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Temporal Appraisal and Molecular Characterization of *Escherichia coli* from Oyun River, Kwara State, Nigeria

Olatunji Matthew Kolawole[1] and Oluwasanmi Anuoluwapo Adeyemi[1*]

[1]Infectious Diseases and Environmental Health Research Group, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author OMK designed the study and supervised the research. Author OAA carried out the research. Authors OMK and OAA wrote the manuscript. Both authors read, reviewed and approved the final manuscript.

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ABSTRACT

Introduction: *Escherichia coli,* an indicator of feacal contamination has been proven to be the cause of several disease outbreaks in countries and continents around the world.
Aim: To determine the genotypic variants of *Escherichia coli* present in Oyun River and provide information regarding the high-risk variants of *E. coli* in Oyun River.
Study Design: The study cuts across the two seasons of Nigeria's tropical climate weather, being the peak of the Harmattan season and the onset of the Rainy season. Three sampling sites (Jimba Oja; Unilorin Dam and Oyun in Ilorin, Kwara State) along the River course were examined for three months (February – April).
Methodology: Heterotrophic counts, coli form counts and molecular characterization via PCR using 16 sRNA primers, of water samples were done using standard microbiological and molecular methods.
Results: Bacteriological results showed monthly mean values of microbial counts, ranged from 23.5 x 10^6 – 45.17 x 10^6 cfu/mL and total coli form count ranged from 53 cfu/100 mL to 256 cfu/100 mLs, both of which exceed the WHO standards of 100 cfu/mL for total microbial count and <1 cfu/100 mLs for total coliforms. A total of forty-eight coliforms were isolated, thirty two of which were

**Corresponding author: E-mail: sanmi_adeyemi@yahoo.com;*

Escherichia coli. Sequencing and BLAST analysis of eleven of the isolates using NCBI's online database revealed five different strains. They include: *Escherichia coli* FAP1 genome (9.1%); *Escherichia coli* strain ST2747 (54.5%); *Escherichia coli* strain EADK4 (9.1%); *Escherichia coli* strain ST540 (18.2%) and *Escherichia coli* strain NCM3722 (9.1%). Correlation of results with previous studies showed that most of the strains identified were pathogenic. The *E. coli* strains isolated, coupled with the bacterial load, coliform count and some physicochemical parameters of Oyun River makes it unsafe for public consumption if not treated. Efforts therefore should be made to treat the water before use, while making frantic efforts to prevent further contamination of Oyun River.

Keywords: Oyun River; Escherichia coli; molecular characterization; 16S rRNA.

1. INTRODUCTION

Water is vital to life and makes up Seventy-one percent (71%) of the Earth's entire surface. Of the Earths water distribution, only 2.5% is freshwater, 98.8% of this freshwater is locked up in ice and groundwater leaving just about 0.3% of freshwater in rivers, lakes, and the atmosphere from which man benefits [1]. The UN [2] estimated that in the year 2050, world population will be 9.7 billion people and exceed 11 billion people by 2100, thus increasing water demand tremendously making conservation and reuse of water imminent. Estimates put the use of water as: 70%, 22%, 8% for irrigation, industrial and household purposes [3,4].

When water is impaired by contaminants of an anthropogenic nature and cannot support a human use or causes harm to aquatic organisms, it is said to be polluted [5]. Water pollution is the leading cause of diarrheal deaths and diseases worldwide, resulting in over 829,000 deaths yearly [6]. Water plays a crucial role in life sustenance and is a major health determinant and lack of good water causes 80% of diseases in developing countries [7]. Polluted water bodies have been known to serve as habitats of pathogenic coliforms and play their role in the disease outbreaks, particularly where there is faecal contamination [8]. Thus, many infectious diseases are transmitted by water through faecal oral contamination. Coliforms are non-spore forming, gram negative, rod-shaped, non motile or motile bacteria which ferment lactose with the production of gas and acid when incubated at 35°C –37°C [9,10]. They are abundant in the faeces of warm-blooded animals and are often found in soil, the aquatic environment and on vegetation; their presence is used to indicate the presence of pathogenic organisms particularly those of fecal origin. Typical genera of coliforms are: *Escherichia, Klebsiella*, *Citrobacter*, *Hafnia, Enterobacter* [10].

Escherichia coli is a gram-negative, facultative anaerobic, bacterium with a rod shape, of the genus *Escherichia* that is a typical member of the bacterial flora of man and warm blooded animals gut (endoderms and are widely used as indicators of faecal pollution in water quality assessment. Wild type *E. coli* causes infections with chronic implications when it ends up in places where it does not belong, such as the urinary tract [11]. Contamination of water sources is possible in all parts of the world, due to the fact that domestic animals, including cattle and pigs, serve as reservoir for the pathogens [12].

Waterborne outbreaks of diseases caused by pathogenic strains of *E. coli* have been recorded in different parts of the world. Such a case occurred in Walkerton in Canada during May 2000, in which the borehole drinking water supply got contaminated by rainwater runoff containing cattle faeces. Six deaths and more than 2000 cases of severe illness were recorded [13]. The pathogen involved was an Enterohemorrhagic *Escherichia coli* (EHEC) strain designated *E. coli* 0157:H7. This particular strain has caused several outbreaks in many parts of the world resulting in severe illness with a high mortality rate and is one of the most feared strains of *E. coli* [13]. *Escherichia coli* O157 is often implicated in food and water borne illnesses in humans worldwide [14]. *Escherichia coli* O104:H4 caused an outbreak of food borne illness in northern Germany from May to June 2011 in which 53 people died and 3,950 people were affected [15,16]. In the United States alone, community-acquired extra intestinal infections with *Escherichia coli* range from 6 to 8 million cases of cystitis each year to 127,500 sepsis cases yearly [17]. The first reported *E. coli* O157 infection outbreak in developing countries was in southern Africa in year 1992 [18], thereafter, outbreaks were recorded in Central African Republic in 1996 and in Cameroon in year 1997 [19]. Although not in outbreak proportions, *E. coli* O157 illness in Nigeria has been reported since

1994 [20]. Effler et al. [18] and Renter et al. [21] have both shown that cattle were carriers of *E. coli* O157 at the Emergence of the bacterium in Africa.

Nigeria is a developing country and water pollution is a battle that is ever raging as the public is still under-sensitized about the dangers of water pollution and the health risks. Previous studies [22] have shown that waste generated in various communities and raw sewage is sometimes directly dumped into water bodies. While provision of potable water for the ever increasing population of Nigeria yet remains a herculean task, it is pertinent that the water bodies which also serve as source of water supply in other areas are free of pollution. Numerous studies [23,24] have been done on bacterial contamination of water bodies and disease outbreaks as a result, even on River Oyun. Risks involved in using polluted water in agricultural practices, reports on the presence and prevalence of *E. coli* O157 in irrigation waters and subsequent transmission via fresh farm produce has been studied and microbial diversity of Oyun River. However no such study has specifically looked at the different strains of *Escherichia coli* along the River Oyun, more so via molecular characterization. Therefore, this research aims to determine the genotypic variants of *E. coli* present in Oyun River and provide information regarding the high-risk variants of *E. coli* in Oyun River.

2. METHODOLOGY

2.1 Study Area

The research was carried in Ilorin, Kwara State. Oyun River is one of the three major Rivers in Ilorin. Oyun River is located at an elevation of 269 meters above sea level Its coordinates are 8°34'60" N and 4°34'0" E in DMS (Degrees Minutes Seconds) or 8.58333 and 4.56667 (in decimal degrees). Over 500,000 persons live along the length of the River.

2.2 Sample Collection

Samples were collected from three strategic points (Jimba Oja - A, Unillorin Dam - B & Oyun bridge - C) along Oyun River, following the WHO approved standards [26]. The distance between points A, B & C is 8.05 Km and 9.5 Km respectively. Samples were collected over a period of 3 months February - April, cutting across the two seasons of the area (rainy & dry season). Activities seen in the area are shown in Table 1.

2.3 Bacteriological Analysis

Total heterotrophic count (THC) was carried out using Nutrient agar, in which Serial dilution was done on samples and 1 mL of selected dilutions was plated out in duplicates on nutrient agar, using the pour plate method. Inoculated plates were then incubated at 37°C for 23 hours. Eosine Methylene Blue (EMB) agar was used for total coliform count (TCC) and selective isolation of *E. coli*. TCC was done using the membrane filtration method, in which 10 mL of the samples was measured and made up to 100 mL. This was then passed through 0.45 μm pore-sized membrane filter, attached to a vacuum pump filtration system. The membranes were then placed with the grid lines facing upwards on EMB agar and incubated at 37°C and observed within a 24 hour period. Positive colonies were streaked onto fresh plates and sub-cultured till pure cultures were obtained. The results obtained were recorded as total heterotrophic count of bacteria in 1 mL of water and total coliform count was expressed in 100 mL of water. Stock cultures were placed on nutrient agar slants and in nutrient broths. Reaction to Gram's staining was also carried out, using young bacterial cultures.

Table 1. Anthropogenic activities at the sampling sites

Code	Sampling sites	Anthropogenic activities
A	Jimba Oja	Farming, cattle rearing, irrigation, washing (cars, clothes and food), bathing, fishing, block making factories, commercial water supply, drinking, domestic use, dump site, bush burning, grass clearing, discharge of effluents, recreational activities.
B	Unilorin Dam	watering of plants, domestic use, bush burning, grass clearing, recreational activities
C	Oyun	Farming, cattle rearing, irrigation, washing, bathing, fishing, block making factories, commercial water supply, drinking, domestic use, dump site, bush burning, grass clearing, discharge of effluents, recreational activities

2.4 Molecular Process

QIAamp DNA Mini Kit (250) cat no 51306 was used for DNA extraction and the manufacturer's instructions were followed. The genetic material was tested for purity using a Thermo Scientific's 'NANODROP 2000' spectrophotometer. The polymerase chain reaction was done using 'GeneAmp PCR system' by Applied Biosystems, prepared gels were read using 'ENDURO^{TM} GDS' by Labnet International Incorporated and molecular weight markers used were 1 kb DNA plus ladder. 16S rRNA primer was used 27F (GAGTTTGATCMTGGCTCAG) and 1525R (AAGGAGGTGWTCCARCCGCA). The PCR protocol used was: Initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds; annealing at 56°C for 30 seconds; Extension at 72°C for 45 seconds (for 30 cycles); final extension at 72°C for 7 minutes, and final holding temperature at 10°C. After the PCR test, the samples were again run on agarose gel electrophoresis to view the results. The dye reaction for sequencing was done using 'BigDye® Terminator v3.1 Cycle Sequencing Kit' and manufacturer's instructions were followed after which it was loaded on '3130xl genetic analyzer' from Applied Biosyetems to obtain the resulting sequence which was BLAST online using National Center for Biotechnology Information (NCBI) database.

2.5 Data entry and Analysis

Data were analyzed with the use of statistical software; SPSS (version 21) statistical data software.

3. RESULTS AND DISCUSSION

The total heterotrophic count (THC) expressed in the mean monthly values ranged from 23.5 x 10^6 to 45.17 x 10^6 cfu/mL (Table 2). The least value recorded was at Jimba Oja in the month of March, while the highest value recorded was found also at Jimba Oja in the month of February. The highest recorded values were seen in February across the three sampling sites. The mean total coliform count (Table 2) recorded in the study ranged from 53 cfu/100 mLs to 256 cfu/100 mLs. The lowest value recorded was at Unilorin Dam in February, while the highest value was recorded at Oyun in April. Forty eight (48) Coliform organisms were isolated and 32 (66.67%) organisms were observed to have growth morphology characteristic of *E. coli*. They all stained negative to Gram's reaction.

Fig. 1 displays the molecular weight of *Escherichia coli* genome using 16S rRNA primer. The resulting bands are at 900bp when compared with the 1 kb marker. Online Blast of sequences obtained revealed five (5) different strains of *E. coli* (Table 3) from the eleven *E. coli* isolates identified: *Escherichia coli* FAP1 genome (9.1%); *Escherichia coli* strain ST2747, complete genome (54.5%); *Escherichia coli* strain EADK4 16S ribosomal RNA gene, partial sequence (9.1%); *Escherichia coli* strain ST540, complete genome (18.2%) and *Escherichia coli* strain NCM3722, complete genome (9.1%). No *Escherichia coli* were isolated from the Unilorin Dam site in this study.

The mean values of the total heterotrophic count showed that the bacterial count was higher during the dry season and lower during the rainy season, which may be due to concentration of the water body due to evaporation, also the anthropogenic activities around the area thus increasing the concentration of microbes in the river with reduced water level. As at the time of the sample collection, many of the afore-mentioned activities were observed (Table 1). On the other hand, the bacterial load was observed to reduce during the onset of the rainy season, possibly because of dilution of the water body by rainfall. Kolawole et al. [24] recorded that human activities such as refusing dumping, swimming, fishing and the like are capable of increasing the bacterial population of the river. The microbial population of both seasons suggests an inverse proportionality of the microbial population to the seasonal variation.

The mean values recorded of the total coliform count showed increased in values from February to April. The total coliform count was rather low during the dry season as opposed to the rainy season. The values recorded were in conformity with the work of Kolawole et al. [24] who also recorded low values of total coliform count at the peak of the dry season and higher values during the rainy season. The values recorded exceeded the WHO guideline of <1 cfu/100 mLs approved for drinking water [25]. It is believed that the increase in River water level, runoffs from farms and dumpsites during the rainy season brought about the abundance of the coliforms in Oyun River. The WHO standards for recreational waters specify > 100 cfu/mL, whereas mean value of counts were still higher than 100 cfu/100mLs, even exceeding 200 cfu/100 mLs.

Table 2. Total heterotrophic count & total coliform count of water samples taken from Oyun river

Parameters	Total heteroptrophic count (cfu/ml) (10^6)			Total coliform count (cfu/100 mL)		
	Jimba Oja	Unilorin Dam	Oyun	Jimba Oja	Unilorin Dam	Oyun
February	45.17±10.18	44.83±12.24	36.50±4.26	55.17±6.47	53.00±7.78	73.83±3.56
March	23.50±3.27	35.00±7.19	29.67±4.48	92.67±24.53	62.00±18.61	126.17±29.84
April	35.25±2.29	32.00±7.63	23.75±2.52	168.00±12.75	154.00±45.56	256.00±32.29
WHO drinking water standard	1.0 X10^2 cfu/ml			0 cfu/100 ml.		

95% confidence interval (n=6)
Key: Jimba Oja - Sample point A on Oyun River (Underneath the bridge at Jimba Oja);
Unilorin Dam - Sample point B on Oyun River (8.05 km from point A);
Oyun - Sample point C on Oyun River (9.50 km from point B – Underneath Oyun Bridge)

Table 3. BLAST results of nucleotide sequences from isolated *Escherichia* species from River Oyun water samples

Isolates	Sampling site	No of bases	Identity (%)	Total score	Accession number	Organism
1	Jimba Oja	1847	97	14771	CP009578.1	*Escherichia coli* FAP1 genome
2	Jimba Oja	998	88	11924	CP007394.1	*Escherichia coli* strain ST2747, complete genome
3	Jimba Oja	712	87	10051	CP007394.1	*Escherichia coli* strain ST2747, complete genome
4	Jimba Oja	1074	86	14570	CP007394.1	*Escherichia coli* strain ST2747, complete genome
5	Jimba Oja	1687	95	17935	CP007391.1	*Escherichia coli* strain ST540, complete genome
6	Oyun	1122	88	15366	CP007394.1	*Escherichia coli* strain ST2747, complete genome
7	Oyun	1495	93	20401	CP007394.1	*Escherichia coli* strain ST2747, complete genome
8	Oyun	172	83	172	KJ473648.1	*Escherichia coli* strain EADK4 16S ribosomal RNA gene, partial sequence
9	Oyun	634	79	6824	CP007390.1	*Escherichia coli* strain ST540, complete genome
10	Oyun	1334	93	10575	CP011495.1	*Escherichia coli* strain NCM3722, complete genome
11	Oyun	1142	97	24981	CP007394.1	*Escherichia coli* strain ST2747, complete genome

The identified bacteria isolates were gram-negative bacteria and all belonged to the Enterobacteriaceae family, 32 organisms with morphological growth of *Escherichia coli* were stocked, while the remaining 16 were thought to include *Pseudomonas aeruginosa* among others. These organisms were isolated from environmental waters in the work done by Kolawole et al. [23] and Kolawole et al. [24] on Asa River, Ilorin, Kwara State. *Escherichia coli* are abundant in human and animal faeces and are found in sewage, treated effluents and all natural waters and soils subject to faecal contamination. Its transmission route remains fecal – oral [26,8]. O'Connor [13] in Walkerton, Canada discovered *E. coli* in their borehole drinking water supply as a result of water runoff from contaminated cattle feaces. *E. coli* has been isolated from fresh farm produce which was grown and irrigated using water contaminated with human or animal feaces in Nigeria [27]. It is worthy of note that although coliforms were isolated from the Unilorin Dam water samples, no *E. coli* was isolated suggesting that, there was no feacal contamination at the area sampled. This may be due to the fact that the university Dam

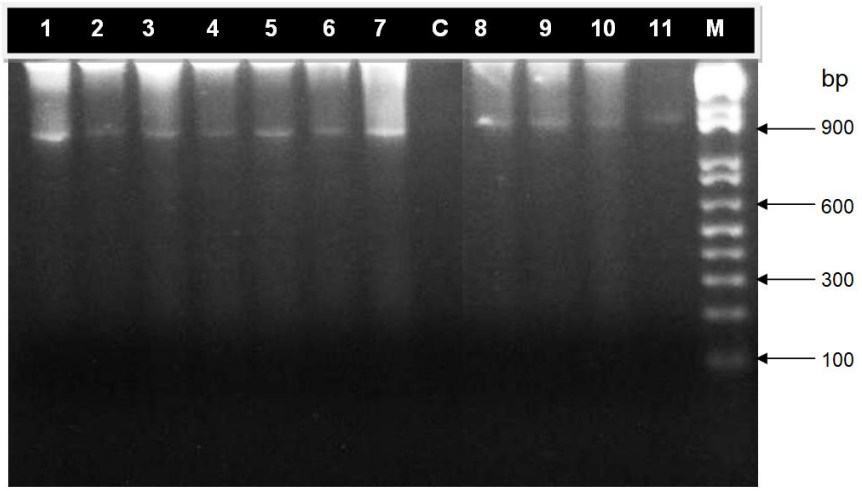

Fig. 1. Agarose gel electrophoresis of amplified DNA from bacterial isolates after PCR
Key: Lane M represents the Marker (1kbp DNA ladder); Lane C is the negative control; Right hand side: Molecular size of the marker measured in base pairs (bp) Lanes 1 - 11: Represents E. coli isolates

site is under constant surveillance by the University of Ilorin security, and movement around the Dam in restricted.

The BLAST of sequenced DNA extracted from 11 of the isolated organisms (Table 3) showed 5 different *E. coli* strains. *Escherichia coli* FAP1 genome (9.1%) has been found to prop up molecularly in cases where two gene determinants are involved. In this study, it was isolated from Jimba Oja and possibly may be a mutant variation of an *Escherichia coli* strain, further research is suggested on this strain as available information at this period is limited. *Escherichia coli* strain ST2747 and *Escherichia coli* strain ST540 complete genome have been proved to be antibiotic resistant (nitrofurantoin resistant) and has necessitated the introduction of older and stronger antibiotics in treating infections caused by it [28]. *Escherichia coli* strain EADK4 16S ribosomal RNA gene has been found to bear very close similarities with pathogenic *E. coli* O157:H7 which has cattle as its main reservoir. This bacterium is considered as a pathogenic agent characterized by producing toxins, which are familiarly known as Shiga-like toxin-1 (Stx1) and Stx2 [29]. *Escherichia coli* strain NCM3722, complete genome wild type has also been known to be pathogenic and is capable of causing gastrointestinal infections [30]. It is particularly noteworthy that 80% of *E. coli* isolates from this study are established high-risk variants and these include: *Escherichia coli* strain ST2747; *Escherichia coli* strain ST540; *Escherichia coli* strain EADK4; and *Escherichia coli* strain NCM3722. They are termed high-risk because, ingestion of such could lead to momentous health challenges in humans, increase horizontal and vertical antibiotic resistance among microorganisms and bear genetically similarities to known strains that have erupted in disease outbreaks.

4. CONCLUSION AND RECOMMENDATION

The study revealed that Jimba Oja, Unilorin Dam and Oyun had unsafe levels of bacteria load and coliforms in the two prominent seasons (rainy and dry seasons) considered. Furthermore, *Escherichia coli* pathogenic in nature and of medical concern were isolated from Jimba Oja and Oyun sampling points along the Oyun River, making it very unsafe for drinking, agricultural purposes and even for recreational activities such as swimming. It was believed that the pollution in Oyun River was more of feacal pollution, other possible contaminants were believed to be agricultural and other domestic activities not leaving out refuse dumping and public toileting.

It is therefore recommended that: Education and sensitization of the populace on safe disposal practices compliant with the developed world is paramount. More water treatment plants should be established by stakeholders to provide

potable water for the populace. There should be constant surveillance (by stakeholders) of all sources of water to prevent and report any form of pollution, whether intending, in progress or already existing; Improved practicable, efficient and functional means of waste collection and disposal, should be introduced by the government to dissuade illegal refuse dumps; Prompt removal of wastes from collection points and subsequent disposal must be ensured to prevent runoffs and leachates from rainfall or flood and remediation of already polluted water bodies such as Oyun River in particular to make the water fit for multiple uses.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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