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Assessment of Potential Efficacy of Coliphage Therapy on Multidrug Resistant Clinical Isolates of *Escherichia coli*

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Abstract

Coliphages are the bacteriophage that attack and lyse Escherichia coli, a bacterium emerging as multidrug resistant, thereby posing serious public health challenge. Thus, searching for alternative therapies, one of which is coliphage therapy is timely. The work was aimed at screening clinical isolates of E. coli for the ability to host the coliphage and to determine the cytopathic effect of the coliphages against the multidrug resistant E. coli hosts. Eight (8) clinical isolates of E. coli were reconfirmed using both conventional and PCR techniques. The isolates were used for the detection and enumeration of the coliphage. The lowest plaque forming unit (PFU/µL) dilution of each of the 5 samples collected, was determined using double agar overlay method. The isolates that successfully hosted the growth of the phage were further screened against 8 commonly used antibiotics, using disc diffusion method. Out of the 8 clinical isolates collected, 5(62.5%) were confirmed as E coli, out of which 3 (60%) supported the growth of the coliphage. The lowest PFU dilution was 1:10^8 and all the 3 isolates of E. coli that supported the growth of the coliphages were found to be Multi Drug Resistant (MDR) and were all (100%) lysed by the coliphages. Phage therapy was found to be effective against even the MDR bacteria as such, can be considered an alternative therapy. However, a cocktail of the phages may be necessary to ensure absolute adsorption and successful lysis of the pathogens.

Key Words: Bacteriophage, Coliphage, Multidrug Resistance, Cytopathic effect

INTRODUCTION

Bacteriophages also known as phages are viruses that infect bacteria. They were discovered independently in the early 20th century by Frederick Twort in England in 1915 and Felix d'Herelle in France in 1917 (Wok, 2001). The potentials of bacteriophages in the therapy of the bacterial infection were discovered prior to the discovery of antibiotics (Morozova *et al.*, 2018), however, the advent of the antibiotics relegated the idea of the phage therapy due to ethical issues (Sarker *et al.*, 2016) and limitations of scientific information necessary for the acceptance of the therapy (Morozova *et al.*, 2018). In spite of the rapid success of antibiotics, the emergence of multidrug resistant bacteria is of public health concern, as some strains of bacteria are resistant to almost all the antibiotics available (Calap and Martinez, 2018; Principi *et al.*, 2019), making it necessary to seek for alternative methods of therapy (Sarker *et al.*, 2016). Some strains of *E. coli* are significant pathogens, causing diarrhoea, urinary tract infections, bacteremia and meningitis; as such, their multidrug resistance is a serious health threat (Delmas *et al.*, 2015). Therefore, coliphages being the phages that infect *E. coli*, are now considered the alternatives in the treatment of the *E. coli* infections (Sarker *et al.*, 2016).The major advantage of phage therapy is their high level of selectivity, minimal possibility of inducing self-resistance, very minute possibility of conferring side effects during or after treatment and their abundance in nature (Morozova *et al.*, 2018; Lin *et al.*, 2017). The study was designed to isolate coliphages from water samples collected from Ahmadu Bello University (ABU) Dam and to determine their cytopathic effect against multi drug resistant *Escherichia coli*

MATERIAL AND METHODS

Collection of Isolates

The study made use of 8 clinical isolates of *E. coli*, collected from the Laboratory of the Department of Microbiology, Ahmadu Bello University, Zaria.

Sub-Culture of the Isolates

Using aseptic techniques, the isolates collected were sub-cultured on Eosin Methylene Blue (EMB) agar plates, and were incubated at 37°C for 24hours.

Conventional Characterization of *E. coli*

Preliminary identification of the isolates were carried out using Gram staining technique and observed under a microscope at 100X objective lens (Cheesebrough, 2009). The isolates were

subjected toother tests including Methyl Red (MR)-Voges-Proskauer (VP), using MR-VP broth (Oxoid), inoculated and incubated overnight at 37°C, subsequently appropriate reagents were added and the reaction was observed. The isolates were further subjected to Indole test, a colony was inoculated into peptone water and incubated at 37ºC for 24 hours, and then 0.5ml of Kovac's reagent was added and the reaction was observed. They were also tested for their ability to utilize citrate by streaking a pure discreet colony from the cultures of the respective isolates, on to Simmon's citrate slants, and were incubated at 37°C for 24 hours (Cheesebrough, 2009).

Molecular Characterization

DNA Extraction

The DNA was extracted from fresh cultures, grown overnight in Eppendorf tubes containing 1ml of nutrient broth. The tubes were centrifuged (Bio-Rad centrifuge) at 10000 rpm for 10min. The supernatants were decanted and the sediments were suspended in 0.5ml of 1X PBS buffer and centrifuged for another 10min at 10000rpm. The supernatant was decanted and the sediments were re-suspended in 0.5ml of sterilized distilled water. The tubes were vortexed and heated in heating block at 100^0C for 10 minutes. The tubes were then incubated in ice for 15min before were centrifuged at 16000rpm for 10min. Then 0.5ml of the supernatants (DNA) were pipetted into sterilized Eppendorf tubes and stored at -2^0C until further analysis (Lindsay *et al.*, 2017).

PCR for the Amplification of *EC*Gene

Each reaction was prepared as 25µl, which contained 12.5µl of the master mix (Biolabs, England), 3µl of the DNA, 0.5µl (10µmol) each of the forward and reverse primers (Inqaba Bio Tech) and 8.5µl molecular grade water. The PCR was run using a pair of primer (F-5''CCAGGCAAAGAGTTTATGTTGA3'R 5'GCTATTTCCTGCCGATAAGAGA3') (Lindsay *et al.*, 2017). The PCR condition was run using a thermo cycler (BioRad, USA): initial denaturation at 94°C for 30sec followed by subsequent denaturation at 94°C for 30sec, annealing at 48°C for 30sec and extension at 68°C for 60sec. The condition (except the initial denaturation) was run for 35 cycles before the final extension at 68°C for 5min. The amplified genes (212bp) were viewed and documented using UV light imaging system (BioDoc-It™).

Antibiotic Susceptibility Testing

The suspension of each of the isolates was prepared in normal saline and standardized to0.5 McFarlandscale. The standardized suspensions were aseptically inoculated onto the Mueller-Hinton agar plates, using a sterile swab stick and allowed for complete absorption. The plates were then seeded with the 8 antibiotic disks namely: gentamicin, streptomycin, ciprofloxacin, amoxicillin, augmentin, trimethoprim/sulfamethoxazole and ofloxacin. The plates were inverted and incubated at 37ºC for 18hours, after which the zone of inhibition was recorded and compared to CLSI manual (Wayne, 2018) for the interpretation.

Isolation of Coliphage

A 10ml of water sample from 5 different locations were collected from ABU Dam. The samples were centrifuged at 5000RPM for 5minutes. The supernatant was filtered using a membrane filter (pore size = 0.22µm). Using peptone water as the diluent, serial dilution of the filtrate was carried out in a microtitre plate; in the first well, the stock was prepared by mixing180µL and 20µLof the peptone water and the filtrate respectively (1:10). Serial dilution was carried out in the subsequent wells to the dilution of 10^{-8}($1:10^8$).

Soft agar was prepared as 0.9% and was kept in a water bath at 50ºC, out of which 3ml was mixed with 10µL of the overnight broth culture of the *E.coli*. The mixture was quickly poured onto nutrient agar plate and was allowed to spread evenly on a flat surface. Then, 10µL from each of the 8 dilutions was point-inoculated on to a different position of the plate and was incubated aerobically at 37ºC for 24hours. The same procedure was repeated to observe the lysis on the multidrug resistant isolates using the lowest PFU dilution (Santos *et al.*, 2009).

RESULTS

Only 5 out of the 8 (6.25%) isolates collected appeared as non-mucoid with metallic green sheen on the EMB plates. The isolates were found to be Gram negative rods, indole and MR positive, VP negative and without ability to utilize citrate as carbon source.

All the five (100%) isolates conventionally identified as *E. coli*, showed *EC* gene band, which is uniquely confirmatory for *E. coli* (Plate I). However, only 3 out of the 5 (60%) isolates (EC1-EC3) supported the growth and the enumeration of the coliphage. The coliphages were recovered from all the 5 (100%) sampling locations, and identified by their ability to lyse (cytopathic effect) the bacteria (*E.coli*); the count was in range of 2.6 x 10^5- 7.7 x 10^6PFU/µL (Table 1).

The isolates showed absolute resistance (100%) against amoxicillin and streptomycin; the rest of the antibiotics showedresistance against 66.7% of the isolates, except Pefloxacin which was effective against 66.7% of the isolates (Table 2).

All the isolates (100%) showed significant MAR indices: EC1 showed the highest index of 1, followed by EC3 which showed 0.75 and EC2 showing 0.375 (Table 3). There was absolute (100%) cytopathic effect in form of cell lysis against all the MDR isolates of *E. coli*, which appeared as clear zone within the colonies (Plate II).

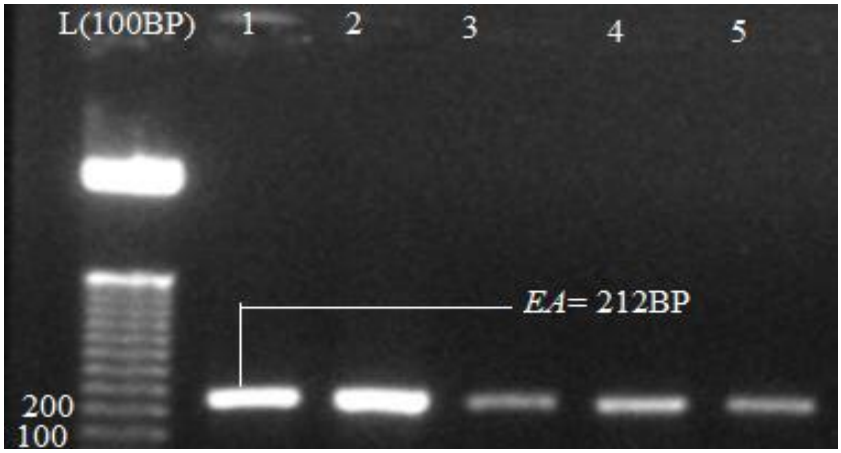

Plate I: amplicons of *EC* gene band of *E. coli*. L was the ladder (100bp) used and lane 1-5 were the samples.

Table 1: Occurrence and Load of Coliphages from different locations within Ahmadu Bello University Dam

Sampling site	EC 1 (PFU/µL)	EC 2 (PFU/µL)	EC 3 (PFU/µL)	E C 4 (PFU/µL)	EC 5 (PFU/µL)
ADL 1	2.3×10^7	3.0×10^7	2.6×10^5	NP	NP
ADL 2	3.1×10^7	3.7×10^6	3.4×10^7	NP	NP
ADL 3	3.8×10^5	4.3×10^7	4.0×10^6	NP	NP
ADL 4	5.9×10^6	6.6×10^5	6.3×10^7	NP	NP
ADL 5	7.2×10^5	8.0×10^6	7.7×10^6	NP	NP

Keys: ADL = ABU Dam Location, NP= No plaque, EC = *Escherichia coli*, PFU/µL = Plaque forming units/microliter

Table 2: Antibiotic Susceptibility Pattern of the Clinical Isolates of *E coli*

Antibiotics (Potency/µg)	Susceptible (%)	Intermediate (%)	Resistant (%)
Amoxacillin (10)	0(0)	0(0)	3 (100)
Pefloxacin (5)	2 (66.7)	0(0)	1(33.3)
Ofloxacin (5)	1(33.3)	0(0)	2(66.7)
Ciprofloxacin (5)	1(33.3)	0(0)	2(66.7)
Trimethoprim/ Sulfamethoxazole (5)	1(33.3)	0(0)	2(66.7)
Streptomycin (10)	0(0)	0(0)	3(100)
Augmentin (10)	0(0)	1(33.3)	2(66.7)
Gentamicin(10)	1(33.3)	0(0)	2(66.7)

Table 3: Multiple Antibiotic Resistance Indices (MARI) of the Clinical Isolates of *E. coli* (N=8)

Isolate Code	No. of Antibiotic Resisted	Resistance Pattern	MAR Index
EC1	8	AM, PEP, OFX, CPX, SXT, S, AU, CN	1
EC2	3	AM, S, AU	0.375
EC3	6	AM, OFX, CPX, SXT, S, CN	0.75

AM = Amoxacillin, PEP =Pefloxacin, OFX =Ofloxacin, CPX= Ciprofloxacin, SXT= Trimethoprim/sulfamethoxazole, S =Streptomycin, AU=Augmentin,CN= Gentamicin.

Plate II: Cytophatic Effect of Coliphagesagainst Multidrug Resistant Clinical Isolates of *E. coli*

DISCUSSION

All the 5 (100%) location sampled in the study were rich in coliphages, indicating their abundance in nature, as previously reported (Pallavalli *et al.*, 2017), this suggests that using phage as an alternative therapy may be cheap, as they are richly around us. However, failure of the phages to cause lysisin up to the 40% of hosts in the study may be an indication to the significance ofreceptor recognition and the pathway undergone by the phage in the success of the therapy. For the phage therapy to be successful, receptor recognition must take place between the host bacteria and the phage, so also the phage must undergo the lytic pathway, which is the pathway that results in the lysis of the bacteria (Sulakvelidze *et al.*, 2001; Haq *et al.*, 2012). Therefore, to improve the success of the phage therapy, a cocktail, carrying various classes of the phages has been suggested; for instance 100% recognition and lysis was reported in a study where cocktail of the coliphages was employed (Sarker *et al*, 2016).

The current findings implicated *E. coli* in posing public health threat, with only perfloxacin as the suitable antibiotic for the treatment, as previously reported (Igwe *et al.*, 2016). This further confirmed how the era of antibiotics is gradually diminishing and the urgency in the need for the discovery and the standardization of the other alternative means of treating the infections caused by the pathogenic bacteria. Furthermore, the potency of even the lowest dilution in lysing the bacterial host in the study, is a promising evidence thatphage therapy is quite efficient and good alternative to antibiotic treatment, as previously reported(Salmon and Fineran, 2015; Sulakvelidze, 2011; Withey *et al.*, 2005; Parfitt; 2005).

CONCLUSION

Phages were abundant in all the locations sampled. The lowest dilution (PFU/µL) that lysed the MDR isolates of bacteria was 1: 10^8, however, the higher the dilutions, the more the plaques. The coliphage-*E. coli* (parasite-host) lytic relationship was determined as 60%. All the isolates of *E. coli* (100%) screened in the study were MDR and were absolutely (100%) lysed by the coliphage.

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