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ABUNDANCE AND ANTIBIOGRAM OF *Escherichia coli* ALONG STAGES OF WATER TREATMENT IN TAMBURAWA WATER TREATMENT PLANT, KANO NIGERIA

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ABSTRACT
Increasing reports of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) being detected in drinking water is of public concern. . This study was aimed at detecting the changes in abundance of Escherichia coli harbouring selected plasmid-mediated ARGs in different stages of water treatment at Tamburawa Water Treatment Plant (TWTP) which has the capacity to deliver 1.50x 10^8L of drinking water per day to the populous Nigerian city of Kano. Water samples from clarification, filtration, disinfection, and storage stages of the plant were subjected to physicochemical and microbiological analyses. Following quantification using membrane filtration technique, standard cultural and biochemical techniques were used for identification and confirmation of E. coli. Susceptibility of the strains to 11 antibiotics (Tetracycline, Ciprofloxacin, Ampicillin, Augmentin, Chloramphenicol, Gentamicin, Ceftazidime, Cefuroxime, Cefixime, Ofloxacin and Nitrofurantoin) was then determined using disk diffusion method according to CLSI breakpoints. Representative strains resistant to β-lactams were chosen from each water treatment stage and screened for the presence of four selected ARGs–bla_{NDM}, bla_{IMP}, bla_{FOX} and bla_{CMY}–using polymerase chain reaction.
The results showed no significant variations ($p < 0.05$) in physicochemical parameters across the water treatment stages. Furthermore, E. coli, which was detected in all the treatment stages, had a high relative abundance of 0.241(24.1%).The isolated E. coli were 100% susceptible to Chloramphenicol and 87.5% susceptible to Ofloxacin and Gentamicin. Of the resistant strains, 33% were detected in storage stage, followed by filtration and clarification stage with 26% each and disinfection with the lowest (15%). It was also revealed that E. coli isolated from disinfection stage were 100% resistant to 5 antibiotics (Ceftazidime, Cefuroxime, Augmentin, Cefixime and Nitrofurantoin). ARGs amplified from plasmid DNAs were harboured by 90% of the E. coli isolated. This study therefore, concludes that resistant E. coli carrying different ARGs persist in drinking water produced in TWTP. These resistant isolates are most abundant at storage stage, and hence, the need for more efficient storage system.
Keywords: Escherichia coli, Tamburawa water treatment plant, antibiotic resistant bacteria, and Antibiotic resistance genes.

INTRODUCTION

The discovery of antibiotics has been one of the greatest ground-breaking developments in medicine and the fight against microorganisms. However, the excessive abuse of these antibiotics in both human and veterinary medicine has led to the emergence of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs), which pose a major public health concern (Walsh, 2003). These ARB and ARGs are found not only in clinical settings, but have also in environmental settings, including but not limited to soil (Marti *et al.,* 2013), sewage (Reinthaler *et al.,* 2003) and water bodies (Dodd, 2012).

In developing countries, water bodies mostly serve as source of raw water to drinking water treatment plants. As a result, large amount of ARB and ARGs have been detected in effluents of waste water treatment plants (WWTP) and drinking water (Hembach *et al.*, 2017).

Escherichia coli is among the major contaminants found in drinking water and play an important role in water treatment (Yu *et al.,* 2014). Despite being part of the natural intestinal flora, *E. coli* also serves as an indicator of faecal contamination in water. Some strains of this bacterium are pathogenic and can cause serious health implications, especially those harbouring plasmid mediated resistance, which enables transfer of ARGs from one bacterium to another through horizontal gene transfer (Khan, 2016). Therefore, it is important to examine the role of drinking water treatment plants in the evolution, persistence and dissemination of ARB and ARGs (Vaz-Moreira *et al.*, 2012).

Tamburawa Water Treatment Plant is located 15km away from Kano city in Dawakin kudu local government of Kano state. It is the largest water treatment plant in West Africa with a storage capacity of one hundred and fifty million litres per day ($150M^3$), and uses treatment methods approved by the World Health Organization; this makes it ideal for the research.

Because there is little knowledge on the fate of ARB and ARGs in TWTP, the need to for this study becomes necessary. Since *E. coli* is globally regarded as a reliable indicator of water quality, the current study soughtto track the abundance and resistance profile of this organism along the treatment line of the plant. This knowledge would benefit operators and policy makers in assessing the quality of the final water in terms of both microbiological and resistome quality. The research is therefore, aimed at investigating the changes in the abundance of *E. coli* and selected plasmid borne resistance genes in the effluents of different treatment stages of Tamburawa water treatment plant.

MATERIALS AND METHODS

Study area

This study was conducted at the following locations:

1. **TWTP:** This is the area where water samples were collected.
2. Kano state pollution control laboratory: this is where analysis of water was carried out. The laboratory is supervised by Kano state ministry of environment and has state of the art equipment for water analysis.
3. Centre for biotechnology research (CBR), Bayero University Kano: all molecular analyses were conducted here.

Sample collection

Water samples from the selected stages of treatment– coagulation and sedimentation (clarification stage), filtration, disinfection, and storage–stages were collected in triplicates using sterilized 2L sampling bottles. Immediately after collection, 2mg/L sodium thiosulphate (Oxoid, United Kingdom) was added to the chlorinated samples to stop the action of residual chlorine (Destiani and Templeton, 2019). All the collected samples were stored in a cool box, transported to the laboratory and processed within 6 hours of collection.

Physicochemical water quality measurements

Temperature, pH, dissolved oxygen, salinity, biological oxygen demand, conductivity and total dissolved solids (TDS) were determined using a portable water quality meter (AZ 86031, China) with multiple probes, which were calibrated before use. 100 mL of sample was poured into a beaker each. The probes were then simultaneously placed into the beaker and the results were read from the LCD monitor. The probes were rinsed with sterile distilled water and allowed to dry before proceeding to the next sample. Residual chlorine and nitrite determination was done using a HACH spectrophotometer (DR3000) according to the manufacturer's protocol.

Determination of relative abundance of *Escherichia coli*

E. coli was isolated using the membrane filtration method according to WHO (2013). Triplicates of water samples (100 mL) collected from each stage at TWTP was filtered through a 0.45 µm pore size mixed cellulose ester filter membrane (Millipore, USA) mounted on membrane filtration apparatus. The membrane was then aseptically removed with sterile forceps and placed on prepared MacConkey agar plates (Oxoid, UK). The plates were incubated at 37°C for 24 hours, after which lactose fermenting colonies typical of *E. coli* were then sub-cultured onto freshly prepared MacConkey agar plates to isolate pure colonies. Relative abundance of *E. coli* was determined by dividing the number of typical *E. coli* colonies on the plate by the total number of colonies.

Microscopic and Biochemical characterization of *E. coli* strains

Gram staining techniques and biochemical tests (Indole. Methyl red, Voges-Proskauer, urease and Catalase) were used to differentiate *E. coli* from other lactose fermenting colonies (Cheesbrough, 2006).

Antibiotic susceptibility testing of *E. coli* strains

Disk diffusion method was employed for the antibiotic susceptibility test using CLSI 2021 breakpoint. Bacterial suspensions were prepared from the overnight grown cultures and compared with McFarland's solution for standardization. The antibiotics used were Tetracycline (30mg); Ciprofloxacin (5mg), Ampicillin (10mg), Augmentin (30mg), Chloramphenicol(30mg), Gentamicin (10mg), Ceftazidime (30mg), Cefuroxime (30mg), Cefixime (5mg), Ofloxacin (5mg), Nitrofurantoin (300mg). Zones of inhibitions were measured and recorded after incubating the plates for 24 hours at 37°C. Quality control testing of the antibiotic discs was done by testing them on ATCC 25922 strains sourced from Aminu Kano Teaching Hospital (AKTH) Microbiology laboratory.

Determination of multi-antibiotic resistance index of *E. coli* strains

Multi-antibiotic resistance (MAR) index was determined using the relation:

MAR = a/b where *a,* is the number of antibiotics to which the test isolate was resistant and *b* is the total number of antibiotics employed (Osundiya *et al.,* 2013).

Detection of plasmid mediated beta-lactam resistance genes from multiple antibiotic resistant *E. coli* strains

Plasmid DNA was extracted using a commercial DNA extraction kit according to the manufacturer's (Zymo, USA) protocol. *E. coli* strains that showed multiple resistances from each water treatment stage were cultured in peptone water and incubated for 48 hours at 37°C. A volume (10 mL) of the suspension was centrifuged at 12,000 rpm for 1 minute and the pellet was used for plasmid DNA extraction. Polymerase chain reaction (PCR) was used to amplify four ARGs (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{CMY}$, *bla*$_{FOX}$) from the extracted plasmid using four specific primer pairs designed by NCBI primer blast and synthesized by Inqaba Biotech firm (West Africa). The PCR was performed by pipetting 2 µL of DNA template, 1 µL of each primer (forward and reverse), 15µL of PCR master mix (*Taq* DNA polymerase, PCR buffer, dNTP, gel loading dye, and fluorescence dye), and 12 µL of nuclease-free water into each PCR tube, making a total of 30µL of the final reaction mixture in each tube. PCR was conducted using Cleaver PCR machine at a cycle of 35 reaction cycles (*bla*$_{NDM}$ and *bla*$_{IMP}$) and 30 reaction cycles (*bla*$_{CMY}$ and *bla*$_{FOX}$). PCR conditions varied for each primer and as presented in Table 1 below.

Table 1.Primer pairs used, their target genes amplicon size, annealing temperatures and number of cycles used

Primer pair	Sequence	Target gene	Amplicon size (bp)	Denaturation temperature (°C)	Elongation temperature (°C)	Annealing temp (°C)	Number of cycles
bla$_{IMP}$	**F**-GAAGGCGTTTAT GTT CAT AC **R**-GTATGTTTCAAG AGT GAT GC	IMP-1	247	95	72	48.16	35
bla$_{NDM}$	**F**-GGGCAGTCGCTT CCA ACG GT **R**-GTAGTGCTCAGT GGCAT	NDM-1	475	94.5	72	57.6	35
bla$_{FOX}$	**F**-GCCGAGCTT ACG GGA TCA AG **R**-CAAAGCGCG TAACCG GAATTG G	FOX-1	247	95	72	50	30
bla$_{CMY}$	**F**-CCGAAGCCT ATG CGT GAA ATC C **R**-GCA ATG CCC TGC TGG AGC G	CMY-2	106	95	72	52.5	30

Table2.Physicochemical parameters of water samples collected from different stages of the water treatment plant

	Temp.	pH	DO (mg/L)	BOD (mg/L)	Conductivity (µs/cm)	Salinity (mg/L)	TDS (ppm)	Nitrite (mg/L)	Residual chlorine (mg/L)
Storage	25.0±0.2	5.18±0.1	12.1±0.2	1.8	194.5±0.3	0.08±0.2	79.7±0.2	0.009	0.225
Disinfection	25.0±0.1	4.85±0.2	5.2±0.3	0.9	379±0.2	0.28±0.05	172±0.1	0.006	0.351
Filtration	26.0±0.1	5.10±0.2	7.6±0.1	1.4	159.2±0.1	0.06±0.1	55.4±0.3	0.000	0.000
Clarification	28.0±0.1	5.13±0.2	9.4±0.1	3.8	170±0.3	0.13±0.2	86±0.2	0.017	0.000

Keys: BOD= Biological Oxygen Demand, OD=Oxygen Demand, TDS= Total Dissolved Solid, Temp. = Temperature

PCR products were resolved by gel electrophoresis using 1% agarose gel (1 g agarose powder in 99 mL Tris-Borate EDTA buffer) under a voltage of 120V and current of 300A for 45 minutes (Lee *et al.,*2012).

Statistical Analysis

Analysis of variance (ANOVA) was used to determine whether physicochemical parameters varied statistically along the stages of water treatment and means were separated using Fisher's LSD.

RESULTS AND DISCUSSION

The physicochemical parameters of water sample collected from each stage revealed that DO, conductivity and TDS varied between the stages (Table 2). Water from all stages was slightly acidic. Conductivity and DO were high in water collected from storage and disinfection stage. A total of 124 bacterial strains were isolated in which 30 (37.2%) were confirmed to be *E. coli* (Figure 1)

Along water treatment stages, temperature among other parameters varied insignificantly with the clarification stage having the highest temperature. This is mostly due to the direct exposure of the clarification tanks to solar radiation as sampling was done during summer (April). During survey of sampling site, it was observed that untreated sewage from tanneries and dyeing companies were emptied at Kano dumpsite located at bank of river Challawa and Tamburawa river confluence. A research conducted in California by United States environmental protection agency (EPA) in 2017 reported industrial pollution and chemical dump as a major cause of acidic pH in surface water; hence the acidic pH observed throughout the process of water treatment may be as a result of presence of these contaminants in source water. The highest pH was recorded after the addition of calcium-hypo-chloride $Ca(OCl)_2$ during disinfection, hence adding to the acidity. This suggests that the pH of the water was not corrected with lime after treatment process as reported by Zakari*et al.,* (2014).

Though all other parameters were within the acceptable limits approved by the WHO (2017), the value of BOD recorded at the storage stage was higher than that of the two stages preceding it; indicating possible contamination and presence of microorganisms.

Throughout the treatment processes, *E. coli* was detected in high quantity (Figure 1) indicating high levels of faecal contamination in source water. Statistically however, there was no significant difference (p= 0.05) in the distribution of *E. coli* along the stages of water treatment as majority of the isolated bacteria were found in storage stage.

Clarification reduced the CFU of *E. coli* in 100 mL of sample by 0% with a 50% decrease in the quantity of the *E. coli* after disinfection. This shows that about half the population of the cultured organisms scaled through disinfection indicating a high chlorine tolerance level. This is in line with the findings of Zhuang *et al.,* in 2014 on the resistance of bacteria to different disinfectants. The study reported that some strains of *E. coli* can tolerate up to 30 mg/L of chlorine after a contact time of 60 minutes which is almost seven times greater than the 4 mg/L concentration approved by WHO.

Results of antibiotic susceptibility test showed a 20% increase in the resistance of *E. coli* to Cefuroxime, Ceftazidime, and Augmentin at the storage stage (Table 3). Antibiotic resistant *E. coli* dominated disinfection stage with organisms showing 100% resistance to 5 antibiotics (Cefuroxime, Ceftazidime, Cefixime, Nitrofurantoin and Augmentin). Antibiotic resistance have shown significant correlation to water quality parameters such as DO, temperature and nutrients abundance. In this study, it can be noted that during physicochemical analysis, disinfection stage had the lowest DO value in comparison to other treatment stages, confirming the findings of Yu *et al.,* (2014) among other studies. Clarification reduced Ampicillin resistance by 50% while increasing the resistance to Nitrofurantoin by 50%. Ofloxacin and Gentamicin resistant *E. coli* were isolated from filtration stage.

ANOVA results showed a significant difference in the resistance of the *E. coli* to the antibiotics along different water treatment stages (p= 7.82×10^{-2} at p= 0.05).

Post disinfection unit harboured the largest population of isolated *E. coli*. It was observed that the storage pipes used in TWTP were mostly made of metal and plastic which are both good substrates that support biofilms adhesion and growth. Therefore, the abundance of *E. coli* in this stage maybe as a result of biofilms formation in the storage pipes as stated in a study conducted by Sharma *et al.,* 2019 showing the biofilms forming potentials of *E. coli* on different surfaces among other bacterial species.

Clarification stage had the highest MAR of 0.63 followed by storage stage with MAR value of 0.54 indicating that these stages harboured most of the multi-antibiotic resistant *E. coli.*

Multiple studies have linked antibiotic resistance to chlorine tolerance, heavy metal resistance and biofilms formation. Destiani and Templeton, 2019 reported that antibiotic resistant bacteria

were able to tolerate concentrations of chlorine 7 times more than susceptible bacterial strains. Similar findings showed biofilms-forming gram negative bacteria had high chlorine tolerance levels in addition to being multidrug resistant when compared with non-biofilm producing strains (Khan *et al*., 2016; Mahapatra *et al.,* 2015; Khan *et al.,* 2019;).

Cephalosporin resistance genes *bla*$_{CMY}$ and *bla*$_{FOX}$ were detected in all isolates subjected to molecular analysis as shown in Table 5.Though *E. coli* identified in this study were not screened for Carbapenems resistance phenotypically, their resistance genes (*bla*$_{IMP}$) were detected in clarification stage; while *bla*$_{NDM}$ was detected in all the stages except disinfection stage using molecular techniques. Therefore, indicating that bacteria resistance to third generation cephalosporins often show resistance to carbapenem antibiotics especially the multidrug resistant strains (Yusuf *et al.,* 2014).

Ma *et al.* (2019)identified the potential antibiotic resistance risk to human health by classifying the drinking water samples into three categories based on the nature of detected pathogens and ARGs; (1) ARG-carrying pathogens (unsafe), (2) ARGs and pathogens, but no ARG-carrying pathogens (probably unsafe), and (3) ARGs only (relatively safe). Based on this classification, *E. coli* strains identified in this study fall in both category one and two thereby rendering drinking water from TWTP a source of concern.

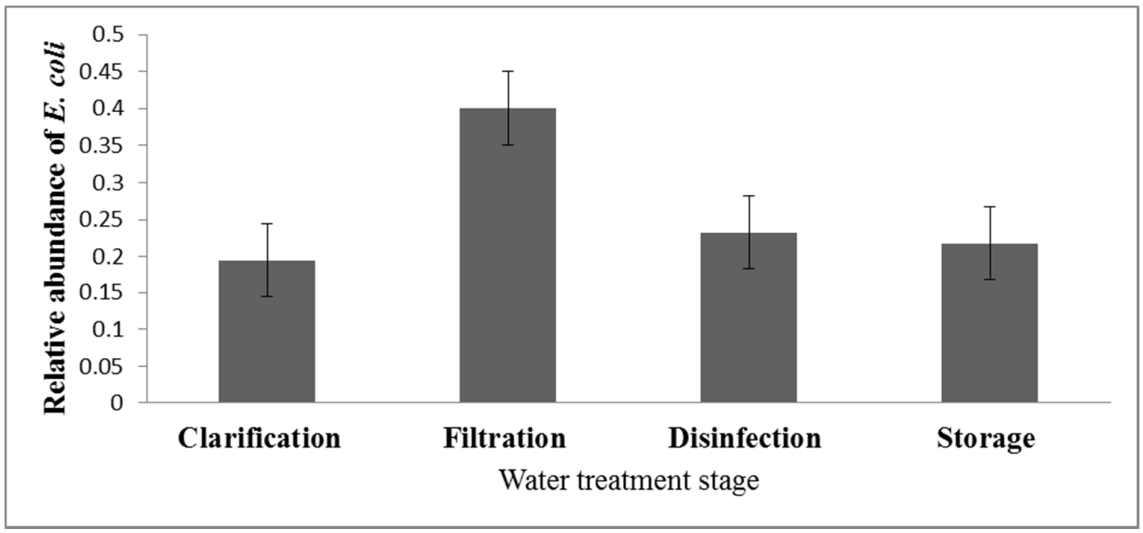

Figure 1. Relative abundance of *E. coli* per 100mL of water sample at each water treatment stage

Table 3. Resistance of *Escherichia coli* to antibiotics at each treatment stage

Antibiotics	Clarification n= 8	Filtration n= 8	Disinfection n=4	Storage n=10
Ceftazidime (30mg)	7 (87.5%)	7 (87.5%)	4 (100%)	9 (90%)
Cefuroxime (30mg)	7(87.5%)	7 (87.5%)	4 (100%)	8 (80%)
Augmentin (30mg)	7(87.5%)	5 (62.5%)	4 (100%)	10 (100%)
Nitrofurantoin (300mg)	2 (25%)	5 (62.5%)	4 (100%)	8 (80%
Cefixime (5mg)	3 (37.5%)	3 (37.5%)	4 (100%)	9 (90%)
Ampicillin (10mg)	5 (62.5%)	1 (12.5%)	1 (25%)	3 (30%)
Tetracycline (30mg)	1 (12.5%)	3 (37.5%)	0	2 (20%)
Gentamicin (10mg)	0	1 (12.5%)	0	0
Chlorampenicol (30mg)	0	0	0	0
Ofloxacin (5mg)		1 (12.5%)	0	0
Ciprofloxacin (5mg)	0	2 (25%)	0	3 (30%)

n is the number of *E. coli* subjected to antibiotic susceptibility test

Table 4. Multidrug resistance index of *E. coli* to tested antibiotics

Treatment stage	No of antibiotics used	No of antibiotics organisms were resistant to	MAR index
Clarification	11	7	0.63
Filtration	11	4	0.36
Disinfection	11	5	0.45
Storage	11	6	0.54

Table 5. Detection of ARGs in selected *E. coli* strains

Stage	Number of tested organisms	*bla*$_{IMP}$	*bla*$_{NDM}$	*bla*$_{CMY}$	*bla*$_{FOX}$
Clarification	2	Detected	Detected	Detected	Detected
Filtration	2	Detected	Detected	Detected	Detected
Disinfection	1	Not detected	Not detected	Detected	Detected
Storage	2	Not detected	Detected	Detected	Detected

CONCLUSION

Water treatment processes in TWTP harboured a significant quantity of multidrug resistant *E. coli.* At the end of the process, there was increase in the population of isolated *E. coli;* indicating significant contamination at the storage units. This study highlights the contribution of each water treatment stage in the reduction of ARB and ARGs and provides basic data for *E. coli* and ARG pollution in TWTP. It indicates that resistant *E. coli* carrying different ARGs persist in drinking water produced in TWTP and highlights the need for a more efficient storage system.

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