phages

Phage name	Plaque diameter (mm)	Number of plaques	Turbidity	Halo
Phage C	8-11	11	C	-
Phage D	1-2	3	C	-
Phage E	2-4	1	T	+

C**:** clear; **+:** presence of halo; **-:** no visible halo

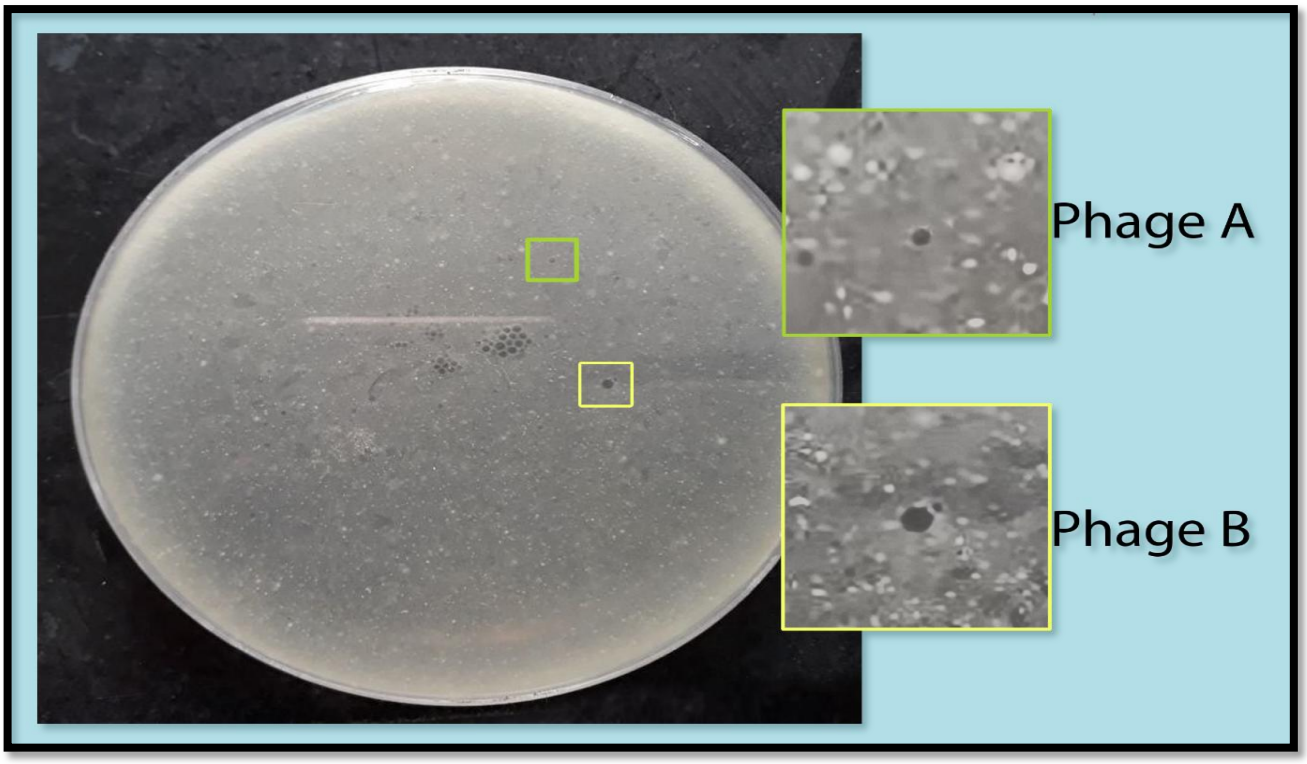

Figure (3-1): Plaques of *K. pneumoniae* phages on nutrient agar after 24 hr. in 37°C

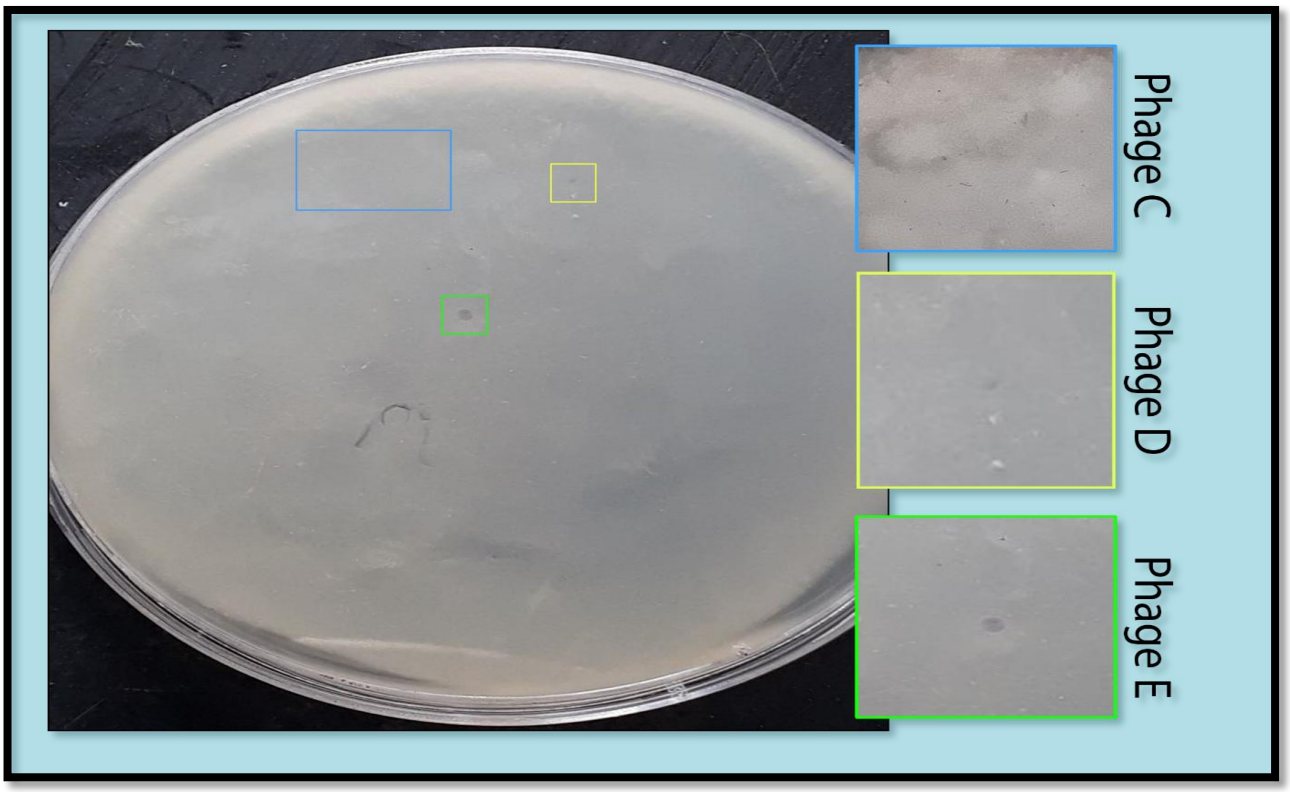

Figure (3-1): Plaques of *K. oxytoca* phages on nutrient agar after 24 hr. in 37°C

Figure [3-4]: Image of plaque on microscope of *Klebsiella*

3-2 Lysis Time:

The present study showed that the isolated phage particles recorded highly significant ability to cause lysis of the bacterial cells and these effects reached to the higher rate after one hours of mixing of phage particles with bacterial cells. Also showed that there is a dramatic decrease in number of bacterial cells with time. Complete lysis time of Phage particles was 5 and half hours as in figure (3-1).

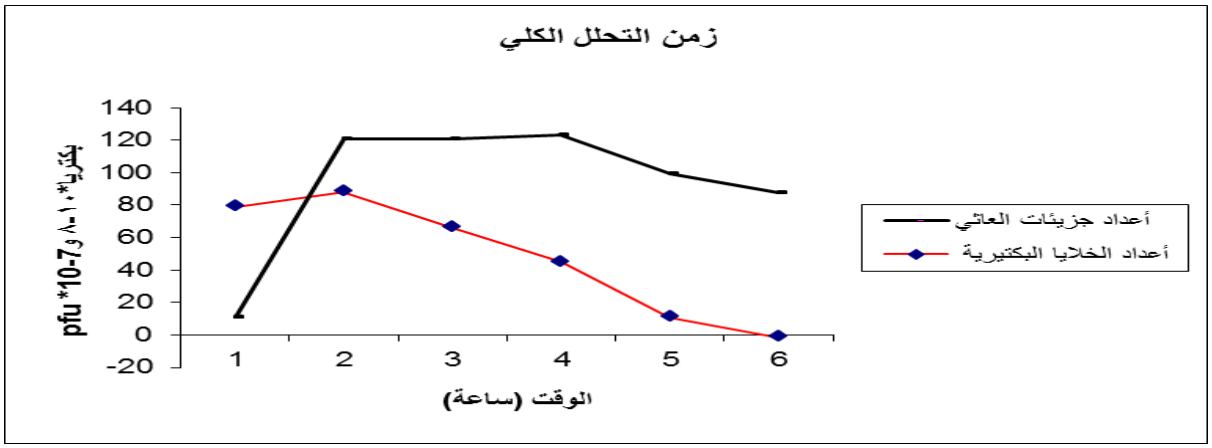

Figure (3-3): Lysis time of *klebsiella*

Bacterial titer 88794566 Phage titer 3368.2785

Chapter Four

Discussion

Discussion

Klebsiella can often cause human-acquired infection, especially in some types of bacteria that show new resistance mechanisms and spread [5]. Microbes are found in humans and animals, in food and environment (water, soil, air). It can be transmitted from human to animal and animal to human and from one person to another [2]. The factors that help spread antimicrobial resistance are had bad infection, eating contaminated food, poor health conditions etc. Antimicrobial resistance appears naturally over time through genetic modification and use of antibiotics is excessive and bad in humans and is often given without professional control [13]. For example *K.peumoniae* account large percentage of hospital-acquired urinary tract infections, septicemia , pneumonia and soft tissue infections and considered medically important type and have shown resistance to carbphenium drug, through produce enzyme marphenozem, which breaks down the drug ,that making it serious danger to human life because of its spread in every place where there are in sewer and dirty water , animals and humans (in the intestines and nasal pharynx), although it is a natural flora on the skin and in the mouth and intestines. Due to the increase cost health care to patients with resistance infections, the cost care for patients with non-resistance infection due to longer illness and procedure additional tests and the use of more expensive drugs [10, 11]. Therefore, the development of effective treatment is one of the top priorities in public health in the world. This study include the use of bacterial viruses to control bacterial population. The phages considered one of the alternatives to antibiotics, all of the bacteriophage isolates collected from sewage water able to infect Klebsiella. Through isolation and diagnostic procedures we have obtained 5 isolated phages designated (A,B,C,D,E) which have been categorized into virus classification criteria which include (plague diameter mm),number of plaques,

turbidity , halo) and four from isolated phages (A ,B ,D ,E) are highly virulent phages which plague were round , clear , no halo and medium sized (1.0-4.0 mm in diameter) plaques , while but isolated phage C produce irregular shaped , turbidity , halo and large sized(8.0-11.0 mm).A,B,C phages were capable to lyse *K .oxytoka* and D,E phages had lysis effect against *K .peumoniae*. Through complete lysis time scheme was noticed that there is a significant decrease in the number of bacteria over time compared to the increment in the number of phages, so we concluded that the isolated phages were infected bacterial cells and were able to lyse it. The C phage isolated in this study was proposed to be chance candidate for medicinal applications due to its strong lytic capability and its broad host range, Phage therapy has many advantages compared with other chemical drugs such as safety , biofilm penetration where Phages, are supported with enzymes (e.g., EPS de-polymerase) on the surface of the capsid that degrade the extracellular polymeric substances and disseminate bacterial biofilms, permitting the phage to reach bacteria embedded within the extra polymer substance matrix[14]. Specificity, in quite disparity with antibiotics phage be very specific against both stains and species .In particular conditions this will be considerable advantage considering the well- authenticated, side effects of broad-spectrum antibiotics on commensal gut microbes, however a lot of identification and characterization trials should be conducted to assure this hypothesis [15].

Chapter Five

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