

## IN VITRO SCREENING OF COLIPHAGE EFFICACY AGAINST MULTIDRUG DRUG RESISTANT CLINICAL ISOLATES OF *ESCHERICHIA COLI*

\*<sup>1</sup>Sulaiman, M. A., <sup>1</sup>Kawuni, J., <sup>1</sup>Madika, A., <sup>1</sup>Musa B., <sup>1</sup>Tijjani, M. B., <sup>1</sup>Atta, H. I. and <sup>2</sup>Musa Hassan Muhammad

<sup>1</sup>Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna State

<sup>2</sup>Department of Microbiology and Biotechnology, Federal University, Dutse, Jigawa State

Corresponding Author's email: [masulaiman@abu.edu.ng](mailto:masulaiman@abu.edu.ng) +2348038937309

### ABSTRACT

The incidence of multi-drug resistance (MDR) in bacteria has become a major problem in this era of modern medicine, making it necessary to look for other options that can be used to treat the infections caused by the MDR bacteria. Scientifically, one of the therapies that is now looking promising, is application of phage therapy. This research work was designed to determine the efficacy of coliphages isolated from sewage, against MDR clinical isolates of *E. coli*. Ten (10) clinical isolates were subjected to both microbiological conventional and PCR techniques for confirmation, thereafter, the confirmed isolates were tested against some 8 commonly used antibiotics using disk diffusion method to identify the MDR among them. The MDR isolates were subjected to various dilutions (1:10-1:10<sup>8</sup>) of coliphage recovered from sewage, sampled from 5 different locations and isolated by using double agar overlay method. Out of the 10 isolates collected, 8(80%) were confirmed as *E. coli*, all (8/8: 100%) of which were MDR. However, 6(75%) out of the 8 MDR *E. coli*, were lysed by the coliphages-observed as cytopathic effect in form of clear zones (plaques) within the bacterial lawn and were recorded from all the dilutions (100%) tested and from all the locations (100%) sampled. Conclusively, infection by MDR *E. coli* may be life threatening, as it is highly prevalent (100% in our study). However, the coliphages were found to be abundant in sewage and the application of their therapy may provide a good alternative treatment against the MDR.

**Keywords:** Bacteriophages, Coliphages, Multi-drug resistance, Cytopathic effect, Plaque, Lytic effect, Phage therapy.

### INTRODUCTION

Although there has been a success in administering antibiotics to treat infectious diseases, the emergence of MDR bacteria is now becoming a setback to that success (Principi *et al.*, 2019). Such MDR bacteria include *Escherichia coli*, known to have some significant pathogenic strains, been associated with urinary tract infections, diarrhoea, meningitis and bacteremia; thus, their resistance to multiple drugs is a serious health challenge (Delmas *et al.*, 2015). This deemed it necessary to seek for alternative methods of therapy (Sarker *et al.*, 2016). Bacteriophages, also called phages are simply referred to as host-specific viruses that target and infect bacteria for replication (Adhikari, 2013). These viruses are so ubiquitous in nature and exist in environments wherever their suitable bacterial hosts exist. Such environments include sewage, soil and natural water bodies (Premarathne *et al.*, 2017). Generally, phage's lytic life cycle results to the lysis of the bacterial host cell (Balcazar, 2017; Dubey *et al.*, 2015; Karthik *et al.*, 2014). Phage therapy is the therapeutic application of phages to treat infections of bacterial etiology (Premarathne *et al.*, 2017), likewise coliphage (a bacteriophage which infect *E. coli*) therapy means application of the coliphage in the treatment of *E. coli* infections (Sulaiman and Usman, 2020). The therapy may be considered a good alternative against *E. coli* infections, especially the ones caused by MDR strains (Sarker *et al.*, 2016). There are nine strains of coliphages, namely, the T-even phages; T2, T4 and T6, and the T-odd phages; T1, T3, T5 and T7 (Shende *et al.*, 2017; Adhikari, 2013). Generally, the therapy is more effective when two or more strains of the phages are used as cocktail (Sarker *et al.*, 2016). The major advantage of phage therapy is that there is only minute possibility of conferring side effect (such as

allergies and reactions to the bacterial endotoxins), they have high level of selectivity and minimal possibility of inducing self-resistance (Morozova *et al.*, 2018; Lin *et al.*, 2017). This work was aimed at *in vitro* screening of coliphage efficacy isolated from sewage against multidrug resistant clinical isolates of *E. coli*.

### MATERIALS AND METHODS

#### Source of Isolates

Ten clinical isolates of *E. coli* from faecal samples of gastroenteritis patients from our previous studies (Sulaiman, *et al.*, 2019) were obtained in nutrient broth and used in the study.

#### Bacterial Isolates Confirmation

The isolates were first sub-cultured by streaking aseptically on Eosine Methylene Blue (EMB) agar plates and incubated at 37°C for 24 hours. The colonies typical of *E. coli* were further characterized by Gram staining carried out aseptically and observed under oil immersion objective lens (Cheesebrough, 2009). The isolates were further subjected to other tests including Methyl Red (MR)-Voges-Proskauer (VP), using MR-VP broth (Oxoid), which was inoculated with pure colony and incubated overnight at 37°C; subsequently 1mL of the inoculated broth was aseptically transferred in to a sterilized test tube (to have a tube each for MR and VP tests). For the MR test, 5 drops of methyl red was added and for the VP test 3 drops of 6% alpha naphthol and 1 drop of 40% potassium hydroxide were added respectively, thereafter the change in colour (red ring developed for MR test and pink colour formed within 15min for VP test) was observed. Additionally, indole test was carried out, where a colony was inoculated into peptone water and incubated at 37°C

for 24 hours, then 0.5ml of Kovac's reagent was added and the reaction inform of colour development was observed. Finally, 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 agar slants, and were incubated at 37°C for 24 hours before the colour change was observed (Cheesebrough, 2009). The isolates re-characterized as *E. coli* conventionally, were further confirmed by using molecular techniques.

### Molecular Characterization Techniques

#### DNA Extraction

The DNA extraction was carried out using boiling method (Slulaiman and Usman, 2020). Fresh cultures of the bacteria, grown overnight in Eppendorf tubes containing 1mL of Luria-Bertani (LB) broth and then centrifuged (Bio-Rad centrifuge, USA) at 6000xg for 10min. The supernatants were decanted and the sediments were suspended in 0.5mL of 1X phosphate-buffered saline (PBS) and centrifuged for another 10min at same 6000xg. The supernatants were decanted and the sediments were re-suspended in 0.5mL of sterilized distilled water. The tubes were vortexed and boiled in heating block at 100°C for 10 min. The tubes were incubated in ice for 20min before were re-centrifuged at 6000xg for another 10min. Then 0.5mL of the supernatants (DNA) were pipetted into sterilized Eppendorf tubes (1.5mL) and stored at -2°C until further analysis (Slulaiman and Usman, 2020).

#### Conventional PCR and Electrophoresis

The pair of primer used (F-5'CCAGGCAAAGAGTTTATGTTGA3'R 5'GCTATTTCTGCCGATAAGAGA3') (Lindsay et al., 2017), were synthesized at Inqaba BioTec Inc (South Africa). Each reaction was prepared as 25µl, containing 0.5µL (10µmol) each of the forward and reverse primer, 12.5µL of the green master mix (Biolabs, England), 3µL of the DNA, and 8.5µL molecular grade water (Biolabs, England). The PCR condition was ran using a thermo cycler (BioRad, USA): initial denaturation at 94°C for 30sec, then subsequent denaturation at 94°C for 30sec, annealing at 48°C for 30sec and extension at 68°C for 60sec, which were run for 35 cycles before the final extension at 68°C for 5min. The gene bands (212bp) were viewed and documented using UV light imaging system (BioDoc-It™).

#### Screening the Isolates for MDR

The isolates confirmed (coded as EC1-EC8) (Table 2) were screened for antibiotic susceptibility using disk diffusion method (CLSI, 2018). Fresh cultures of the isolates were suspended in 0.5ml of sterilized 1X physiological saline and the turbidity was adjusted to 0.5 McFarland standard scale. A 0.1ml from each of the standardized suspensions was streaked aseptically on to Mueller-Hinton agar plates and allowed for complete absorption before were seeded with the following antibiotics (Oxoid): sparfloracin (10µg), ciprofloracin (30µg), amoxicillin (30µg), augmentin (10µg), gentamycin (30µg), pefloracin (30µg), ofloxacin (10µg), and streptomycin (30µg). The plates were incubated overnight at 37°C before were examined for zone of inhibitions, which were recorded and interpreted using CLSI manual (CLSI, 2018). The isolates that were resistant to at least 2 antibiotics of different classes (MDR) were further tested against the coliphage.

#### Sample (sewage) Collection for Isolation of Coliphages

The samples were collected from 5 different locations (coded as AL1-AL5) within ABU Zaria, using sterile containers (100mL). The containers were aseptically opened and lowered into the sewage collecting compartments. The bottles were removed when filled to the 50mL and transported immediately (under room temperature protected from sun and UV light) to the laboratory, Department of Microbiology, ABU Zaria for analysis.

#### Preparation of phage suspension

Phage suspensions were prepared by transferring 10mL of the samples each into a sterile test tube and centrifuged at 2000xg for 5 min. After centrifugation, the supernatants were carefully and aseptically transferred each into a sterilized conical flask and aseptically filtered through a 0.22µm pore sized membrane filter (MF-Millipore)™ to remove particulates and bacterial cells (Beaudoin et al., 2007).

#### Preparation of the Phage Dilutions

The stock was prepared by mixing 20 µL of the phage suspension with 180µL peptone water in a microtitre plate well (1:10). Furthermore, 7 more wells were dispensed each with 180µL of peptone water and from the stock 20µL was used to carry out serial dilution from 10<sup>-2</sup>-10<sup>-8</sup>.

#### Determination of the Cytopathic Effects of the Coliphage Dilutions on MDR *E. coli*

Plaque Assay was used to determine the cytopathic effect of the coliphages against the clinical isolates of *E. coli* (Cappuccino and Sherman, 2014). A 10µL from 24 hours broth cultures of the MDR isolates each, was aseptically pipetted into the 5mL of the soft agar (prepared as 0.9% in distilled water, kept in water bath maintained at 50°C). The mixtures were shaken gently and each was overlaid aseptically onto a nutrient agar plate. The overlaid soft agar was allowed to solidify, and marker was used to divide each of the plates in to 8 parts. Then, 10µL from each of the eight different dilutions (1:10-1:10<sup>8</sup>) of the phage suspensions was inoculated on a different part on the plates. The plates were incubated aerobically at 37°C for 24 hours and examined for cytopathic effects.

### RESULTS

Out of the 10 isolates collected, 8 (80%) were confirmed as *E. coli*, being non-mucoid, blue-black colony with green metallic sheen on EMB agar. Also, they were Gram negative rods, Indole and MR positive, but VP and citrate negative. Furthermore, all the 8 isolates confirmed as *E. coli* by microbiological conventional techniques, were found to carry EC gene (Plate I), which is unique for *E. coli*.

Absolute resistance (100%) of the isolates was observed against augmentin and amoxicillin, which is followed by 75% resistance against sparfloracin, pefloracin and ofloxacin. Furthermore, a very high level of resistance of the isolates was recorded against streptomycin (62.5%) and ciprofloracin (50%), with good level of efficacy (62.5%) only observed in the activity of gentamicin (Table 1).

Furthermore, the evidence of the antibiotic resistance of the individual isolates was more pronounced, as all of them (100%) showed significant MAR index (≥0.2). The MAR index was highly significant, ranging from 0.625 (in isolates that resisted 5 out of the 8 antibiotics tested) to 1 (in isolates resistant to all the antibiotics tested) (Table 2).

Coliphages were abundantly found to occur in all the 5 sewage locations (100%) sampled (Table 3). Out of the 8 MDR *E. coli* tested, only 6 (75%) were recognized, hosted and subsequently

lysed by the coliphage (Table 3). however, even the lowest dilution of the phage suspension (1:10<sup>8</sup>) was found to have enough coliphage to lyse the MDR *E. coli* isolates (Plate II)

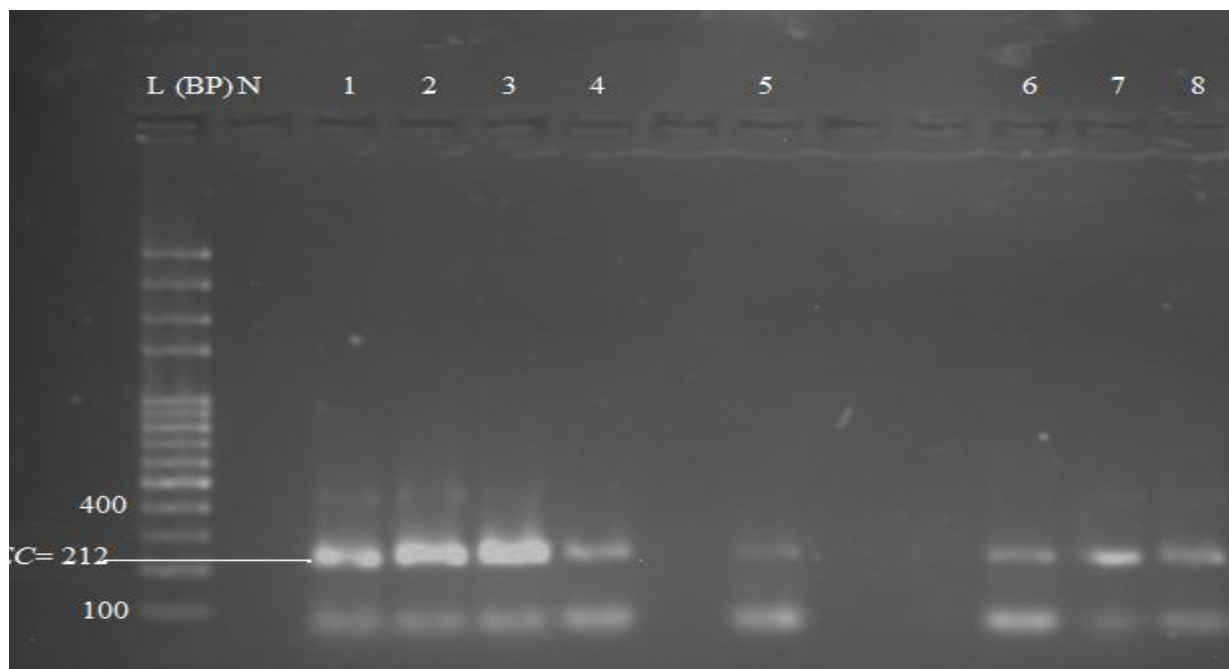


Plate I: well L= DNA ladder (100bp), N= negative control (PCR-mix without DNA), well 1-8= Bands of the EC Genes obtained from the Amplicons of the Samples (EC1-EC8)

Table 1. Antibiotic Sensitivity Profile of the Clinical Isolates of *E. coli*

Susceptibility Pattern			
Antibiotics (Content/ $\mu$ g)	No. Sensitive (%)	No. Interm. (%)	No. Resistant (%)
SP (10)	2(25%)	0(0%)	6(75%)
CPX (30)	4(50%)	0(0%)	4(50%)
AM (10)	0(0%)	0(0%)	8(100%)
AU (30)	0(0%)	0(0%)	8(100%)
CN (10)	5(62.5%)	0(0%)	3(37.5%)
PEF (30)	2(25%)	0(0%)	6(75%)
OFX (10)	2(25%)	0(0%)	6(75%)
S (30)	3(37.5%)	0(0%)	5(62.5%)

Key: AU = Augmentin    CN = Gentamicin    SP = Sparfloxacin    PEF = Pefloxacin, CPX = Ciprofloxacin    OFX = Ofloxacin  
 AM = Amoxicillin    S = Streptomycin,    Interm.= intermediate

**Table 2: Multiple Antibiotic Resistance (MAR) Index of the Clinical Isolates of *E. coli* (N=8)**

Isolate Code	No. of Antib. Resisted	Resistance Pattern	MAR Index
EC1	6	SP, AM, AU, CN, PEF, OFX	0.75
EC2	6	CPX, AM, AU, PEF, OFX, S	0.75
EC3	7	SP, CPX, AM, AU, CN, PEF, OFX	0.875
EC4	8	SP, CPX, AM, AU, CN, PEF, OFX, S	1
EC5	5	SP, AM, AU, PEF, S	0.625
EC6	6	SP, AM, AU, PEF, OFX, S	0.75
EC7	5	SP, CPX, AM, AU, S	0.625
EC8	6	CPX, AM, AU, PEF, OFX, S	0.75

Key: AU = Augmentin CN = Gentamicin SP = Sparfloxacin PEF = Pefloxacin, CPX = Ciprofloxacin OFX = Ofloxacin  
AM = Amoxicillin S = Streptomycin, Antib.=Antibiotics

**Table 3: Occurrence of Coliphages in Sewage Samples Using *E. coli* as Host**

Isolate Code	Sampling Location Code				
	ASL1	ASL2	ASL3	ASL4	ASL5
EC1	+	+	+	+	+
EC2	-	-	-	-	-
EC3	+	+	+	+	+
EC4	+	+	+	+	+
EC5	+	+	+	+	+
EC6	+	+	+	+	+
EC7	+	+	+	+	+
EC8	-	-	-	-	-

Key:+= Plaque observed, - = no plaque observed

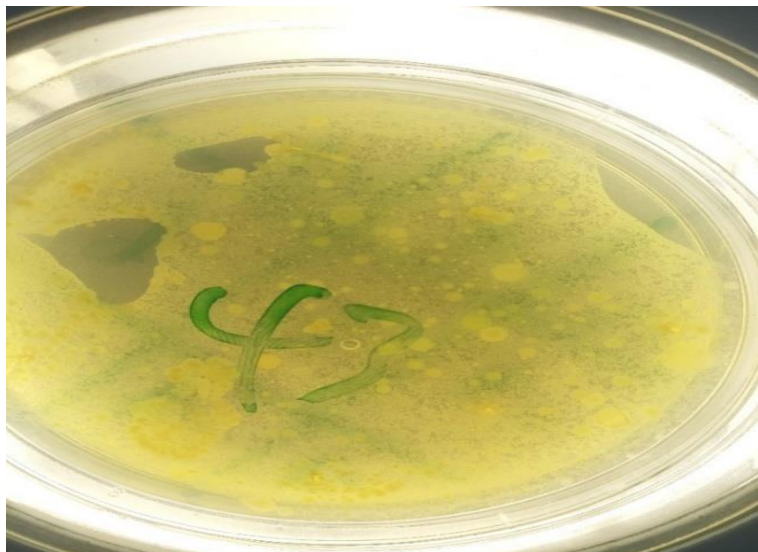


Plate II: Cytopathic Effect of the Various Dilution of Coliphages on MDR *E. coli*

## DISCUSSION

From our findings, out of the 8 antibiotics tested, only gentamicin and ciprofloxacin showed high level of efficacy, indicating the level of failure of antibiotic therapy! As in conformity, with numerous studies that have recently reported a very high level of bacterial MDR (Alanazi *et al.*, 2018; Yadav and Prakash 2017; Igwe *et al.*, 2016; Dibua *et al.*, 2014; Zhang *et al.*, 2012). Recording much higher significant MAR index in all the isolates used in the study, further suggests that the MDR resistance might have resulted from inappropriate use of antibiotics-an attitude that induce antibiotic resistance but not bacteriophage therapy resistance (Sarker *et al.*, 2016; Sulakvelidze, 2011).

Additionally, from our findings, coliphages are very abundant in nature and the possibility of isolating them from sewage is absolute (100%). Furthermore, diluting the coliphage up to (1:10<sup>8</sup>) did not reduce the lytic efficacy of the phage suspension, suggesting the possibility of success of coliphage therapy even when a very low dilution of the coliphage suspension is administered. In conformity with our report, coliphages were previously reported to be abundant in various environments including sewage, regulating the growth of their host bacteria (Sarker *et al.*, 2016).

However, the failure of the coliphage to lysed 2 (25%) of the *E. coli* tested, may suggest that a change in the receptors of the bacteria that recognize that of the coliphage for the successful adsorption of the phage onto the bacterial host, may be one of the determinants of the success of the coliphage therapy, which is in agreement with the work of Shende *et al.* (2017). It was reported that for the bacteriophage therapy to be successful, receptor recognition must take place between the bacterial host and the specific phage, furthermore, the phage must be able to undergo the lytic pathway, which is associated with the lysis of the bacteria for the release of the progenies (Sulaiman and Usman, 2020). Thus to get the best out of the phage therapy, a cocktail suspension, carrying various classes of the phages has been suggested; for instance absolute (100%) success was reported in a study where cocktail of the coliphages was administered (Sarker *et al.*, 2016).

## CONCLUSION

The era of successful antibiotic therapy is coming to an end, and inappropriate use of the antibiotics may be one of the factors accelerating it. Coliphages are very abundant in sewage environment and a dilution as low as 1:10<sup>8</sup> may supply the phages needed for the phage therapy. Implementation of phage therapy can serve as an alternative in the treatment of MDR isolates. A cocktail containing various strains of the phage may improve the success of the therapy.

## RECOMMENDATION

More studies should be carried out to identify the phages that can lyse wider range of bacterial host. There is a need for the Nigerian government and other stakeholders to invest in this area of research, because the need for alternative to antibiotic therapy may be now!

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