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ISOLATION, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF BACTERIOPHAGES AGAINST MULTIPLE ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* INVOLVED IN URINARY TRACT INFECTIONS

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ABSTRACT

Bacteriophages, as bacterial phages, are very species-specific with regard to their hosts and usually only infect a single, bacterial species, or even specific bacterial strains within a species. The study was carried out using 820 samples comprised of 100 stool and 720 urine from UTI infected patients. Four hundred twenty isolates of *E.coli* (51%) were isolated. It was found to be more frequent in female (73.17%) and less prevalent in males (28.82%) as determined by microbiological, biochemical and vitek-2 system and confirmed by detection of 16SrRNA. The sensitivity test carried out on *E.coli* isolates against 16 different antibiotics revealed resistance at a high ratio for more than nine antibiotics (data presented in a separate report). As a consequence of the challenge of multiple antibiotic resistance to the public health worldwide, a highly effective phage alternative therapy is sought in this study. The antibacterial activity of coliphages were assessed against 15 multiple antibiotic resistant *E.coli* isolates obtained from children with UTI. All isolates (100%) tested were sensitive to bacteriophge lysis effect. This indicated that bacteriophge activity as anti-bacterial therapy was specifically effective against *E. coli* isolates. The electron microscopy revealed three types of viruses named: Siphoviridae, Podoviridae, and Microviridae. The three types of colipahges were assessed for their stability, physical, chemical sensitivity/durability. Coliphages stability with no change in titre was its best at 4ºC, then declines at other temperatures tested at 25, 37, and 45°C. The phage titer decreased with the length of exposure time to sun light from clear (10^{11}) to 150. Incubating phage

suspensions with alcohol, chloroform, detergent and hypochlorite at 37ºC resulted in reduction of phage activity by 10% at 12h with chloroform. On the other hand, ethanol showed the lowest inhibiting effect on the infectivity of tested coliphages. Detergent has reduced infectivity by 10% at different time exposure. While hypochlorite had a 1.25% effect on phage infectivity.

KEYWORDS: Urinary tract infection, *Bacteriophage, E. coli*; multiple antibiotic resistant.

INTRODUCTION

Antimicrobial resistance is one of the major health problems worldwide resulting from overuse of antimicrobials in clinical situations where they are not necessary or in prolonged courses of therapy when shorter durations are as effective. Urinary tract infections (UTIs) are considered one of the most frequently acquired infections in the community, hospitals and other health care set-up, causing a huge amount of antibiotic consumption not only for treatment but also for prophylaxis of infectious complications after urological interventions that leads to a worldwide increase of uropathogens resistant to the first line antibiotics, such as cotrimoxazole, fluoroquinolones and cephalosporin's and to the emergence of multiresistant bacterial strains (Wagenlehner and Naber, 2012; Tandogdu *et al*., 2014).

Bacteriophage therapy was introduced much earlier prior to the discovery of the sulphonamides and antibiotics into medical practice then their uses as an alternative therapy have been only on a limited scale. However, the lytic activity of bacteriophages *in vitro* enabled some investigators to use specific bacteriophages for the differentiation of various species of bacteria (McGrath and Sinderen, 2007). Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics in the USA (Thiel, 2004).

MATERIALS AND METHODS

Subjects and Sampling

There were 820 samples was collected, including 720 samples urine samples were collected from patients with symptoms of UTI. In addition, 100 stool samples were collected from healthy individuals and used as controls. All patients' samples were obtained from emergency units of Ibin Al-Balady, Central Teaching Hospital of Pediatrics, Al-Yarmouk, Fatima Al-Zahraa hospitals during the period from December 2018 to August 2019. Samples cultivated on blood agar base medium and MacConkey agar medium, then isolated on EMB

agar medium and incubated in 37°C for 24 hours. *E. coli* was identified according to Colony shape and Gram strains and its positivity for Catalase, Indole and methyl red, while negative for Oxidase, voges proskauer and citrate test (Collee *et al*., 2005).

Antibiotic Susceptibility Testing

E.coli isolates were tested for their sensitivity to 16 antibiotics by Kirby-Bauer disk (Perilla *et al*., 2010). The results recorded by measuring the inhibition zone (in millimeters) and interpreted according to clinical and laboratory standards (CLSI, 2017).

Isolation and Enrichment of Bacteriophages From Urine

The phage was enriched by mixing 200 ml of fresh samples urine with 20 ml of bacteriophage broth, 20 ml of a mixture of a seven multidrug resistant *E. coli* and 20 ml of brain heart infusion broth to 1 liter sterile flask and incubated at 37°C for 48h with shaking at 120rpm. After incubation, the mixture was centrifuged at 10000 xg for 10 min to remove solid matters, then the supernatant was filtered by using 0.2 µm pore size Millipore filter (Kumar *et al.*, 2011).

Isolation and Enrichment of Bacteriophages From Stool

The stool samples (10g) were homogenized in tryptone salt (8.5g of NaCl and 1g tryptone per liter) to a final volume of 30 ml. Stool samples were centrifuged for 15 min at 14.500 ×g and filtered through a Millex AP20 prefilter followed by a 0.45-µm Minisart filter. Subsequently, the samples were stored at 4°C (Mattila, 2015).

Determination of Phage Titer From Stool

The titer of phage was determined according to a method described by Kropinski *et al.*, (2009) by preparing ten-fold serial dilutions (1-10) using agarose overlay method. The dilution factor that gave the best countable number of plaques was selected and used for all other experiments. The phage activity was determined against multiple antibiotic resistant *E.coli* isolates by using spot assay according to Karumidze *et al*., (2013). The presence of a clear zone on the bacterial lawn was considered as a complete lysis.

Plaque Assay

One milliliter of phage filtrate was transferred into a sterile tube, then, 50 µl of the respective *E.coli* suspension was added and mixed well. It was left for 10 minutes at ambient temperature for allowing phage to adsorb to the host. After 10 minutes, 3 ml of 0.7% Molten

agar (at 50°C) was added, mixed well, and poured over the surface of Eosin Methylene blue agar plate. It was allowed to set at room temperature and incubated at 37°C for 24 hours. Plates were observed and scored positive if there was a presence of clear zone (plaque formation) over the surface of the agar plate. Plaques were counted from all positive samples and recorded as a plaque forming unit (pfu/ml) (Naghavi, 2013).

Spot Testing

A plate is inoculated with *E.coli* to form a lawn, then small drops of phage filtrate were placed on the surface. After incubation a zone of bacterial lysis indicated the presence of phage function (Gill, 2010).

Purification of Phages

For purification of the phages, separate clear plaques were selected and cut off from the agar surface using sterile pipette tips and then mixed in 10 ml SM-buffer with agitation in a vortex mixer. The agar and cell debries were removed by centrifugation at 3000 rpm for 30 min, followed by filtration of the supernatant through a 0.22 µm pore sized syringe filter. The resulting filtrate (phage lysate) was preserved at 4°C until processing (Ahmed, 2013).

Determination of Bacteriophage Host Range

The host range of isolated phages was determined by spot test using bacteria. The plate was marked to allow identification of each phage. A sterile cotton swab was moistened with the broth culture and lawn culture was made on the surface of nutrient agar plate (HiMedia Laboratories, India) from each bacterial strain. Five microliters (5 µl) of each phage lysate was spotted on the marked area of the bacterial lawn on an agar plate. Lysates were allowed to dry then incubated at 37°C for 24 hours. Plates were observed for lytic zone formed on the spotted area and the effectiveness of individual phage was measured (Ibrahim, 2017).

Effect of Culture Media on Plaque Formation

The morphology of plaques made by coliphages was examined on seven different media. These media include blood, nutrient, LB, EMB, TCBS, Muller hiton and MacConkey agars media by double layer agar technique (Hyman *et al*., 2009). An aliquot of 0.1 ml phage lysate was mixed with 0.1 ml of the host culture in 3ml of semi-solid media. This mixture was poured on to the surface of the respective medium. The plates were incubated at 37°C for 24h then the development of plaques and their morphology was observed and recorded.

Stability of Coliphages to Some of Physical Condition

Five temperatures (4, 20, 25, and 45°C) were used to study the thermal tolerance of phage in nutrient broth. An aliquot of phage was taken every 10 min for 12, 24, 36, 48 h, and tittered immediately by a double-layer agar plate method (Chandra *et al*., 2011).

Stability of Coliphages to some of chemical agents

To analyze phage chemical stability, phage were subjected to treatment with various concentration of chloroform at 10, 30, 60 and 100 % (v/v) for 12, 24, 36 and 48 h at 4°C. Treatment was also carried out using hypochlorite at 2.5, 1.25, 3, 75 and 5% (v/v) for 12, 24, 36 and 48 h. In addition, phages were also exposed to alcohol at 10, 30, 70 and 100% for 12, 24, 36, 48 h and phage was treated with detergents at 10, 30, 60 and 100 % (v/v) for 12, 24, 36, 48 h. Then tittered immediately by a double-layer agar plate method (Chandra *et al*., 2011). The results were stated as the percentages of existence phages from original particles in each treatment.

Transmission Electron Microscopy (TEM)

Bacteriophage filtrate was centrifuged at 25000 xg for 1h., where one drop of phage filtrate (10^9) was placed on carbon-coated copper grid and stained negatively with 2% uranyl acetate. Then morphology of purified phages was examined by using the transmission electron microscopy (Cerveny *et al*., 2002).

RESULTS

Isolation and Characterization of Bacteriophages by Plaque Assay

Urine, stool/urine combined and stool samples were used for screening and isolation of different bacteriophages to be tested for its activity on multiple antibiotic resistant (MAR) *E. coli* isolates using the plaque assay test and spot techniques. According to the diameter of the plaques, three types of phage's plaques were observed. These types represented by three plaques: large plaques with a diameter equivalent to 4.5mm, two medium plaques with a diameter of 3mm and one small plaque of 2.5mm. Three different single plaques with different plaque morphologies and diameters designated as A, B and C were picked and selected for further identification and characterization Figure (1).

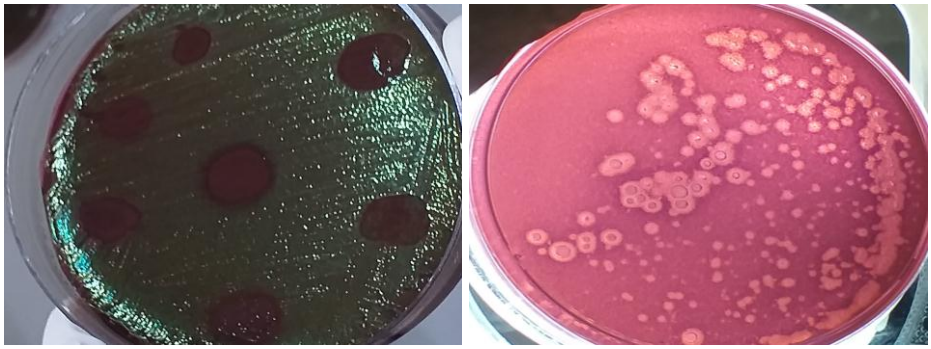

Figure (1): Formation of large and small plaques by bacteriophages derived from stool tested against *E. coli* isolates derived from UTI patients.

Identification of Bacteriophages by Electron Microscopy

As examined by electron microscopy (EM), three types of phages (A, B and C) were seen from samples isolated from stool, mixture (urine and stool) and urine. The first type was belong to *Podoviridae* viruses which contain a short tail of a 100nm in size isolated from stool Figure (2A). The second type was belong to the family of *Siphoviridae* family viruses which contained a long non contractile, cubic face icosahedral with a 220nm in size and head tail isolated from a mixture of urine and stool Figure (2B). The third type was *Microviridae* containing no tail and was 25-27nm in size Figure (2C).

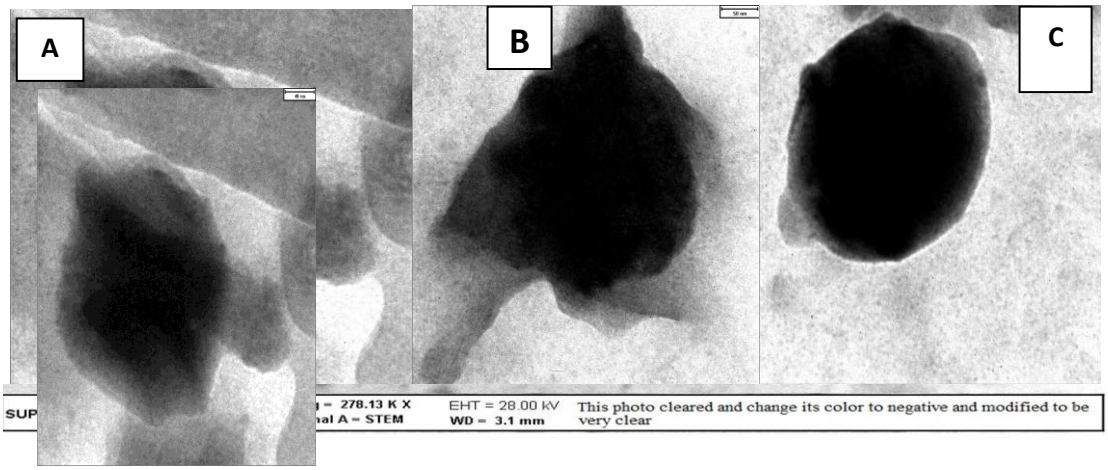

Figure (2): Electron micrographs images of the three types of bacteriopahges isolated from local *E coli* isolates which appeared to be belong to *Podoviridae* (A), *Siphoviridae* (B) and *Microviridae* (C) viruses.

Antibacterial Activity of Isolated Bacteriophages Assessed by Plaque Assay

This study included determination of phage titer by using 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, $10^{-9}$and 10^{-10}) and counting the number of plaque forming units (PFU). Dilutions 10^{-1}, 10^{-2} and 10^{-3} showed complete bacterial lysis and cannot be

counted. The other dilutions showed lysis of 5.65×10^{7}, 1.8×10^{8}, 1.45×10^{10}, 4×10^{10}, 7.5×10^{10}, 2×10^{11}, and 0, respectively. These results indicate that the 10^{-6} dilution gave the best countable number of plaques. This dilution factor was then used for all other experiments of this part of the study. When comparing activity of bacteriophages derived from stool, urine/stool and urine alone, it appeared that stool bacteriophages were the most effective in bacterial lysis, followed by the combination, and then bacteriophages obtained from urine alone Table(1).

Table 1: Antibacterial activity of bacteriophage obtained from stool by plaque assay.

Plate no.	Dilution factor	Plaque per plate	Dilution titer
1	10^{-1}	Clear	Clear
2	10^{-2}	Clear	Clear
3	10^{-3}	Clear	Clear
4	10^{-4}	565	5.65×10^{4}
5	10^{-5}	180	1.8×10^{4}
6	10^{-6}	145	1.45×10^{4}
7	10^{-7}	75	7.5×10^{3}
8	10^{-8}	40	4×10^{3}
9	10^{-9}	20	2×10^{3}
10	10^{-10}	0	0

Table 2: Sources and lysis activity of coliphages derived from stool, stoo/urine and urine of patients with UTI.

Plate number	Plaque per plate		
	Urine	Urine / stool	Stool
1	Clear	Clear	500
2	Clear	Clear	200
3	Clear	Semi	120
4	565	150	90
5	180	130	65
6	145	70	50
7	75	32	15
8	40	0	0
9	20	0	0
10	0	0	0

Clear, complete bacterial lysis by bacteriophages.

Clear, complete bacterial lysis, clear bacteriophage fully lysed bacteria. Titer was calculated by plaque number × dilution factor divided by 0.1

Specificity of Bacteriophages Tested Against *E. coli* Isolates

For this study the three types of bacteriophages (A, B, and C) were assessed for their activities and produced similar results, therefore results for phage A will be presented here.

Phage A from stool was selected to assess host specificity where it showed no activity on bacterial species tested because isolates such as S*higela, Staphylococcus. epidermis, Staphylococcus.. scursi, Staphylococcus aureus, Proteus mirabilis,, Klebsella pneumonae, Salmonella Typhi, Pantoea Spp, Staphylococcus haemolyticus, Acinetobacter baumanni complex, Streptococcus pyogen, and Micrococcus*. These bacteriophages were only worked by lysing its bacteria host of *E.coli* Table (3).

Table 3: Specificity of bacteriophage lysis against a number of bacterial isolates.

Type of bacteria	Bacteriophage lysis ability
E.coli	+
Staphylococcus aures	-
Staphylococcus homenes	-
Staphylococcus epiderms	-
Staphylococcus haemolyticus	-
Shigela Spp	-
Salmonella Typhi	-
Pantoea Spp	-
Enterococcus cloacac	-
Proteus mirabilis	-
Klebsella pneumonae	-
Proteus mirabilis	-
Acinetobacter baumannii complex	-
Streptococcus pyogen	-
Micrococcus Spp	-
Staphylococcus scursi	-

Ability of Different Growth Media to Support Plaques Formation

Eight different types of culture media (EMB, Nutrient, blood, TCBS, XLD, LB, MacConkey and Muller Hinton agars) were used to assess their ability to better support plaque formation. Plaques phenotypes appeared as clear and transparent round zones and uniformed per each plate only on four different media types of EMB agar, nutrient agar, blood and Muller Hinton. On the other hand, they appeared as a multiform on MacConkey agar. The average plaque diameter was 1-3, 1-4, 1-6, 1-8 and 2-4 mm but when cultured on Muller Hinton, MacConkeyy, EMB, blood, Muller Hinton agars, respectively. The results concluded that phage responded differently host on different growth media and the best media in this cases was Muller Hinton followed by EMB, and blood agar Table (4).

Table 4: Effect of different culture media on effectiveness of bacteriophages activity.

Dilutions Of Coliphages				
Media	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Muller Hinton	Clear	Clear	95	75
EMB	565	180	145	75
Nutrient agar	NL	NL	NL	NL
Blood agar	29	28	21	20
LB agar	Semi clear	135	NL	NL
MacConkey agar	105	67	NL	NL
TCBS	NL	NL	NL	NL
XLD	NL	NL	NL	NL

NL, no lysis of bacteria by coliphage

Effect of temperature on the infectivity of isolated phages

Phages A were highly sensitive to heat inactivation after growth of phages at 45°C for 12, 24, 36 and 48h. The appearance of plaque formation (bacterial lysis) was better observed when culture kept at 4°C. The viability decreased as the temperature increases. Stability of coliphage titer was its best at 4ºC, then declines at other temperatures tested at 25, 37, and 45°C Figure (3).

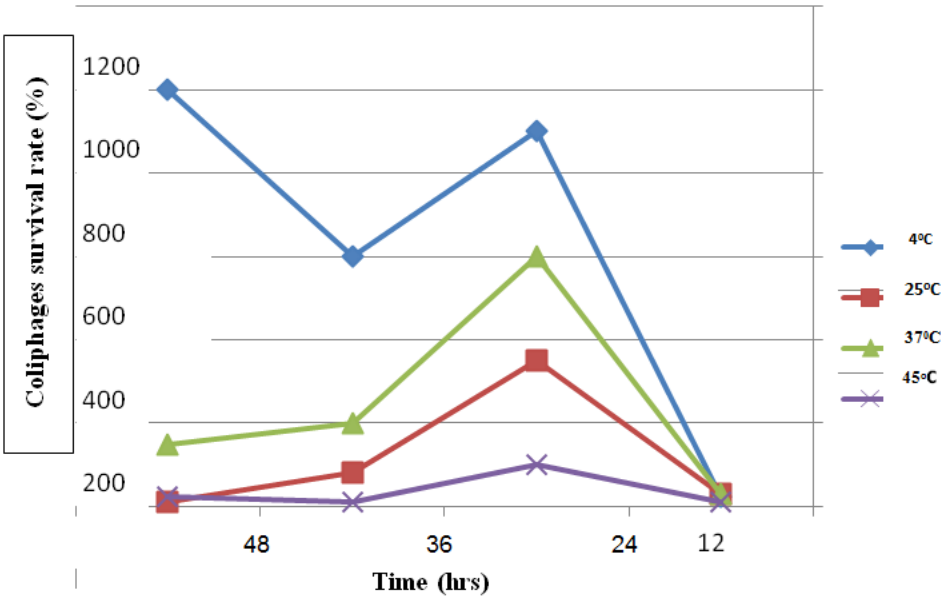

Figure (3): Stability of coliphages after treatment with different temperature ranges (4, 25, 37, and 45°C).

Sensitivity of Coliphages to Sunlight

When the effect of sun radiation on the phages was evaluated, phage counts decreased when exposed directly to sunlight at almost constant ambient temperature for 12, 24, 36, and 48h.

The phage titer decreased as the length of exposure time to sun light increases from clear at 12 h to 150 after 48 h Table(5).

Table (5): Stability of coliphages after exposure to sunlight.

Times (hours)	Sun light
12	Clear
24	Semiclear
36	Semiclear
48	150

Effect of Organic Solvents on the Infectivity of Coliphages

Incubating phages A suspensions with alcohol, chloroform, detergent and hypochlorite at 37ºC, resulted in the reduction of phage activity by 10% at 12h with chloroform. On the other hand, ethanol showed the lowest inhibiting effect on the infectivity of phage A which showed reduction of infectivity by 30-60% at different time points. Detergents have reduced infectivity by 10% at different time exposure. While hypochorite had a limited effect (1.25%) on phage infectivity.

Effect of pH on The Infectivity of Coliphages

The optimum pH for the phages A infectivity were determined by double layer technique and incubation of phage suspensions at different pH concentrations ranging from 4 to 14 for 30 min at 37°C. pH14 was the optimum for bacteriophage stability. The infectivity of the phage was rapidly decreased as pH changed toward acidic (lower than pH6). Moreover, plaque formation of phageA was more sensitive to pH 2, 5, 7, and 9 Figure (4).

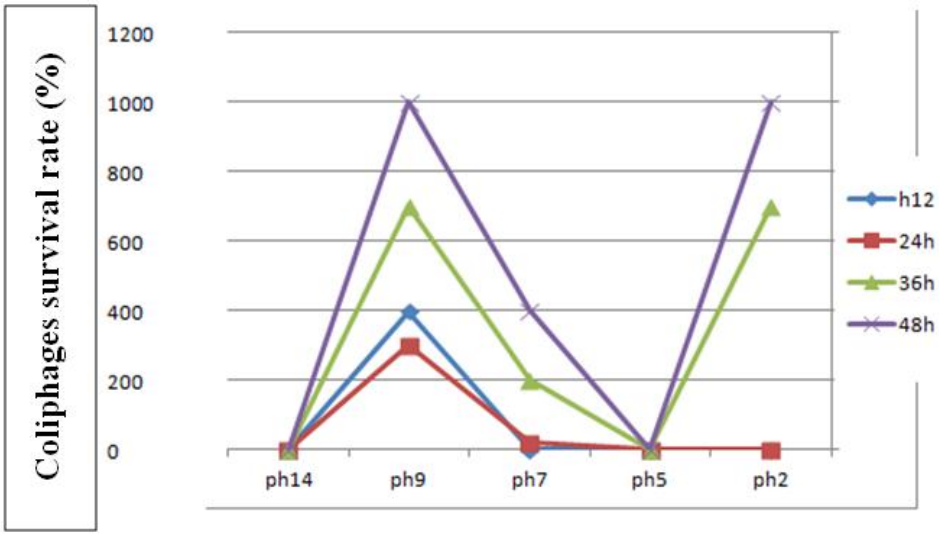

Figure (4): Stability of coliphges after treatment with different pH values.

Effect of Glycerin on Coliphages Infectivity

The storage of phageA at -20 and 4ºC showed the least effect on its infectivity after treatment with 100% glycerin at 4ºC. The ability of phages to form plaques after different storage temperatures were slightly affected Table (6).

Table (6): The effect of glycerin on phages infectivity and titer.

Temperatures	Glycerin concentration	Number of plaques after exposure to glycerin			
		12h	24h	36h	48h
4ºC	20%	clear	clear	clear	clear
	50%	clear	clear	clear	clear
20ºC-	20%	520	216	130	29
	50%	460	224	100	7
	100%	840	420	210	21

DISCUSSION

The successful isolation of *E. coli*-specific phages in 100% of stool samples with higher capability of lysing bacteria compared to urine samples was interesting and this may suggest a substantial ability of the bacteriophage to persist in stool samples, possibly due its ability to replicate off the host bacteria population. *E. coli*-specific phages appear to be naturally abundant enough to achieve phage titration, even though urine and stool \ urine, even if present, appeared at very low levels. This is expected as *E. coli* can be naturally found in the guts of human (Gill and Hyman 2010). The phages have successfully persisted and maintained their populations by replicating off new infections of bacteria. Alternatively, bacteriophages have been shown to have a higher persistency than host bacteria, therefore remained viable (Kudva *et al.*,1998). This raises the possibility that bacteriophage distributions in humans may be decided mainly by infection and opportunities for phage shedding and transmission. The lower distribution of coliphages in urine was, in general, attributed to diluted viral abundance which decreases the rate of phages encountering and lysing od the bacteria (Weinbauer 2004). In contrast, stool phage may simply be more compact, leading to a higher encounter rate between phages and host bacteria compared to other sources. A high encounter rate was observed with stool phages (Garcia *et al.* 2009, Niu *et al.* 2009, Oot *et al*. 2007).

The host range results of bacteriophages revealed that phages derived from stool, urine/stool and urine exhibited specificity to *E.coli* only. Since adsorption to the host cell is controlled by molecular interactions between the phage tail fibers and the cell surface binding site. The host

range is usually determined by the orientation of the G segment in the phage, where the G positive orientation enables *E. coli* to specifically infect its host only. The differences in bacteriophages host range may be associated with diminishing adsorption to an acceptable bacterial receptor. Both evasion systems were seen in *Salmonella* or *E. coli* serotypes (Snyder, 2012)**.**

This might be attributed to required for the changes in polysaccharides constituting the receptivepoints scattered on the host cell wall (Ashbolt *et al.,* 2001 and Samhan, 2005). *E. coli* specifically up-regulates the expression of specific proteins and down-regulates others. These physiological and genetic changes have a dramatic effect on the susceptibility of *E.coli* to coliphage infection. These results agree with previous studies (Stanek & Lacy, 1997).

High temperature degrees might be responsible for destruction of virus receptors that consequently declined its infectivity found that number of plaques and phage titers during 10, 20, 30, 40, 50, and 60 min of chloroform treatment (Al-Mola and Al-Yassari, 2010). In contrast Samhan *et al.,* (2016) found the viability of phages was unaffected by different temperatures. This phenomenon relies on the fact that some phages contain lipids in their virions and these lipids are essential for maintaining virus ability to infect new host cells (Bertani and Bertani (1974)**.** In contrast, Ajoke *et al.*, (2019) found chloroform to remain stable in 10% chloroform after incubation for 1 h. However, the addition of chloroform resulted in a 40% reduction in phage titer. Non-polar solvents are capable of denaturing proteins by disrupting the hydrophobic interaction between proteins, which is part of phage viability (Bertani and Bertani, 1974)**.** Work showed all tested organic solvents inactivated phages at different ratios under certain conditions (Wang and Lin, 2001). In contrast, Geun and Mark (2011) found the titer phage was affected in 250 ppm hypochlorite. Hypochlorite is electronegative, and therefore oxidises peptide links and denatures proteins. Hypochlorite and chloramine in water produce hypochloric acid, which then decomposes. Both chlorine and oxygen are involved, and thiol groups are oxidised. Exposure of E. coli strains, Pseudomonas spp. and Staphylococcus spp. to lethal doses of hypochloric acid decreases ATP production.

We have shown that bacteriophages were very much affected by ethanol (100%) while unaffected by the use of 10% and 60%. These results were similar to those described by Ochieng *et al., (*2020), and Kurek (2016). Detergents generally have less effect on phage than they do on bacteria although those enveloped phages are shown to be quite sensitive. It has been found that 5% SDS reduced phage infectivity (Fister *et al.,* 2016). Another report

viruses had attributed this finding due to that SDS may be capable of denaturing the capsid proteins of non-enveloped.

Freezing at -20ºC in the presence of glycerol have yielded reasonably a good survival rate of various bacteriophages to avoid losses due to aggregation. However, bacteriophages vary in their sensitivity to freezing and freeze-drying where the large phages are more vulnerable than the small, osmotic shock-resistant phages. Thus, appropriate storage conditions need to be determined for each phage (Ashbolt, 2009).

The abilities of phages isolated from stool to form plaques after different storage periods were inversely proportioned to incubation temperature. The infectivity processes or propagation cycles were not properly completed, reflecting a decrease in the total viability of phages.

CONCLUSION

This study presented a well characterized three types of bacteriophages isolated from stool, stool/urine and urine derived from patients with UTIS. It showed that phage obtained from stool had higher lysis activity of multi-antibiotic resistant *E. coli* than the stool/urine and urine phages. Therefore, phage stool would be more effective alternative antibacterial therapy to treat multidrug resistant *E. coli* in UTI patients.

ACKNOWLEDGMENTS

Conflict of Interest

Authors' contribution

REFERENCES

1. **Ahmed**, D.; Islam, M.S.; Begum, Y.A.; Janzon, A.; Qadri, F. and Sjoling, A. Presence of enterotoxigenic *Escherichia coli* in biofilms formed in water containers in poor households coincides with epidemic seasons in Dhaka. *J Appl Microbiol*, 2013; 114: 1223–1229.
2. **Ajoke** Akindolire Muyiwa and Collins Njie Ateba (2019). Isolation and characterisation of bacteriophages with lytic activity against virulent *Escherichia coli* O157:H7: potential bio-control agents doi:10.20944/preprints201901.0132.v1.
3. **Al-Mola, G. A. and Al-Yassari, I. H.** Characterization of *E.coli* phage isolated from sewage. *AL-Qadisiya Journal of Vet. Med. Sci*., **2010; 9**(2): 45-52.

4. **Ashbolt, N., Grabow, W. and Snozzi, M. (2001)** Indicators of microbial water quality. "*Water Quality: Guidelines, Standards and Health*," (Grabow 1996); 289–316.
5. **Bertani, G. and Bertani, L. E.** Constitutive expression of bacteriophage P2 early genes resulting from a tandem duplication. *Proc. Natl. Acad. Sci. U S A*, **1974; 71**(2): 315–319.
6. **Cerveny** KE, DePaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by Vibrio vulnificus in iron-dextran-treated mic. Infect Golkar, Z., Bagasra, O., and Pace, D. G. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J. Infect Dev. Ctries, 2014; 8: 129–136.
7. **Chandra** M.; Thakur S.; Narang D. and Saxena H. M. Isolation of a bacteriophage against Salmonella Dublin and determination of its physical resistance under varied in vitro conditions, African Journal of Microbiology Research, 2011; 5(15): 2044-2047.
8. **CLSI.,** Performance standards for antimicrobial susceptibility testing (27th) informational supplement. M100-S27. Clinical Laboratory Standards Institute, 2017; 27(1): 42-67.
9. **Collee, J.**; Fraser, A.; Marmion, B. and Simmons, A. (1996). Mackie and
10. **Fister** Susanne, Christian Robben, Anna K. Witte, Dagmar Schoder, Martin Wagner and Peter Rossmanith. Influence of Environmental Factors on Phage–Bacteria Interaction and on the Efficacy and Infectivity of Phage P100. Journal of Frontiers in Microbiology, 2016; 7: 1152. doi: 10.3389/fmicb.2016.01152.
11. **Geun** Woo and Mark D. Sobsey Simultaneous Comparison of Murine Norovirus, Feline Calicivirus, Coliphage MS2, and GII.4 Norovirus to Evaluate the Efficacy of Sodium Hypochlorite Against Human Norovirus on a Fecally Soiled Stainless Steel Surface. FOODBORNE PATHOGENS AND DISEASE, 2011; 8. DOI: 10.1089/fpd.2010.0782.
12. **Gill**, J., and Hyman, P. Phage choice, isolation, and preparation for the phage therapy. Curr. Pharm. Biotechnol, 2010; 11: 2–14. doi: 10.2174/138920110790725311.
13. **Hyman** P. and Abedon S. Practical methods for determining phage growth parameters. In: Clokie MRJ, Kropinski AM (eds) Bacteriophages: methods and protocols, volume 1: isolation, characterization and interactions, 501. Humana Press, New York, 2009; 175–202.
14. **Ibrahim**, I. A.; Al-Shwaikh, R. M. and Ismaeil, M. I. Virulence and Antimicrobial Resistance of Escherichia coli Isolated from Tigris River Children Diarrhea. Infect Drug Resist, 2014; 7: 317-322.
15. **Karumidze** N., Kusradze I., Rigvava S., Goderdzishvili M., Rajakumar K., Alavidze Z. Isolation and characterisation of lytic bacteriophages of *Klebsiella pneumonia* and

Klebsiella oxytoca. *Curr. Microbiol*, 2013; 66 251–258. 10.1007/s00284-012-0264-7422–426. doi: 10.3923/jbs.2013.422.42.

16. **Kropinski** AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol., 2009; 501: 69–76. doi:10.1007/978-1- 60327-164-6_7
17. **Kudva, I. T., K. Blanch and C. J. Hovde.** Analysis *of Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. Applied and Environmental Microbiology, 1998; **64**: 3166-3174.
18. **Kumari**, S; Harjai, K. and Chhibber, S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by Klebsiella pneumoniae B5055.*J Med Microbiol.*, 2011; 60(Pt 2): 205-10.
19. **Kurek -Jurczak**,A.; Gąsior,T.; Nejman-Faleńczyk,B.; Bloch,S.; Dydecka,A.; Topka,G.; Necel, A.; Jakubowska-Deredas, M.; Narajczyk,M.; Richert,M.; Mieszkowska,A.; Wróbel, B.; Węgrzyn, G.; Węgrzyn, M. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. Scientific Reports, 2016; 6: 34338 | DOI: 10.1038/srep34338.
20. **Mattila** S, Ruotsalainen P, Jalasvuori M. On-demand isolation of bacteriophages against drug-resistant bacteria for personalized phage therapy. Front. Microbiol, 2015; 6: 1271. Microbiology Research, 5(15): 2044-2047.
21. McCartney. Practical medical microbiology, 14: 413-424.
22. **McGrath** S. and vanSinderen D. Bacteriophage: Genetics and Molecular Biology, 1st edition. Caister Academic Press, Norfolk, UK., 2007; 445-514.
23. **Naghavi**, N. S., Golgoljam, M., and Akbari, M. (2013). Effect of three sewage isolated bacteriophages on the multidrug resistant pathogenic bacteria. J. Biol. Sci., 2002; 70(11): 6251-62.
24. **Niu, Y. D., T. A. McAllister, Y. Xu, R. P. Johnson, T. P. Stephens, and K. Stanford**. Prevalence and impact of bacteriophages on the presence of *Escherichia coli* O157:H7 in feedlot cattle and their environment. Appl. Environ. Microbiol, 2009; **75**: 1271-1278.
25. **Ochieng.** Joseph M.' Oduor, Ermir Kadija, Atunga Nyachieo, Marianne W. Mureithi and Mikael Skurnik, Bioprospecting Staphylococcus Phages with Therapeutic and Bio-Control Potential. Journal of viruses, 2020; 12: 133. doi:10.3390/v12020133.
26. **Oot, R. A., R. R. Raya, T. R. Callaway, T. S. Edrington, E. M. Kutter, and A. D. Brabban.** Prevalence of *Escherichia coli* O157 and O157:H7-infecting bacteriophages in feedlot cattle feces. Lett. Appl. Microbiol, 2007; **45**: 445–453.

27. **Perilla MJ**, AJello G, Boop C, Elliot J, Facklam R, Popovic T, Wells, J. Manual for laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in developing World. CDC, Atlanta, Georgia, USA, 2010.
28. **Samhan, F. A. (2005)** Coliphages as indicators and test organisms for assessment of hygienic safety of water, wastewater and liquid organic wastes, PhD Dissertation, University of Hohenheim, Germany. Verlag Grauer. Beuren and Stuttgart. ISBN 3861865033.
29. **Snyder**, A. B. (2012). Isolation and characterization of bacteriophage for the control of enterohemorrhagic *Escherichia coli* on fresh produce. *PhD. Thesis*, 2012 Ohio State University, USA.
30. **Stanek, J. E. and Lacy, G. H. (1997)** Development of a rapid coliphage detection assay. *PhD. Thesis.* Virginia Polytechnic Institute and State University, Blacksburg, VA., U.S.A.
31. **Thiel**, K. "Old dogma, new tricks—21st Century phage therapy". Nature Biotechnology, 2004; 22(1): 31-36.10.3855/jidc.3573Immun.
32. **Wagenlehner, F.**; and Naber, K. Editorial commentary: asymptomatic bacteriuria—shift of paradigm, 2012.
33. **Wang**, A. and Lin, K. Effects of N-acetylglucosamine and α -methylglucoside on bacteriophage T4 adsorption to *Escherichia coli* B23, *Journal of Experimental Microbiology and Immunology (JEMI)*, 2001; 1: 54–63.
34. **Weinbauer, M. G.** Ecology of prokaryotic viruses. FEMS Microbiol. Rev., 2004; **28**: 127-181.