Journal of Multidisciplinary Sciences
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Biological characterization of two bacteriophages infecting *Klebsiella pneumoniae*

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Received: 08 February 2020; Accepted: 10 April 2020; Published online: 18 April 2020

Abstract. *Klebsiella* became an increasingly important source of community-acquired and nosocomial infections. Extensive broad-spectrum utilization of antibiotics in hospitalized patients has contributed to both increased carriages of *Klebsiella* and the development of multidrug-resistant strains. Many of these strains are highly virulent and show a strong tendency to spread. Bacteriophages viruses because bacterial lysis can serve as a valuable tool for *Klebsiella* infection control. In this study, two lytic phages designated as ØKPAS1 and ØKPAS2 infecting multidrug-resistant *K. pneumoniae* were isolated from sewage samples collected in Assiut, Egypt transmission electron microscopy, the morphology of isolated phages was characterized, and their host range was determined. The morphological analysis revealed that both phages belong to the Podoviridae family. The ØKPAS1 has ahead of about 50 ± 5 nm in diameter and a short tail of 20 ± 2 nm in length, while the ØKPAS2 has ahead of about 53 ± 5 nm in diameter with a short tail of 19 ± 2 nm in length. ØKPAS1 phage showed a broader host range within genus *Klebsiella* since it could lyse 8 out of 15 different *Klebsiella* cultures while ØKPAS2 could lyse only 5 out of 15. Both phages could not infect bacteria from other genera such as *Escherichia coli* and *Salmonella typhi*. The isolated phage ØKPAS1 could survive at a temperature up to 50 ºC and was infective in a pH range between 4.0-9.0, while ØKPAS2 could survive at a temperature up to 60 ºC and was stable over the pH range of 4,0 to10,0. Both phages were stable in chloroform. One-step growth curves of ØKPAS1 and ØKPAS2 revealed that the latent period was 10 min for either phage, with burst sizes of about 120 and 245 pfu/ml for ØKPAS1 and ØKPAS2, respectively.

Keywords: Bacteriophages, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhi*, multidrug resistant

Cite this as: Rawy, D.K., Hemida, S.K., Askora, A., El-Mokhtar, M.A. & Yousef, N. (2020). Biological characterization of two bacteriophages infecting *Klebsiella pneumoniae*. J. Multidiscip. Sci. 2(1), 1-10.

1. Introduction

Klebsiella is one of the most common Gram-negative bacillus bacteria in the Enterobacteriaceae family. Medically, the most important species of this genus is *K. pneumoniae*, followed by *K. oxytoca*. Many *Klebsiella* virulence factors have been characterized, including polysaccharide capsules, fimbriae, serum resistance factors, lipopolysaccharides, and siderophores [1]. *K. pneumoniae* is responsible for hospital-acquired bacterial infections, including pneumonia, bacteremia, urinary tract infections, liver abscess, and wound infections in immune-compromised patients [1,2]. It is also a particular problem for patients with diabetes Mellitus leading to 'diabetic foot' infections and osteomyelitis [1].

Pneumonia caused by *K. pneumoniae* is usually associated with a high mortality rate. Despite the development of broad-spectrum antibiotics, mortality rates greater than 50% have been reported in those infected with *Klebsiella pneumoniae* [3]. Also, treating *Klebsiella pneumoniae* has become more difficult due to the worldwide increase in multidrug-resistant (MDR) strains [4-8]. In effect, about 80% of the nosocomial infections caused by *K. pneumoniae* are due to these-MDR strains [9,10]. At a more destructive end of the spectrum, *Klebsiella*-associated pneumonia, unless treated promptly, results in necrosis of lung tissue,

severe inflammation, and hemorrhage, with consequent potential production of thick, bloody, mucoid sputum that resembles red currant jelly.

The extensive spread of MDR-strains of *Klebsiella* spp. has encouraged the interest in phage therapy, i.e., the use of bacteriophages as a safe and effective modality for dealing with MDR- pathogens [11]. Bacteriophages are viruses that parasitize bacteria; their ability to rapidly kill or lyse bacteria and their specificity make them active antibacterial agents. Also, bacteriophages seem incapable of infecting eukaryotic cells so that they can serve as safe therapeutic agents [12]. Hospital-associated outbreaks of MDR-*Klebsiella* infections are increasingly reported. Therefore, the present work aimed at isolation and identification of bacteriophages from sewage and to test the ability of the isolated phages to induce lysis against different pathogens, particularly *K. pneumoniae*.

2.Materials and methods

2.1. Isolation of *K. pneumoniae*

Different strains of *K. pneumoniae* were isolated initially on MacConkey medium [13] from various clinical specimens, [blood, urine, and sputum] from different infected patients admitted from Assiut University Hospital, Assiut, Egypt. Three additional isolates were obtained from sewage samples. The strains were identified and confirmed as *K. pneumoniae* by the VITEK 2 C1 system (version 04.01, Biomérieux, France) in Clinical Pathology Lab in Assiut University Hospital, Assiut. The isolated strains were stored in Brain Heart Infusion Broth containing 30% glycerol at -70 ºC.

2.2. Antibiotic sensitivity test for *K. pneumoniae* strains

K. pneumoniae strains were tested for their response as resistant, intermediate or sensitive against 10 different antibiotic discs [Amikacin (AK, 30µg), Imipenem (IPM, 10µg), Ampicillin (AM, 10µg), Norfloxacin (NOR, 10µg), Meropenem (MEM, 10µg), Tetracycline (TE, 30µg), Cefoperazone (CEP, 75µg), Ceftriaxone (CRO, 30µg), Amoxicillin-Clavulanate (AMC, 20/10µg) and Aztreonam (ATM, 30µg)] all from Oxoid (England). Antimicrobial sensitivity testing was performed using the Kirby-Bauer disc diffusion method, where the National Committee interpreted the inhibition zone for Clinical Standards guidelines [Clinical and Laboratory Standards Institute [14].

2.3. Isolation, propagation, and purification of *K. pneumoniae* phages

Bacteriophages were isolated from sewage water samples obtained from Assiut, Egypt, by the enrichment technique according to [15]. Phages, if any recovered from lysis zones, were purified and propagated according to [16]. Purified phages were stored at 4 ºC until used.

2.4. Electron Microscopy

A drop of each purified phage suspension (10^8 pfu/ml) was placed on 200 mesh copper grids with carbon-coated formvar films, and the excess was drawn off with filter paper. A saturated solution of 1%Na-phosphotungstate, pH 4.5, was then placed on the grids, and the excess was drawn off as before. The specimens were examined by transmission electron microscope JEOL (JEM -1400 TEM) at 80 KV in the Electron Microscope Unit, Faculty of Agriculture, Mansoura University.

2.5. Determination of phage host range

The host range of the isolated phages was assessed on 15 *Klebsiella* strains and 2 other bacterial strains [*Escherichia coli* and *Salmonella typhi*] kindly provided from Microbiology laboratory, Faculty of Science, Zagazig University, Egypt. Lytic activity was determined against all strains listed in Table 3. Bacterial lawns of isolated *K. pneumoniae* and other bacterial strains were propagated on nutrient plates, and 10µl of isolated and purified phage was spotted on the lawns of tested bacterial strain separately. Following overnight incubation at 37 °C, plates were observed for a clear spot in the bacterial lawn.

2.6. Single-step growth experiment

The single-step growth curve of phages was determined according to the method [17]. Phages were added at an MOI of 1 to the cells of K. pneumonia and allowed to adsorb for 10 min at 37 ºC. The mixture was then centrifuged (10,000 × *g*, 10 min), and the pellet containing infected cells was suspended in 10 ml nutrient broth followed by incubation at room temperature. Then

samples of 1ml were taken at equal intervals (10 min) for 80 min, immediately diluted, and titrated by the double-layer technique. The first set of samples was immediately diluted before titration. The second set of samples was treated with 1 % (v/v) chloroform to release intracellular phages to determine the eclipse period.

2.7. Effect of chloroform on phage stability

Aliquots of phage suspensions with high titers 10^8 $PFUmL^{-1}$ mixed with chloroform and incubated at 4 °C for 24h.The phage titers were tested by the double-layer agar method.

2.8. Physical properties of the isolated phages

2.8.1. Effect of heat on phages

The effect of temperature on bacteriophages was studied at different temperatures (30, 40, 50, 60,70, 80, 90, and 100 °C). A known titer of purified samples of bacteriophages was incubated at different temperatures for 10 min each. Phages infectivity was assayed by the double overlayer agar technique [15].

2.8.2. Effect of pH on the phage stability

Each phage sample with known titer was incubated at different pH values [3, 4, 5, 6, 7, 8, 9, 10, 11 and 12] adjusted using 0.1mol/L HCL or NaOH] for 16 hours at 4 °C and then checked for survival by the double overlayer agar technique [15].

2.9. Statistical analysis

The results were, whenever needed, analyzed statistically to one-way ANOVA by SPSS 19.0 software program. Tests were considered statistically significant at a p-value of ≤ 0.01.

3. Results and discussion

3.1. Identification of *K. pneumoniae*

Fifteen strains of *Klebsiella* bacteria isolated in the present study were identified as *K. pneumoniae* by VITEK 2 C1 system. Sources of the isolated strains are blood, urine, sputum, and sewage (Table 1). Biradar et al. [18] isolated *K. pneumoniae* from blood, urine, sputum, and pus and throat swab. *K. pneumoniae* was also detected in fecal contamination water from Modimola dam, South Africa [19].

Table 1. List of *Klebsiella* strains and their sources

***Klebsiella* strain**	**Source**
KP16	Blood
KP80	Blood
KP87	Blood
KP43	Urine
KP81	Urine
KP111	Urine
KP114	Urine
KP38	Sputum
KP53	Sputum
KP77	Sputum
KP143	Sputum
KP126	Sputum
KP59	Sewage
KP61	Sewage
KP75	Sewage

Numerous pathogenic bacteria carry genes that make them resistant to most known antibiotics [20]. Moreover, the ability of these bacteria to form biofilms further strengthens their ability to resist antibiotics [21]. Hospital-acquired pneumonia caused by *Klebsiella pneumoniae* is always a threat and a fastidious public, human health problem [22]. Despite advances in antimicrobial

therapy, morbidity and mortality remain high and out of control. **Table 2** indicates that *k. pneumoniae* strains, numbers 14, 14, and 13 were resistant to Cefoperazone, Ceftriaxone, and Azetreonam, respectively. Also, but in the second class, 10 strains showed resistance against Ampicillin and Amoxicillin Clavulanate. On the other hand, strains: KP143, KP80, KP111, and KP59 of *K. pneumoniae* exhibited resistance response against 10, 9, 8, and 8 antibiotics, respectively. However, 70% of antibiotics were active on the KP16 strain and 60% on either KP87 or KP53 strains. These results are in line with those reported by Shawkey et al. [23]. All of their *K. pneumoniae* isolates were resistant to all tested beta-lactamase inhibitor combinations. Therefore, the need for an alternative to antimicrobial becomes an urgent necessity to combat this MDR pathogenic bacteria. Bacteriophages are considered a possible alternative to many antimicrobials against multi-drug pathogens [24]. Bacteriophages constitute most biological organisms on Earth and have essential effects on bacterial evolution [25-27].

Table 2. Antimicrobial susceptibility test of K. pneumoniae strains.

Antibiotic / *Klebsiella* strains	Amikacin	Meropenem	Tetracycline	Ampicillin	Amoxicillin-Clavulanate	Norfloxacin	Cefoperazone	Aztreonam	Imipenem	Ceftriaxone
KP16	S	S	S	R	S	S	R	S	S	R
KP80	R	R	I	R	R	R	R	R	R	R
KP87	S	S	S	S	S	I	R	R	S	R
KP43	S	S	S	R	R	I	R	R	I	R
KP81	I	R	I	R	R	S	R	R	R	R
KP111	I	R	R	R	R	S	R	R	R	R
KP114	S	I	R	S	R	S	R	R	I	R
KP38	S	R	S	I	S	S	R	R	I	R
KP53	S	S	S	S	I	S	R	R	S	R
KP77	R	R	S	R	I	S	R	R	S	R
KP143	R	R	R	R	R	R	R	R	R	R
KP126	I	R	R	I	R	I	R	R	R	R
KP59	I	R	R	R	R	S	R	R	R	R
KP61	S	R	I	R	R	S	R	R	R	R
KP75	S	I	I	R	R	S	I	S	I	I

S: sensitive strain, **R**: resistant strain, **I**: intermediate strain

3.2. Morphological characterization of the isolated *K. pneumoniae* phages

Morphological features of purified phages designated as ØKPAS1 and ØKPAS2, which are visualized by TEM, are shown in Figures 1 and 2.

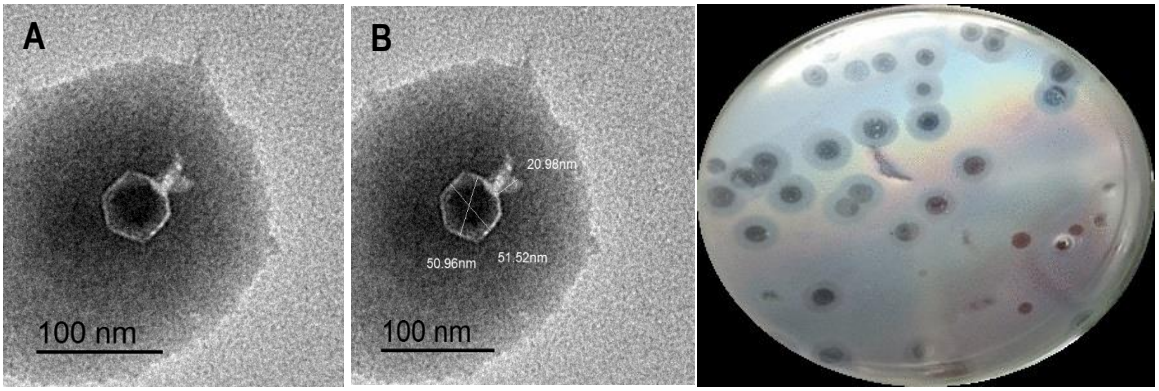

Figure 1. Electron micrographs **(A+B)** and plaque morphology **(C)** of ØKPAS1

Both phages (ØKPAS1and ØKPAS2) seemed to belong to the Podoviridae family, characterized by the icosahedral head with a short non-contractile tail [28]. Figure (1A, B) showed that the head of the ØKPAS1 has a diameter of 50 ± 5 nm and a tail with 20 ± 2 nm. Those measures of ØKPAS2 were 53 ± 5 nm and 19 ± 2 nm for head and tail, respectively (Figure 2A, B). Regarding plaques morphology, ØKPAS1 appeared with the turbid center and clear area (Figure 1C) whereas, ØKPAS2 plaques of phage exhibited with turbid center and area (Figure 2C).

The halo around the plaque indicates the decapsulation of the bacterial host cell by phage-produced soluble enzymes such as depolymerase [29]. Early studies showed that specific *Klebsiella pneumoniae* bacteriophages produced depolymerase during phage proliferation and released the enzyme from infected bacteria that targeted another bacterial capsular polysaccharide (CPS) [30]. Morphologically the two phages were classified as podoviruses according to the electron micrographs. The dimensions of the isolated podoviruses were similar and resembled those previously isolated for *K. pneumoniae*. Earlier works on phages such as Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22 against *Klebsiella pneumoniae* indicated that these phages belong *Podoviridae* [31]. Morphological characteristics are considered necessary in phage taxonomy [32], and 96% of all phages investigated in the last 45 years have turned out to be members of the *Siphoviridae*, the *Myoviridae*, or the *Podoviridae* [33].

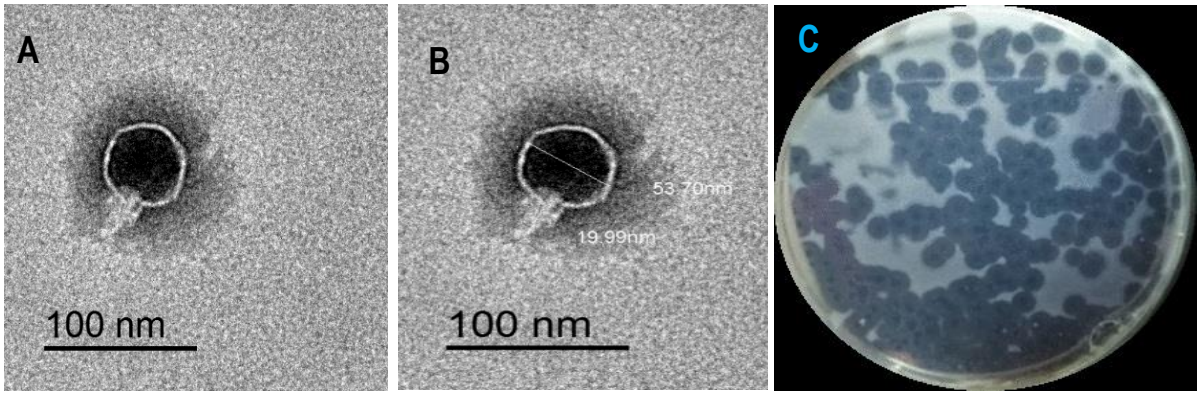

Figure 2. Electron micrographs (A+B) and plaque morphology(C) of ØKPAS2

3.3. Host range of *K. pneumoniae* phages

ØKPAS1 and ØKPAS2 phages were spotted on 17 strains of bacteria; these were *K. pneumoniae* (15 strains), *E. coli* (1 strain), and *S. typhi* (1 strain). Results in Table 3 showed that ØKPAS1 phage exhibited to some extent a broad host range within *Klebsiella* strains, where it was able to lyse 8 of 15 strains. On the other hand, ØKPAS2 phage was able only to infect 5 strains. Both phages could not infect *E. coli* and *S. typhi.* Results also revealed that both phages share their ability to infect KP87, KP53, KP143, and KP75 strains, whereas 4 *Klebsiella* strains were lysed ØKPAS1 not with the other phage. The host range is an essential characteristic factor, making the bacteriophage a potential therapeutic agent against bacterial infections [34]. The presented results were consistent with previous documents that bacteriophages are highly specific and can only infect a single species of bacterium, usually a subset of strains within that species [35,36]. Additionally, results seem to indicate a broad host range within genus *Klebsiella,* and this correlates with the idea that some phages have strain level specificity. In contrast, others have a broader host range infecting only multiple species' strains to closely related several species [37].

3.4. Phage growth characteristics

As described above, phages ØKPAS1 and ØKPAS2 are lytic podoviruses with broad host ranges, to some extent infecting strains of *K. pneumoniae*. One-step growth experiment allows identifying the effect of changes in the yield of virus per infected host cell and chemical and physical properties on the period of an infectious cycle [15]. Through the one-step growth experiment, the latent period and burst size were measured for ØKPAS1 and ØKPAS2phage. These two parameters are influenced by the host culture, the incubation temperature, the medium on which the experiment was done, and the specific growth rate [38]. Figure 3 shows the results of the one-step growth experiment of ØKPAS1 and ØKPAS2. The two phages exhibit a short latent period of 10 min, followed by a rise period of 20 min. Moreover, the one-round cycle of infection for both phages took approximately 30 min. However, the average burst size was ~120 $\times 10^{10}$ pfu per infected cell for ØKPAS1. While for phage ØKPAS2 the average burst

size was approximate ~245×10⁹ pfu per infected cell. These results are similar to those of a previous study conducted by Abedon [39] relating the phages with a short latent period that can utilize the high bacterial host more quickly to proliferate. Therefore, it indicates *Klebsiella pneumoniae* in high density in the sewage water sample where rich nutrient supports the rapid growth of host culture.

Table 3. Host range of *K. pneumoniae* phages against different strains.

phage / *klebsiella* strain	ØKPAS1	ØKPAS2
KP16	-	-
KP80	-	-
KP87	+	+
KP43	-	-
KP81	-	-
KP111	+	-
KP114	+	-
KP38	+	-
KP53	+	+
KP77	+	-
KP143	+	+
KP126	-	-
KP59	-	-
KP61	-	-
KP75	+	+
E. coli	-	-
S. Typhi	-	-

(+) This indicates that the phage gave lytic area. (-) indicates that no lytic area

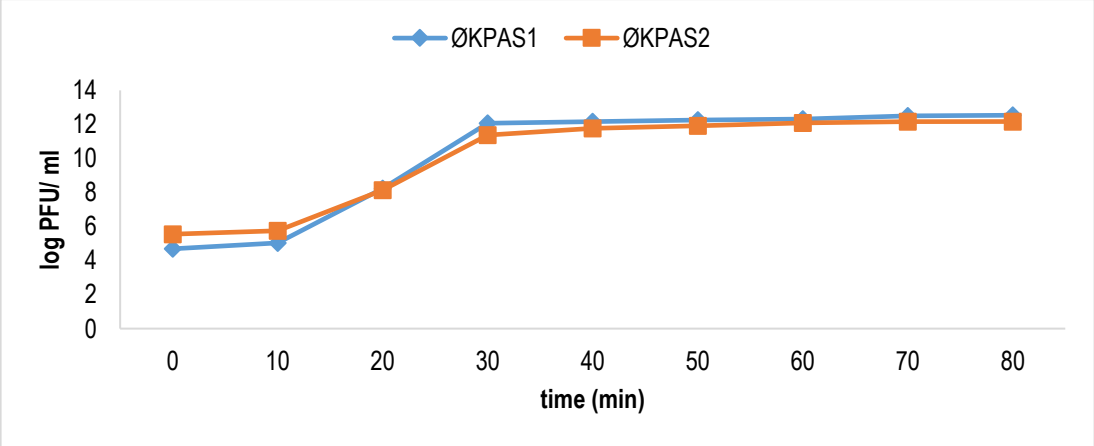

Figure 3. Single-step growth curve for *K. pneumoniae* phages. ØKPAS1, the plaque-forming units (PFUs) per infected cell in cultures of *K. pneumoniae* at different times post-infection are shown. Samples were taken at intervals. ØKPAS2, the plaque-forming units (PFUs) per infected cell in cultures of *K. pneumoniae* at different times post-infection are shown.

3.5. Effect of temperature

ØKPAS1 phage was found to be near heat-stable, over 10 min between the temperature range of 30-60 ºC where no significant loss in phage activity was obtained. ØKPAS2 was also found to nearly heat stable as over 10 min but between a temperature range of 30-70 ºC. The percentage of phage survival was not significantly affected (Figure 4). Temperature is a crucial factor affecting the stability of bacteriophages [40,41]. Moreover, the temperature is controlling in viability, occurrence, and storage of phages [42]**.** The infectivity of ØKPAS1and ØKPAS2 was entirely inhibited by temperatures above 60 ºC and 70 ºC,

respectively. Interestingly, ØKPAS1 and ØKPAS2 phages were survived at 37 °C, without significant loss in phage particle number, which is a significant parameter for phages considered for therapeutic application. These results agree with those previously revealed by Fang Cao et al. [43]; their phage 1513 was stable for short time storage at 37 °C.

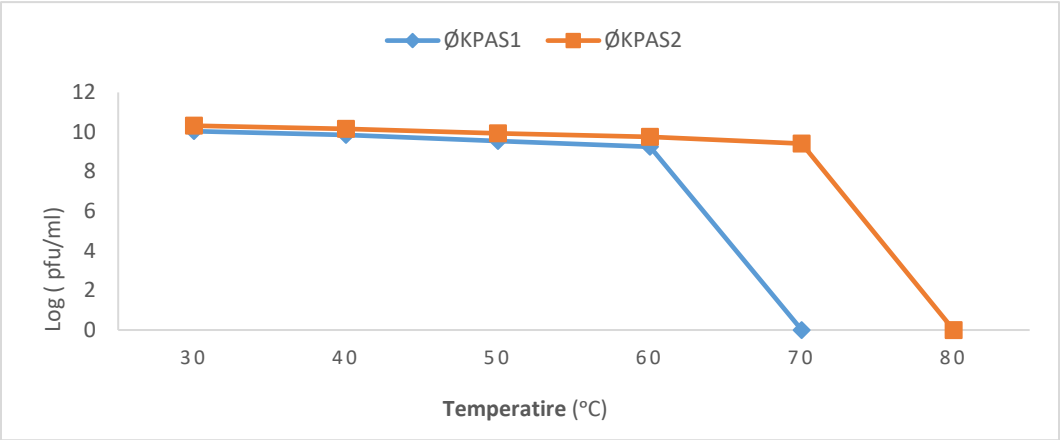

Figure 4. Effect of heat on the *K. pneumoniae* phages stability (ØKPAS1 and ØKPAS2)

3.6. Effect of pH values on phage infectivity

Results represented in Figure 5 showed that the survival of 100% of ØKPAS1 and ØKPAS2 infectivity was at pH 6.0 and pH 7.0; respectively, the infectivity of either phage was decreased by acidity or alkalinity. It was worth mentioning that ØKPAS2 remained active till pH 10, wherever the infectivity of both phages was completely diminished at pH 3.0 and pH 11. The acidity or alkalinity of the environment is another crucial factor in phage survivability [44]. Our findings are in agreement with the results reported by Fang Cao et al. [43], where phage 1513 was susceptible with strong acid and alkaline pH (pH < 3 or pH > 10) and was relatively stable at pH between 6 and 9 at 4 °C for 10 h. As ØKPAS1 and ØKPAS2 are stable for a wide range of pH, they can be used as a therapeutic agent for *Klebsiella pneumoniae* induced Urinary Tract Infection (UTI). It can be used in the impregnation of urinary catheters to inhibit bacteriological biofilm, as previously suggested by Verma et al. [45].

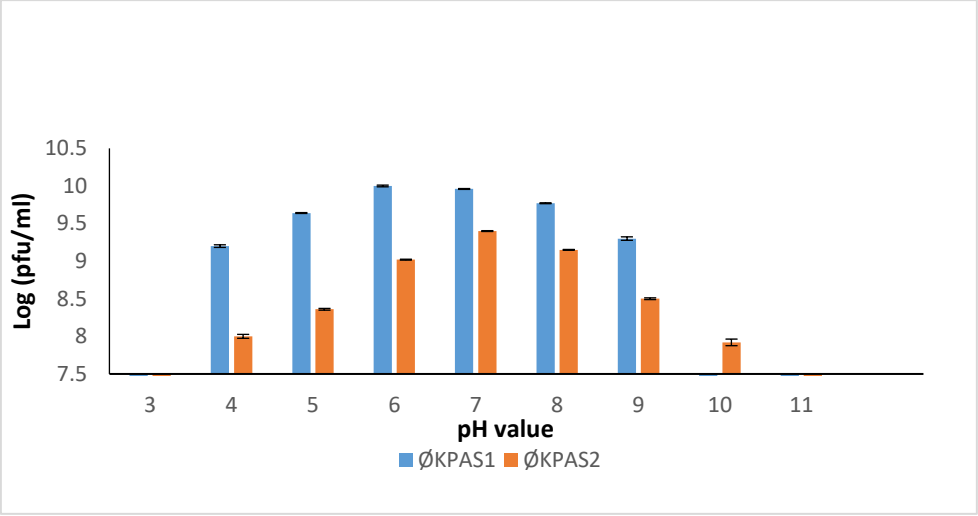

Figure 5. Histograms represent the effect of pH values on infectivity of ØKPAS1 and ØKPAS2

3.7. Effect of chloroform on phage infectivity

The stability of phage in the different organic solvents depends mainly on the stability of its protein, which is influenced by the solvent's ability to strengthen or destroy specific inter and intramolecular hydrophobic and electrostatic interactions [46]. Results in Figure 6 showed that ØKPAS1 and ØKPAS2 phages were not chloroformed sensitive, where no significant changes in phage infectivity were recorded. The best concentration of chloroform for phage preparation and preservation is (1/10) (v/v). ØKPAS1 and ØKPAS2 phages were found to be resistant upon exposure to chloroform in a chloroform-aqueous environment which paves the way for storage of ØKPAS1 and ØKPAS2 in chloroform which will keep the phage solution from bacterial contamination as chloroform is a known antimicrobial agent. It has been used often in preparation of phage stock. Findings are correlated with the results reported by Othman et al. [47], where chloroform did not affect φBM phage viability for 30 min at room temperature. They also mentioned that titers before and after chloroform treatment were the same, indicating chloroform resistance of the φBM phage. The presented results also agreed with Fang Cao et al. [43], where phage 1513 was not chloroformed sensitive, which is vital since chloroform was involved in the purification procedures.

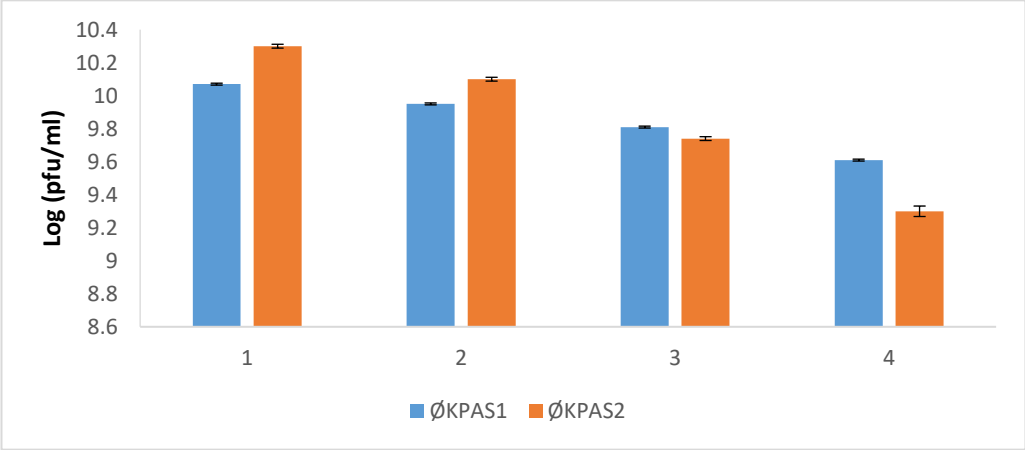

Figure 6. Histograms showed the effect of chloroform on a different volume of ØKPAS1 and ØKPAS2.

4. Conclusion

Bacteriophages could be an essential alternative to antibiotics, especially for the treatment of multidrug-resistant bacteria. Two phages infecting strains of *K. pneumoniae* were isolated from sewage collected in Assiut, Egypt. The results demonstrated that the two *Klebsiella* phages are pretty stable under pH fluctuation, heat-stable, and remain stable in Chloroform. ØKPAS1 and ØKPAS2 Phages were found to be specific to *Klebsiella pneumoniae* but not to *E. coli* and *S. typhi*. The larger bust size and a shorter latent period of the two phages improved the efficiency of phage therapy. Based on these results, the two phages could be suitable for antimicrobial agents against multidrug-resistant *K. pneumoniae*.

Conflicts of interest. The authors declare that there is no conflict of interest.

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