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Biological and physical characterization of bacteriophage JHA against multidrug-resistant *Acinetobacter baumannii*

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Abstract: Due to the emergence of antibiotic resistance, bacteriophage therapy appears to be an ideal weapon to utilize against pathogenic bacteria. This study aimed to isolate, identify and characterize the lytic bacteriophage effective against the multidrug-resistant *Acinetobacter baumannii* clinical isolates. The isolated bacteriophage caused lysis by applying the double-layer agar technique on *A. baumannii* up to 99% in 18 hours of incubation at 37ºC. The bacterial growth reduction assay exhibited that JHA phage had high adsorption rates and could rapidly inhibit bacterial growth. The pH and thermal stability testing showed that JHA phage was stable in vast ranges of pH from 5 to 9 but its activity was highest at pH7 (1860000±1000 pfu/mL). It was stable in broad ranges of temperatures from 25ºC to 60ºC but the highest activity was found at 37ºC (1300000±30000 pfu/mL). One-step growth test results showed that it has a short latent period, strong lytic ability, high burst size, and adsorption rates and was host specific. Scanning electron microscopy (SEM) of JHA phage demonstrated icosahedral heads and tailless particles. Transmission electron microscopy (TEM) revealed JHA phage belongs to *Tectiviridae* family. All the characteristics of JHA phage possess lytic activity against *A. baumannii* strains and exhibit novel candidates to use as an alternative competitor to antibiotics in controlling such infections.

Keywords: *Tectiviridae,* Lytic bacteriophage, MDR, scanning EM, transmission EM, *Acinetobacter baumannii*.

INTRODUCTION

Acinetobacter is one of the clinically significant pathogen emerging as MDR bacteria that pose threatening remarks in hospitals, immunosuppressed patients and nursing homes. These bacteria have developed resistant mechanisms against numerous antibiotics, including carbapenems and third-generation cephalosporins which are the best accessible antibiotics for treating multi-drug resistant bacteria. *A. baumannii* is known as the most imperative opportunistic pathogens in nosocomial infections especially, in intensive care units (ICUs) (Cerqueira & Peleg, 2011). In recent years, *A. baumannii* becomes "red alert" because of a broad range of antibiotic resistance rates and became a challenge for medical professionals and researchers (Cerqueira & Peleg, 2011).

Bacteriophages are omnipresent on our earth. Their highlighted host-specific nature only targets the bacteria against which they are purified or isolated (Clokie *et al*., 2011). Phage therapy is the administration of bacteria-specific viruses (phages), as a bactericidal agent to combat uncontrolled, and undesired multidrug-resistant bacteria that cause multiple infectious diseases (Kutter *et al*., 2010). The worldwide proliferation of disease-causing bacteria that are resistant to a majority of antibiotics alarmed to reduce modern medicine to a state similar to the pre-antibiotic era (N Ahmed *et al*., 2019). To break this dangerous cycle, it will be compulsory to adopt chemotherapy-independent salutary strategies to treat bacterial infections (Matsuzaki *et al*., 2005).

Bacteriophages that lyse the Gram-positive organisms interact with peptidoglycan components, lipoteichoic acids, and related proteins (Clokie *et al*., 2011; Parveen *et al*., 2020). While phage interaction in gram-negative organisms exhibited protein-membrane present outside like porins, lipopolysaccharide components, and transport proteins, the polysaccharide K antigens of the capsule and pili or flagella (Haq *et al*., 2012). Bacteriophage activity against *A. baumannii* which is a gram-negative organism works in this way. The specific property of receptors present on the single phage strain could identify its targeted host selection (Zahra *et al*., 2021). Some phages show specificity up to the strain level, whereas various phages are more broad-spectrum and can infect many strains (Ahmed *et al*., 2020; Yang *et al*., 2010). Keeping in view the significance of the emerging prevalence of resistant strains of *A. baumannii* this research work was designed to ascertain the effect of bacteriophage against clinical isolates of *A. baumannii* and to discourage the use and misuse of antibiotics in order to combat antibiotic resistance globally.

MATERIALS AND METHODS

Sample collection and bacteriophage isolation

The JHA phage was isolated from two different sewage

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samples (250mL each) in sterile screw-tight bottles from tertiary care hospitals in Lahore, Pakistan. The sewage water samples were centrifuged at 4000 rpm for 5 minutes and the supernatant was collected. For phage isolation, 5 X Luria Bertani broths medium was used followed by 1% chloroform to kill bacterial cells. The Spot test was performed for confirmation of the presence of phage before the double-layer agar technique and to ensure the lytic activity of phage (Bibi *et al*., 2016). Plaques with the same morphology were picked and stored at 4°C or -80°C in 5% glycerol (Yang *et al*., 2010).

Host bacterial strains

All the bacterial isolates of *A. baumannii* (n=10) used in this study were collected from a tertiary care hospital in Lahore, Pakistan and were sub-cultured onto LB agar Petri plates followed by incubation at 37°C for 24 hours. For further confirmation, API 10S strip was used to evaluate the biochemical profile of *A. baumannii*. Furthermore, antibiotic susceptibility testing was done to select MDR bacterial strains for this study. For this purpose, imipenem (IPM) 10µg, meropenem (MEM) 10µg, cotrimoxazole (CTX) 30µg, piperacillin/ tazobactam (TZP) 110µg, gentamicin (CN) 10µg, chloramphenicol (C) 30µg, ertapenem (E) 10µg, amikacin (AK) 30µg, ampicillin (AMP) 30µg, ciprofloxacin (CIP) 5µg, cefuroxime (CXM) 30µg, ceftriaxone (CRO) 30µg, tetracycline (TE) 30µg, amoxicillin/clavulanate (AMC) 25µg was used.

Host range determination of JHA phage

For the determination of host specificity, the JHA spot test was performed against each of the 10 MDR species. All these MDR bacterial strains were clinical pathogens obtained from Microbiology Laboratory, University of Central Punjab including Acinetobacter spp (n=10), E. coli spp (n=10), MRSA spp (n=10) and Pseudomonas spp (n=10). For the spot test, 10µl of phage lysate spots were placed over the bacterial lawn and incubated at 37°C for 24 hours. A clear zone of inhibition indicates the presence of lytic phage against that specific MDR bacterial strain (Yang et al., 2019).

Thermal and pH stability testing of JHA phage

The thermal and pH stability testing of isolated phage was checked by incubating it at varying temperature and pH conditions with its host followed by determination of virus titer and statistical analysis. Briefly, JHA phage lysate (1×10^9pfu/mL) was incubated at 37°C for one hour in different pH ranges from (5.0 to 9.0). Similarly, for thermal stability testing the JHA phage (1×10^9 Pfu/mL)was incubated at different temperatures (4°C, 25°C, 37°C, 45°C and 60°C) for one hour. The incubated phages were combined with bacterial culture and the double-layer agar method was performed to calculate differences in titer (Capra *et al*., 2006). All data were expressed as Mean pfu/mL ± standard deviations (SD) and was statistically analyzed.

One-step growth curve

For this experiment, exponentially grown bacteria were taken in 25 ml of LB broth at 37°C until optical density (O.D) at 600nm reached 0.2 to 0.3. Cells were collected by centrifugation at 4000rpm for 10 mins and re-suspended in 500µl of LB broth followed by the addition of bacteriophage lysate (1×10^8pfu/mL). The mixture was centrifuged at 11000 rpm for 30 seconds to remove unabsorbed free phage and the virus titer was then evaluated by the double-layer agar method. The pellet was collected and re-suspended in 25ml of fresh LB broth and incubated at 37°C. Later than every 5 minutes, 2ml of sample was withdrawn and their virus titer was achieved by the double-layer agar technique (Kutter *et al*., 2010).

Bacterial growth reduction assay

An important method to evaluate the lytic potential of phage growth, a growth reduction assay was performed. Briefly, two flasks of 50ml of autoclaved LB broth were taken and 100µl of bacterial culture was each added to each flask. Then, 100µl of JHA phage lysate was added to one of the flasks while 2nd flask was kept as a control. Both flasks were incubated with continuous shaking of 120 rpm at 37°C for 12 hours. Repeatedly after every 2 hours, 2ml of the sample was taken from each flask and O.D was checked at 600 nm using a spectrophotometer. The graph was plotted between O.D and incubation time to analyze the lytic ability of the isolated JHA bacteriophage (Haq *et al*., 2012).

Morphological examination of bacteriophage by SEM and TEM

The bacteriophage lysate sample was prepared and syringe filtered (0.45 and 0.20 µm (Minisart, Sartorius Stedim Biotech). The lysate activity was checked by positive control. The filtered sample was sent for Scanning Electron Microscopy. The facility was provided by Interdisciplinary Research Centre in Biomedical Materials COMSATS Institute of Information Technology-Lahore.

For TEM, the sample was prepared by collecting phage buffer containing phage sample (10ml), followed by centrifugation at 12000 rpm for 10min, supernatant (10µl) was collected, diluted and syringe filtered. The new lysate viability was also checked by positive control and the filtered lysate was analyzed by transmission electron micrograph (TEM) for morphological examination of bacteriophage. The facility was utilized commercially by the National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan.

After evaluating the results of SEM and TEM, the JHA bacteriophage was allocated to the respective family concerning proposed guidelines of the International Committee on Taxonomy of Virus (ICTV) charged with leading examination of virion particle morphology (ICTV2005).

Storage in different conditions

The isolated phage was stored at different temperatures (-20°C, 4°C, 37°C and room temperature) for one month to evaluate the most appropriate storage states for its survival. The difference in titre was evaluated by the double-layer agar technique.

STATISTICAL ANALYSIS

The data was recorded in SPSS software version 22.0 for statistical analysis. The data were analyzed for mean and standard deviations (SD) values first. Two-way ANOVA test was run to check the significance of the data. A p-value of < 0.05 was considered statistically significant.

RESULTS

The phage was isolated from the sewage water of Jinnah hospital and against *A. baumannii* strain therefore it was named JHA (J for Jinnah, H for hospital and A representing the *A. baumannii*bacteria). JHA phage shows clear plagues over the lawn of the host, which shows the virulence ability of isolated phage.

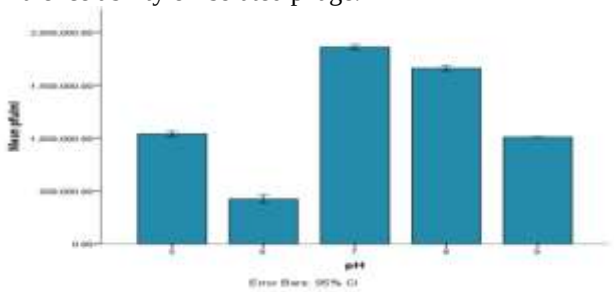

Fig. 1: The effect of pH on JHA phage stability treating at different pH 5, 6, 7, 8 and 9 incubated for 60 minutes and represented by the phage titer by double-layer agar technique.

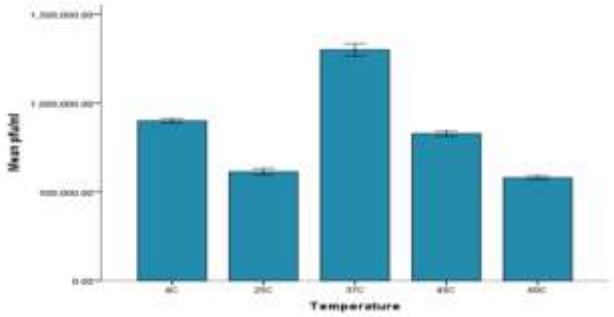

Fig. 2: Effect of temperature on JHA phage by giving stress at 4°C, 25°C, 37°C, 45°C and 60°C for 60 minutes and represented by phage titer by double-layer agar technique (p< 0.05).

Host specificity and antibacterial activity by spot test

The isolated JHA phage showed a zone of inhibition against specific *A. baumannii spp.* exhibiting clear round spots (plaques) of almost 2-3 mm diameter by double-layer agar technique. While none of the other tested MDR strains of *MRSA, Ecoli, Pseudomonas spp.* and *A. baumannii* was susceptible to infection by JHA bacteriophage. Host range determination test results showed that JHA is very host-specific having no lytic potential with other test strains.

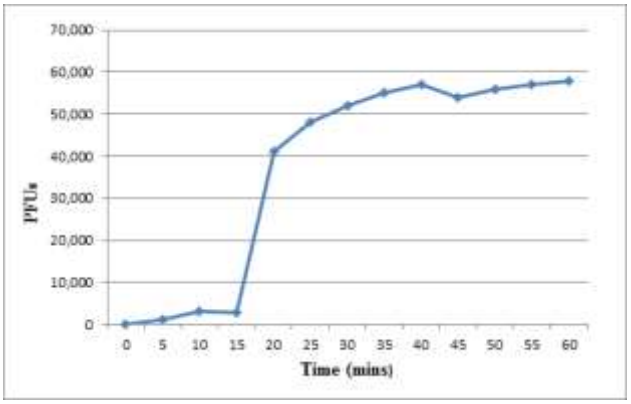

Fig. 3: One-step growth curve of JHA phage to estimate the Latent period and growth pattern of phage using double layer agar technique (p< 0.05).

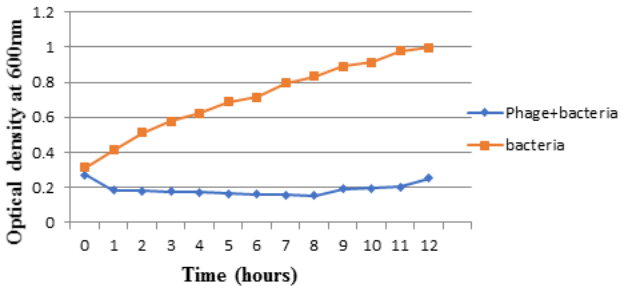

Fig. 4: The effect of JHA phage on the growth of MDR *A. baumannii* with the time calculated by spectrophotometer at O.D 600nm (p< 0.05).

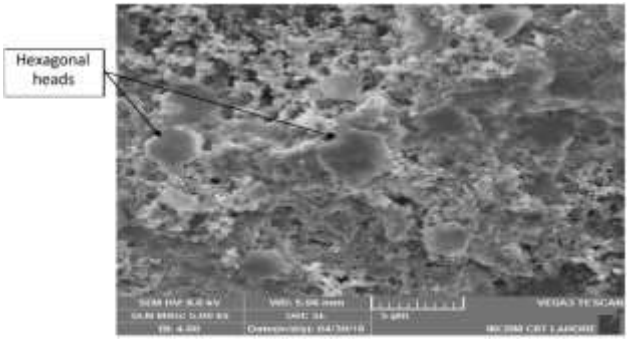

Fig. 5: Scanning electron micrograph of JHA phage exhibiting hexagonal heads.

Physical characterization of phage

The isolated JHA bacteriophage was characterized by physiological parameters including pH stability and thermal stability using the double-layer agar technique. The phage titer was calculated at each physiological parameter. All data were expressed as mean pfu/mL ± standard deviations (SD). The pH stability testing results showed that JHA phage was stable in both acidic and

basic pH conditions from pH 5 to pH 9 but the highest mean titre stability (Mean pfu/mL ±S.D)was obtained when incubated at pH 7 (1860000±1000 pfu/mL) while least mean titre stability (Mean pfu/mL ±S.D) at pH 6 (423333.3333 ± 15275.25232 pfu/mL) ($p \leq 0.05$). Plagues were found in the highest number showed active infectivity at pH 7 as represented in fig. 1.

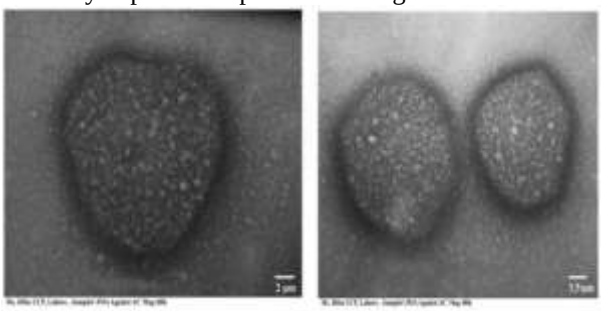

Fig. 6: Transmission Electron Micrograph of JHA phage (a) 2µm (b) 3.5µm. Non enveloped icosahedral head with the absence of a contractile tail.

Similarly, the best temperatures noted for JHA phage lytic activity was 37°C (1300000±30000 pfu/mL) but it was also stable in broad ranges of temperatures from 4°C to 60°C. Gradually the phage titre declined from 45°C (828333.3333±12583.05739 pfu/mL) to 60°C (580000±1000 pfu/mL) indicating inactivation of phage ($p \leq 0.05$). The graphical representation of plagues at different temperatures is represented in fig. 2.

One-step growth curve

One-step growth analysis of JHA phage with *A. baumannii* was done for one-hour interval with every 5-minute evaluation by double-layer agar technique (fig. 3). It was observed that JHA phage has a short latent period of about 5 minutes, high burst size and good adsorption rates. Almost 99 percent phages were adsorbed within 10 minutes and few free phages were found on the double-layer agar technique.

Bacterial growth reduction assay

Bacterial growth reduction assay was calculated by comparing it with the positive control bacterial growth after every one hour for 12 hours as represented in fig. 4. The control culture showed a normal growth pattern by increasing OD with time for 12 hours (red line in the graph). The O.D in phage treated culture decreased in the first 8 hours and after that, it was slightly increased up to 12 hours (blue line in the graph).

Scanning electron micrograph (SEM)

To analyze the surface topology and 3D structure of the JHA phage SEM was performed. The micrograph revealed the existence of hexagonal heads which reflects bacteriophage lysis on bacterial cells.

Transmission electron microscopy (TEM)

Transmission electron microscopy creates an image of tail-less icosahedral particles. The lack of a visible tail structure suggested no relationship with the members of the order Caudovirales, the most common type of bacterial virus but it could fit into either one of the three families of tail-less bacteriophages with isometric capsids, namely Tectiviridae, Corticoviridae, or Leviviridae. By studying its morphology, it fits into the *Tectiviridae* family which has key features of ds DNA, cubic, lipid, double-coated and non-enveloped.

DISCUSSION

The strong antibiotic resistance mechanism of *A.baumannii* made it a worldwide threat (Zahra *et al*., 2021). The phage AB-1 was also described previously as being very host-specific and lacking any lytic activity against any other bacteria (Yang *et al*., 2010).

A very specific host range determination test showed that the newly isolated JHA phage is not active against any other strain of bacteria in the present study not even with the strains of *A. baumannii*, other than its host. At a wider pH range, stability of phages is important for their preservation and clinical use as phage therapy (Matsuzaki *et al*., 2005). The assessment of stability to physical parameters including temperature and pH is another important characteristic of phages. The pH stability showed that JHA phage was stable in broad ranges of pH (5.0 to 9.0) but it was working efficiently on neutral pH (7.0) while any pH level lower than this (pH5) was lethal for the phage activity. Similarly, a wide range of pH stability (>90%) was retained at pH 4 to pH 10 for up to 30 days was also reported for phage B-C62 (Jeon *et al*., 2016). The phage was able to cause lysis in both acidic and basic conditions, Thermal stability testing of the JHA phage indicates its optimum stability and good activity in a broad range of temperatures as it indicates a bit of thermal resistance. A broad range of heat stability was also found for phage ZZ-1 in another study, which is a stable temperature between 50°C to 60°C (Jin *et al*., 2012). The long-term storage of phage is also important and JHA phage was stable at 4°C months and for prolonged storage, it was kept at -20°C but it was not stable at 37°C for a long time. Several experiments need to be performed to understand the mechanism for storage.

The "one-step growth curve" test of JHA phage was performed and the results were evaluated by the double-layer agar technique. JHA phage showed a short latent period, high burst size and good adsorption rates. Almost 99 percent of phage was adsorbed within 10 minutes and few free phages were found on the double-layer agar technique. Phage Abp53 has also a high burst size of 150 pfu per infected cell and strong lytic ability and narrow host range. Among the *A. baumannii* strains (n=26) tested, 7 (27%) including Ab53 support clear spots (Lee *et al*., 2011).The bacterial growth reduction assay of JHA phage with *A. baumannii* was performed and the optical

density showed that the growth of bacteria including JHA phage was continuously decreased and the growth of bacteria started to increase due to the decreasing activity of JHA phage.

The morphological studies and 3D structure of JHA phage when examined on an electron microscope, it revealed that JHA phage fit into the *Tectiviridae* family, which has structural features of dsDNA, cubic and double coated.while in another study, the preliminary approximation of the JHA phage capsid size was based on TEM which gives the consensus characteristic of the member of the Tectiviridae family (Cerqueira & Peleg, 2011; Montefour *et al*., 2008). It was also observed that the JHA phage showed the nonexistence of a contractile tail.

CONCLUSION

Based on our results, it is concluded that isolated novel JHA phage's host exclusive nature has the power to act against specific MDR strains of *A. baumannii*. After transmission electron microscopy it is culminates that our JHA bacteriophage belongs to the *Tectiviridea* family. It is also concluded that further studies are required to analyze JHA phage as a potential agent for phage therapy and it can be used as an alternative candidate to antibiotics for treatment against MDR *A. baumannii* diseasesas it shows very high specificity against *A. baumannii*.

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