Molecular Characterization of Sewage Bacteriophage and Their Efficiency in Treatment of Antibiotic Resistant Bacteria

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Abstract

Background: The increasing prevalence of multi-drug resistant of bacteria strains, has become a global problem and therefore alternative method to antibiotics must be found. Bacteriophages are considered an alternative agent to control infection and contamination of the bacteria. **Method**: We used RAPD-PCR technique in present study, and used restriction endonuclease conformed presence unknown DNA fragments. **Results:** In this study, we described the isolation and character development of lytic bacteriophages capable of infecting bacteria specifically. Lytic bacteriophages, specific to *Escherichia coli, Klebsiella pneumonia, Salmonella typhi*, clinical strains, was first isolated from sewage. We have analyzed the DNA (RAPD)-PCR technique to produce unique and reproducible band from 21 different bacteriophages infecting *E.coli, K. pneumonia, S. typhi* . Used restriction endonuclease conformed presence unknown DNA fragments. **Conclusion:** Phages was capable of eliciting efficient lysis of studied bacteria, revealing its potential as a non-toxic sanitizer for controlling bacterial infection and contamination in both hospital and other public environments.

Keywords: *Bacteriophages, phage therapy, RAPD-PCR, Restriction endonuclease.*

Introduction

The discovery of antibiotics has revolutionized the treatment of infectious diseases, and because of their overuse, resistance to antibiotics has increased and has become a global problem that must be paid attention to, and to continue to follow the side effects of antibiotics, as at least (2) million people are infected with bacteria that are resistant to some antibiotics every year in The United States alone, and at least 23,000 patients die every year from infection[1]. The danger of these antibiotics do not distinguish or differentiate between beneficial bacteria and bacteria harmful to human. the misuse of these antibiotic enables bacteria to strengthen their resistance against these antibiotic, and they become stronger and more resistant to them[2]. Therefore, the scientists tended to phage therapy. Bacteriophages are viruses found naturally in the environment and have been studied to treat bacterial infections nearly 100 years ago. These phages invade and kill target bacteria by decomposition and do not attack mammalian cells. Phages are specialized for different bacteria. They bind to receptors on the bacterial cell walls to inject the phage DNA into the cell and eventually the cell lysis this in lytic phages[3] . During the lifecycle of the lysogenic bacteriophages, the DNA phage integrate into their host genome and evolve to coexist with bacteria. RAPD-PCR uses a randomized, purified DNA technique for extracted and purified DNA to help describe bacteriophages. RAPD-PCR is also used to assess the genetic diversity of phages[4].

The RAPD reaction is a PCR technique that works to duplicate DNA segments that are unknown and random, using one short primer (8-12 nucleotides), and does not require any prior knowledge of the genome sequence. It also uses very low amounts of DNA, moreover, it is very sensitive, allowing the discovery of a variety of DNA

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as well as mutations. This technique is widely applied in various fields of research (medicine, forensic science, genetically modified organisms, genetic diversity, taxonomy). In addition, the RAPD technique was also applied to detect genetic instability in tumors and DNA changes caused by toxic compounds in animals, bacteria, and plants [5].

The aim of study: Isolation of bacteriophages and study of their genetic content.

Materials and Method

Microbiological culture media and chemicals. The main microbiological culture media we used in this research were BHA (Oxoid,UK), BHI (Oxoid,UK), EMB agar (Oxoid, UK) MacConkey's agar (Oxoid,UK) and Blood agar (Oxoid,UK). The SM buffer [(Tris-HCL, 10 Mm & pH = 7.6;MgSO 4 7H 2 O, 10 Mm; NaCl, 100 Mm and gelatin solution 2%(w/v), 5mL; distilled water, up to 1000 mL] (Merck, Germany). The chemicals we used were Methyl red, SIM, Urea agar, TSI and Simmon's citrate agar that provided from (Syrbio/Syria(.

Sample Collection: Collected samples from various sources including wounds, burns, urine, stool, spinal fluid and blood, were collected under aseptic conditions from Al- Anbar hospitals during the period (September to December 2019). The bacterial samples were carried immediately to the laboratory complex, Microbiology Branch, College of Science, Anbar University, Anbar, Iraq at 4◦C.

Isolation of bacterial species and their characterization

Isolated bacterial strains and exanimated by the compact vitek2 automated method according [6] .

Antibiotic Sensitivity Test: Bacteria strains was subjected to antibiotic resistance evaluation against a set of antibiotic discs including: Tetracycline(TE;10µg), imipenem (IPM;10µg), Meropenem (MEM;10µg), Ciprofloxacin (CiP;10µg), Ceftriaxone(CRO;10µg), Piperacillen (PRL;100µg), Gentamicin (GN;10µg), Chloramphenicol (C;10µg), Trimethoprim(T;12.5µg), and Nitrofurantion(F;100µg) all from(Bio analyses/ Turkey). Antimicrobial sensitivity testing was performed for strains of *K. pneumoniae, E.coli* and *S. typhi* using the disk diffusion method in accordance with National Committee for Clinical Standards guidelines (Clinical and Laboratory Standards Institute, 2019). The antibiotics chosen are usually used for the treatment the multi-drug resistance of bacteria.

Enrichment and isolation of bacteriophages: To isolate bacteriophages, public wastewater of Al-Anbar, Iraq was used as a possible resource .A total of 10 mL of wastewater for 20 min at 3000 g was centrifuged, and the supernatant was filtered using a 0.45µL syringe filter. For the enrichment of bacteriophages, 100 µL overnight bacterial culture and 10 mL wastewater filtrates were applied to 40 mL of BHI flask and then incubated for 24 h at 37C at 120 rpm shaking pace. In the same conditions, 10mL of BHI broth flask was centrifuged and filtered after 24 h. Then for the next stage the supernatant was stored at 4◦C. In order to confirm the presence of phages, 10 µL of phage filtrate was dropped on BHA 's bacterial lawns and then incubated for 24 hours at 37◦C.[7] .

Plaques purification and bacteriophage tittering: Double-layer agar method was used to purify the isolated phages and determine the phage titer. Briefly, phage filtrate was diluted to 10^{-12} using SM buffer then mixed 100 µL of each dilution and 100 µL of overnight bacterial culture and added to 5mL of 45bhc molten BHA top agar (0.5% agar). Then the mixture vortexed and quickly overlaid with 1.5 % agar on BHA media plates. The plates were incubated for 24 h at 37°C and the plaques of bacteriophage were enumerated on each plate. This method was executed in triplicates [8] .

Phage DNA isolation: Phage DNA was extracted as described previously from 100 mL of purified phage stocks previously dialyzed against SM buffer [9].

Genomic fingerprinting by RAPD analysis: Random amplification of polymorphic DNA was carried out according to a modification of the method described previously (Johansson et al., 1995). Primers OPL5(50-ACGCAGGCAC-30) and RAPD5 (50-AACGCGCAAC-30) were assayed at concentration (8 mM). PCR reactions were performed using PureTaq TM Ready To-Go TM PCR Beads (GE Healthcare, Munich, Germany) adding 10ng of purified phage DNA. Reactions were supplemented with 3 mM magnesium oxalacetate and/or 5% v/v dimethyl sulfoxide (DMSO). PCR was performed in a thermocycler (Bio-Rad, Hercules) under the following thermal cycling conditions: one cycles at 94 1 C for180 s; 40 cycles at 94 1 C for 30 s,35 1 C for 45 s and 72 1 C for 30 s (the extension step was increased by 1 s for every new cycle); and a final step of 5 min at 72 1 C.

Results

The identity of bacteria strains was confirmed to be *E.coli,S.typhi* and *K.pneumoniae* by VITEK2 compact system . The antibiotic sensitivity of E.coli, S.typhi and K.pneumoniae isolate was tested using the disc diffusion method and the results showed that all bacteria isolate was sensitive to Meropenem, imipenem but resistant to Ciprofloxacin, Ceftriaxone, and Nitrofurantion .

In Fig.1,2 showed 50 water samples were collected from different sources and 21 isolates of Bacteriophages specific for 9 *S.typhi*, 7 *K.pneumoniae* and 5 *E.coli* bacteria were isolated .by spotted method on solid media and double layer agar method. And the liquid media method.

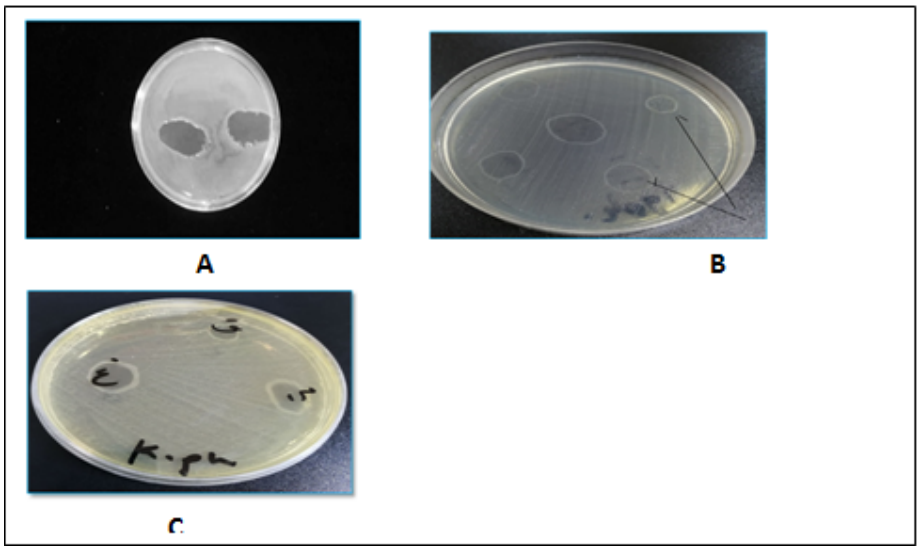


Figure 1. Plaque formation of (A) *E.coli*, (B) *S.typhi*, (C) *K.pneumonia* phages after 24h incubation at 37 °C on BHA

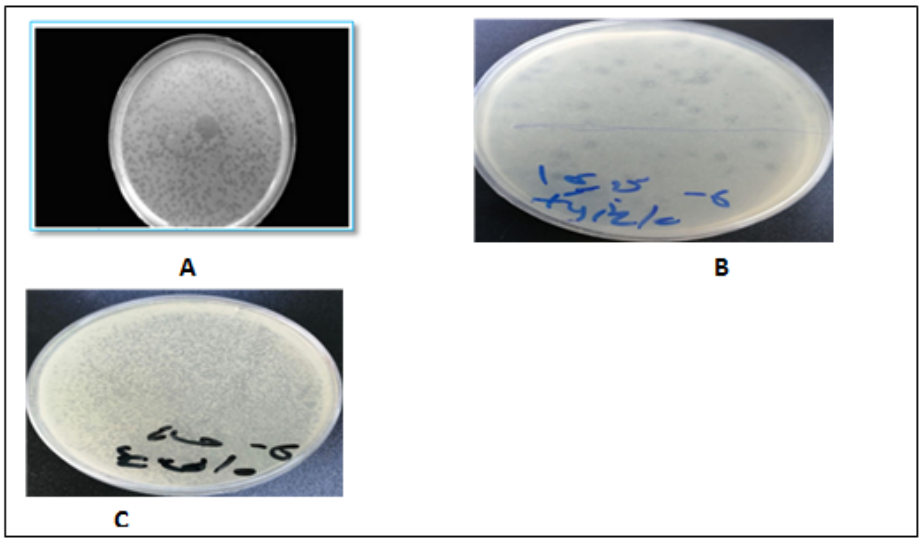


Figure 2. The total titer of (A) *K.pneumonia*, (B) *S.typhi*, (C) *E.coli* specific phages after 24h incubation at 37◦C on BHA.

Extraction genomic DNA: DNA was extracted for all selected bacteriophages with 21 phages, which were isolated from sewage from various sources. The extraction results highlights the presence of DNA bands for all studied phages . The integrity of the extracted DNA was confirmed after gel electrophoresis 1.5%. Fig. 3.

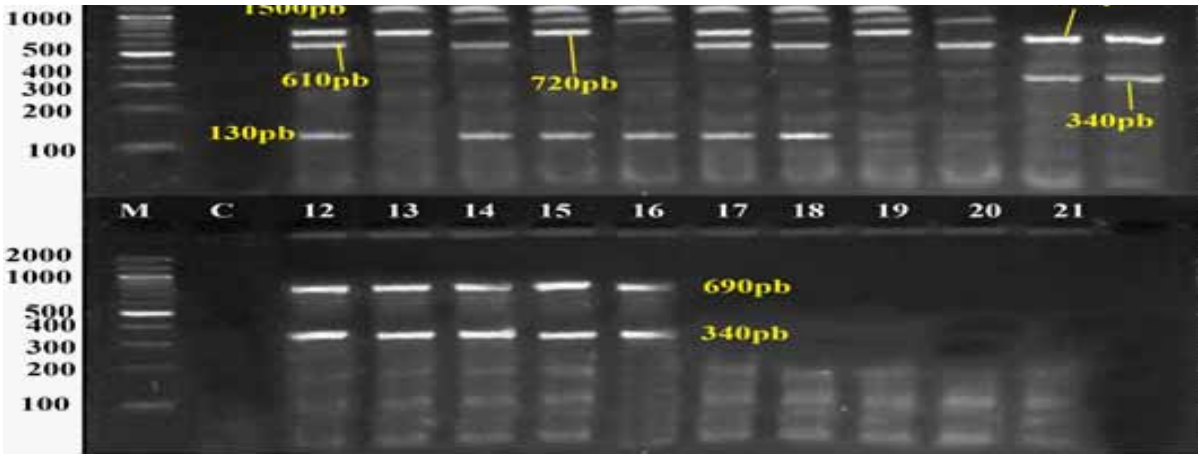
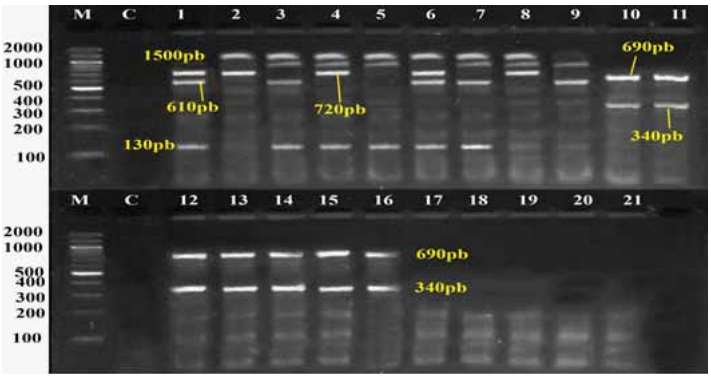
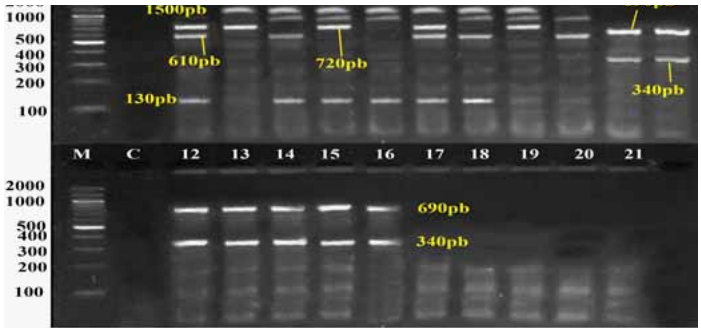


Figure 3. 21 Bands of genomic DNA and (M): DNA ladder (100 bp), agarose 1.5%, at 70 volt for 1 hr.

Random amplification of polymorphic DNA (RAPD-PCR) technique: Fig. 4 shows RAPD-PCR random amplification technique was performed using two primers OPL5, RAPD5 for all phages . This technique showed obvious difference in the number of DNA bands and a marked variation in their molecular weights according to the primer used. In Fig.4a, *S. typhi* phages (1-9) and *K. pneumonia* phages (10-16) showed positive results with OPL5 primer, while *E.coli* phages (17-21) showed negative results. But when using RAPD5 primer showed positive results of all the phages, Fig. 4b.



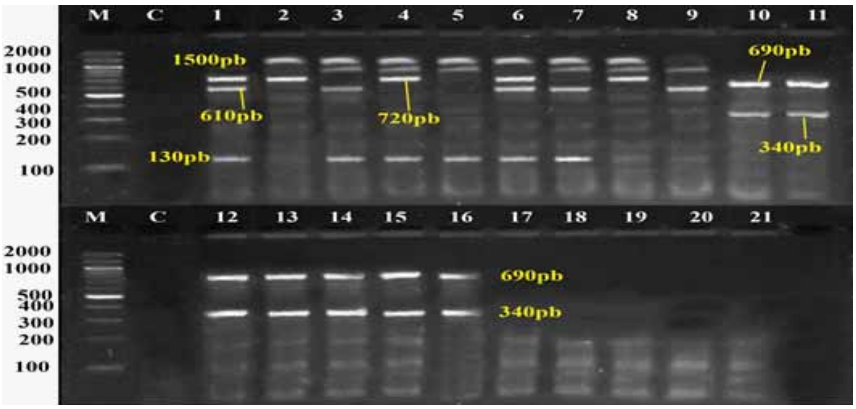
A



B

Figure 4. RAPD PCR products, Gel electrophoresis at 70 volt and 1h on 1.5% agarose gel showing [A] using OPL5 primer lanes positive of *S. typhi* phages(1-9), lanes positive *K.pneumonia* phages (10-16), negative lanes of *E.coli* phages(17-21). [B] using RAPD5 primer, lanes positive of *S. typhi* phages (1-9), lanes positive *K.pneumonia* phages (10-16), positive lanes of *E.coli* phages(17-21). Lane M: DNA ladder (100bp), C: negative control.

Restriction endonuclease enzymes: Four Restriction endonuclease enzymes (NdeI, SspI, Swa I, TaqI) were performed for all phages. The enzymes (Swa I, TaqI) did not produce positive result, while the enzymes (NdeI, SspI,) digested the DNA at more than one site and showed different bands and different weights as positive results. Fig5a, showed *S.typhi* phages(1-9) similar due to similar DNA bands and also similar all *E.coli* phages(17-21), while showed *K.pneumonia* phages (10-16) negative results with using NdeI enzyme. As shown Fig4b *S.typhi* phages(1-9) and *E.coli* phages(17-21) positive results, and negative results of *K.pneumonia* phages (10-16) after using SspI enzyme.



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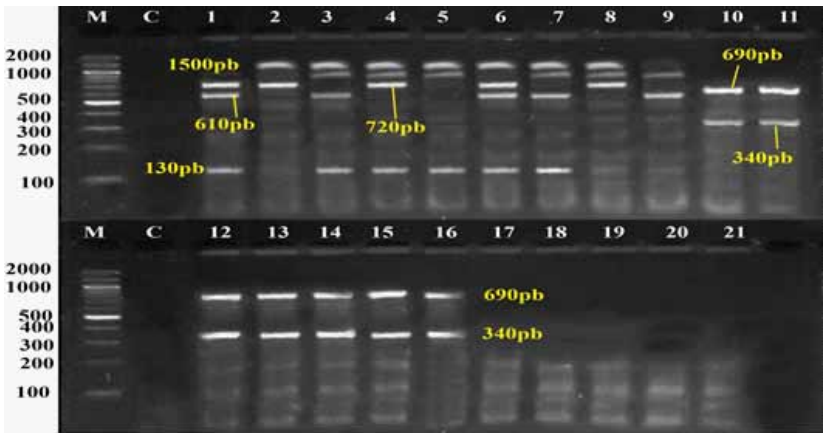

B

Figure 5. Restriction endonuclease products, Gel electrophoresis at 70 volt and 1h on1% agarose gel showing [A]using NdeI enzyme lanes positive of *S.typhi* phages(1-9), lanes negative *K.pneumonia* phages (10-16), positive lanes of *E.coli* phages(17-21). [B] using SspI enzyme, lanes positive of *S. typhi* phages (1-9), lanes negative *K.pneumonia* phages (10-16), positive lanes of *E.coli* phages (17-21). Lane M: DNA ladder (100bp). C: negative control.

Discussion

Increase of multidrug-resistant bacteria and other losses, supporting alternative therapy development. Phage therapy may be seen as an effective method for the prevention and control of bacterial infection [11] . The current study, the lytic and specific phages to the bacterial strains were isolated from different environmental sources; sewage was the main source; this finding is consistent with other studies[12].

The phages are the most abundant organisms in water, feces, soil and sewage water, therefore they are considered good indicators of bacteria presence[13].

RAPD-based method do not require sequence information when designing the PCR primers. We are therefore highly dependent on laboratory conditions such as DNA template concentration, PCR and electrophoretic environments, were selected to test several experimental conditions in order to produce reproducible RAPD patterns and gain a preliminary insight into the power of this approach to discrimination[14].

As shown in Fig. 4 OPL5, RAPD5 primer produced distinct amplicon band patterns . in current study Fig.4a shown S.typhi phages were different among them Whereas, *K.pneumonia* phages were similar these results are disagree with the researchers' findings [15]. The results shown in Fig.4b showed *S.typhi* phages (1-6) were similar after use RAPD5 primer in the RAPD-PCR technique . The phages of *S.typhi* (7,5,3) was similar too. *S.typhi* phages 4,8 differed from the other phages, The results of the current study are consistent with the findings [16] .

Fig.5a, Restriction endonuclease (NdeI, SspI) showed the enzyme's effectiveness in digesting the phage DNA fragments, SspI was more efficient and producing DNA bands. Enzymes can digest DNA at Recognition site or more than in the same strand. NdeI was digested of DNA in the same site and bands of the same molecular weight appeared for *S.typhi* phages lanes(1-9) and *E.coli* phages lanes (17-21), which means that the phages are similar. But *K.pneumonia* phages(10-16) was negative results with using NdeI enzyme, these results disagree with results [17] .

In Fig.5b Endonuclease SspI digesting the DNA phages of *S.typhi*(1-9) and *E.coli* phages(17-21) and produces different bands pattern,which means that the phages are different among them. These results are consistent with [18]. *K.pneumonia* phages(10-16) was negative results after digesting with SspI enzyme.

Conclusions

Phages characterization has shown that it has been very successful in lysing *E.coli, K.pneumonia, S.typhi*, it can be a good candidate for use as an alternative non-toxic green sanitizer. Host range research, however, revealed that phages did not infect other clinical strains of *E.coli, K.pneumonia, S.typhi* included in this review, indicating that more virulent bacteriophages unique to various bacterial strains will be screened and collected in the future. In potential phage applications a pool of lytic phages may be more effective against *E.coli, K.pneumonia, S.typhi* strains.

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Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

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