Research Article

Isolation of Bacteriophage and Assessment of its Activity against Biofilms of Uropathogenic *Escherichia coli* in Jimma Town, South Western Ethiopia

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Abstract: *Escherichia coli* is one of the most commonly associated bacteria with urinary tract infections (UTIs) in humans and many times are antimicrobial resistant. Production of biofilms further makes matters worse in UTIs. Alternative therapy using bacteriophages was known in the past. This study was aimed to isolate lytic bacteriophages from sewage samples and assess their activity against biofilm of uropathogenic *E. coli* (UPEC). Lytic phage was isolated from sewage water collected in Jimma town following standard enrichment method against UPEC. *E. coli* was isolated from UTI suspect patients using standard protocol. Microtiter plate technique was used to determine bacterial biofilm formation. Biofilm degrading efficacy of phage was assessed by treating biofilm developed on cover slip with standardized number of lytic phages or gentamicin compared with the control *E. coli* (untreated cells). Of 30 UPEC strains isolated from patients, 29 (96.6%) of them displayed biofilm forming phenotype. The strains with strongly biofilm positive were 76.6%. Generally, antibiotic resistance for biofilm producing *E. coli* was found to be high. Virulent phage (ΦJS4) was isolated which was effective against a strong biofilm former UPEC strain. Application of ΦJS4 phage or gentamicin to established biofilms have caused significant reduction of the cells within 3 hours of application and almost complete eradication of the cells within 36 hrs of incubation at 37°C. These results uphold the efficacy of phage against biofilm of UPEC and suggest that ΦJS4 phage may be a potential therapeutic alternative to antimicrobials on inanimate and animate surfaces.

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Introduction

Urinary Tract Infections (UTIs) caused mainly by bacterial etiology creates a serious health threat associated with antibiotic resistance and high recurrence rates [1]. From bacterial aetiologies, *Escherichia coli* is one of the most prevalent pathogens capable of causing complicated and uncomplicated UTIs [2]. According to Foxman [3], uropathogenic *E. coli* (UPEC) is the primary cause of community-acquired urinary tract infections (70%-95%) and a large portion of nosocomial UTIs (50%). Mulvey and his colleague in 2000 and Bower and co-workers in 2005 have reported that UPEC strains act as opportunistic pathogens, which colonize the bladder of the urinary tract, causing cystitis and ascend through the ureters to cause pyelonephritis [4,5]. This bacterium forms intracellular bacterial communities which equivocally are biofilm like properties within the epithelium [6].

Biofilms have a role in up to 60% of human infections and they are difficult to eradicate with antimicrobial treatment. *In vitro* susceptibility tests have shown considerable increase in resistance of biofilm cells to killing [7]. Its formation regarded as a universal strategy for bacterial survival. Biofilm uses largely polysaccharides for adherence, which prevents access of antibacterial agents, antibodies and white blood cells. Comparatively planktonic bacterial cells are highly susceptible to antibiotics than the sessile bacterial cells in the biofilms, which can withstand the host immune responses [8,9].

The world is closer to the end of the antibiotic era with Gram-negative pathogens such as *A. baumannii* and *E. coli* [10] are becoming highly resistant. Equally worrying is the fact that new antibiotics are not being developed at a rate sufficient to replace those drugs that are becoming less useful. Spellberg and his colleagues [11] surveyed 15 largest multinational pharmaceutical companies and they found that only five antibacterial agents were currently undergoing development, none of which represented new classes of antibiotics. It is therefore apparent that the prospect of new antibiotics in the next decade is likely low. This has become the driver in the search for alternative strategies to treat infections caused by antibiotic resistant bacterial pathogens.

An alternative to antimicrobial therapy would be of great relevance to treat those multidrug resistant pathogens including bacterial pathogens of UTIs [12] as well as to safeguard the efficacy of antimicrobial agents for life-threatening infections. In this regard, bacteriophages are one of the best candidate to eradicate multi drug resistant bacteria and their biofilms separately or in combination with drugs [13].

Bacteriophages are bacterial viruses that majority of the strains are capable to kill their bacterial hosts. These viruses can also penetrate the extracellular matrix that binds macromolecules and prevents their diffusion into the biofilm [14] and kill cells [15-17]. Phages are probably the most numerous and most diverse group of viruses. The estimated global population of phages is estimated to be: in the aquatic environment is above 10^{31}, land ecosystems demonstrate 10^7 phage particles per a gram of soil, and the total number of phages in sewage is $10^8–10^{10}$ per milliliter. As a result of these, they may constitute the most dominant form of life in the biosphere that in every place there may be a well adapted virulent virus that can destruct bacteria that may be even multidrug resistant [18,19].

In Ethiopia, there was no study reported about the use of bacteriophages except one recently conducted work. It was on isolation of bacteriophages and its use as alternative antibiotics against lethal doses of *E. coli* infected mice models [20]. This study was therefore aimed at isolation of bacteriophages from sewage sources in Jimma town and assessing its effectiveness as natural antimicrobial agents against biofilm of UPEC.

Materials and methods

Study area and period

The study was conducted in Jimma town, which is located 346 km southwest of Addis Ababa. The town is found at an average altitude of 1,780 meters above sea level with geographical coordinates of approximately 7°41' N latitude and 36°50' E longitude. It lies in the climatic zone locally known as 'Woyna Dega' (areas located between 1,500 - 2,400 m above sea level) which is considered ideal for agriculture as well as human settlement. The town has generally a mean annual maximum temperature of 30°C and mean annual minimum temperature of 14°C. The annual rainfall ranges from 1,138 to 1,690 mm. Maximum precipitation occurs during the three months period, June to August, with minimum rainfall in December and January [21]. According to the Ethiopian central statistical agency (ECSA) report of 2007, the total population of the town is 130,254 [22]. The study period was conducted in between July 2016 - Sep 2016 in Medical Microbiology laboratory, college of health sciences, Jimma University.

Study design

Institutional based cross sectional design was employed with a total of 130 UTI suspected and admitted patients attending Jimma University specialized hospital between July, 2016 and September, 2016. Ethical clearance was obtained from Jimma University, Health Institute Ethical Review Committee. Each study participant was informed about purpose of the study and verbal or written consent was obtained to confirm their willingness to participate in the study. Names of patients were replaced by codes. All data obtained in the course of the study was kept confidential, and was used solely for the purpose of the study.

Culturing and identification of host bacterial strains

The bacterial strain used in this study was *Escherichia coli* clinically isolated from catheterized urine sample from inpatients of Jimma University Specialized Hospital (JUSH). Each study participant was informed about purpose of the study and verbal or written consent was obtained to confirm their willingness to participate in the study.

Standard operation procedure was used to obtain fresh urine that was aspirated from the sampling port of urine catheter with a sterile syringe and needle after cleansing the port with a disinfectant (ethanol 70%). Then sterile urine cap was placed over syringe hub and transferred. The Urine was brought to Medical microbiology laboratory of Jimma University within maximum of an hour of collection. Well mixed urine sample of 0.001ml was inoculated onto MacConkey agar, blood agar (trypticase soya agar enriched with 5% sheep blood) and manitol salt agar using calibrated wire loop. Then, plates were incubated at 37°C aerobically for 24 hours. From grown culture plates, uropathogens were identified based on colonial characteristics, gram-staining reaction and biochemical tests [23,24].

All *E. coli* strains were included in this study and analyzed for the production of biofilm and antimicrobial susceptibility pattern [25,26].

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were done on Mueller-Hinton agar for 24hours using Kirby Bauer

disk diffusion method [27]. The antibiotics tested were tetracycline (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), cephalothin (30μg), ceftriaxone (30μg), and ampicillin (30μg). All discs were obtained from Oxoid Ltd, Hampshire, England. The reading for zone of inhibition was interpreted according to Clinical and Laboratory Standards Institute protocol [28].

Bacteriophage Isolation and Amplification

Isolation of Bacteriophages specific against *E. coli* was carried out from sewage samples according to the standard protocol described by Twest and Kropinski [29]. Briefly, sewage samples were collected in sterile 500ml containers from five different areas around the university compound based on high organic contaminations so as recovery of phage was known to be good: namely, Jimma University Hospital (one area), College of Natural Sciences (two areas) and Jimma Kochi compound (two areas). The samples were processed by the enrichment method in medical microbiology laboratory of Jimma University. Fifty milliliters of each sample was centrifuged at 10,000rpm for 10 minutes to remove particulate materials. The supernatants were filter sterilized by passing through a 0.45-micrometer membrane filter (MERK, EUROLAB [PTFE], U.S.A). The filtrate (50 ml) along with 5ml log phase grown *E. coli* strain was then mixed with equal volume of sterile double strength Nutrient broth containing 2mM MgSO4 in a 250 ml Erlenmeyer flask. The flask was incubated overnight at 37 ^{O}C in static incubator shaking in between every 2 to 4 hours. Next day, the mixture was centrifuged at 10,000rpm at 4°C for 15 minutes. Then the supernatant containing phage was passed through a 0.45-micrometer pore membrane filter under aseptic condition. The pellet was discarded whereas the filtered was held for amplification of phage. From this step, 50 ml of the filtrate was mixed with equal volume of double strength nutrient broth containing 2mM MgSO4 and incubated with 5ml of the indicator strain (*E. coli*). The mixture was incubated at 37°C shaking every two to four hourly overnight for 20 - 24 hrs. The next day, the mixture was centrifuged at 10,000 rpm at 4°C for 15 minutes. The supernatant considered to contain phages was filtered and sterilized through a 0.45-micrometer pore membrane under aseptic condition.

Bacteriophage Activity Detection and Purification

The amplified filtrate obtained was tested for phage activity against the *E. coli* by using spot assay as described elsewhere [30,31]. The indicator cell (0.1ml) was added to sterile molten soft agar (0.75%) prepared and maintained at 45-50°C in water bath and quickly mixed. The contents were poured on previously prepared nutrient agar plate surface. Around 12-15 microliter (two drops) of amplified filtrate was spotted on each plate at 2 different places peripherally. The plates were allowed to dry at room temperature and then incubated at 37°C overnight for 24 hours. The plates were examined next day for clearance at the spotted area. All the isolated phages were purified by successive single plaque isolation until homogenous plaque was obtained according to the protocol followed by Schuch and co-workers [32]. A single plaque was picked aseptically by using sterile tooth pick and transferred into tube containing 5ml broth and fresh log phase grown indicator strain. Another tube containing indicator strain was left as a control. Both tubes were incubated at 37°C under shaking condition until complete lysis occurred in the test preparation.

Phage host mixture and control preparation was centrifuged at 10,000rpm for 15 minutes at 4°C. The supernatant was filtered by passing through sterile 0.45 micrometer pore membrane to remove bacterial contaminants. The filtrate was serially diluted and assayed for plaques. The procedure was repeated for three times to ascertain the purity of isolated phage. The plaque recorded

and scored as phage that resulted in confluent lysis with some halo formation. From five samples processed, purified phage (ΦJS4) was stored for subsequent tests.

Quantitative Assay of Bacteriophage (Titration)

Titer of the phage preparation [plaque forming units per milliliter (PFU/ml)] was estimated by the soft agar overlay method as described by Adams [33]. High titer was prepared by adding phages to early log phase host culture at an MOI (multiplicity of infection) of 1 and incubating at 37ºC, until complete clearance was obtained. The large plaque forming sample content was selected for the next processes. Serial dilution of the bacteriophage sample was made in sterile phosphate buffer solution. A 0.1ml of bacteriophage suspension from each dilution was mixed with 0.1ml of the indicator host cells of *E. coli* and added to about 7 to 8ml of molten soft agar held at 45ºC. This was thoroughly mixed without air bubbles and quickly poured over previously moisture dried nutrient agar plate. The overlays allowed to solidify upright for few minutes at room temperature and then incubated in inverted position at 37ºC overnight. Next day plates with 30 to 300 plaque forms were selected and counted. Original phage count (titer) was determined by using the following formula for calculation of phage number.

$$\text{Number of plaques} = \frac{\text{Plaques counted x Reciprocal of the dilution}}{\text{Amount of plated in milliliter}}$$

= PFU/ml (plaque forming units per millimeter)

Characterization of isolated phages

The ability of bacteriophage to survive and infect the host cells was tested under the following different conditions; different pH value and temperature with predetermined phage count. The isolated bacteriophage was exposed to pH values adjusted from 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 for one-hour incubation. Furthermore, phage along with the indicator host was kept overnight at temperature range from 10°C to 60°C. The adsorption rate, plaque size, sensitivity to chloroform, ether and absolute ethanol, and its host ranges against all biofilm forming *E. coli* and other species of bacteria was determined essentially according to the method of Adams [33].

1) Bacteriophage adsorption

Bacteria in logarithmic growth phase were diluted in nutrient broth to an optical density (OD600) of 1.0. Afterwards, 10 ml of the bacterial suspension and 10 ml of phage solution was mixed in order to give a multiplicity of infection (MOI) of 0.01. The mixture was incubated at room temperature with shaking (150 rpm) and samples were collected every minute during a total period of 15 min. Samples were immediately chloroform-treated, diluted and plated on nutrient agar plates. After overnight incubation at 37 ºC phage plaques were counted.

2) pH stability assay of bacteriophage

The stability of bacteriophage at different pH values were evaluated by adjusting the pH of the nutrient broth with either HCl (1 M) or NaOH (1 M) to a pH range of 1–12. The titer of bacteriophage lysate was determined to prepare bacteriophage suspension having a titer of 10^7 PFU/ml. Then 100 µl of this bacteriophage suspension was added into the respective 9.9 ml of nutrient broth adjusted to pH

ranging from 2 to 12. The preparation was properly mixed and incubated at 37^0C for 1h. Phage titer was estimated quantitatively by agar overlay method. Finally, phage suspension held at pH 7 was taken as control to calculate the percentage of surviving phages at the respective pH value.

3)   Temperature stability assay of bacteriophage

The stability of the bacteriophages at different temperatures (10, 20 30, 37, 40, 50, 60^0C) were checked using the method of Verma and his co-workers [34]. The titer of bacteriophage suspension at • 10^6 PFU/ml was prepared. Then sterile test tubes were labeled and pre warmed at the respective temperature. One milliliter of the phage suspension was incubated at different temperatures (10, 20, 30, 37, 40, 50, or 60^0C) for 1h. The phage titer was enumerated at the end of incubation. Finally, the phage suspension held at 10^0C was taken as control to calculate the percentage of infective phage after exposure to respective temperatures.

Biofilm formation assay protocol

The micro titer plate assay of biofilm involves the analysis of bacterial adhesion and biofilm formation on clear sterile polystyrene plastic wells by measuring the optical density at wave length of 570 nm after certain period of incubation [26]. Brain heart infusion broth (Oxoid, UK) supplemented with 1% glucose was prepared freshly. Then, bacterial suspension was directly added to the broth and diluted to 0.5 McFarland turbidity standards. In each microtiter well, 200 µL was performed in triplicates for each isolate and incubated at 37^oC for 24 h. Following incubation, the content of each well was aspirated, and washed 3 times gently with sterile distilled water to remove planktonic bacteria. The attached bacteria was fixed with 200 µL of methanol per well and then 250 µl of 0.1% crystal violet solution was added to each well, and was kept for 10 min at room temperature. Each content of the microtiter well was spent and washed with sterile distilled water to remove the staining solution. Then, plates were allowed to air-dry and 250 µl of 95% ethanol per well was added to solubilize the crystal violet dye by covering plates and incubating for 15 min at room temperature. The experiment was performed in triplicate.

Finally, the OD of each well was measured at 570nm by using automatic ELISA Reader (HumaReader HS, German). The measured optical density (OD) from the triplicate wells was then averaged and standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixated and dyed wells were averaged and subtracted from all test values. Briefly, the cut-off optical density (ODc) was calculated and defined as three standard deviations above the mean OD of the negative control. Based on the average OD produced by bacterial films at a wavelength of 570 nm, isolates were classified as: bacterial OD < ODc = biofilm non-producer; OD > ODc, but < 2 ODc = weak biofilm producer; OD > 2 ODc but < 4 ODc = moderate biofilm producer and > 4 ODc = strong biofilm producer as described by Stepanovi• and his colleagues [26].

Development of biofilm on glass cover slip

Biofilm was grown on air-liquid interface using batch culture model of Hughes and his team [25] in a static condition. It is also known as Tip-box batch culture method. Petri plate was suitably modified and converted in to a batch culture vessel. Inner tip holder was suitably modified to hold glass cover slips vertically. The tip holder was placed in inverted position in the petri plate with cover slips (22 × 22 mm) in place. Whole system was autoclaved carefully so that cover slips did not move from their fixed place. About 200 ml of brain heart broth was autoclaved separately in a flask. To the cooled brain heart infusion broth, 100 µl of bacterial culture in the log phase (10^8 CFU/ml) was added. Inoculated broth was poured aseptically into the Tip-box and incubated at 37^0C for maximum of

36hrs without media renewal and incubation under static condition. Each time desired number of cover slips were removed aseptically and were washed using sterile sodium phosphate buffer (pH 7.2).

Biofilm treatment on glass cover slip

The biofilm developed on cover slip as mentioned above were used to treat with gentamicin, bacteriophage, or normal saline (as a control). Biofilm of desired duration (36h) was removed aseptically from the Tip-box and rinsed thoroughly with sterile normal saline. Then it was incubated with either gentamicin (32µg/ml) or bacteriophage or normal saline for 3 hrs and 36 hrs in a petri plate. After the respective treatment, biofilm laden cover slip was washed gently with sterile sodium phosphate buffer (pH 7.2) and stained with (0.1%) crystal violet for microscopic examination.

Staining of biofilm on cover slip

Biofilm after particular duration of treatment on cover slip was washed by submerging three times in sterile sodium phosphate buffer (pH 7.2). The preparation was then allowed to air dry and stained with 0.1% Crystal Violet. Preparation was allowed to stand at room temperature for 10 minutes. Stained biofilm was rinsed with sterile distilled water after which it was allowed to air dry. The stained biofilm on cover slip was put on a clean microscope slide and visualized using a compound light microscope under high power and oil immersion objective.

Quality Control

The reliability of the study findings was guaranteed by implementing Quality control (QC) measures throughout the processes of the laboratory works. Aseptic techniques have been employed in all the steps of specimen collection and culture media preparation to minimize contamination. All culture media were prepared according to the directions of the manufacturers and tested for expiry date, sterility and performance. Control bacterial strains: *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) was used in controlling the potency of the drugs.

Statistical analysis

Statistical analysis was performed to describe different variables and parameters in the research, and to describe relationship with each other as well. Calculation of mean value and standard deviation (SD) were made for physiochemical properties of the isolated phages, biofilm forming capability and antimicrobial resistance of the selected bacterial cells. Descriptive statistics were used to derive percentage, standard deviation, and to tabulate tables and graphs.

Results

Bacteria Isolation, Biofilm Forming Capabilities and Antimicrobial susceptibility testing

From the total of 130 patient urine specimens processed in this study, 30 of them were culture positive for *E. coli* contributing for 23.1% of urinary tract infection. Among the total *E. coli* isolates (n=30) tested for biofilm formation in the current study, 29 (96.7%) of them were capable of producing biofilms. Strong and weak biofilm formation was observed in about 77% and 20% of the uropathogenic *E. coli* isolated, respectively (Table 1).

Table 1 Biofilm status of isolated uropathogenic *E. coli,* Jimma, 2016.

Biofilm status	No. (%)
Strong	23(76.6)
Weak	6(20.0)
None	1(3.3)

Note: Data points represent an average of two independent experiments, each with 3 replicate wells making 6 repeated testing for each of *E. coli* isolate.

Antibiogram effect on biofilm producing uropathogenic *E. coli*

All the biofilm forming strains showed maximum resistance to Ampicillin, 29 (100%), followed by Amoxicillin-clavulanate, 27(93.1%); Cephalothin, 22 (75.9%); Gentamicin, 20 (68.9%); Ceftraxone, 18 (62.1%); Tetracycline, 17 (58.6%); Ciprofloxacin, 15 (51.7%) (Table 2).

Table 2 Antibiotic susceptibility result of biofilm producing uropathogenic *E. coli*, Jimma, 2016.

| Antibiotics	Drug susceptibility (n=29)	
	Resistance (No. (%)	Sensitive No. (%)
Gentamicin	20(68.9%)	9(31.1%)
Ampicillin	29(100%)	_____
Ceftraxone	18(62.1%)	11(37.9%)
Amoxicillin-clavulanate	27(93.1%)	2(6.9%)
Tetracycline	17(58.6%)	12(41.37%)
Cephalothin	22(75.9%)	7(24.1%)
Ciprofloxacin	15(51.7%)	14(48.3%)

Isolation of *E. coli* – specific phages (ΦJS4) with their therapeutic potential

Of five areas of sewage samples processed in the current study, one lytic bacteriophage was isolated producing large and clear plaque possessing phage which was used in subsequent study, indicating that this phage can absorb efficiently to the indicator host and producing large plaques (Figure 1). It was named as ΦJS4.
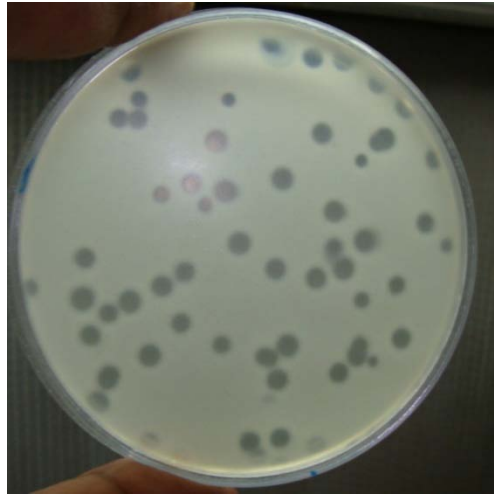
Figure 1 Purified phage lysate titrated and formed plaques of ΦJS4.
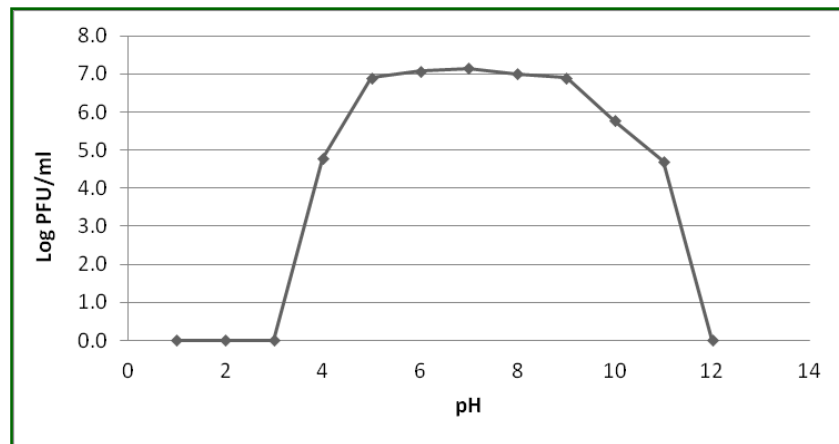
Figure 2 Exposure of isolated phage (ΦJS4) to varying pH values.

Phage characterization

Bacteriophage ΦJS4 was propagated tested for lysis rate, adsorption rate, plaque size, sensitivity to chloroform, ether and alcohol, its specificity to biofilm former *E. coli* isolated from patients as well as other selected bacteria other than *E coli*, and the effect of different pH-values and temperature of the isolated phage was determined.

The *in vitro* characteristic for complete lysis rate in broth was about 4 hours at MOI of 1. The phage has a burst plaque size of 3mm to 5mm diameter. Adsorption time for ΦJS4 was 12 minutes where about 98 to 99% of the phage particles were adsorbed onto the bacterial cells. The phage specificity was checked by dropping the phages on *E. coli* other than the host bacterium isolates including all biofilm former *E. coli* isolated from the patients, *Salmonella* Paratyphi A, *Pseudomonas* spp. and *E. coli* ATCC 25922 forming no plaques or infection was detected, except on the specific

host *E. coli* coded as C152. The phage is resistant to chloroform and ether which rules out that it's not enveloped virus. However, 100% of the phage was denatured by the use of absolute ethanol exposure.

The stability study of the phage held at different pH for 1 h and kept at room temperature showed that the phage ΦJS4 tested lost their infectivity completely at pH 2, 3 or 12. Maximum phage stability was observed between pH 5 to 9 (Figure 2).

The effect of different temperature on the phage was determined by agar overlay method (Table 3). The isolated phage ΦJS4 has maximum infection at temperature between 10^0C-40^0C but has no activity at temperature around 50^0C and above.

Table 3 Phage (ΦJS4) stability after exposure to different temperature conditions, 2016.

Plate number	Temperature (^{o}C)	Plaque per plate	Titer: plaque-forming units (PFU) per ml
01	10^0C	13	$1.3*10^7$
02	20^0C	14	$1.4*10^7$
03	30^0C	14	$1.4*10^7$
04	37^0C	15	$1.5*10^7$
05	40^0C	10	$1.0*10^7$
06	50^0C	0	0
07	60^0C	0	0

Eradication of Established Biofilms on cover slip by Phage ΦJS4

UPEC isolate C152 was selected to determine the effectiveness of the isolated phages to treat established strong biofilms. Phage ΦJS4 treatment of preformed biofilms of *E coli* yielded reductions in biofilm mass compared to untreated control (Figure 3). The phage displayed, a steady reduction of biofilm biomass over time with the largest percentage of biofilm reduction in biomass was observed after 36 hrs of incubation (Figure 3).

After 3hrs and 36hrs of phage ΦJS4 or gentamicin treatment at 37°C, average biofilm biomass were compared with saline treated control samples (100%). Three independent experiments were performed, and each produces similar appearance where upon subculture to sterile agar plates, only the control produced a viable *E. coli* colonies but no growth for the 36 hours phage or genatamicin treated cover slide.
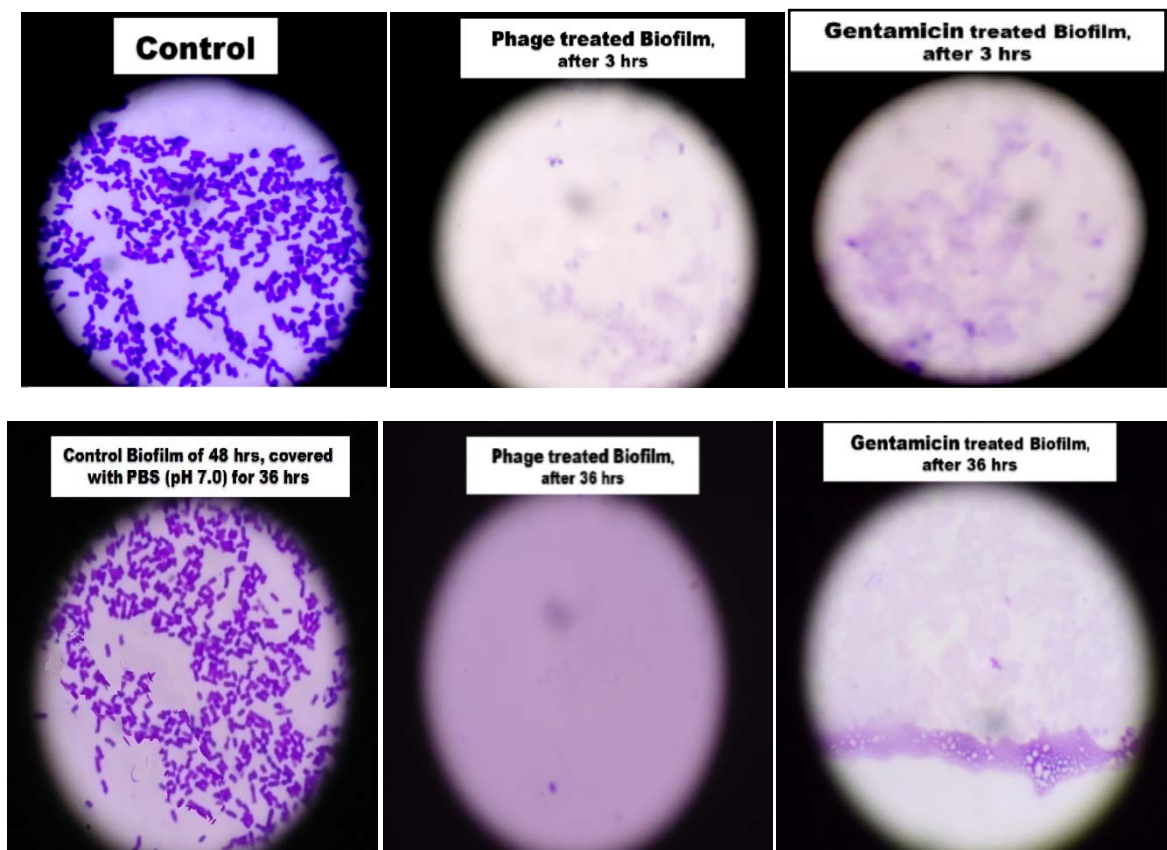
Figure 3 Treatment of established *E. coli* biofilm using antibiotics and phage ΦJS4. Biofilms grown on glass cover slips and exposed for 3hrs (a) and 36hrs (b) were inoculated with 107 PFU/ml of phages ΦJS4.

Discussion

Urinary tract infections due to bacterial strain are one of the most frequent problems known in human. From gram negative bacteria, *E. coli* is one of the frequent and leading aetiology of urinary tract infections that are biofilm former and drug resistant [35]. All *E. coli* isolates (30/130) were isolated from urinary tract symptomatic patients visiting the hospital. Among the isolates, only one strain tested for biofilm production was negative or non-biofilm producer whereas the remaining 29 (96.7%) isolates were positive for biofilm formation. This is in contrast to a previous study [36] where 53% of the bacterial strains in UTI produced biofilms *in vitro*.

        Using virulent bacteriophages as potential chemotherapy-independent schemes, drug-resistant bacteria can be destructed using microbial interference. In the present study, virulent phage was isolated against uropathogenic *E. coli*, and it was experimentally evaluated for eradication of established biofilm on cover glass. Since phages are obligate parasites, a host must be provided in order to isolate and enumerate them from any environment samples. Providing a host and counting the number of phages is most easily accomplished by using an agar overlay technique [33]. In samples where phages were present, they multiplied and lyse the bacteria, causing a zone of clearing (a

plaque) on the plate. Theoretically, each plaque is formed by one virus and the number of plaques multiplied by the dilution factor is equal to the total number of viruses in a test suspension. This is analogous to bacterial cell enumeration guidelines to apply to plaque forming units (PFUs) [33].

The selection of best phages was done according to results of lytic spectrum. The phage plaque size was used to evaluate the lytic clearance and its size was measured. On the basis of this, among the samples processed for phage isolation, environmental sample number four obtained from one of the local area in Jimma was selected on the basis of big plaque size formation which indicates its effectiveness in lysing bacteria.

The effect of different pH-values on the phage with respect to its infectivity capacity was shown in the results. It was apparent that the infectivity was stable in the range between pH 5 and 6. However, the isolated phage was very unstable below pH 4. This is because phages are sensitive to protein denaturation in acidic condition [37]. While on the alkaline side, the isolated phage was unable to survive above pH 11. The maximum stability seemed to be at pH 6 followed by a decline in infectivity below pH 5 and greater than pH 9. This result is consistent with the previous study that survival of most phages is within pH 5 to 9 that may maintain the native virion structure and stability [38].

The results from exposure of isolated phage to varying temperatures ranging from 10°C to 60°C was tested and shown that the isolated phage was stable in the temperature range of 10°C to 40°C. However, it became less stable when the numbers of phage count were significantly reduced to zero following exposure to 50°C and 60°C. The region of optimum stability was close to human body temperature (37°C) as the phage appeared to survive in this range. The isolated phage was unable to grow well in temperature that is higher than body temperature although the loss was not significant at 40°C and most phages did not have the ability to survive at temperature above this temperature. Thus, it was seen that the isolated phage was rapidly inactivated at 50°C temperature. This result could show that extreme condition might affect the phage structure and cellular functions [13].

Phages have high specificity for specific bacterial strains, a characteristic which requires careful targeting [39]. Therefore, phage therapy can be used to lyse specific pathogens without disturbing other normal bacterial flora [40]. From a clinical standpoint, phage therapy appears to be very safe if further preclinical and clinical tests can be ruled out. Efficacy of natural phages against antibiotic-resistant *Streptococci, Escherichia, Pseudomonas, Proteus, Salmonella, Shigella, Serratia, Klebsiella* [31], *Enterobacter, Campylobacter, Yersinia, Acinetobacter* and *Brucella* were being evaluated by researchers [13]. Our study also proved that the ΦJS4 were specific to the selected *E. coli* (C152) that produced strong biofilm but not even to the other *E. coli* strains isolated from other patients.

In this study the purified phage and gentamicin were applied immediately to treat established *E. coli* biofilm and effective results were observed. First the isolated phage (ΦJS4) or the antibiotics (gentamicin) were applied on established biofilm for 3hrs and both were effective in eradication of the biofilm when compared with the control cover slip (untreated). On the other hand, application of the phage (ΦJS4) or gentamicin for 36hrs was more effective in eradication of the established biofilm when examined under the microscope. This finding indicates that giving longer

time was more effective than the short time treatment of bacterial biofilm by using phages. On the other hand, either phage ΦJS4 or gentamicin has equally eradicated an established biofilm of *E. coli* in this *in vitro* experiment. There is also a need for in-depth characterization of the bacteriophage to understand more about the biology of the virus.

Conclusion

All of UPEC isolated were resistant to Ampicillin. The phage was naked virion having stability at wider pH ranges between 5 and 9 and temperatures between 10 and 40°C. The candidate phage has adequate lytic spectrum with maximum adsorption capability for therapeutic purposes. Since phages are ubiquitous, it can be readily isolated from the environment using simple, low-cost techniques than that of developing a new antibiotic. The results of this study indicated that phage has a potential to degrade biofilm of UPEC. This will help in giving great attention in use of phage therapy for bacterial infections.

Acknowledgments

References

1. Anderson G, Martin SM, Hultgren SJ. Host subversion by formation of intercellular bacterial communities in the urinary tract. *Microbes Infection*. 2004, 6:1094-1101
2. Rodriguez-Bano J, Navarro MD, Romero L, Romero L, Muniain MA, Perea EJ. Clinical and molecular epidemiology of extended-spectrum beta-lactamase-producing *Escherichia coli* as a cause of nosocomial infection or colonization: implications for control. *Clin Infect Dis*. 2006, 42: 37-45
3. Foxman B. Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *Dis-a-Mon.* 2003, 49:53-70
4. Bower JM, Danelle SE, Matthew AM. Covert operations of uropathogenic *Escherichia coli* within the urinary tract. *Traffic*. 2005, 6:18-31
5. Mulvey MA, Joel DS, Juan JM, Scott JH. Bad bugs and beleaguered bladders: interplay between uropathogenic *Escherichia coli* and innate host defenses. *Proc Natl Acad Sci USA.* 2000, 97: 8829-8835
6. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. Intracellular bacterial biofilm like pods in urinary tract infections. *Science*. 2003, 301:105-107
7. Spoering AM, Lewis K. Biofilm and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol.* 2001, 138:6746-6751
8. Costerton JW, Cheng KJ, Geesey GG. Bacterial biofilms in nature and disease. *Ann Rev Microbiol.* 1987, 41:435-464
9. Crouzet M, Claverol S, Lomenech AM, et al. Pseudomonas aeruginosa cells attached to a surface display a typical proteome early as 20 minutes of incubation. *PLoS One*. 2017, 12:e0180341

10. Coelho J, Woodford N, Turton J, Livermore DM. Multiresistant Acinetobacter in the UK: how big a threat? *J Hosp Infect.* 2004, 58: 167-9

11. Spellberg B, Powers JH, Brass EP, Miller LG, Edwards Jr JE. Trends in antimicrobial drug development: implications for the future. *Clin Infect Dis*. 2004, 38:1279-86

12. Czaja CA, Hooton TM. Update on acute uncomplicated urinary tract infection in women. *Postgrad Med.* 2006, 119: 39-45

13. Kutter E, Sulakvelidze A. Bacteriophage: Biology and Applications. **CRC Press,** Inc, Boca Raton, Fla. p. 437-494; 2005

14. Lacroix-Gueu P, Briandet R, Leveque-Fort S, Bellon-Fontaine MN, Fontaine-Aupart MP. In situ measurements of viral particles diffusion inside mucoid biofilms. *C R Biol.* 2005, 328: 1065-1072

15. Hanlon GW, Denyer SP, Ollif CJ, Ibrahim LJ. Reduction in exo-polysaccharide viscosity as an aid to bacteriophage penetration through Pseudomonas aeruginosa biofilms. *Appl Environ Microbiol.* 2001, 67: 2746-2753

16. Sillankorva S, Oliveira R, Vieira MJ, Sutherland IW, Azeredo J. Bacteriophage • S1 infection of *Pseudomonas fluorescens* planktonic cells versus biofilms. *Biofouling.* 2004, 20:133-138

17. Curtin JJ, Donlan RM. Using bacteriophages to reduce formation of catheter associated biofilms by Staphylococcus epidermidis. *Antimicrob Agents Chemother.* 2006, 50: 1268-1275

18. Sharp R. Bacteriophages: biology and history. *J Chem Technol Biotechnol*. 2001, 76: 667-672.

19. Ashelford KE, Day MJ, Fry JC. Elevated abundance of bacteriophage infecting bacteria in soil. *Appl Environ Microbiol.* 2003, 69: 285-289

20. Leta A, Yohannes M, Kassa T. Assessment of Therapeutic Potential of Bacteriophages to Control *Escherichia Coli* Infection in Swiss Mice Model. *Ethiop J Sci & Technol*. 2017, 8: 73-83

21. Jimma City Administration Strategic Plan (JCASP). Oromia National Regional State, Jimma *City Administration Strategic Plan*, Jimma, Ethiopia; 2006

22. ECSA. Ethiopian Central Statistical Agency. Population and Housing Census of Ethiopia administrative report: The Projected result for Oromia Region. *Addis Ababa*, 2010

23. Cheesbrough M. District laboratory practice in tropical countries, part II. 2nd edition: England: *Butterworth-Heineman LTD*, pp105-15; 2006

24. Collee JG, Fraser AG, Marmion BP, Simmons A. (eds). Mackie and Mc Cartney practical medical microbiology. 14th Ed. New York: *Churchill Livingstone*; 1996

25. Hughes KA, Sutherland IW, Clark J, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology.*1998, 144: 3039-3047

26. Stepanovi• S, Vukovi• D, Hola V, Di Bonaventura G, Djuki• S, • irkovi• I. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*. 2007, 115:891-9

27. Bauer AW Kirby WMM Sherris JC Turck M. Antibiotic susceptibility testing by standard single disc method. *A J Clin Pathol.* 1969, 45: 493-496

28. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute, Twenty-seventh Information Supplement, 2016; 37(1), M100-S26:1-249

29. Twest R, Kropinski AM. Bacteriophage enrichment from water and soil: Methods in molecular biology 501. 1:15-32. In: Clokie MRJ, Kropinski AM, editors. Bacteriophages methods and protocols: isolation, characterization, and interactions. New York Springer protocols: *Humana Press*; 2009

30. Cerveny KE, DePaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infect Immun.* 2002, 70:6251-6262

31. Kumari S, Harjai K, Chhibber S. Isolation and characterization of Klebsiella pneumoniae specific bacteriophages from sewage samples. *Folia Microbiol.* 2010, 55:221-227

32. Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills *Bacillus anthracis*. *Nature.* 2002, 418:884-889

33. Adams, M.H. Methods of study of bacterial viruses, In: Bacteriophages. London, United Kingdom: *Interscience Publishers*;1959

34. Verma V, Harjai K, Chhibber S. Characterization of a T7–like lytic bacteriophage of *Klebsiella pneumoniae* B5055: A potential therapeutic agent. *Curr Microbiol.* 2009, 59: 274-281

35. Kibret M, Abera B. Prevalence and antibiogram of bacterial isolates from urinary tract infections at Dessie Health Research Laboratory, Ethiopia. *Asian Pac J Trop Biomed*. 2014, 4:164-8

36. Rijavec M, Müller-Premru M, Zakotnik B, Zgur-Bertok D. Virulence factors and biofilm production among *Escherichia coli* strains causing bacteraemia of urinary tract origin. *J Med Microbiol.* 2008, 57: 1329-1334

37. Ackermann HW. Classification of bacteriophages. The Bacteriophages, Ed. Calendar R, ISBN 0-19-514850-9, New York, USA, *Oxford University Press*; 2006

38. Jamalludeen N, Kropinski AM, Johnson RP, Lingohr E, Harel J, Gyles CL. Complete Genomic Sequence of Bacteriophage • EcoM-GJ1, a Novel Phage That Has Myovirus Morphology and a Podovirus-Like RNA Polymerase. *Appl Environ Microbiol.* 2008, 74(2): 516-525

39. Merril R, Scholl D, Adhya L. The prospect for Bacteriophage therapy in Western medicine. *Nat Rev Drug Discov.* 2003, 2:489-497

40. Sulakvelidze A, Alavidze Z, Morris JG Jr. Bacteriophage therapy. *Antimicrob Agents Chemother*. 2001, 45:649-659