

How to Cite:

Mutlk, S. T. (2022). Isolation and characterization of MDR- *Klebsiella pneumonia* phage from Euphrates River in Al-Anbar city. *International Journal of Health Sciences*, 6(S2), 5115–5123. <https://doi.org/10.53730/ijhs.v6nS2.6208>

Isolation and characterization of MDR- *Klebsiella pneumonia* phage from Euphrates River in Al-Anbar city

Saad. T. Mutlk

Department of Biology, College of Sciences, University of Anbar, Al-Anbar Province, Iraq

Corresponding author email: saad.t.mutalk@uoanbar.edu.iq

Abstract---In Iraq, there is no active action for Al-Ramadi teaching hospital for maternity and children waste which is straightforwardly discharge in to Euphrates River in Al-Anbar city. As a result, the numbers of multi-drugs resistant bacteria were raised specially in Enterobacteriaceae family (*Klebsiella pneumonia*). Nevertheless, modern studies are using phages isolated from similar ecosystem as a biologic alternate for antimicrobial remedy. The present work has an aim to isolate *Klebsiella pneumonia* and categorise its antibiotic resistance elements from Euphrates River in Al-Anbar city, Iraq. As well as isolation of phages from the similar collected river water specimens then examining their repressive consequence on MDR-*Klebsiella pneumonia* isolates. Water specimens were taken from 5 sites in to Euphrates River near to the residues of sewage estuaries that run straight to the main river for many regions of Ramadi city. These specimens were inoculated on MacConkey agar plates for morphologic appearance, and biochemical characters by API- 20E test and confirmed by VITEK 2. Disc diffusion technique was performed to identify antibiotic sensitivity. The *Klebsiella pneumonia* phage isolated from L4 that showed lytic activity against *Klebsiella pneumonia* was further confirmed that *Klebsiella pneumonia* is ESBL producers by phenotypic assay using the VITEK 2 systems version 08.01.

Keywords---Euphrates River, Al-Anbar city, antimicrobial agents, wastewater, phage therapy.

Introduction

Up to date there is an unplanned and extreme use of antimicrobial agents as a treatment for bacterial infections in both humans and animals ¹. This led to a rise in the numbers of multi-drugs resistant bacteria ². In general, river can be

contaminated by several types of sources. These contaminant includes manufacturing, farming, household waste ³. The relation between the increase in the number of multi-drugs resistant bacteria and rivers' pollution have become a problematic and worries all over the world. In the same way, antimicrobial agents are disposed straight into the rivers throughout sewages, particularly from public hospital sewage ⁴. Furthermore, antimicrobial agents and their metabolites are similarly dropped to the wastewater leading into contamination of the rivers water ⁵. The Euphrates River enters Al Anbar province and it is addressed as a major water source in Al Anbar province. A recent study indicated that muds area closes to the residues of waste water are highly contaminated with Enterobacteriaceae family (*Klebsiella pneumonia*) in Euphrates river in Al Anbar city ⁶.

This bacterium known as a significant marker for pollution of the water environments, soil and food ^{7,8}. *Klebsiella pneumonia* is well known as a core member in the Enterobacteriaceae family, it is Gram negative, rod, non-motile, It has a mucoidal lactose fermenter appearance on MacConkey agar plates ⁹. This bacterium naturally occupy the gastrointestinal tract of several mammals such as birds, reptiles, livestock, and humans ¹⁰; categories high among the causative agents of epidemics foodborne diseases. Bacteriophages are the promising alternate treatment for multidrug resistant bacteria, as these viruses specially attacks particular bacteria led to their lysis ¹¹. Newly, they are used as controller for antibiotic resistant foodborne bacterial pathogen. Bacteriophages might provide a normal, harmless, viable treatment for controlling human foodborne causative agents ¹¹. This study aims to isolate bacteriophages and test their ability to inhibit (therapy) the growth of multidrug resistant *Klebsiella pneumonia* isolated from the Euphrates River in Al Anbar province by means of the double agar overlay method.

Materials and Methods

collection of samples

Water samples were divided into 5 sites, from each site 250ml were taken from 1m Euphrates River near to the residues of sewage estuaries that run straight to the main river for many regions of Ramadi city in Anbar Governorate, 50 cm in deepness. Over a distance of approximately 2.36 Km, these samples were collected in January 2020, collected into sterile flagons, kept at a temperature of 4 °C, transferred to the laboratory and examined on the same day of collection.

Isolation and identification of host bacterium

Form each site 15 ml of water sample was centrifuged for 10 min at 5000 rpm, screened for *Klebsiella pneumonia* by culturing 50 µl on MacConkey agar plates for 24 hours at 35°C. This followed by for biochemical characters by API- 20E test and confirmed by VITEK 2 systems version 08.01 for the lactose fermenter with mucoidal colonies appearance.

Antimicrobial sensitivity test

Disc diffusion technique was performed to identify antibiotic sensitivity according to the typical procedure of Kirby-Bauer and rendering to CLSI article ¹². The previously isolates of *Klebsiella pneumonia* were cultivated on MacConkey agar for 16 hours at 37°C. Three to five colonies of were mixed with normal saline to obtain an inoculum with optical density equivalent to 0.5 MacFarland turbidity values. A cotton swab was used for spreading of each isolated *Klebsiella pneumonia* on Mueller-Hinton agar, let to dry for 10 minutes at room temperature. The antibacterial discs used for the sensitivity tests were Amikacin (AK-10 µg), Cefotaxime (CTX-30 µg), Gentamicin (GEN-10 µg), Ciprofloxacin (CIP-10 µg), Trimethoprim/Sulfamethoxazole (SXT-1.25/23.75 µg), Imipenem (IPM-10 µg), Azithromycin (AZM-15 µg), and Tobramycin (ToB-10 µg). Furthermore, these antibiotic discs were sited on the agar's surface, plates were cultivated for 24 hours at 37°C. After that, the inhibition zone (diameter) was estimated over each disc. Advance identification of *Klebsiella pneumonia* isolate was achieved through the use of BioMerieux VITEK 2 Compact system consistent with the manufacturer's protocol.

Isolation and purification of bacteriophages from river samples

Each of the previously centrifuged water samples were similarly filtered by using 0.45 and 0.22 µm Millex-GP syringe filter unit (Merck) to eliminate bacteria and confirmation of phages on specimen only. These samples were kept at 4°C. The identified *Klebsiella pneumonia* isolates were cultivated on MacConkey agar (Oxoid) for 24 hours at 37°C. From each isolate 3 to 5 colonies were postponed in normal saline solution to reach the optical density equal to 0.5 McFarland values, 100 µl of phage (each filtrated sample) was transmitted into a new sterile tube. Then, 200 µl of each *Klebsiella pneumonia* isolated solution (each location) was added individually and mixed well. The mixture was allowed for 10 minutes at room temperature to ensure of phage attachment to its host.

After that, the mixtures of bacteria+phage were assorted with 4 ml molten agar (0.7% agar) and spilled onto the top of nutrient agar plate (N.A). these plates were let to still at room temperature for about 30 minutes. Plaques were obtained after incubation of these plates for 24 hours at 37 °C. A cleared zones around the top of the agar plate indicated the existence of lytic plaques (bacteriophages). These phages were purified by picking up a well-defined plaque from the agar plate by using sterilized tips and then propagated into 5 ml magnesium salt solution (NaCl 5.8 g, MgSO₄·7H₂O 2 g, Tris-Cl 1 mol/ L1, and 2% Gelatine in 1 L of distilled water, pH 7.5) and assorted with vortex. The remains of cells and agars were eliminated by centrifugation at 5000 rpm for 20 minutes. The resilient was filtered by using 0.22 µm Millex-GP syringe filter unit (Merck) and transmitted to new sterilise tubes. The lysate phages were kept at 4 °C.

Results and Discussion

Identity of host *Klebsiella pneumonia* in river water

A total of five water specimens were collected from Euphrates River near to residues of sewage estuaries that run straight to the main river for many regions around the Al-Ramadi teaching hospital for maternity and children, Ramadi city in Anbar Governorate, Iraq as seen in Figure-1. The overall distance was approximately 2.36 km, these places were named as follows: 1: Al-Tamim centre for water treatment, 2: residentiary region, 3: Dream Land Restaurant, 4: Al-Ramadi teaching hospital for maternity and children and 5: residentiary region.



Figure 1. Allocation of selected points close to residues of sewage estuaries that run straight to the main river for many regions around the Al-Ramadi teaching hospital for maternity and children, Ramadi city in Anbar Governorate, Iraq. Keys features are marked.

In consonance with samples selection in the Euphrates River indicates a higher concentration of *Klebsiella pneumonia* (MDR) at location 4, which is the closest location to the Al-Ramadi teaching hospital for maternity and children waste. The morphologic test displayed that these bacteria were Gram negative, rod, non-motile, it has a mucoidal lactose fermenter appearance on MacConkey agar plates as shown in Figure 2, confirmed by API- 20E test and VITEK 2 systems version 08.01.



Figure 2. Morphological appearance of *Klebsiella pneumonia* (MDR) at location 4. it has a mucoidal lactose fermenter appearance on MacConkey agar plates.

Antimicrobial susceptibility patterns of bacterial isolates

A pure isolate of *Klebsiella pneumonia* were taken from each location, examined for antimicrobial sensitivity toward 12 antimicrobial agents as indicated in Table 1. Following results analysis, it was founded that the isolated *Klebsiella pneumonia* in location L4 acts as a multidrug resistant bacterium, as mentioned in Table 1, in which *Klebsiella pneumonia* displays a higher resistance to a wide range of antibacterial agents which used as a cure for mammalian diseases ^{13,14}. This is due to a rising in the number of the existences of antibiotic resistant bacteria strains in different environments (sewage, soil and water)¹⁵. In addition, hospitals sewage has a direct relation in to the spread of multidrug resistant bacteria in to rivers. A newly study has shown drinking water is contaminated with coliform bacteria in Euphrates River in Iraq ¹⁶.

Table 1. Antibacterial susceptibility test of *Klebsiella pneumonia*.

Antimicrobial agents	<i>Klebsiella pneumonia</i> isolates from different locations				
	L1	L2	L3	L4	L5
Piperacillin	R	R	R	R	R
Amikacin	S	S	S	S	S
Ceftazidime	I	S	S	R	S
Cefepime	S	S	S	S	S
Aztreonam	R	R	R	R	R
Imipenem	S	S	S	S	S
Amikacin	S	S	S	S	S
Gentamicin	S	R	S	R	S
Tobramycin	S	I	S	I	S
Ciprofloxacin	S	S	S	I	S
Minocycline	R	R	R	R	R
Trimethoprim/Sulfamethoxazole	S	S	S	R	S
Meropenem	S	S	S	S	S

Isolation of bacteriophage

After filtration of Euphrates River water for each location mix culture of bacteriophages were isolated at location 4, which was very close to the Al-Ramadi teaching hospital for maternity and children waste as indicated in Figure 1 and 3. This incidence of bacteriophages in the ecological environment is correlated with their biological host (bacterial cell) and it delivers a valuable data explaining how rivers water was contaminated by infective bacteria, especially intestinal viruses. A recent Iraqi study resolved that sewage of Al-Jumhori hospital in Kirkuk city preformed as a good example of environment for bacteriophages in comparison with other location in the same city. In which each isolated bacteriophage has an inhibitory activity alongside one type of the examined infective microorganisms. Another Iraqi study in Kerbala city used two simple technique for bacteriophage isolation from Sewage water, these isolated bacteriophages displayed a lytic activity against MDR-bacteria ¹⁷. From the overall distance was approximately 2.36 km (5 locations), bacteriophages were isolated from the river samples collected in location 4, in which these bacteriophages displayed a full *Klebsiella pneumonia* cell lysis activity due to growing of plaques as seen in Figure 3. One of the most abundant organisms on the earth is bacteriophages or phages, which infect bacteria. Bacteriophages are usually present in every environment as long as their bacterial host exists (Keen, 2015).

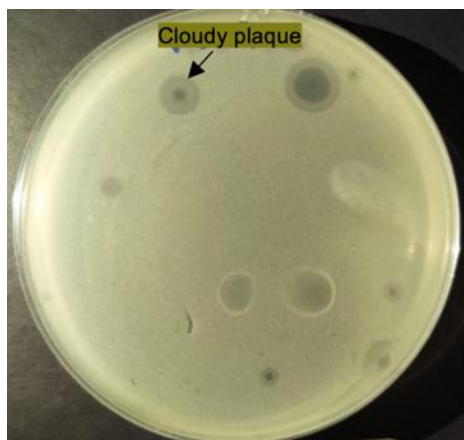


Figure 3. Isolation of bacteriophages. the filtrated water sample in location 4 displayed plaques forming by using double agar overlay method

Purification of *Klebsiella pneumonia* phage

For this purpose, one plaque was taken from petri dish and the refined phage was examined on *Klebsiella pneumonia* by overlay technique in 3 plates as mentioned in Figure 4. After that, five ml of isolated phages were added to SM solution containing 20% glycerol, then kept at -80°C .

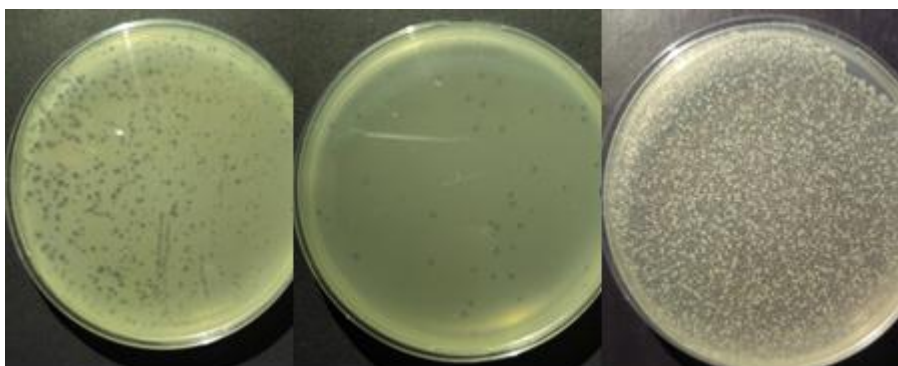


Figure 4. Purification of bacteriophages. Pure plaques were selected from the double agar by sterile tip, re-suspended in SM solution. supernatant fluid was filtered throughout 0.22 μ m sterile syringe filter to acquire phage lysate.

Identification of Extended Spectrum Beta-Lactamase *Klebsiella pneumonia*

The *Klebsiella pneumonia* phage isolated from L4 that showed lytic activity against *Klebsiella pneumonia* was further confirmed that *Klebsiella pneumonia* is ESBL producers by phenotypic assay using the VITEK 2 systems version 08.01. The result was interpreted according to the CLSI guideline criteria¹⁸. This bacterium has a multidrug resistance activity to Ticarcillin, Aztreonam, Minocycline, Piperacillin, Trimethoprim/Sulfamethoxazole and Intermediate to Ticarcillin/Clavulanic acid, Ceftazidime, Tobramycin, Ciprofloxacin while it was susceptible to Piperacillin/ tazobactam, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin. The highly dominant type of antimicrobial resistance mechanism in *Klebsiella pneumonia* is Extended Spectrum Beta-Lactamase^{19,20}.

The purified *Klebsiella pneumonia* from L4 was identified as Extended Spectrum Beta-Lactamase bacterium recovered from Al-Ramadi teaching hospital for maternity and children waste. The highly incidence of *Klebsiella pneumonia* microbes in river sample reveals that this ecosystem has been contaminated with faeces of humans or other animals. The relocation of antibiotics resistant genes could lead to spread in bacterial populaces throughout special approaches like plasmids, transposons or integrons^{14,21}. Bacteria have established special tools to resistant several types of antibiotics and *Klebsiella pneumonia* is considered as a one of major reservoir for Extended Spectrum Beta-Lactamase encoding genes²². Consequently, these phages can be used as an alternate cure in state of antimicrobial agents.

Conclusions

Isolation of bacteriophages can be achieved easily by using very simple procedure from Euphrates River in many regions around the Al-Ramadi teaching hospital for women and children, Ramadi city in Anbar Governorate, Iraq. As a result, the selected bacteriophage delivered an effective lytic activity in contradiction of multi-drug resistant *Klebsiella pneumonia* isolated from the similar location.

Conflict of Interest

No conflict of interest was declared by the author that has an influence on the work reported in this paper.

Acknowledgments

The author would like to thank University of Anbar, College of Science, Iraq for its support in the present work.

Source of funding

The author confirmed that the present work has not gained any funding.

References

1. Alanis, A. J. Resistance to antibiotics: are we in the post-antibiotic era? *Arch. Med. Res.* 36, 697–705 (2005).
2. Zhang, S. *et al.* Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. *Chemosphere* 135, 138–145 (2015).
3. Fawell, J. & Nieuwenhuijsen, M. J. Contaminants in drinking water: Environmental pollution and health. *Br. Med. Bull.* 68, 199–208 (2003).
4. Azam, M. *et al.* Pharmaceutical disposal facilitates the mobilization of resistance determinants among microbiota of polluted environment. *Saudi Pharm. J.* 28, 1626–1634 (2020).
5. Lazăr, V. *et al.* Antibiotic resistance profiles in cultivable microbiota isolated from some romanian natural fishery lakes included in Natura 2000 network. *BMC Vet. Res.* 17, 52 (2021).
6. Jihad, D. M. & Aasim, J. H. Bacterial And Algal Components Of Sediments Close To Sources Of Sewage Drainage In The Euphrates River, Anbar Governorate. *Eur. J. Mol. Clin. Med.* 7, 1383–1392 (2020).
7. Abed HH, ZY, K., Abed SM & Mahdi ZS. Levels of bacterial and chemical pollutants in Euphrates river in Samawah, Iraq. *World J Exp Biosci* 66–69 (2016). doi:2313-3937
8. Popa, L. I. *et al.* Multidrug Resistant *Klebsiella pneumoniae* ST101 Clone Survival Chain From Inpatients to Hospital Effluent After Chlorine Treatment. *Front. Microbiol.* 11, 3403 (2021).
9. Kradin, R. L. & Mark, E. J. 8 - Pathology of Pulmonary Infection. in (ed. Kradin, R. L. B. T.-D. P. of I. D. (Second E.) 143–206 (Elsevier, 2018). doi:<https://doi.org/10.1016/B978-0-323-44585-6.00008-4>
10. Gundogan, N. *Klebsiella*. in (eds. Batt, C. A. & Tortorello, M. L. B. T.-E. of F. M. (Second E.) 383–388 (Academic Press, 2014). doi:<https://doi.org/10.1016/B978-0-12-384730-0.00172-5>
11. Anand, T. *et al.* Phage therapy for treatment of virulent *Klebsiella pneumoniae* infection in a mouse model. *J. Glob. Antimicrob. Resist.* 21, 34–41 (2020).
12. Mahdi Yahya Mohsen, S., Hamzah, H. A., Muhammad Imad Al-Deen, M. & Baharudin, R. Antimicrobial Susceptibility of *Klebsiella pneumoniae* and *Escherichia coli* with Extended-Spectrum β -lactamase associated Genes in

- Hospital Tengku Ampuan Afzan, Kuantan, Pahang. *Malays. J. Med. Sci.* 23, 14–20 (2016).
13. Effah, C. Y., Sun, T., Liu, S. & Wu, Y. *Klebsiella pneumoniae*: an increasing threat to public health. *Ann. Clin. Microbiol. Antimicrob.* 19, 1 (2020).
 14. Navon-Venezia, S., Kondratyeva, K. & Carattoli, A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol. Rev.* 41, 252–275 (2017).
 15. Alharbi, N. M. & Ziadi, M. M. Wastewater as a fertility source for novel bacteriophages against multi-drug resistant bacteria. *Saudi J. Biol. Sci.* 28, 4358–4364 (2021).
 16. H. Abed, H., Kadhim, Z., Abed, S. & Mahdi, Z. Levels of bacterial and chemical pollutants in Euphrates River in Samawah, Iraq. *World J Exp Biosci* 4, 66–69 (2016).
 17. Mohammed, S. H. *et al.* Isolation of various local Bacteriophages via Simple Methods and their Effects against Multidrug Resistance Bacterial Isolates. *kerbala J. Pharm. Sci.* (2018).
 18. Cao, F. *et al.* Evaluation of the efficacy of a bacteriophage in the treatment of pneumonia induced by multidrug resistance *Klebsiella pneumoniae* in mice. *Biomed Res. Int.* 2015, 752930 (2015).
 19. Demirdag, K. & Hosoglu, S. Epidemiology and risk factors for ESBL-producing *Klebsiella pneumoniae*: a case control study. *J. Infect. Dev. Ctries.* 4, 717–722 (2010).
 20. Demir, S., Soysal, A., Bakir, M., Kaufmann, M. E. & Yagci, A. Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in paediatric wards: a nested case-control study. *J. Paediatr. Child Health* 44, 548–553 (2008).
 21. Wyres, K. L. *et al.* Distinct evolutionary dynamics of horizontal gene transfer in drug resistant and virulent clones of *Klebsiella pneumoniae*. *PLoS Genet.* 15, e1008114 (2019).
 22. Ferreira, R. L. *et al.* High Prevalence of Multidrug-Resistant *Klebsiella pneumoniae* Harboring Several Virulence and β -Lactamase Encoding Genes in a Brazilian Intensive Care Unit. *Front. Microbiol.* 9, (2019).