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Inhibitory Effect of Bacteriophages Isolated from Sewage Water in the City of Kirkuk on some Types of Human Pathogenic Bacteria

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Abstract:

Most approaches to combat antibiotic resistant bacteria concentrate on discovering new antibiotics or modifying existing ones. However, one of the most promising alternatives is the use of bacteriophages. This study was focused on the isolation of bacteriophages that are specific to some of commonly human pathogens namely *E. coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella spp*. and *Klebsiella pneumoniae*. These bacteriophages were isolated from sewages that were collected from four different locations in Kirkuk City. Apart from *S. pyogenes*, bacteriophages specific to all tested bacteria were successfully isolated and tested for their effectiveness by spot test. The most effective bacteriophages that were isolated from sewages and sewage water of Al-Jumhori Hospital compared to other sites. It is concluded that the sewage water of hospitals represents a perfect environment for these bacteriophages.

Keywords: Bacteriophages, sewage, Kirkuk General Hospital.

Introduction:

The discovery of bacteriophages is dated back to 1915 when a British microbiologist, Frederick Twort, tried to look for "essential substances" needed to grow vaccinia virus on artificial culture media [1, 2]. However, the term bacteriophage was only coined by another scientist, Félix d'Herelle, a French-Canadian microbiologist, who in 1917 independently confirmed Twort's discovery [1, 2]. Bacteriophages or simply "phages" are a group of different viruses that have the ability to infect, multiply and eventually kill bacteria [3]. Like all viruses, phages are simple organisms that consist of a core of genetic material (nucleic acid) surrounded by a protein capsid. The nucleic acid may be either DNA or RNA and may be double-stranded or single-stranded [4]. Bacteriophages are natural, widely diverse and ubiquitous component of nature [5].

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Because they are obligatory intracellular; therefore, they are usually found whenever bacteria and archaea exist such as in soil, open oceans, ocean sediments, river tributaries and sewage water [5]. They are host specific and have been found in essentially all groups of bacteria [6]. Due to their bactericidal ability, bacteriophages had been used to treat bacterial infections in the pre-antibiotic era in early 1900. The first attempt to use bacteriophage to treat human infection was conducted by Félix d'Hérelle in France (1919). He used bacteriophage to treat a 12 - years old boy presented with hemorrhagic dysentery [7]. Although his trial was successful and supported by several other studies thereafter, the efficacy of "Phage Therapy" in humans remained a matter of controversy and after the discovery of antibiotics in the 1940s, it was virtually abandoned in western countries [7]. Nowadays, with the rise of antibiotic-resistant bacteria, the therapeutic potential of phages has received renewed attention. Recent studies have shown the success of the use of this technology to treat infections in plants [8,9,10,11], animals [11,12,13,14,15,16] and humans [11,17,18,19,20]. In contrast to the chemical therapeutic agents, phages can evolve to counter phage - resistant bacteria and are likely to be resistant to strains of non-targeted bacteria [21].

Globally, the emergence of antibiotic-resistant bacteria represents a major health challenge with both clinical and financial burden on public health system [22]. Unfortunately, our country is not an exception from this problem. In fact, previous reports from Iraq indicated that this problem is probably more striking than many other countries [23]. Hence, very hard work to reduce the burden of antibiotic resistant bacteria in this region of the world is needed. Probably one of the most promising solutions is the use of bacteriophages as alternatives to progressively failing antibiotics [23]. In this context, this study was conducted to examine the possible inhibitory therapeutic effect of bacteriophages isolated from multiple locations in the city of Kirkuk on different pathogenic bacteria isolated from patients attending general hospitals.

Materials and Methods:

This research was conducted at Azadi (Al-Jumhori) General Hospital in Kirkuk City, Iraq between the periods of December 2015 to April 2016. All the media and reagents used during the course of the study were obtained from the College of Science, University of Kirkuk, Iraq.

Host bacterial cultures (preparation of bacterial suspension)

Bacteria used in this research as phage hosts were obtained from Microbiology Lab at Al-Jumhori General Hospital in Kirkuk City. These bacteria were isolated, diagnosed and identified by conventional bacteriological methods and by ABI system using standard protocols. The isolated bacteria were *E. coli, Streptococcus pyogenes, Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella spp.* and *Klebsiella pneumoniae.* The day prior to bacteriophage isolation, 5 ml of autoclaved nutrient broth (NB) were inoculated with a loopful of prospective bacterial culture in a sterile tube [24]. Meanwhile, 5 ml of autoclaved brain hearts infusion broth were inoculated with a loopful of *S. pyogenes culture* in a sterile tube [24]. After inoculation the tubes were incubated overnight at 37 ° C with shaking [24].

Isolation of Bacteriophages

Treatment of sewage water

About 1 L of sewage water samples were collected in sterile dark containers from different locations of the city of Kirkuk (Rahim Awa, Al-Sayada, Ras Al-Jisser and Al-Jumhori Hospital). In the laboratory; the sewage samples were first filtered through course filter papers to remove debris. Isolation of the bacteriophages from the sewage was done according to the protocol described by Pitt and Gaston [25] with slight modification. 5 ml sample of sewage was mixed with 5 ml of double strength nutrient broth (DSNB) and 0.1 ml of overnight nutrient broth (NB) of the enrichment bacterial strain in sterile flask (in case of *S. pyogenes* brain heart infusion was used). DSNB (a broth with double nutrient broth concentration) is richer in nutrients than single nutrient broth and therefore provides better growth for small inocula. The mixture is left for 24-48 hours at 37 ° C with gentle mixing (50 rpm). During this incubation, phages in the sewage capable of binding to respective microorganism, can replicate in and eventually lyse the bacteria. Thus, this step serves as a means of amplifying the phage that can infect bacteria. Meanwhile, gentle agitation allows phages to come out and distributed into the suspension. Following incubation, the flasks were taken out of the incubator and left at room temperature for 15 minutes to allow the suspension to settle. The suspension was then subjected to centrifugation at 2500 rpm for 3 consecutive times and supernatant was collected after each centrifugation step. Each time the centrifugation step should cause the bacteria and other cell debris to form a pellet at the bottom of the tube. Following centrifugation, the supernatant was filtered through 0.22 mm Millipore filter and mixed with chloroform at 10-1 concentration to kill any residual bacteria left in the supernatant. Finally, the filtered supernatant was centrifuged again at 3000 rpm, then collected in sterile tubes and stored at 4 ° C [26] (with modification).

Testing of bacteriophages (Spot test)

The demonstration of bacteriophages was carried out using spot test [27]. In brief, 1 ml of overnight bacterial growth on nutrient broth was spread on nutrient agar medium and allowed to dry for 30 minutes at room temperature (blood agar was used in case of *S. pyogenes*). Thereafter, 5 µl of bacteriophage filtrate was poured on the agar at different places and incubated at 37 ° C for 24 hours. At the same time, 5 µl of chloroform alone without phage filtrate was poured onto each plate to serve as negative control and the experiments were done on duplicate sets. If the filtrate contains bacteriophage specific against the inoculated microorganism, the effect will appear as spots of inhibitions (inhibition plaques). The areas of inhibition were then measured in mm using a ruler. The center of the plaques was then scraped by a sterile loop, transferred into another pure culture broth of bacterial pathogen and incubated at 37 ° C for 24 hours with gentle shaking. This step is important to purify the bacteria-specific phages since the phage population may consist of several

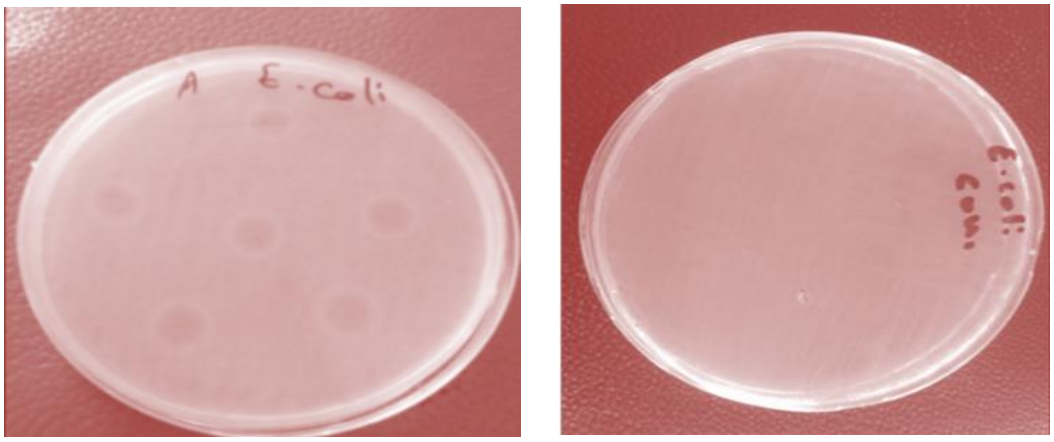
phage strains. Hence there is a need to obtain pure strains since phage filtrate might contain more than one type of phage. Same steps were then repeated again to isolate each bacterial filtrate and retained at 4 ° C until used.

Results:

The results of spot test are shown in Table 1. Apart from the *Streptococcus pyogenes*, all the other six tested bacteria were sensitive to bacteriophages isolated from sewage water in four different locations of Kirkuk City with areas of inhibitions ranged from 7-13 mm. The largest areas of inhibition zones were seen with bacteriophages isolated from sewage water of Al-Jumhori Hospital (13 mm for *Proteus mirabilis, Pseudomonas aeruginosa, Salmonella spp.* and *Klebsiella pneumoniae* and 11 mm for *E.coli* and *Staphylococcus aureus* respectively). Bacteriophages isolated from sewage water of Ras Al-Jisser and Al-Sayada were less effective against tested bacteria with inhibition zones ranged from 8-11 mm. However, the least effective bacteriophages were isolated from Rahim Awa area with inhibition zones ranged from 7-10 mm respectively. No *Streptococcus pyogenes* - specific bacteriophages could be isolated from sewage water in four different areas. The sensitive cultures belong to the genera *E. coli, S. aureus, P. mirabilis, K. pneumoniae, P. aeruginosa* and *Salmonella spp.* were illustrated in Fig. 1, 2, 3, 4, 5 respectively.

Table 1. Sensitivity of tested bacteria (mm) to bacteriophages isolated from sewages from various locations in Kirkuk City

Isolated bacteria	Al-Jumhori Hospital	Ras Al-Jisser	Al-Sayada	Rahim Awa
E. coli	11 mm	10 mm	8 mm	7 mm
S. pyogenes	0 mm	0 mm	0 mm	0 mm
S. aureus	11 mm	9 mm	8 mm	9 mm
P. mirabilis	13 mm	10 mm	9 mm	8 mm
P. aeruginosa	13 mm	8 mm	9 mm	8 mm
Salmonella	13 mm	9 mm	11 mm	7 mm
K. pneumoniae	13 mm	11 mm	11 mm	10 mm



A **B**

Figure 1. Bacteriophage for *E.coli* isolated from sewage water of Al-Jumhori Hospital. A) Inhibition (Plaque) areas on test plate. B) Negative control

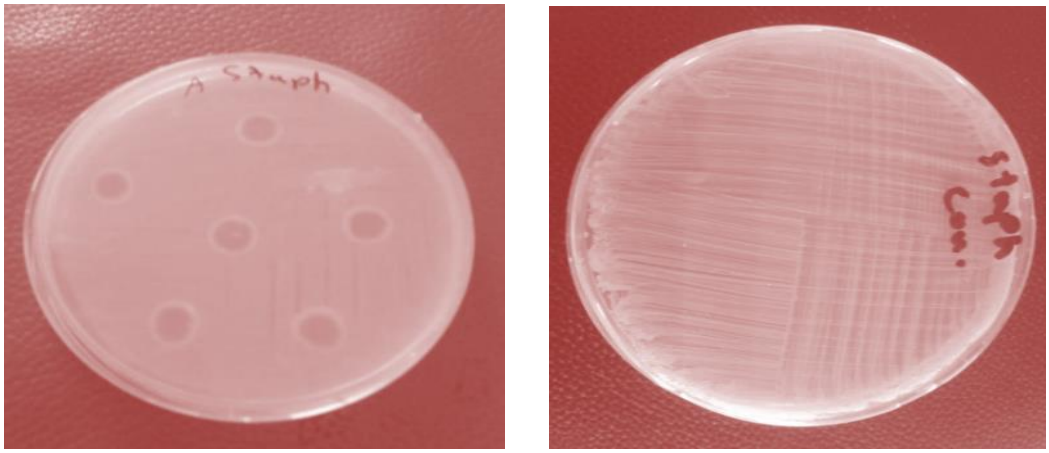


Figure 2. Bacteriophage for *Staphylococcus aureus* isolated from sewage water of Al-Jumhori Hospital. A) Inhibition (Plaque) areas on test plate. B) Negative control

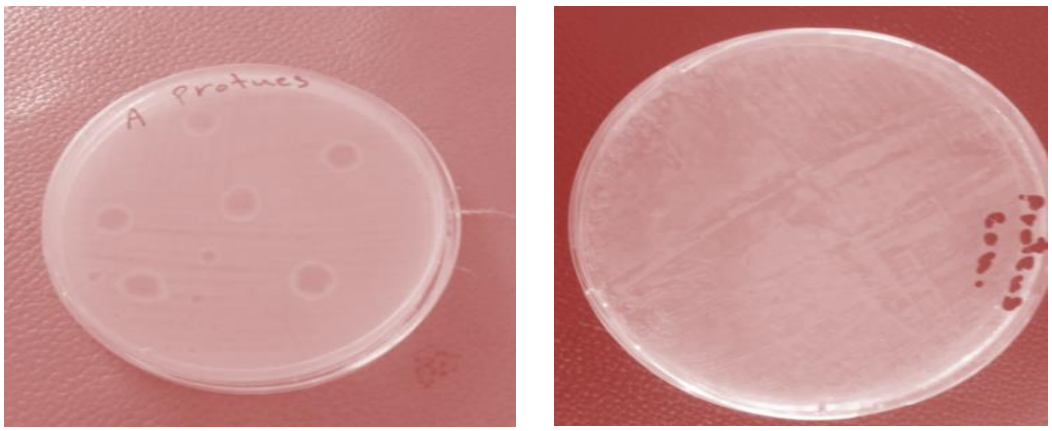


Figure 3. Bacteriophage for *Proteus spp.* isolated from sewage water of Al-Jumhori Hospital. A) Inhibition (Plaque) areas on test plate. B) Negative control

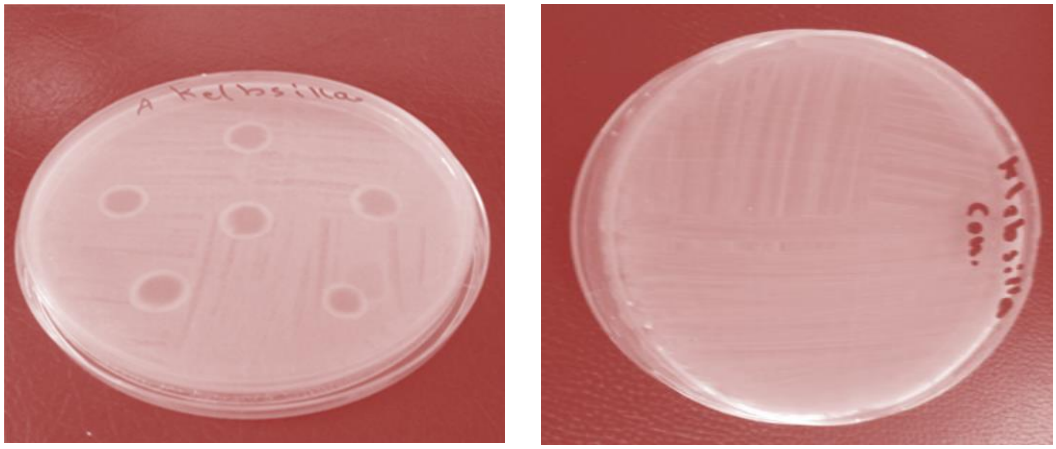


Figure 4. Bacteriophage for *K. pneumoniae* isolated from sewage water of Al-Jumhori Hospital. A) Inhibition (Plaque) areas on test plate. B) Negative control

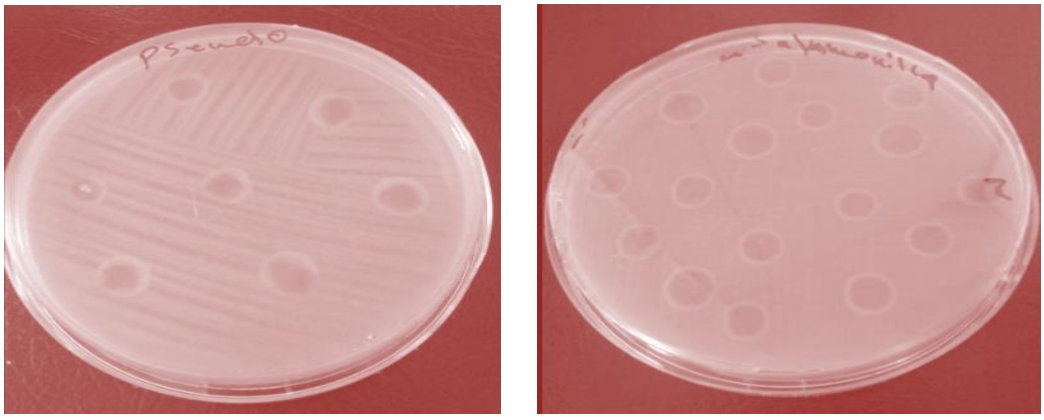


Figure 5. Bacteriophage for A) *P. aeruginosa* and B) *Salmonella spp* isolated from sewage water of Al-Jumhori Hospital

Discussion:

In pre-antibiotic era, bacteriophages have been used as therapeutics in the '20s and '30s of the 20th century [28]. During that time, they were being employed to treat a wide range of topical, internal and respiratory bacterial pathogens [7]. However, with the discovery of antibiotics in 1940, focuses on "Phage Therapy" research in western countries became gradually less important and eventually stopped in early 70s [28]. The picture was different in Eastern Europe (Formal Soviet Union, Poland and Georgia) where it seems that the use of bacteriophages to treat human infection was authorized and maintained on a large scale [29]. Nowadays, with the looming spectra of antibiotic resistance, the use of bacteriophages as therapeutics to treat human infection has been re-evaluated and re-vitalized by western scientists [30]. Antibiotic resistant bacterial infections are global health problem and continue to be major causes of morbidity and mortality both in society and hospitals worlds wide. Each year, about two million

people become infected with bacteria that are antibiotic resistant in USA with 23000 deaths as a direct result of infection with these bacteria [21]. This problem costs the US health system more than US $20 billion in excess costs annually. Therefore, the emergence of these antibiotic resistant bacteria led the scientists to actively search for successful and effective ways to combat these life threatening bacteria. Most approaches concentrated on the discovery of new chemical antimicrobials, use bacterial adjuvants or use multiple rather than single antibiotic. Unfortunately less attention has been received to surrogate therapies. Given that post antibiotic era is expected to reach soon [23]; therefore, searching for alternative approaches is not only justifiable but also highly indicated. In this context, "Phage Therapy" could provide a safe and effective alternative to treat bacterial infections. In fact, "Phage Therapy" has many advantages over conventional antibiotic therapy. First, phages are highly specific i.e. they affect only the targeted bacterial *spp*. while avoiding harming the normal flora [31]. This is in sharp contrast to antibiotics which target both the pathogenic and normal flora of the host leading to bacterial imbalance and increase risk of secondary infections. Second, unlike antibiotics which might be toxic, phages are relatively safe and therefore no serious side effect have been described; moreover, phages may be employed prophylactically with little fear of adversely affecting patients [32, 33]. The third advantage of phages is that they often are capable of replicating to higher concentration at the site of infection [33]. This is a striking distinct from antibiotics which can be metabolized and eliminated from the body without necessarily being concentrated at the site of infection. Therefore, delivering too high or too small doses of phages is of less concern. Fourth, phages are ubiquitous, highly numerous and widely diverse. Hence, novel phages with novel activities can be decremented and disclosed in a matter of days or weeks. Finally, although cases of phage - resistant bacteria are reported, phage resistance is not nearly as disquieting as antibiotic drug resistance [34]. Like bacteria, phages can mutate and therefore can evolve to counter phage-resistant bacteria [34]. This means that phage resistant bacteria may still remain susceptible to other phages having similar target range. Furthermore, the development of phage resistance can be minimized altogether if phages are used in cocktails (preparations containing multiple types of phages) and/or in conjunction with antibiotics [34]. Apart from antibiotic resistance, several other reasons have driven the scientists to give a renewed attention to bacteriophages. For example, a viral genome is constantly seen within multidrug resistant (MDR) bacterial DNA indicating that these viruses can still recognize and infect these bacteria despite their antibiotic resistance [35]. Evidence from ecological studies revealed that each bacterial species in the environment can be infected by more than 10 phage species suggesting that these viruses playing critical roles in shaping the nutrient cycling and evolutionary dynamics of the organisms they infect [36].

In Iraq antibiotic resistant bacteria are common, perhaps due to antibiotic malpractices. Some reasonable efforts to isolate and examine, and evaluate bacteriophages in the treatment of some pathogenic and MDR bacteria were carried out by some researchers in our country [23, 37, 38, 39]. Our study is therefore, an additional ring of research series in this field in our country. The current research had focused on the isolation of bacteriophages effective against some of the common human bacterial pathogens namely *Staphylococcus aureus*, *Streptococcus pyogenes*, *E.coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Salmonella* spp. Bacteriophages were segregated from Sewage water from four different locations in Kirkuk City including sewage water from Al-Jumhori Hospital as well as sewages from Rahim Awa, Al-Sayada and Ras Al-Jisser areas. Although bacteriophages can be isolated from diverse sources such as soil, intestine of animals, pond, river and ocean waters, un-treated sewage water was chosen in this study. Un-treated sewage is an ideal source to isolate bacteriophages since it contains high numbers of diverse bacteria making it an optimal environment for exploring viral density [40]. With the exception of *S .pyogenes*, bacteriophages specific to all tested bacteria were successfully isolated from sewages collected from all four different locations in Kirkuk City. However, the highly sensitive bacteriophages were seen in sewage collected from Al-Jumhori Hospital with inhibition zones ranged 11-13 mm. This finding is, in fact, not surprising since all the studied bacteria were isolated from this hospital. Hence, pathogens excreted into hospital sewage perhaps reflect the true infections that have been transmitted from the hospital population and mostly include the viral pathogens that are transmitted through faecally contaminated water, food or hospital waste products. On other hand, failure to isolate bacteriophages specific to *S. pyogenes* might be a weird finding since bacteriophages are stable and widely distributed in environment. Like other viruses, bacteriophage virion or viral DNA can be affected by some external factors such as temperature, pH, and ionic strength [41, 42]. Moreover, some authors even assume a relationship

between phage morphology and its stability and diversity in the environment [43]. Jończyket et al (2011) concluded that the ability of the bacteriophages to resist unfavorable environmental conditions is different not only among different families but also among them. Therefore, one or more of the above mentioned factors could be a contributing factor for failure of isolation *S. pyogenes* bacteriophages.

Conclusion:

Hospital sewages are the best source to isolate bacteriophages against human pathogens.

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التأثير المثبط للفيجات المعزولة من مياه المجاري في مدينة كركوك على بعض أنواع البكتيريا المسببة للأمراض البشرية

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[3] قسم علوم البيئه، كليه علوم البيئه وتقناتها، جامعة الموصل، العراق

الخلاصه:

تركز معظم النهج لمكافحة البكتيريا المقاومة للمضادات الحيوية على اكتشاف المضادات الحيوية الجديدة أو تعديل القائمة منها. ومع ذلك يعد استخدام الفيجات المنتشره بشكل واسع في الطبيعه واحدة من أكثر البدائل الواعدة .لذلك ركزت هذه الدراسة على عزل الفيجات المضاده لبعض انواع البكتريا المسببه للأمراض البشرية مثل الايشريشيا القولونية، المكورات العنقوديه الذهبية، المكورات العقديه ، المتقلبات، الزوائف الزنجاريه، السالمونيلا و الكليبسيلا الرئوية. تم عزل هذه الفيجات من المجاري التي تم جمعها من أربعة مواقع مختلفة في مدينة كركوك وهي مناطق المستشفى الجمهوري، رحيم اوى، الصياده، و رأس الجسر. تم بنجاح عزل جميع الفيجات المضاده للبكتريا الوارده ذكرها اعلاه باستثناء الفيج المضاد لجرثومه المكورات العقديه فلم نتمكن من عزلها. تم اختبار فعاليه جنيع الفيجات المعزوله من خلال اختبار النقط و كانت اكثر الفيجات فعالية تلك المعزوله من مياه الصرف الصحي والمجاري التابعه لمستشفى الجمهوري مقارنة بالمناطق الاخرى. تم الاستنتاج ان (مياه الصرف الصحي للمستشفيات هي التي تعد البيئه الملائمه لتلك الفايروسات).

الكلمات المفتاحية: الفيجات، مياه الصرف الصحي، مستشفى كركوك العام.