Research Article

Isolation of *Escherichia Coli* Phages from Waste Waters

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Abstract | This research was designed to isolate and test the lytic action of a bacteriophage specific to *Escherichia coli*. Both *Escherichia coli* and bacteriophages were isolated from farmlands and residential wastewaters. The isolated *Escherichia coli* were confirmed through phenotypic and biochemical tests. Antimicrobial susceptibility test to nine antibiotics was determined. Multi-antibiotic resistance index (MARI) was assessed. Spot assay was done to determine lytic action of the bacteriophage on the lawns of the *Escherichia coli*. The phenotypic and biochemical tests confirmed *Escherichia coli* isolates. There was metallic green sheen on EMB agar. The isolates were citrate negative and indole positive. The isolates were found to be resistant to amoxicillin (100 %), meropenem (100 %), and ceftriaxone (100 %). The Multi Antibiotic resistance index (MARI) of the isolates was calculated to be 0.33. The formation of plaques (clear zones of inhibition) on the lawn of the plates of *Escherichia coli* was confirmed. The isolation of *Escherichia coli* phages from residential and farm wastewaters is a promising molecular tool for *E. coli* tracking in the environment.

Introduction

Enterobacteriaceae are a large and diverse family of gram-negative non-spore forming bacilli, some of which are normal flora and some are pathogenic. *Escherichia coli* is a member of Enterobacteriaceae that can either be a commensal or cause intestinal or extraintestinal infections, such as diarrhoea, bleeding colitis, hemolytic uremic syndrome, thrombocytopenic purpura or death (Zared *et al.*, 2021). *Escherichia coli* are one of the most studied bacteria in the world. This rod-shaped organism is normally discharged in the environment through open defecation of humans and animals or through waste-water treatment plants. Under optimal conditions, it can replicate in 20 minutes making it an ecological threat (Jang *et al.*, 2017). There are six intestinal pathotypes causing a variety of human diseases and leading to an average of two million deaths a year. They are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), shiga

toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC) (Jang *et al.*, 2017). In recent times, metagenetics has been studying the active genetic exchange from a mixed community of organisms in the natural environment inducing the spread of virulence and resistance determinants (Klassert et al., 2021). Among the different antibiotic-resistant mechanisms developed by ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*) pathogens, the ones found by Enterobacteriaceae are more diverse. Multidrug-resistant *E. coli* have become a global threat due to its ease of transmission among humans and from animals to humans through the faecal-oral route. *E. coli* has a dual functionality of acting as a donor of genetic material or recipient of resistant genes from other microorganisms (Galindo-Mendez, 2020). In the current era of increasing antibiotic resistance and lack of new antimicrobials, the re-emergence of bacteriophages becomes attractive. Bacteriophages are the most prolific biological form on Earth specific for eliminating bacteria. Each bacterial species is preyed by one or multiple bacteriophages which makes them a promising alternative for pathogenic control and ecological stability. This selectivity of bacteriophages is leveraged by environmental microbiologists in microbial source-tracking or monitoring (Rogovski *et al.*, 2021; Attama *et al.*, 2017). They are being used as indicators in fecal contamination (Poluri *et al.*, 2021). This study aims to isolate coliphages from the community for further characterization as a molecular tool in environmental tracking of *E. coli*.

Materials and Methods

Study area and sample collection

The study was conducted in Nsukka, Enugu State, Nigeria. Samples of waste water were taken from ninety-two farms and residential areas over a period of three weeks. A 10 ml sample was collected in sterilized Bijou bottles and taken to the research laboratory for bacteriological analysis. Samples were randomly collected.

Isolation of Escherichia coli from the wastewater

The wastewater samples were diluted 10-fold with distilled water. A 0.1 ml of the diluted samples was dropped at the centre of different Petri dishes containing MacConkey agar. A spreader was used to spread the sample on the surface under aseptic conditions. The inoculated plates were incubated at 37 °C for 24 h in an autoclave.

Purification of isolated Escherichia coli

The overnight culture was purified by picking a colony from each plate and sub-culturing using the streak plate method on MacConkey agar and incubating for 24 h at 37 °C. Distinct colonies from each colony were then sub-cultured on Eosin Methylene blue (EMB) agar and incubated for 24 h at 37 °C to identify the *Escherichia coli*. Pure *E. coli* isolates were then stocked in double strength nutrient agar pending further tests.

Biochemical tests

Citrate and indole tests were carried out on 24 h broth cultures to confirm the presence of *Escherichia coli*.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of *E. coli* isolates to aztreonam (ATM), chloramphenicol (CPL), gentamicin (GTN), tetracycline (TTE), sulphamethoxazole/trimethoprim (SXT), amoxicillin/clavulanic acid (AMC), meropenem (MEM), ciprofloxacin (CIP), ceftriaxone (CRO) was determined by the Kirby Bauer disk diffusion method according to CLSI guidelines (CLSI, 2014).

Multiple antibiotic resistance index (MARI)

Multiple Antibiotic Resistance Index (MARI) was determined using the procedure as described by Krumperman (1983). A MARI for an isolate was calculated as:

$$\frac{\text{The number of antibiotics to which isolate is resistant}}{\text{The total number of antibiotics against which isolate was tested}} \quad \text{...(1)}$$

Isolation of bacteriophages

The *E. coli* phages were isolated from the farm or residential wastewater by centrifuging at 15,000 rpm for 10 min to separate the phages from other particles. The supernatant was filtered through 0.45μm syringe filter units. The filtrates were stored in the freezer.

Phage enrichment and filtration

The pure stocked isolates of *E. coli* were inoculated into 10 ml nutrient broth and incubated overnight. For the enrichment, the reaction mixture in a sterile test tube consisted of 0.4 ml of the overnight broth culture, 0.5 ml of 5x Luria Bertani (LB) broth, 0.04 ml calcium chloride ($CaCl_2$), and 1.2 ml of the phage filtrate. This was incubated for 48 h at 37 °C. After incubation, the

mixture was centrifuged for 15 min at 15,000 rpm. The supernatant was filtered using 0.45µm syringe filter units and the filtrates were stored at 4 °C.

Spot assay

A suspension of the 24 h bacterial culture, adjusted to 0.5 McFarland was swabbed on the surface of Mueller-Hinton agar plates and allowed to incubate at room temperature for 15 min. A drop of the enriched phages was spotted on the lawn of the bacterial isolate and allowed to incubate at room temperature for 15 min. The plates were incubated at 37 °C overnight. After incubation, the plates were observed for the presence of plaques.

Results and Discussion

Isolation and biochemical tests

From a total of ninety-two (92) isolates collected from different locations of waste waters, twenty-one (21) isolates were suspected to be *E. coli* because of the pink appearance on MacConkey agar. These 21 isolates were then subcultured on EMB agar and only 8 isolates had the distinctive metallic green sheen which is 8.69 % of the total samples collected. All eight isolates were subjected to biochemical tests and the results are shown in Table 1. A negative reaction was obtained with the citrate utilization test while a positive reaction was detected in the indole test. Identification was based specifically on the positive indole reaction. It was thought that *E. coli* cannot survive outside the intestinal tract for long periods of time but recent studies have shown that this bacterium can naturalize and reproduce in soil, sand and sediments in tropical, subtropical and temperate climates (Byappanahalli *et al.*, 2015). Contamination of drinking water by this waste water through surface run-offs or flooding could cause illnesses such as urinary tract infection, meningitis, septicaemia or intestinal infections when harbouring toxins from the *E. coli*. Adapted *E. coli* in the environment have been detected with high levels of eaeA, which encodes intimin, a virulence factor. Presence of eaeA in the environment is a great concern for public health. *Escherichia coli* O157: H7 have also been known to persist for months in manure and on green leafy vegetables such as spinach and lettuce and cause outbreaks (Ishii *et al.*, 2014). Some ready-to-eat foods and vegetables grown on and irrigated with these waste waters get contaminated with *E. coli* at different stages from pre- to postharvest (Luna-Guevara *et al.*, 2019). Boiling of drinking water at 100 °C for some minutes, handling and good storage of water becomes important in households as a preventive measure (WHO, 2017). Basic hygiene and handling of foods for personnel at farms, abbatoirs and restaurants is equally essential to keep *E. coli* infection to a minimum (WHO, 2017).

Table 1: *Biochemical tests to confirm presence of E. coli in the environment.*

S/n	Isolate	Source	Citrate	Indole
1	42	Res	-	+
2	48	Res	-	+
3	78	Farm	-	+
4	82	Farm	-	+
5	83	Res	-	+
6	85	Res	-	+
7	89	Farm	-	+
8	90	Res	-	+

Key: Res(Residential), +(positive), – (negative).

Antimicrobial susceptibility testing

The isolated strains of *E. coli* were screened against aztreonam (ATM), chloramphenicol(CPL), gentamicin(GTN),tetracycline(TTE),amoxicillin/ clavulanic acid (AMC), sulphamethoxazole/ trimethoprim (SXT), ciprofloxacin (CIP), meropenem (MEM), and ceftriaxone (CRO). There was high susceptibility of the strains to ciprofloxacin (CIP), followed by sulphamethoxazole/ trimethoprim and chloramphenicol respectively as shown in Table 2. However, there was 100 percent resistance to amoxicillin/ clavulanic acid, meropenem and ceftriaxone. The role of environmental factors in the antibiotic resistance transmission and migration dynamics of *Escherichia coli* is not well understood. However, the ecological consequences of the antimicrobial resistance are difficult to control because of close interactions between interfacing environments (Guangshui et al., 2018). The increased antimicrobial resistance in the environment could be attributed to increased anthropogenic activities, some inappropriate, involving antimicrobial compounds and subsequent release into receiving environments such as soil and water. Researchers are undertaking studies on the chemicals and pollutants being released into the environment and the pressure of resistant pathogens being selected. It behoves policy-makers to study these findings and take appropriate actions to control the unwanted effects (Stanton *et al.*, 2020). *E. coli* has evolved different mechanisms of resistance to antibiotics mostly plasmidic and which transfers

Table 2: *Antimicrobial susceptibility test of Escherichia coli to antibiotics of choice.*

S/n	Iso-late	Source	ATM 30μm	CPL 30μm	GTN 10μm	TET 30μm	SXT 25μm	AMC 30μm	MEM 10μm	CIP 5μm	CRO 30μm
1	42	Res	15 I	16 S	9 I	12 I	18 S	0 R	0 R	29 S	0 R
2	48	Res	13 I	18 S	6 I	10 I	20 S	0 R	0 R	30 S	0 R
3	78	farm	11 I	16 S	7 I	9 I	17 S	0 R	0 R	28 S	0 R
4	82	farm	12 I	16 S	9 I	8 I	19 S	0 R	0 R	26 S	0 R
5	83	Res	13 I	19 S	10 I	11 I	16 S	0 R	0 R	29 S	0 R
6	85	Res	14 I	23 S	13 I	10 I	19 S	0 R	0 R	34 S	0 R
7	89	Farm	10 I	17 S	13 I	7 I	16 S	0 R	0 R	34 S	0 R
8	90	Res	7 I	20 S	6 I	13 I	17 S	0 R	0 R	30 S	0 R

Key: S-susceptible; I-Intermediate; R-Resistant.

resistance genes encoding extended spectrum β-lactamases (ESBLs) horizontally to other species including pathogenic bacteria (Peterson and Kauer, 2018). The most commonly seen ESBLs in *E. coli* are CTX-M enzymes. There is now an emergence of carbapenemase-producing enterobacteriaceae against carbapenem which has been used as the first-line drug for ESBLs. Current carbapenemases are Ambler Class A *Klebsiella pneumonia* carbapenemase (KPC); Class B metallo-β-lactamases (MBLs) such as New Delhi MBL (NDM), Verona integrin-encoded MBL (VIM), and imipenemase (IMP); and Class D oxacillinases (OXA)-type enzymes such as OXA-48-like carbapenemases. A novel boron-containing serine-β-lactamase inhibitor called Varborbactam is being studied which has inhibitory activity against Ambler Class A and C serine carbapenemases such as KPC. It is used synergistically in combination with meropenem as a carbapenem-β-lacatamase inhibitor with susceptibility rates ranging from 66.2 to 100 % (Sheu *et al.*, 2019). World Health Organization (WHO) announced that one of the top ten global health threats is Antimicrobial resistance (AMR) (WHO, 2020). It has been forewarned that by, 2050, antibiotic resistance will be responsible for the death of 10 million people (Sharma *et al.*, 2021). In another study, AMR was projected to drain the global economy of US$100 trillion in the same period (Mogasale *et al.*, 2021). From the antibiotic susceptibility study, *Escherichia coli* exhibited moderate to high resistance pattern with a MARI of 0.3. MARI is a dependable and reproducible method of tracking antibiotic resistance in the environment. The major factors causing the high resistance of bacteria to multiple antibiotics is the indiscriminate use of antibiotics in homes and farms (Ayandele *et al.*, 2020).

Table 3: *Showing the number of plaques formed by the bacteriophages.*

S/N	Isolates	Code	Source	Phage filtrates Spots/Plaques			
				1	2	3	4
1	42	R	RES	+	-	-	+
2	85	S	Sand	+	-	-	+

Spot assay

The Muller-Hinton agar plates containing lawns of eight confirmed *E. coli* isolates were spotted with the enriched bacteriophages in four different spots. After 24 h incubation, we observed only two plates with plaques as seen in Table 3. This test was to done to determine the susceptibility of *Escherichia coli* to a lytic concentration of a specific bacteriophage(s). The spot test revealed that the enriched phages were capable of infecting some of the isolated *Escherichia coli* from different environmental waste waters. The phages showed clear plaques and no overgrowth was observed. An increasing number of studies have examined the use of phages in *Escherichia coli* tracking or as indicators of fecal contamination in an environment. This has been done through phage-induced bacterial lysis using quantitative image analysis to achieve detection of *Escherichia coli* at low concentrations (Yang *et al.*, 2020). An imaging study using rhodamine isothiocyanate or DNA dye Syto 13 fluorescently labeled phages and its *Escherichia coli* receptor, LamB have also been studied to reveal the role of space and time in the evolutionary dynamics of phage control with high throughput outcomes (Wang *et al.*, 2020; Low *et al.*, 2020). A study has also investigated the use of transposon insertion sequencing (INSeq) screens as a rapid, high throughput tool to identify candidate phage receptors (bacterial genes involved in phage binding) for previously well characterized

phages T2, T4, T6 and T7 (Kortright *et al.*, 2020). In Bangkok canals, the microbial quality was assessed using the fluorescent bacteriophage assay (FBA) and fluorescence in-situ hybridization (FISH) using oligonucleotide probes targeted to *Escherichia coli* (Takehiko *et al.*, 2006).

Conclusions and Recommendations

The phages isolated from farms and residential waste waters were specific to the *Escherichia coli* isolates. The re-emergence of phages as an environmental tool for *Escherichia coli* tracking is both an iterative and attractive development for ecologists. Further studies would be done to characterize the phages isolated.

Novelty Statement

The study contributes to the increasing support in the future advancement and utilization of bacteriophages in common biological processes. Specifically here, the use of specific bacteriophages as a tool for identification, monitoring, and control of specific *Escherichia coli* strains in the environment.

Author's Contribution

The study was conceptualized by Ebele Benedette Onuigbo and Anthony Amaechi Attama. Experimentation and data collection was done by Stephen Chijioke Emencheta and Adaora Angela Agubata. Initial manuscript was prepared by Ebele Benedette Onuigbo and Stephen Chijioke Emencheta. Manuscript was reviewed for intellectual content by Ezinwanne Nneoma Ezeibe and Anthony Amaechi Attama.

Conflict of interest
The authors have declared no conflict of interest.

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