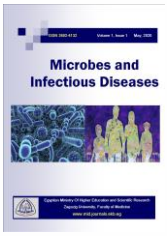
Microbes and Infectious Diseases 2024; Article-In-Press, **DOI:** 10.21608/mid.2024.252551.1710



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Occurence of extended spectrum beta lactamase (ESBL) producing *Escherichia coli* in wastewater from two hospitals in Akure

***Tolulope Gbenga Emoruwa*[*1] , *Funmilola Olufunmilola Omoya* [2] , *Ayokunle Itunu Okewale* [3], *Kehinde Oluyemi Ajayi* [4]**

1- *Federal University of Technology Akure Department of Microbiology*
2- *MICROBIOLOGY DEPARTMENT, FEDERAL UNIVERSITY OF TECHNOLOGY, AKURE*
3- *Microbiology, Faculty of Science, Federal University of Technology Akure, Nigeria*
4- *Microbiology, Federal University of Technology Akure, Nigeria.*

ARTICLE INFO

Article history:
Received 12 December 2023
Received in revised form 4 January 2024
Accepted 10 January 2024

Keywords:
Antibiotic susceptibility
Wastewater
Escherichia coli

ABSTRACT

Background and rationale: Hospital wastewater is heavily contaminated with different microorganisms, resulting in a major public health threat in the developing world. This study was conducted to detect the presence of extended spectrum beta lactamase (ESBL) producing *Escherichia coli (E. coli*) in hospital wastewater of University of Medical Science (UNIMED) teaching hospital and University health center FUTA. **Methods**: Wastewater was collected from outlets in different wards, laboratories, laundry, and pipe borne water was collected as a control. The wastewater underwent bacteriological analysis using membrane filtration, identifying all bacteria isolates based on cultural, morphological, and biochemical characteristics. Zones of inhibition were interpreted to screen *E. coli* isolates for antibiotic susceptibility. Molecular detection of ESBL in *E. coli* isolates survivability at various pH, temperature, and salt concentrations were examined as well. **Results**: It was observed that the total bacterial counts in wastewater collected from UNIMEDTH and FUTA Health Center ranged from 51.96±0.76 cfu/ 100 ml (tap water) to 865.44±2.06 cfu/ 100 ml (Microbiology Laboratory) and 74.77±0.21 cfu/ 100 ml (nurse station) to 416.41±2.86 cfu/ 100 ml (Wound treatment ward) respectively, while the total coliform counts in UNIMED and FUTA health centre ranged from 4.40±0.07 cfu/ 100 ml (tap water) to 375.32±1.33 cfu/ 100 ml (Microbiology Laboratory) and 5.54±0.11 cfu/ 100 ml (tap water) to 80.41±0.48 cfu/ 100 ml (doctors' station) respectively. The least and most frequent bacterial isolates were *Aeromonas hydrophila* and *E. coli* respectively. Septrin, chloramphenicol, amoxicillin, augmentin and gentamicin had lower zones of inhibition against *E. coli* isolates. **Conclusion**: This study revealed that hospital wastewater could serve as an important source for exposure and dissemination of ESBL producing *E. coli*, which could pose a health risk to the people in the hospital environment and surrounding water bodies.

Introduction

Water is essential for the hygiene and functioning of hospitals. The wastewater discharged by hospitals presents health and environmental risks because of the nature and the importance of the substances they contain [1, 2]. The management of hospital wastewater is a real problem in developing countries, due to the inexistence of wastewater

DOI: 10.21608/MID.2024.252551.1710
* *Corresponding author:* Tolulope Gbenga Emoruwa
E-mail address: emoruwatolu09@gmail.com

treatment and purification stations [3]. This hospital wastewater is continuously discharged into natural receptacles such as the ocean and lake which may result in ecotoxicity [4]. There is ignorance of the quality of the wastewater produced by hospitals in Nigeria, hence they are continually discharged into the environment. Therefore, it is of utmost importance to know the current quality of the hospital wastewater discharged into the environment [1].

Wastewater from hospitals contains potentially pathogenic micro-pollutants. The presence of these substances in the environment is considered an emerging subject due to uncertainties related to the risk that they pose for terrestrial and aquatic ecosystems [5]. Despite the health and environmental risks of hospital wastewater, there are almost no regulations on their treatment before discharge into the environment [6].

The release of hospital wastewater in the environment without prior treatment has become a subject of global concern in the fields of the environment and public health and arouses the interest of scientists and public authorities. In recent decades, the scientific community has focused on the biological, physical and chemical characteristics of hospital wastewater in order to assess the potential risks associated with their discharges into aquatic environments. Pollutants such as total and fecal coliforms, chemical residues (detergents), pathogens (*Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* and *Vibrio*) and potentially toxic metals (cadmium, copper, cyanide, iron, gadolinium, nickel, lead, platinum, zinc, phenol, etc.) have been quantified in hospital wastewater. The chronic exposure of aquatic organisms to these substances explains the emergence of various phenomena such as hormonal imbalances, resistance to antibiotics, and all other harmful impacts on the environment [2, 7]. It, therefore, becomes important to know the quality of the wastewater produced in university hospitals which are the greatest producers of hospital wastewater.

Escherichia coli belong to Enterobacteriaceae that are facultative anaerobic, and Gram-negative rods known to inhabit the gastrointestinal tract of humans and animals. *Escherichia coli* strains are of biological significance to humans. On the basis of genetic and clinical criteria can be broadly classified into three major *E. coli* groups; commensal *E. coli*, intestinal pathogenic (diarrheagenic) *E. coli*, and extra intestinal *E. coli* (ExPEC) [6].

The presence of multidrug-resistant (MDR) and Extended-spectrum β-lactamase-producing (ESBL-producing) bacteria, especially in wastewater, poses a great risk to the environment when such wastewater is discharged into the environment [8].

Materials and methods

Study design and area

This case-control study was carried out in selected hospitals in Akure, and microbiological analysis was conducted at the Department of Microbiology, The Federal University of Technology, Akure (FUTA). Wastewater (200 ml) was collected from the University of Medical Science (UNIMED) teaching hospital and University Health Center FUTA. These two hospitals were selected because they are the only hospitals affiliated with tertiary institutions in Akure, the state capital. A letter of introduction was collected from the Head of Department, Department of Microbiology, FUTA, and the student identity card was used as a valid means of identification in the hospitals where samples were collected in Akure.

Isolation of bacteria from wastewater

Freshly collected wastewater sample was shaken and 100.0 ml of the water samples was filtered with 0.45 µm membrane filter, the filter was aseptically placed on molten agar (nutrient agar, eosin methylene blue agar (EMB) and macConkey agar) [9]. Incubation was carried out at 37oC for 24 hours.

Colony counting was carried out visually by counting the number of visible colonies that appeared on the agar plates and plate with a distinct colony was used. Calculation of colony forming unit (CFU) per mL was based on the formula (equation 1):

CFU = Number of colonies × mL of the sample suspended …… equation 1

Dilution factor

The number of colonies on each plate was recorded. Different colonies observed on the plates used for the isolation of bacteria were picked and streaked on sterile prepared agar plates for pure culture. These were incubated at 37^{o}C for 24 hours. Different bacterial pure cultures were then inoculated into double strength Nutrient agar slants, incubated at 37^{o}C for 24 hours in order to ensure proper growth,

and then kept as stock cultures in the refrigerator at 4ºC. Biochemical characterization and identification of the bacteria were carried out using methods of [10]. The scheme of identification is recorded in the result section of this work.

Antimicrobial susceptibility testing

CLSI (5th edition) guidelines recommend the Kirby–Bauer disk diffusion method, which involves using Muller–Hinton agar (MHA) to inoculate bacterial suspensions [11]. The disc diffusion method was used to determine the susceptibility and resistance of the organisms to the antimicrobials. Twenty milliliters (20 ml) of sterile Mueller-Hinton agar were aseptically poured into sterile Petri dishes and allowed to gel. A bacterial suspension equivalent to the 0.5 McFarland turbidity standard was prepared for inoculation. Each plate was seeded with the test organism before aseptically introducing the antibiotic disc with sterile forceps onto the surface of the solidified Mueller Hilton agar plate and incubated at 37ºC for 18 hours. After incubation, the diameter of clear zones around the disk was measured in millimeters and recorded as the zones of inhibition. Diameters of the zone of inhibition were measured with a calibrated ruler and then compared with the clinical and laboratory standards institute (CLSI) standard interpretative chart for their sensitivity, intermediate, or resistance. Seeded plates without antibiotic disks served as the control. The antibiotic sensitivity profile was carried out in triplicates. The antibiotics used were; augmentin® (30 µg), amoxicillin (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), cefixime (30 µg), nitrofurantoin (300 µg), ofloxacin (10 µg), ciprofloxacin (10 µg), gentamicin (20 µg), tetracycline (30 µg), chloramphenicol (30 µg), septrin (25 µg) (Oxoid Ltd., Basingstoke Hampshire, England).

Standardization of test bacteria inoculum

Method modified by [10], was used to prepare the McFarland 0.5 turbidity standard which was used to measure the density of bacterial cells. In this method, fifty milliliters (50 ml) of a 1.175% (wt/vol) dehydrated barium chloride (BaCl2.2H2O) solution was added to 99.4 ml of 1% (vol/vol) sulfuric acid. The McFarland standard tube was then sealed with paraffin to prevent evaporation and stored in the dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1 cm light path. The 0.5 McFarland standards were vigorously agitated before use.

A loopful of the bacterial culture was aseptically inoculated into 10.0 mL freshly prepared sterile nutrient broth and incubated at 37°C for 24 hours sterile distilled water was dispensed into the broth to standardize the culture to 0.5 McFarland's standard (106 cfu/ml) before use as described by [10], modified by [11].

Determination of physicochemical parameters of water

Different physicochemical parameters of collected raw hospital wastewater samples were determined according to American Public Health Association (APHA, 5th edition) standard methods. These parameters include pH, dissolved oxygen, Biochemical Oxygen Demand (BOD), and Chemical Oxygen Demand (COD) [12].

Detection of extended spectrum Beta-lactamases (ESBLs)

The isolates that were resistant to any of the tested third-generation cephalosporins (ceftazidime and cefotaxime) were screened for ESBL production using the combined disk method. This involved the use of cephalosporin discs (cefotaxime 30 µg and ceftazidime 30 µg) with and without 10 µg clavulanic acid placed on Mueller Hinton agar earlier inoculated with the test organisms [11]. An increase in the inhibition zone diameter of ≥ 5 mm in cephalosporin disk combined with clavulanic acid, compared to cephalosporin alone, indicates ESBL production. *Klebsiella pneumoniae* ATCC 700603 and non-ESBL-producing strain *E. coli* ATCC 25922 were used as positive and negative controls.

Extraction of bacteria genomic DNA

The manufacturer specification stated in the manual of Quick-DNA Miniprep Plus Kit (D4068 and D4069) Zymo was followed. A 1.5 ml of 24-hour old bacterial broth culture with cell counts of 5 x 106 cfu/ml was dispensed in centrifuge tube and centrifuged at 10,000 rpm for 2 minutes, the supernatant containing media was discarded and to the pellet 1 ml of distilled water was added and dissolved the pellet completely and again centrifuged at 10,000 rpm for 2 min, the procedure was repeated for two times. The supernatant was discarded and the pellet which is the residue (200 µl) was transferred to a microcentrifuge tube, equal volume (200 µl) of BioFluid and cell buffer solution was added, and then incubated at 55ºC for 10 minutes for complete digestion of cells. After the incubation, 420 µl of genomic binding buffer was added to the digested sample and mixed thoroughly,

it was then transferred to Zymo-Spin column in a collection tube and centrifuge at 12,000 rpm for 1 minute after which the collection tube was discarded with the flow through. The column was transferred into another collection tube and 400 µl of DNA pre-wash buffer was added and centrifuged at 12000 rpm for 1 minute, the collection tube was emptied and 700 µl of genomic-DNA wash buffer was added and centrifuged at 12000 rpm for 1 minute, and collection tub was emptied then 200 µl of genomic-DNA wash buffer was an added and centrifuge for 1 minute, the collection tube and the flow through were discarded. The column was transferred into a new microcentrifuge tube and 50 µl of DNA elution buffer was added and incubated at 55ºC for 5 minutes and centrifuged at 12000 rpm for 1 minute to elute the DNA. The eluted DNA was stored at -20°C. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer, 1 µl of DNA was checked at absorbance of ratio of 260 nm / 280 nm and 260 nm / 230 nm, the concentration ranged from 97 to 178 µg/µl.

Amplification of extended-spectrum β-lactamase gene (blaTEM),

The obtained DNA suspension was used as a template for polymerase chain reaction (PCR). All isolates that were phenotypically positive for ESBL production were screened by PCR, using TEM specific primers as described by [13]. The total reaction volume of 20 µl consists of nuclease-free water, master mix (New England Bioline 'NEB'), forward primer, reverse primer, and DNA template 5, 10, 1, 1, and 3 µl respectively. Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 denaturation cycles at 94°C for 1 minute, annealing at 52°C, extension at 72°C for 1 minute, and final extension at 72°C for 3 minutes. The positive and negative controls were *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 respectively, the primer used is presented in **table (3.1).** The amplified gene was examined on 2.0% agarose gel, 100 and 1 kbp DNA ladder (NEB) were used as control, 1 and 5 µl of loading dye (bromophenol blue) and amplicon respectively were mixed and loaded on solidified agarose gel and 1X TAE buffer was used for the electrophoresis.

Amplification and sequencing of 16S rRNA gene of bacteria

The 16S rRNA gene was amplified using primers 5'AGAGTTTGATCCTGGCTCAG 3' and 5'GACGGGCTGTGCGTTCA 3' forward and reverse respectively. The total reaction volume of 20 µl consists of nuclease-free water, master mix (New England Bioline), forward primer, reverse primer, and DNA template 5, 10, 1, 1, and 3 µl respectively. Amplification reactions were performed under the following conditions: initial denaturation of 2 minutes at 94 °C (pre heating) followed by 25 cycles were run on a thermal cycler, each comprising 1 min at 94°C (denaturation), 1 min at 94 °C (annealing) and 1.5 minutes at 94 °C (extension), followed by a final extension of 10 minutes at 94 °C for utilization of extra dNTPs in the PCR mixture. The amplified gene was examined on 2.0% agarose gel, 1 kbp DNA ladder (NEB) was used as control, 1 and 5 µl of loading dye (bromophenol blue) and amplicon respectively were mixed and loaded on solidified agarose gel and 1X TAE buffer was used for the electrophoresis. The electrophoresis was carried out at 90 v for 60 minutes after which the DNA bands were observed by the gel documentation system.

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. The sequences were compared with sequences available in GeneBank (U43165), derived sequence aligned by Basic Local Alignment Search Tool (BLAST) algorithm, the highest S-ab value with identified species in the Sequence match search. Using the results received through BLASTn a phylogenetic tree is created using the BLASTn web-pageBio- Edit software and MEGA X were used for all genetic analysis. The bacterial sequence was compared with those that have been isolated from blood in a phylogenetic tree.

Survival of ESBL producing *E. coli* isolates in different salt concentration, pH and temperature

Survival of ESBL producing *E. coli* subjected to different environmental conditions (pH, temperature, and salt concentration) were examined as described by [13]. For the influence of pH on survival of *E. coli*, each isolate was inoculated into sterile test tubes with 9 ml of Nutrient broth (with the following pH adjusted to 3, 4, 5, 6, 7, 8, 9, and 10) and were incubated at 37 °C for 24 hours, the

influence of temperature on survival of *E. coli* was carried out by inoculation 9 ml of nutrient broths and the tubes were incubated at different temperature (ranging from 4 to 40^{o}C) for 24 hours. Also, salt concentrations ranging from 0 to 30% v/v were prepared in test tubes and inoculated at 37^{o}C for 24 hours to determine the survival of *E. coli* at different salt concentrations. The microbial growth was observed after 24 hours using a spectrophotometer at absorbance of 600 nm and 0.1 ml of each preparation was poured on nutrient agar and incubated at 37^{o}C for 24 hours after which the colonies were counted.

Statistical analysis

Data was analyzed using IBM-Statistical Package (IBM-SPSS) version 20. Relationships between parameters were evaluated and *p*-value <0.05 was considered significant. Differences in the mean of parameters were compared using Duncan's Multiple Range test at p <0.05. MEGA 6 and Bioedit software were used for the sequence alignment and phylogenetic tree.

Results

Bacterial counts of wastewater collected from selected hospitals in Akure

The result in **table (1)** showed the bacterial counts of wastewater collected from UNIMED, it was noted that total bacterial counts of wastewater ranged from 51.96±0.76 cfu/100 ml (water source) to 865.44±2.06 cfu/100 ml (microbiology laboratory) while the total coliform bacterial counts ranged from 4.40±0.07 cfu/100 ml (water source) to 375.32±1.33 cfu/100 ml (microbiology laboratory). Bacterial counts of wastewater collected from FUTA is shown in **table (2).** The result showed that 59.06±1.33 cfu/100 ml (laundry) to 416.41±2.86 cfu/100 ml (wound treatment ward) while the coliform bacterial counts ranged from 5.54±0.11 cfu/100 ml (water source) to 80.41±0.48 cfu/100 ml (doctor's station). Statistically, there were significant (p<0.05) variations in the total bacterial and coliform counts of all the wastewater samples.

Cultural and biochemical characteristics of bacteria isolated from wastewater at UNIMED Teaching Hospital and FUTA Health Center Akure

Table 2 shows the identity of bacteria isolated from hospital wastewater, it was noted that eleven (11) different bacterial species were identified and they include; *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus (S. aureus).*

Occurrence of bacterial isolates in hospital wastewater

Table 3 revealed the occurrence of bacterial isolates in hospital wastewater samples. The result showed that *E. coli* was isolated from all samples collected at UNIMED. Among fifty-one bacterial isolates from UNIMED wastewaters, *E. coli* (23.53%), *S. aureus* (17.65%), *Bacillus subtilis* (13.73%) and i (13.73%) were the most frequently isolated bacteria while among the twenty (20) bacterial isolates from FUTA wastewater, *S. aureus* (25%), *E. coli* (15%) and *Pseudomonas aeruginosa (P. aeruginosa)* (15%) were the most frequently isolated bacteria. The least frequent bacterial isolates from UNIMED were *E. aerogenes, P. mirabilis, P. aeruginosa* and *Salmonella typhi* while the least frequent bacterial isolated from FUTA are *A. hydrophila, B. subtilis* and *P. mirabilis*.

Physicochemical parameters of wastewater from UNIMED teaching hospital and FUTA Health Center Akure

Table 4 shows the physicochemical parameters of all wastewaters collected from selected hospitals in Akure. It was noted that the pH (9.82±1.33) of wastewater sample collected from laundry in FUTA health centre was significantly (p<0.05) higher than pH of others and the least pH (5.31±0.62) was observed in wastewater collected from post-natal ward at UNIMED while there is no significant (p<0.05) between pH of other wastewater samples. The value of DO and COD of wastewater ranged from 2.01±0.02 mg/l in UNIMED laundry to 8.31±0.11 mg/l in UNIMED water source and 5.11±0.05 mg/l in UNIMED water source to 931.44±5.06 mg/l in UNIMED laundry respectively. Also, the BOD was between 3.68±0.07 mg/l in FUTA water source to 14.57±0.55 mg/l in FUTA laundry while the ratio of BOD and COD was between 0.01in wound treatment ward to 0.94 in UNUMED water source.

Antibiotic susceptibility profiles of Escherichia coli Isolated from wastewater in UNIMED teaching hospital Akure

Figure 1 showed the antibiotic susceptibility profiles of *E. coli* isolated from UNIMED wastewater, it was observed that all the isolates were less susceptible to septrin (1.01±0.01 mm in isolate from pharmacy to 5.03±0.21 mm in isolate from

antenatal ward), chloramphenicol (0.00±0.00 mm in isolate from MLS laboratory to 20.07±0.13 mm in isolate from water source) and sparfloxacin (1.01±0.01 mm in isolate from MLS laboratory to 21.40±0.20 mm in isolate from blood bank). The isolates were more susceptible to ciprofloxacin (20.04±0.02 mm in isolate from MLS laboratory to 26.11±0.04 mm in isolate from blood bank) and tarivid 13.32±0.11 mm in isolate from community clinic to 24.06±0.31 mm in isolate from antenatal ward. Generally, the isolates from MLS laboratory and postnatal ward showed least susceptibility to all the antibiotics used.

Antibiotic susceptibility profiles of *Escherichia coli* isolated from wastewater in FUTA Health Center Akure

Figure 2 showed the antibiotic susceptibility of *E. coli* isolated from FUTA health centre, it was observed that the isolates were least susceptible to septrin (2.03±0.01 mm in isolate from nurses' station to 3.02±0.11 mm in isolate from laundry), chloramphenicol (2.71±0.01 mm in isolate from water source to 12.07±0.33 mm in isolate from laundry), amoxicillin (2.10±0.02 mm in isolate from water source to 11.04±0.10 mm in isolate from laundry) and augmentin® (2.22±0.05 mm in isolate from nurses' station to 18.06±0.30 mm in isolate from laundry). The *E. coli* isolates showed higher susceptibility to spafloxacin, gentamycin, ciprofloxacin, tarivid, streptomycin and pefloxacin.

Multiple antibiotic resistant patterns of *Escherichia coli* isolated from wastewater in UNIMED teaching hospital and FUTA Health Center Akure

Table 6 revealed that the isolates of *E. coli* had higher resistance against septrin (66.67%), amoxicillin (40%), augmentin® (40%), chloramphenicol (26.67%) and streptomycin (26.67%) and showed no resistance to sparfloxacin, pefloxacin and tarivid. The MARi of isolates from MLS laboratory, postnatal ward and eye clinic were 0.7, 0.6 and 0.4 respectively which were higher than others.

Occurrence of extended spectrum Beta Lactamase (ESBL) producing E. coli isolated from wastewater in UNIMED Teaching Hospital and FUTA Health Center Akure

Table 7 showed the occurrence of ESBL producing *E. coli* in hospital wastewater, it was noted that phenotypically, 60% and 40% of the isolates were positive for ESBL using disc diffusion and double disc synergistic while molecular revealed that 26.67% of the isolates (*E. coli* isolated from postnatal ward, laundry in UNIMED, water source in UNIMED and nurses' station in FUTA) were positive for ESBL. The agarose gel electropherogram of the ESBL positive isolate is shown in **plate (1).**

Molecular identity of ESBL producing *E. coli* isolated from hospital wastewater in Akure

Plate 2 revealed the amplification of 16S rRNA gene of ESBL producing *E. coli* at 1500 bp. **Table 8** showed that two isolate of ESBL producing E. coli were 100% homologous to *E. coli* k-12 strain MG1655 substrain while it was 96.65% and 94.21% similar to *E. coli* 0121 strain and *E. coli* type 131 strain respectively.

Effects of temperature on ESBL producing *Escherichia coli* isolated from different wastewater sources in selected hospitals in Akure

Figure 3 revealed that ESBL producing *E. coli* isolates survive temperature range 20oC to 40ºC, there was gradual increase in cell counts as temperature increased from 20ºC to 40ºC except E. coli isolated from nurses' station that had reduction in cell counts at temperature above 35ºC.

Effects of salt solution on ESBL producing *Escherichia coli* isolated from different wastewater sources in selected Hospitals in Akure

Figure 4 shows that ESBL producing *E. coli* isolates survive salt concentration of 0 to 10%, however, gradual decrease in cell counts as the salt concentration increased from 2% to 10% was observed in all the isolates, optimum growth was observed at 2% salt concentrations except the isolate from nurses' station that had optimum growth at 4% salt concentration.

Effects of pH on ESBL producing *Escherichia coli* isolated from different wastewater sources in two hospitals in Akure

Figure 5 showed that isolate of ESBL producing *E. coli* isolate survived pH range of 5 to 9 with optimum cell growth at pH of 7.0. Also, the isolate from water source and nurses' station survived pH above 10.0.

Table 1. Bacterial counts of wastewater collected from UNIMED teaching hospital Akure

Wastewater sampling points	**Total bacterial counts (cfu/ 100ml)**	**Total coliform counts (cfu/100 ml)**
Chemical Laboratory	726.31±1.36^{e-}	303.05±0.05^{h}
Microbiology Laboratory	865.44±2.06^{f}	375.32±1.33^{i}
Eye clinic	742.33±1.03^{e}	180.01±0.76^{f}
Community clinic	681.24±0.69^{d}	11.11±0.21^{b}
Blood bank	720.00±0.00^{e}	201.03±0.45^{g}
MLS laboratory	751.04±0.77^{e}	323.04±1.33^{h}
Antenatal	762.41±0.32^{e}	32.07±0.59^{d}
Post natal	782.01±1.33^{ef}	25.12±1.31^{c}
Accident and Emergency	852.09±1.21^{ef}	50.60±0.79^{e}
Pharmacy	498.09±0.82^{c}	21.22±0.45^{c}
Laundry	262.03±1.24^{b}	17.16±1.21^{c}
Water source	51.96±0.76^{a}	4.40±0.07^{a}

Values are presented as mean ± standard error, values in the same column carrying same superscript are not significantly different at $p<0.05$ using new Duncan Multiple Range test

Table 2. Bacterial counts of wastewater collected from FUTA Health Centre Akure

Wastewater sampling points	**Total bacterial counts (cfu/100 ml)**	**Total coliform counts (cfu/100 ml)**
Nurses' station	74.77±0.21^{b}	8.20±0.51^{a}
Children's ward	110.33±1.27^{c}	35.34±0.45^{c}
Doctor's station	211.71+1.37^{d}	80.41±0.48^{e}
Laundry	59.06±1.33^{a}	12.41±0.55^{b}
Health center entrance	312.07±1.22^{e}	60.04±1.25^{d}
Wound treatment ward	416.41±2.86^{f}	7.32±0.67^{a}
Water source	87.40±1.32^{b}	5.54±0.11^{a}

Table 3. Factors related to mortality among patients with COVID-19.

Wastewater sampling points (UNIMED)`	***Aeromonas hydrophila***	***Bacillus cereus***	***Bacillus subtilis***	***Citrobacter freundii***	***Enterobacter aerogenes***	***Escherichia coli***	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Pseudomonas aeruginosa***	***Salmonella* Typhi**	***Staphylococcus aureus***
Chemical Laboratory	-	+	-	-	-	+	-	-	+	-	+
Microbiology Laboratory	-	+	-	+	-	+	-	+	-	+	+
Eye clinic	-	+	+	-	-	+	+	-	-	-	+
Community clinic	-	-	+	+	-	+	+	-	-	-	-
Blood bank	-	+	+	+	-	+	-	-	-	-	-
MLS laboratory	-	+	-	-	+	+	-	-	-	-	+
Antenatal	-	+	+	+	-	+	+	-	-	-	+
Post natal	-	+	-	-	-	+	-	+	+	+	+
Accident and Emergency	-	-	+	-	-	+	-	-	-	-	+
Pharmacy	-	-	+	-	+	+	+	-	-	-	+
Laundry	-	-	+	-	-	+	-	-	-	-	+
Water source	-	-	-	-	-	+	-	-	-	-	-
Total = 51	0(0)	7(13.73)	7(13.73)	4(7.84)	2(3.92)	12(23.53)	4(7.84)	2(3.92)	2(3.92)	2(3.92)	9(17.65)
Wastewater sampling points (FUTA health center)`											
Nurses' station	-	-	-	+	-	+	-	+	-	-	+
Children's ward	-	-	-	-	-	-	-	-	+	-	+
Doctor's station	-	-	-	-	+	-	-	-	-	-	+
Laundry	-	-	-	+	-	+	-	-	-	-	+
Health center entrance	-	+	-	-	+	-	-	-	-	-	-
Wound treatment ward	+	+	+	-	-	-	-	-	+	-	-
Water source	-	-	-	-	-	+	-	-	+	-	+
Total = 20	1(5.00)	2(10.00)	1(5.00)	2(10.00)	2(10.00)	3(15.00)	0(0)	1(5.00)	3(15.00)	0(0)	5(25.00)

Key: + = present in the sample, - = absent in the sample

Table 3.1: Primers used for amplification of ESBL genes

Gene	Primer	Sequence (5'-3')	Size(bp)
TEM	TEM-F	TTTCGTGTCGCCCTTATTCC	403
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG	

Source: Asgar *et al*. (2017)

Key: F = forward, R = reverse, ATCG = nucleotides, bp = base pair

Table 4: The result of physicochemical parameters analysis of wastewater from UNIMED Teaching Hospital and FUTA Health Center Akure

Sample source	pH	DO (mg/l)	COD (mg/l)	BOD (mg/l)	BOD:COD
A	5.41±0.41^{ab}	5.13±0.43^{b}	280.03±2.32^{d}	8.92±0.82^{b}	0.03
B	7.38±0.31^{b}	6.04±1.83^{bc}	170.11±3.07^{d}	4.86±0.11^{a}	0.03
C	8.42±0.03^{b}	4.02±0.04^{b}	123.31±3.41^{d}	9.07±0.08^{b}	0.07
D	5.53±0.46^{ab}	3.10±0.55^{a}	143.06±1.03^{d}	11.06±0.26^{b}	0.08
E	8.26±0.12^{b}	5.06±0.07^{b}	219.04±5.07^{d}	9.02±0.33^{b}	0.04
F	8.42±0.22^{b}	8.11±0.64^{d}	280.33±4.03^{d}	5.01±0.39^{a}	0.02
G	7.11±0.05^{b}	6.82±1.32^{c}	7.86±0.82^{a}	4.29±0.06^{a}	0.55
H	5.31±0.62^{a}	7.53±0.32^{d}	8.31±1.02^{a}	4.88±0.05^{a}	0.59
I	6.48±0.01^{b}	4.16±0.07^{b}	155.39±4.06^{d}	8.63±0.51^{b}	0.06
J	6.77±0.01^{b}	5.62±0.41^{b}	293.06±2.22^{d}	7.03±0.04^{b}	0.02
K	7.03±0.03^{b}	2.01±0.02^{a}	931.44±5.06^{e}	11.73±0.93^{b}	0.01
L	7.41±0.48^{b}	8.31±0.11^{d}	5.11±0.05^{a}	4.81±0.22^{a}	0.94
M	8.29±1.32^{b}	6.54±0.71^{c}	9.42±0.31^{b}	5.22±1.28^{a}	0.55
N	8.93±0.74^{b}	6.93±0.07^{c}	6.17±0.71^{a}	4.82±0.93^{a}	0.78
O	7.16±0.86^{b}	7.32±0.61^{d}	10.32±0.42^{b}	5.02±0.11^{a}	0.49
P	5.93±0.12^{b}	6.34±0.73^{c}	11.63±1.06^{b}	5.38±0.08^{a}	0.46
Q	9.82±1.33^{c}	2.04±0.61^{a}	34.16±2.65^{b}	14.57±0.55^{c}	0.43
R	6.83±0.06^{b}	5.72±0.54^{b}	43.26±1.32^{bc}	10.31±0.03^{b}	0.24
S	6.59±0.05^{b}	5.32±1.03^{b}	721.05±0.63^{e}	9.42±0.52^{b}	0.01
T	7.31±0.22^{b}	8.22±0.55^{d}	5.32±0.07^{a}	3.68±0.07^{a}	0.69
EPA STANDARD	**7.0 - 8.5**	**6 - 9.5**	**3.0 – 900**	**<5.0**	

Values are means ± SE for samples. Values in the same column carrying the same superscript are not significantly different at ($p \leq 0.05$) using Duncan's New Multiple Range test

KEY: A: Chemical Laboratory, B: Microbiology Laboratory C: Eye clinic, D: Community clinic E: Blood bank, F: MLS laboratory, G: Antenatal, H: Post-natal, I: Accident and Emergency, J: Pharmacy, K: Laundry (UNIMED), L: Water source (UNIMED), M: Nurses' station, N: Children's ward, O: Doctor's station, P: Oda Road, Q: Laundry (FUTA), R: Health center entrance, S: Wound treatment ward, T: Water source (FUTA), EPA: environmental protection agency Standards. DO: Dissolved Oxygen, COD: Chemical Oxygen Demand, BOD: Biochemical Oxygen Demand

Table 5: Multiple Antibiotic Resistant Patterns of Escherichia coli Isolated from Wastewater in UNIMED Teaching Hospital and FUTA Health Center Akure

Wastewater sampling points (UNIMED)`	**Antibiotics used**											**Multiple antibiotic resistant index**
	Septrin	**Chloramphenicol**	**Sparfloxacin**	**Ciprofloxacin**	**Amoxacillin**	**Augmentin®**	**Gentamycin**	**Pefloxacin**	**Tarivid**	**Streptomycin**		
Chemical Laboratory	R	R	S	S	S	S	S	S	S	S		0.2
Microbiology Laboratory	I	I	S	S	R	R	I	I	I	I		0.2
Eye clinic	R	I	I	I	R	R	R	S	S	S		0.4
Community clinic	I	I	I	S	I	I	I	I	I	R		0.1
Blood bank	R	R	S	S	S	S	S	S	S	R		0.3
MLS laboratory	R	R	S	R	R	R	R	I	I	R		0.7
Antenatal	I	I	I	S	I	I	I	I	S	I		-
Post natal	R	R	S	S	R	R	R	S	S	R		0.6
Accident and Emergency	S	S	S	S	S	S	S	S	S	I		-
Pharmacy	R	I	I	S	I	I	I	I	I	I		0.1
Laundry	R	I	S	S	R	R	I	I	I	I		0.3
Water source	R	S	S	S	S	S	I	S	S	S		0.1
Wastewater sampling points (FUTA health center)`												
Nurses' station	R	S	S	S	R	R	I	S	S	I		0.3
Laundry	R	I	I	S	I	I	I	I	I	I		0.1
Water source	I	I	S	S	I	I	I	S	S	I		-
Percentage resistance	10(66.67)	4(26.67)	0	1(6.67)	6(40.00)	6(40.00)	3(20.00)	0	0	4(26.67)		

Key: R – resistance, S – susceptible, I – intermediate

Table 6: Occurrence of extended spectrum Beta Lactamase (*ESBL*) producing *E. coli* isolated from wastewater in UNIMED teaching hospital and FUTA Health Center Akure

Sources of isolates	**Method used for detection**		
	Disc diffusion	**Double disc synergistic test**	**Molecular**
Chemical Laboratory	-	-	-
Microbiology Laboratory	+	+	-
Eye clinic	+	+	-
Community clinic	+	+	-
Blood bank	-	-	-
MLS laboratory	+	-	-
Antenatal	+	+	-
Post natal	+	+	+
Accident and Emergency	-	-	-
Pharmacy	-	-	-
Laundry (UNIMED)	+	-	+
Water source (UNIMED)	-	-	+
Nurses' station	+	+	+
Laundry (FUTA health center)	-	-	-
Water source (FUTA health center)	+	+	-
Total (%)	9(60.00)	6 (40.00)	4 (26.67)

Table 7. Molecular identity of *ESBL* Producing *E. coli* isolated from hospital wastewater in Akure

Cultural and biochemical identity	**Molecular identity**	**Percentage similarity**	**Accession number**
E. coli	*E. coli* 0121 strain	96.65	CP051632.1
E. coli	*E. coli* k-12 strain MG1655 substrain	100.00	CP097883.1
E. coli	*E. coli* k-12 strain MG1655 substrain	100.00	CP097883.1
E. coli	*E. coli* Type 131 strain	94.21	CP026358.1

Figure 1. Antibiotic susceptibility profiles of *Escherichia coli* isolated from wastewater in UNIMED Teaching Hospital Akure

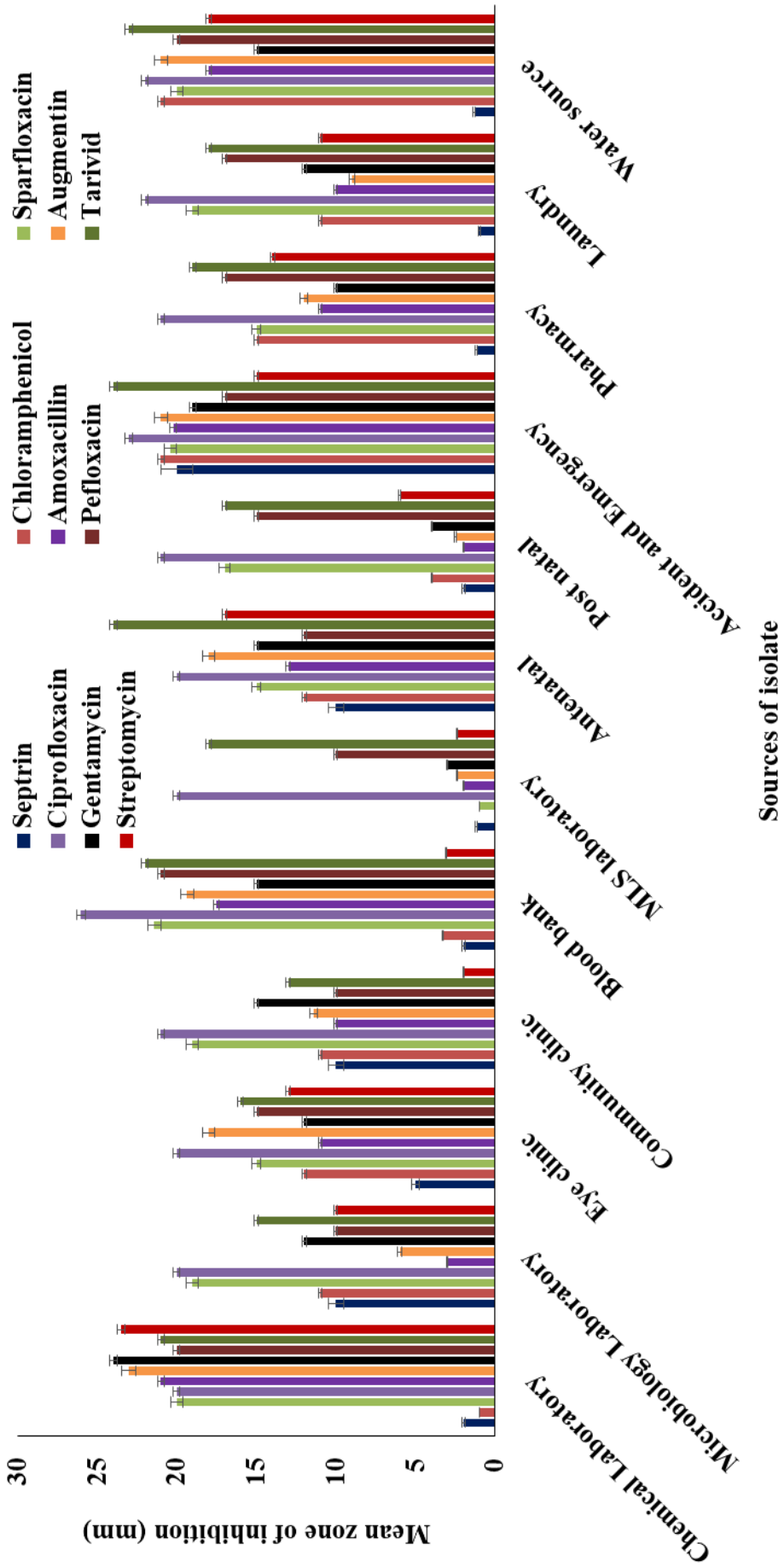


Figure 2. Antibiotic susceptibility profiles of *Escherichia coli* isolated from wastewater in FUTA Health Center Akure

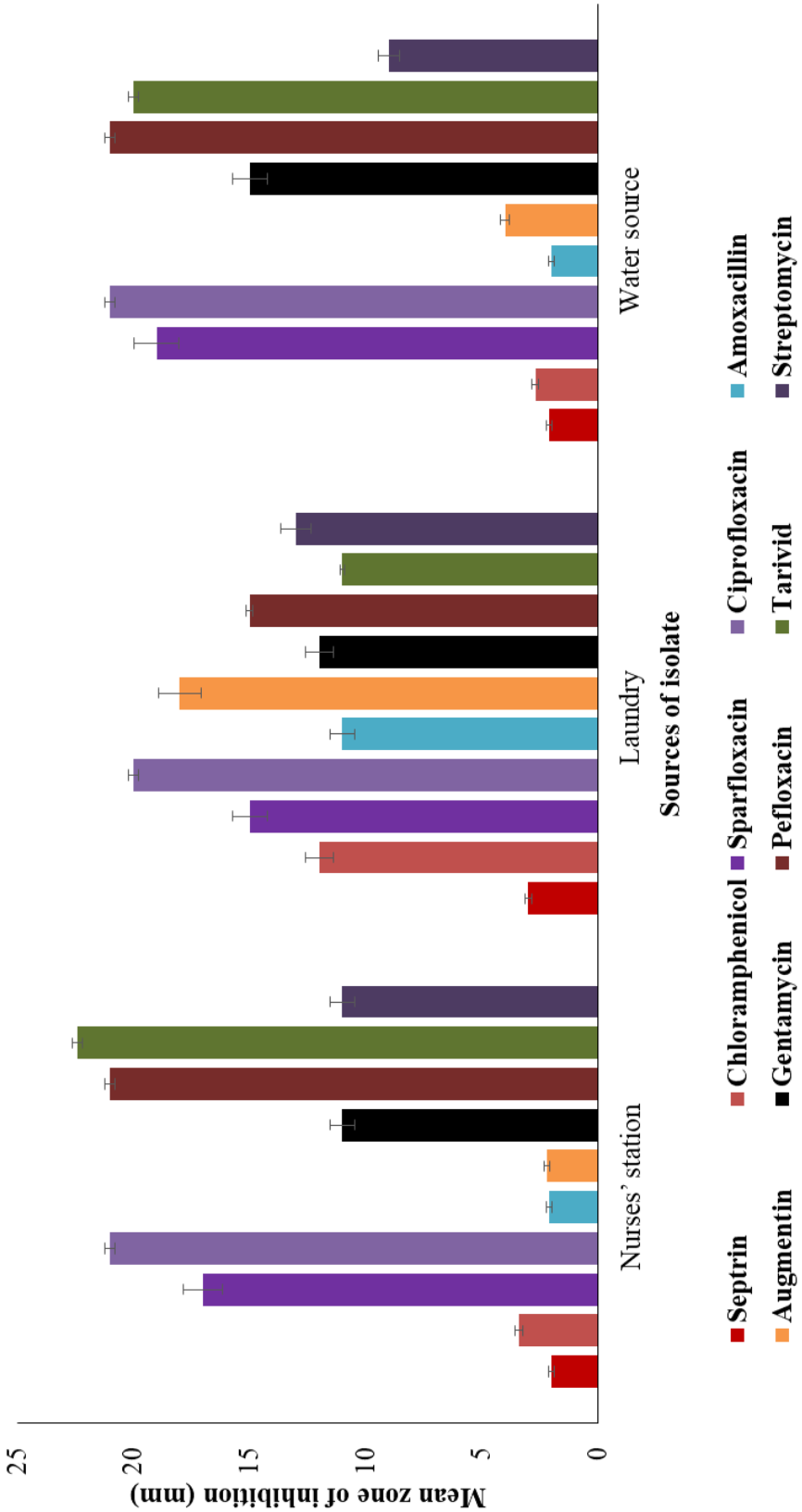


Figure 3: Effects of temperature on *ESBL* Producing *Escherichia coli* isolated from different wastewater sources from two hospitals in Akure

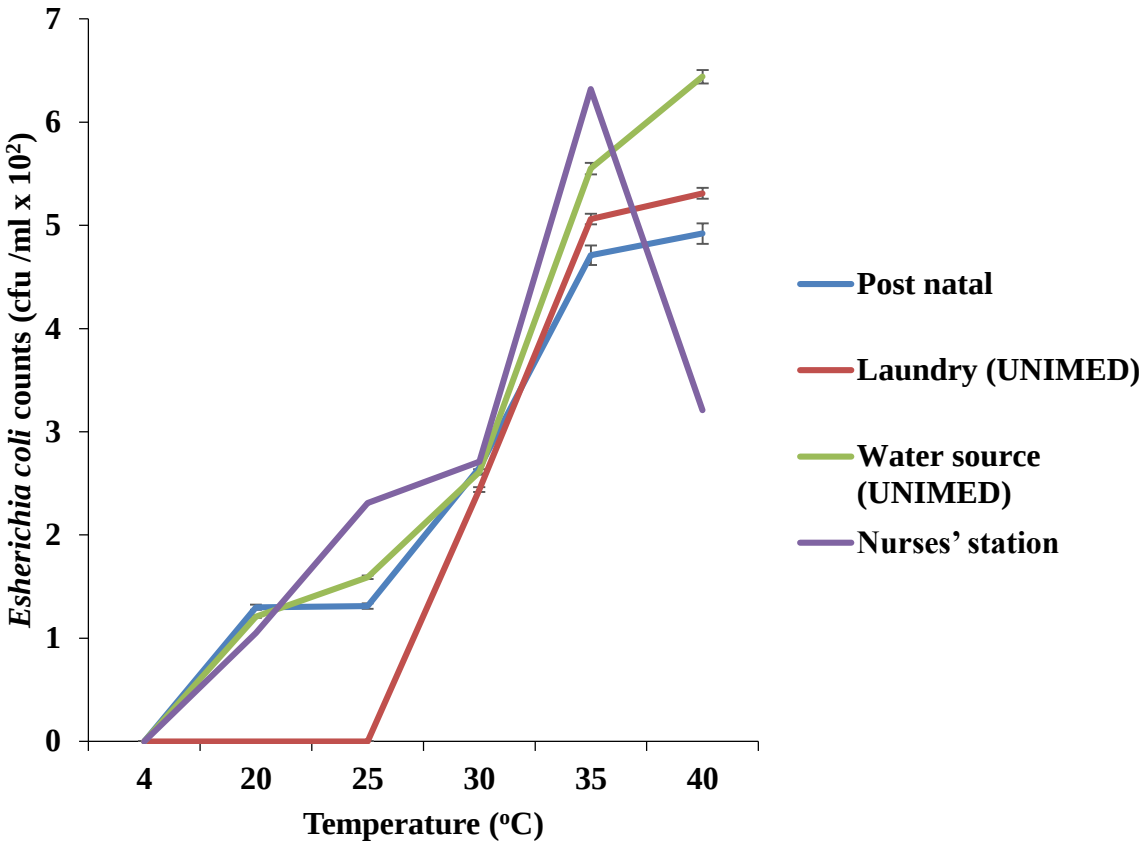


Figure 4. Effects of salt concentration on ESBL Producing *Escherichia coli* isolated from different wastewater sources from two hospitals in Akure

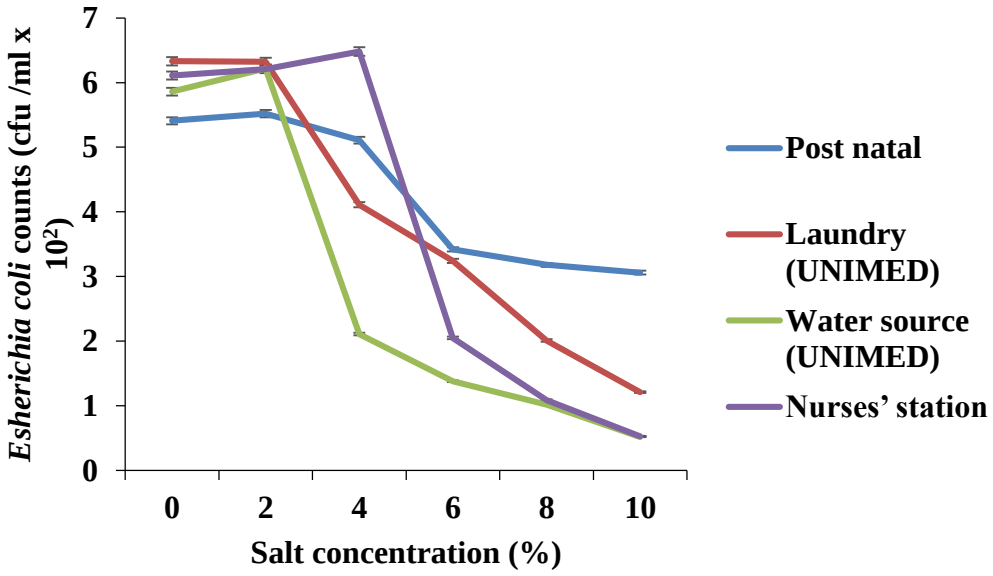


Figure 5. Effects of pH on ESBL producing *Escherichia coli* isolated from different wastewater sources from two hospitals in Akure

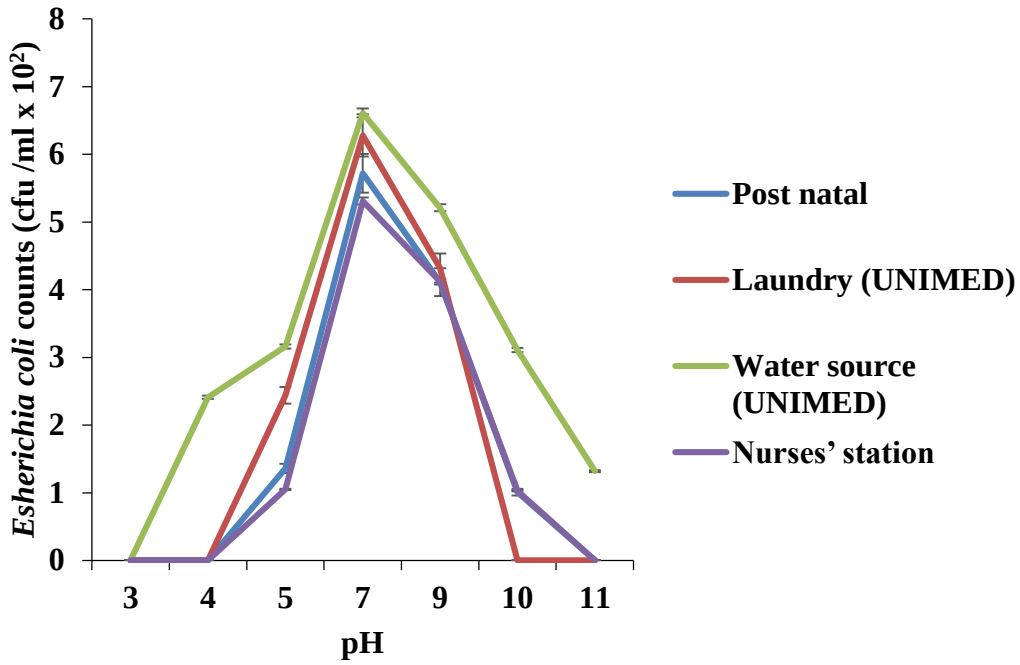


Figure 6. Agarose gel electrophoresis.

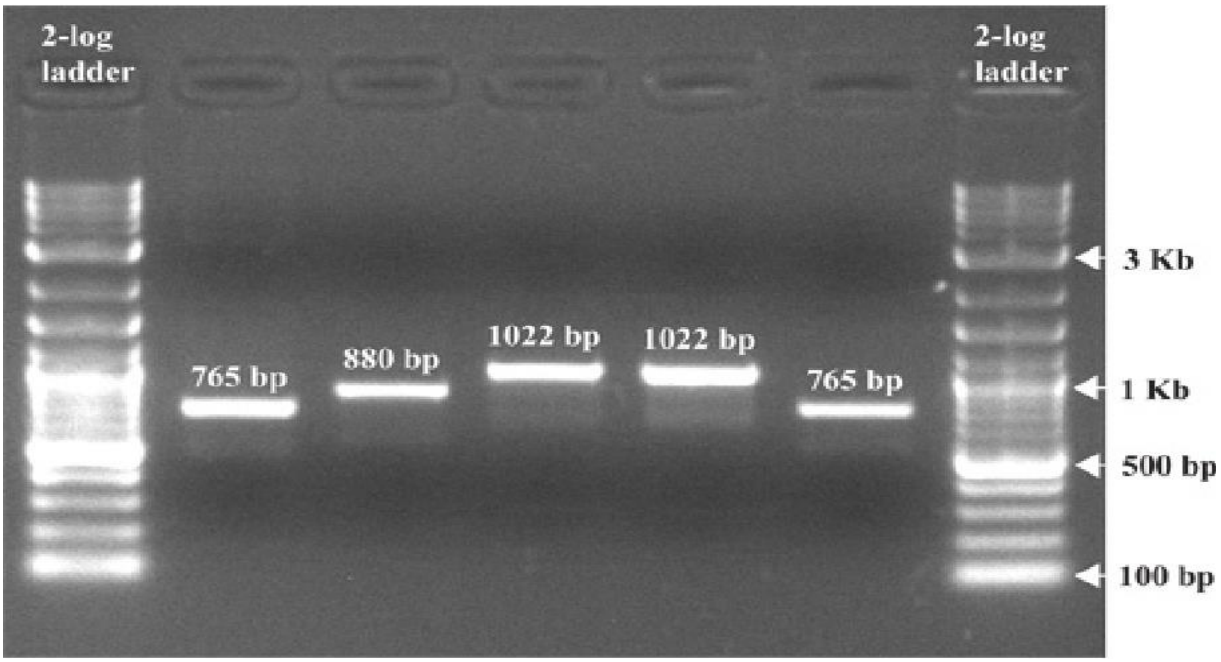
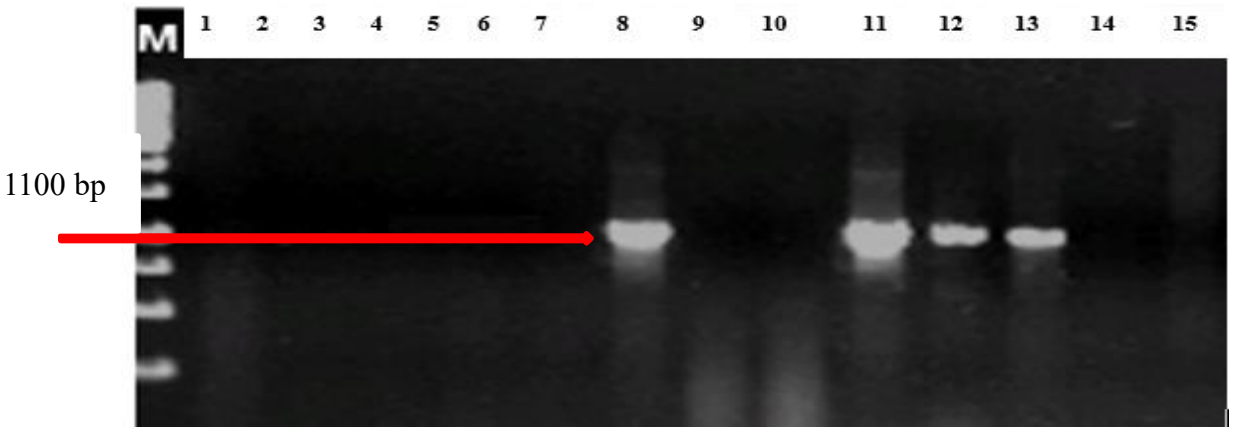
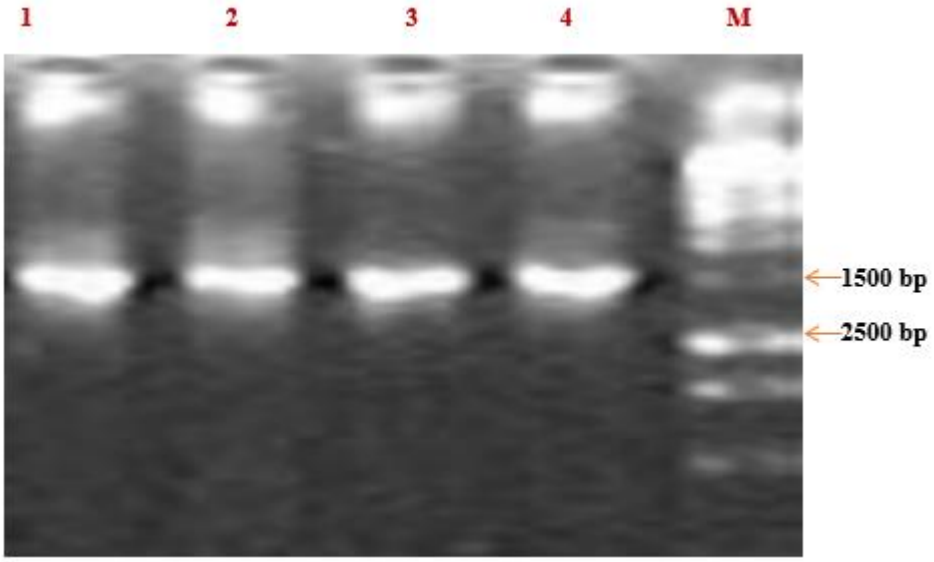


Plate 1. Agarose gel electropherogram of amplified blaTEM gene of *Escherichia coli* isolated from wastewater in UNIMED teaching hospital and FUTA Health Center Akure



Key:
Line 1 – 15 = Bacterial isolates
M = Marker
Line 8, 11, 12 and 13 were positive for ESBL

Plate 2. Agarose gel electropherogram of 16S rRNA gene of *Escherichia coli* isolated from wastewater in UNIMED teaching hospital and FUTA Health Center Akure



Key:
Line 1 – 4 = Bacterial isolates
M = Molecular marker 1kb DNA ladder

Discussion

Microbiological analyses of hospital wastewater have shown the presence of germs such as total *Coliforms*, fecal *Coliforms*, fecal *Streptococcus*, *Staphylococcus*, yeast and *Clostridium perfringens* [2]. The mean values of coliforms in wastewater from all hospitals were above the WHO standard recommendation (0 cfu/ml) for the discharge to the environment. Other studies had reported that hospital wastewaters are highly polluted and their discharges without any treatment, can present serious risks to human health and the environment [14]. These authors claimed that the wastewater contained large amount of fecal coliforms and fecal streptococcus. The presence of fecal contamination indicators such as total coliforms shows that the wastewater are subject to

anthropogenic microbiological pollution. Releasing this wastewater into the environment without any treatment will increase the potential epidemiological risk [2]. Coliforms are an indicator of the pollution degree of water and also an indirect indicator of the presence or absence of antibiotics or disinfectants in wastewater [14]. The lack of proper treatment of wastewater and the high level of medical activity in these hospitals could explain the microbiological quality of these wastewater. Also, high bacteria loads and the presence of *Coliform* bacteria in water source at hospitals in this study could be a thing of concern. This could be that the water sours in these hospitals have been contaminated from underground or through the tank or piping system therefore the contamination observed in the wastewater may not only originated as a result of the activities in the hospital but water source also contributed to microbial quality.

Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella Typhi and *Staphylococcus aureus* were isolated from wastewater in this study. Larger proportion of these bacterial isolates (*Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Salmonella Typhi*) were enteric. Wastewater from hospital have been reported as potential source of infectious organisms like enteric bacteria [15-17]. However, there were differences in bacterial profiles of wastewater in this study compared with the study of [15-17], they reported the presence of *Seratia* sp. [2, 15, 16], *Vibrio* sp. [2, 16] and *Acinetobacter johnsonii* [16, 17] in addition with those reported in this study. The variation in the bacterial profile/ community could due to differences in hospital operational parameters and quality/composition of the wastewater [2].

Escherichia coli, S. aureus, B. subtilis and *B. cereus* were the most frequently isolated bacteria in UNIMED while in FUTA wastewater, *S. aureus, E. coli* and *P. aeruginosa* were the most frequently isolated bacteria. This differences could be as a result of differences in hospital environment, operational parameters, and physicochemical properties of wastewater, type of antibiotic or disinfectant used and genetic make-up of the bacterial isolates. *Escherichia coli, S. aureus* and *Salmonella typhi* were mostly reported to be the dominant bacteria in hospital wastewater [2, 15, 16]. The lack of proper treatment of wastewater and the high level of medical activity in these centers could explain the microbiological quality of this wastewater.

The physico-chemical characterization of hospital wastewater includes the evaluation of different parameters. Among these parameters, the most commonly used to assess the presence and the loads of inorganic/organic matter in the effluent are the conductivity, the biochemical oxygen demand, the chemical oxygen demand, the suspended matter and the total nitrogen [18]. The results of the physicochemical characteristics in this study revealed that there were variations in all the parameters, some were higher while some were lower than the standards for the discharge of wastewater into the environment. The results were in agreement with those reported in the literature [19-21]. It is therefore necessary to properly treat this wastewater before its release into the environment [21]. The higher pH value obtained from laundry could be influenced by the nature of soap used for the laundry activity. Other parameters like DO, BOD and COD of some hospital wastewater in this study were higher than the recommended standard. Hospital wastewaters have high levels of nutritive salts, including nitrogenous compounds. The presence of ammonia in large quantities is indicative of anthropogenic contamination probably due to the transformation of the speed of urea into ammonia [2]. The ration of COD/BOD in this study were less than 3 which means that they are biodegradable. [21] reported that when the value of wastewater COD/BOD is less than 3, the wastewater is biodegradable. Therefore, degradation of organic contents of the wastewater in the study area is paramount.

Antibiotic susceptibility profiles of *E. coli* isolates from UNIMED wastewater had lesser susceptibility to septrin, chloramphenicol and sparfloxacin and isolates from MLS laboratory and postnatal ward showed least susceptibility to all the antibiotics used while *E. coli* isolates from FUTA health centre showed least susceptibility to septrin, chloramphenicol, amoxicillin and augmentin® . This could be that the *E. coli* had gained resistance to these antibiotics as reduction in zones of inhibition of antibiotics is related to bacterial resistance [11].

Also, generally *E. coli* isolate had higher resistance against septrin, amoxicillin, augmentin®, chloramphenicol and streptomycin. Resistance to

the antibiotics observed in this study and other antibiotics has been reported in different parts of the world in previous studies and that hospital wastewater is a huge source of drug-resistant pathogens in the environment [2, 4, 22]. The MARi of isolates from MLS laboratory, postnatal ward and eye clinic were higher than 0.3, MARi higher than 0.3 has been reported to originate from environment where antibiotics are been used continually [23, 24]

This study also revealed the presence of ESBL gene in *E. coli* isolates in wastewater from postnatal ward, laundry in UNIMED, water source in UNIMED and nurses' station in FUTA Previous studies have also reported ESBL production among bacteria isolated from hospital wastewater [2, 21]. ESBLs are enzymes capable of hydrolyzing penicillins, oxyimino-cephalosporins and monobactams, can be transferred by mobile genetic elements or might be chromosomally mediated and are usually multi drug-resistant. The existence of ESBL producers *E. coli* in hospital wastewater is worrisome considering that this water is discharged into environment without treatment [23].

Molecular identity revealed *E. coli* k-12 strain MG1655, *E. coli* 0121 strain and *E. coli* type 131 strain. These *E. coli* strains have been reported to cause infection in man and have ability to cross blood brain barrier [25].

ESBL producing *E. coli* isolates survive wide range of temperature, salt concentration and pH range. This is an indication that these isolate could survive changes in environmental conditions during the sewage treatment process and still enter the environment, also, these isolate could survive wide range of environmental conditions when disseminated. Therefore, the high prevalence of these strains in hospitals and consequently in the community could be considered as a potential threat to public health.

Authors' contribution

''FOO designed and supervised the study. ''TGE developed the methodology, literature, conducted the study, acquired, analyzed and interpreted the data obtained. TGE wrote the first draft. ''TGE, YKA, AIO previewed and fine-tuned the draft before submission''. The paper has been read and approved by all authors.

Acknowledgements

The Federal University of Technology Akure Health Center's (FUTAHC) technical personnel provided support to the authors during the sample collection for this study, which is greatly appreciated.

Competing interests

The authors declare that they have no competing interests

Funding

No funding was obtained for this study.

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