

## Original Article

# Prevalence and Genetic Diversity of *Escherichia coli* Isolated from Sewage Samples of Hospitals in Different Regions of Bangladesh

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*Escherichia coli* are a large and diverse group of bacteria found in the environment, foods and intestines of humans and animals. This study was carried out to observe the genotypic variations in *E. coli* found at different regions in Bangladesh using Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S rDNA and RAPD (Random Amplified Polymorphic DNA). Sewage samples were collected from different medical college hospitals and a total of 118 samples were observed in this study of which 26 were identified as *E. coli* based on their cultural, biochemical and molecular characteristics. Antibiotic susceptibility test was carried out where most isolates were resistant to Amoxicillin, Rifampicin and Erythromycin; and some were resistant to kanamycin, ceftriaxone, Chloramphenicol and Azithromycin; besides 73% (N=19 out of 26) of the test isolates were multi-drug resistant. Results of Polymerase chain reaction (PCR) showed that none of the isolates carried *Stx1*, *Stx2* and *LT* gene. Two genotypic groups were found from Amplified Ribosomal DNA Restriction Analysis (ARDRA) by *AluI* restriction enzyme and seven different Random Amplification of Polymorphic DNA (RAPD) patterns were found. After analyzing, it was found that 24 out of 26 isolates showed similarity with *Escherichia coli* strain NBRC 102203 and the remaining two isolates showed similarity with *Escherichia coli* strain Mui T5 based on the restriction patterns of ARDRA and sequences of 16S rDNA. From the study, it was concluded that *Escherichia coli* strain NBRC 102203 was more prevalent than *Escherichia coli* strain Mui T5 isolated from sewage sample of hospitals from different regions of Bangladesh.

**Keywords:** *Escherichia coli*, genetic diversity, 16S rDNA, ARDRA, RAPD, multidrug resistance.

## Introduction

*Escherichia coli* (*E. coli*) found in the environment, foods, and intestines of human and animals are a large and diverse group of bacteria. Although most strains of *E. coli* are harmless, some can be harmful and cause diarrhea, urinary tract infections, respiratory illness and pneumonia, as well as other illnesses<sup>1</sup>. *Escherichia coli* can grow both aerobically and anaerobically, preferably at 37°C and can be non-motile or motile with peritrichous flagella<sup>2</sup>. It is part of the normal flora of the gut and protects the intestinal tract from other bacterial infection, plays role in digestion and produces small amounts of vitamins B12 and K<sup>3</sup>. Many of the pathotypes are major public health concern as they have low infectious doses and are transmitted through food and water<sup>4</sup>. Antimicrobial resistance is one of the leading public health concerns of the 21<sup>st</sup> century which is a serious threat to the treatment of infectious diseases<sup>5</sup>. Antibiotics resistance has become a concern for both pathogenic and commensal bacterial species; especially for enteric pathogens such as *Escherichia coli*<sup>6</sup>. As diarrheal disease causes death in massive scale, antimicrobial resistance property of these enteric pathogens has become a particular interest in developing countries like Bangladesh.

It has been reported that *E. coli* exhibits high genetic diversity and complex population dynamics in the primary (humans and warm-blooded animals) and secondary (environment outside the host) habitats<sup>7</sup>. For instance, several studies using different fingerprinting methods have revealed enormous genetic diversity in *E. coli* strains obtained from different animals. Similarly, extensive genetic diversity was also found in *E. coli* strains isolated from various aquatic environments<sup>8,9</sup>.

In this study, the genetic diversity of *E. coli* was determined using ARDRA and RAPD; and dendrogram was constructed based on Jaccard similarity coefficient. To detect the presence of *Stx1*, *Stx2* and *LT* gene PCR was carried out and to identify the isolates 16 S rDNA gene was sequenced.

## Materials and Methods

### Sample collection

Sewage samples were collected from different medical college hospitals, such as Dhaka Medical College Hospital (Dhaka), Osmani Medical College Hospital (Sylhet), Chittagong Medical College Hospital (Chittagong), Sir Salimullah Medical College Hospital (Dhaka), Bangabandhu Sheikh Mujib Medical

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University (Dhaka), Rajshahi Medical College Hospital (Rajshahi) and Sher-E-Bangla Medical College hospital (Barisal). At least sixteen (16) samples were collected from each location differed from one another in terms of climatic factors (temperature, humidity, pH) which might be responsible for genetic diversity of *Escherichia coli*. Samples were transported to the Laboratory of the Department of Microbiology, University of Dhaka maintaining proper conditions.

#### Sample processing

Sewage samples from different hospitals of Bangladesh were collected in sterile McCartney bottle. The samples were filtered through sterile Grade 1 Whatman filter paper and then, the samples were spread onto EMB agar plates and the plates were incubated at 37°C for 24 hours, metallic sheen colonies on EMB agar plates were subsequently streaked on Mac-Conkey agar plates. After 24 hours incubation at 37°C, pink colonies on Mac-Conkey agar plates were subjected to biochemical and molecular testing to confirm as *E. coli* and then were stored in glycerol broth for stock<sup>10, 11</sup>.

#### Morphological, Microscopic and Biochemical characterization

After observing colony morphology on two different agar media, microscopy was done after performing gram staining following standard methodology<sup>12</sup>. Isolates were subjected to different biochemical tests such as Methyl Red (MR), Indole Production, Voges-Proskauer (VP), Citrate utilization, Catalase, Oxidase, Motility Indole Urease (MIU) and Kligler's Iron Agar (KIA); using standard procedures and test results were observed after incubation at 37°C for 24-48 hours.

#### Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed following standard guidelines by Kirby-Bauer disc diffusion method using discs of 15 different antibiotics<sup>13</sup>. Antibiotics used in this study were amoxicillin (30 µg), ampicillin (30 µg), azithromycin (15 µg), cefixime (5 µg), cefotaxime (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifamycin (5 µg), streptomycin (10 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (25 µg)<sup>14</sup>.

#### Molecular Characterization of the isolates

DNA of the isolates was extracted using standard phenol-chloroform-isoamyl alcohol method<sup>15</sup>. The concentration and quality of the extracted DNA were measured using Nanodrop™ spectrophotometer at absorbance 260 nm with adjusted working concentration of 50 µg/ml.

PCR was performed for the molecular characterization using primers listed in Table 1. All PCR other than RAPD, was performed with reaction set up each consisting 12.5 µl of 2X Taq PCR master mix (Promega, USA), 1 µl each forward and reverse primers, 4 µl of template DNA and 6.5 µl of nuclease free water. The thermocycling conditions were 95°C for 5 minutes for initial denaturation, subsequently 35 cycles of denaturation at 95°C for 45 seconds, followed by annealing at different temperatures (mentioned at Table 1) for different genes for 45 seconds, extension at 72°C and the extension time was different for different genes, with a final extension at 72°C and the extension time varied for different genes. PCR products of 16S rDNA were purified by using DNA clean-up kit (Qiagen, Germany).

For RAPD, primer concentration was 20 pmol/µl and the thermocycling conditions were 94°C for 5 minutes for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 39°C for 1 minute and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. A template-free negative control was also used during PCR. The PCR products were subjected to gel electrophoresis in 1.5 % agarose gel which was stained with ethidium bromide and observed under UV-transilluminator (AlphaImager, USA) followed by photograph of gel was taken.

For 16S rDNA sequencing, PCR products were sent to First Base Malaysia and the sequences were processed using Sequencer v5.4. Sequences were further analyzed using MEGA v6.0 software package and Basic Local Alignment Searching Tool (BLAST) of National Centre for Biotechnological Information (NCBI). For ARDRA of the 16S rDNA, the PCR products were digested overnight with *AluI* (Thermo Fisher Scientific, USA) and resolved in a 1.5% agarose gel. Afterwards the gel was visualized under UV-transilluminator (AlphaImager, USA) after staining with ethidium bromide followed by photograph of gel was taken<sup>16</sup>.

**Table 1.** List of the primers used in the present study

Gene	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon Size (bp)
<i>Lt</i>	Forward	GCACACGGA GCTCCTCAGTC	62	218
	Reverse	TCCTTCATCCTTTCAATGGCTT		
<i>Stx 1</i>	Forward	ATAAATCGCCATTCGTTGACTAC	60	180
	Reverse	AGAACGCCCACTGAGATCATC		
<i>stx2</i>	Forward	GGCACTGTCTGAAACTGCTCC	65	255
	Reverse	TCGCCAGTTATCTGACATTCTG		
16S rDNA	Forward	AGAGTTTGATCMTGGCTCAG	55	1466
	Reverse	CGGTTACCTTGTTACGACTT		

## Results and Discussion

After analyzing morphological, biochemical and molecular characteristics, 26 isolates were confirmed as *E. coli*. Isolates showed characteristic metallic sheen colonies on the Eosin Methylene Blue (EMB) agar plates and pink colonies on MacConkey agar plates were presumptively selected as *Escherichia coli*. Gram-stained isolates were observed as gram negative rod under light microscope. All the isolates were catalase, Methyl red, indole and motility test positive; and oxidase, citrate, urease and VP negative. All these morphological and biochemical test results were very similar to the results of the previously reported *E. coli* strains isolated from environmental samples<sup>17</sup>.

PCR was carried out to detect the presence of *Stx1*, *Stx2* and *Lt*. But none of the isolates were found positive for these genes in PCR (Figure 1).

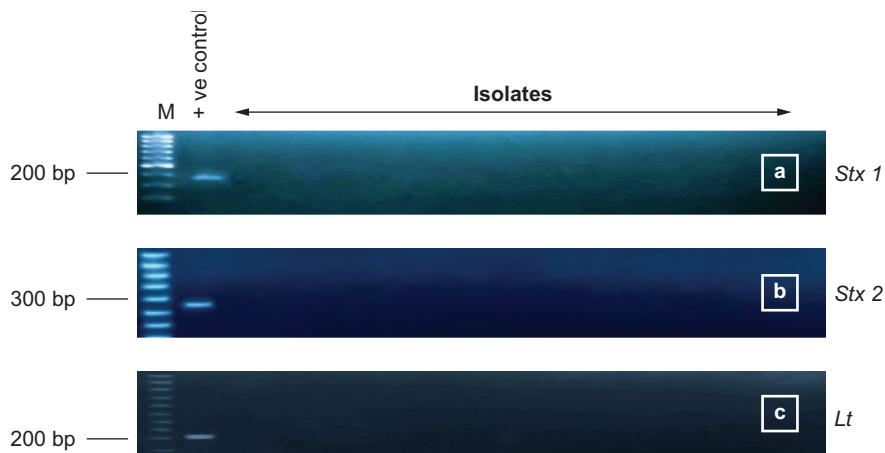
16S rDNA of the isolates were amplified and *AluI* restriction enzyme was used to digest amplified 16S rDNA products for ARDRA analysis. The ARDRA patterns showed two groups of *E. coli* where 22 and 4 isolates belong to group 1 and group 2 respectively (Figure 2). For further confirmation 16S rDNA genes of different isolates were sequenced and the sequences were analyzed by using BLAST of NCBI. After analysis, it was found

that ARDRA group 1 belonged to *Escherichia coli* strain NBRC 102203 and the ARDRA group 2 belonged to the *Escherichia coli* strain Mui T5. In many studies amplified ribosomal DNA restriction analysis (ARDRA) was also used for strain typing, determining genetic diversity and for screening clone libraries to identify phylogenetic clusters within a microbial community<sup>18</sup> as we used in this research.

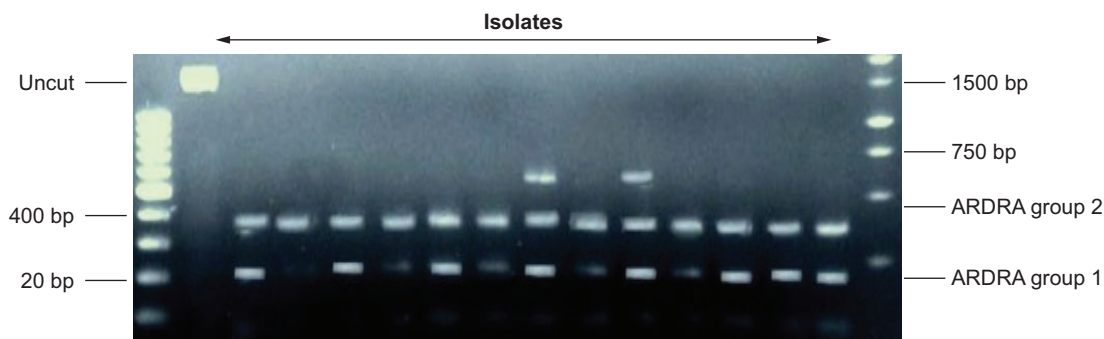
A dendrogram was constructed using 16S rDNA sequences of the isolates based on Jaccard similarity coefficient (Figure 3). Here, S1 and D1 denotes ARDRA group 1 and 2 respectively.

RAPD (Random Amplified Polymorphic DNA) showed seven different groups based on banding patterns of the isolates where group I, III and V include 2 isolate each; group II and VI include 6 isolates each; and group IV and VII include 4 isolates each. RAPD indicated that the isolates have immense genetic diversity among themselves. In many studies, RAPD analysis was also used as alternative, rapid, reproducible, and powerful method for the genetic typing of bacterial spp.<sup>19</sup>.

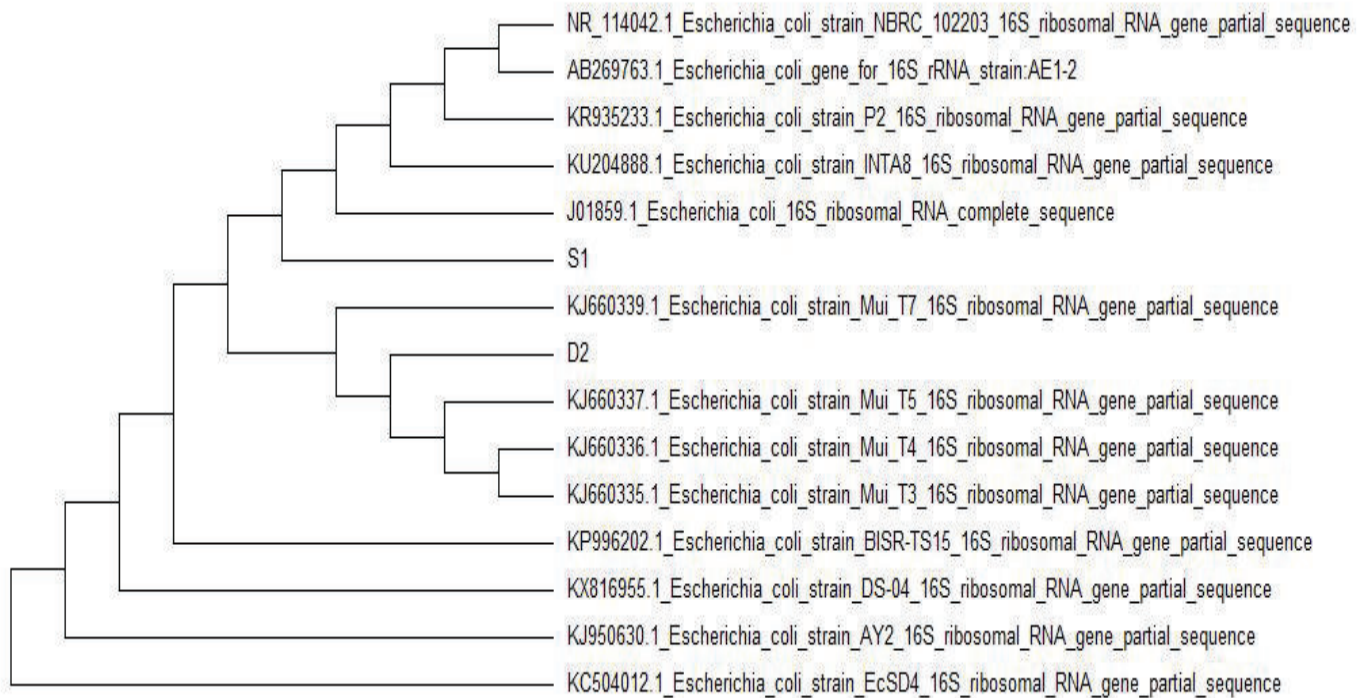
After performing antibiotic susceptibility test, isolates were found as resistant, intermediate and susceptible according to the CLSI guidelines (CLSI, 2013). In this study the percentages of resistant isolates to different antibiotics- Amoxicillin (80%), Erythromycin (76%), Nalidixic acid (56%), Streptomycin



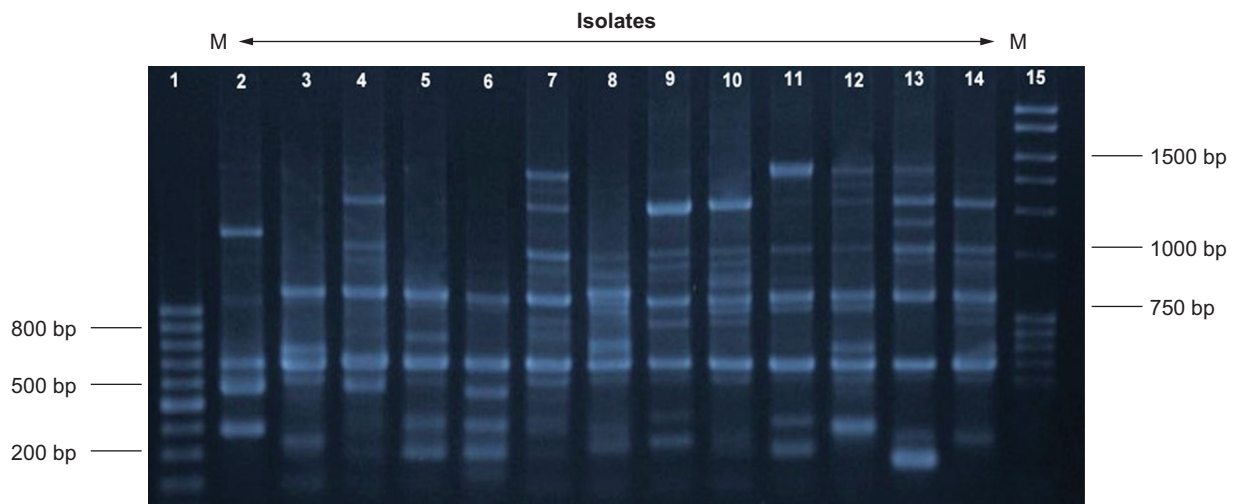
**Fig. 1.** PCR for detection of (a) *stx1* gene, (b) *stx2* gene and (c) *Lt* gene in isolates. Lane M- Marker.



**Fig. 2.** ARDRA profile of *Escherichia coli* isolates after digestion with restriction enzyme *AluI*. (Lane M- Marker).



**Fig. 3.** Dendrogram constructed using sequences of the isolates of two different ARDRA group.



**Fig. 4.** RAPD profile of the isolates showing seven different patterns: lane 1 and 15- Marker(M); lane 2- group I; lane 3,5 and 6- group II; lane 4 - group III; lane 7 and 13- group IV; lane 8- group V; lane 9, 10 and 14- group VI; and, lane 11 and 12- group VII.

(36%), Ciprofloxacin (56%), Kanamycin (12%), Tetracycline (32%), Trimethoprim (12%), Cefotaxime (32%), Chloramphenicol (16%), Ampicillin (52%), Ceftriaxone (32%), Azithromycin (20%), Cefixime (64%) and Rifampicin (88%). 18 isolates showed resistant to three or more classes of

antibiotics and thus designated as multi-drug resistant (MDR). Recent studies also demonstrate that surface waters in developing and developed countries have become major reservoirs of multi-drug resistant pathogenic microbes due to indiscriminate use of antimicrobials<sup>20</sup>.

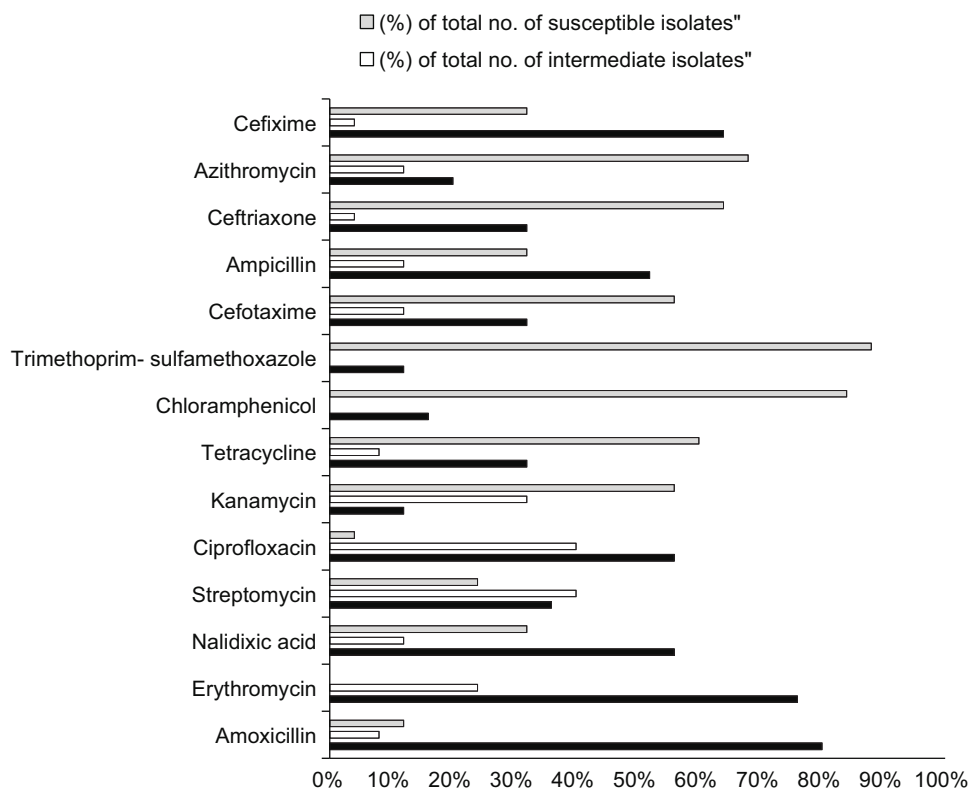


Fig. 5. Antibiotic resistance pattern of the isolates.

## Conclusion

In this study, it was found that *E. coli* isolates present in sewage samples were genetically diverse. Most of the isolates were resistant to different antibiotics and some of isolates showed multidrug resistance. Multidrug-resistant organisms may transfer their antibiotic resistance gene(s) to more virulent strains and that is a matter of public health concern. As none of the three virulent genes was found in any of the isolates, it is presumable that isolates were non virulent. In our study, from the analysis of ARDRA and 16S rDNA sequence it was observed that the *E. coli* strain NBRC 102203 was more prevalent in different regions of Bangladesh as compared to *E. coli* strain Mui T5.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgement

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## References

- Manges AR and Johnson JR. Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clinical infectious diseases*, 2012;**55**(5):712-719.
- Roy K, Hilliard GM, Hamilton DJ, Luo J, Ostmann MM and Fleckenstein JM. Enterotoxigenic *Escherichia coli* EtpA mediate adhesion between flagella and host cells. *Nature*, 2009;**457**(7229):594-598.
- Ko Y, Ashok S, Ainala SK, Sankaranarayanan M, Chun AY, Jung GY and Park S. Coenzyme B12 can be produced by engineered *Escherichia coli* under both anaerobic and aerobic conditions. *Biotechnology journal*, 2014;**9**(12):1526-1535.
- Van Elsas JD, Semenov AV, Cost R and Trevors JT. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. *The ISME journal*, 2011;**5**(2):173-183.
- Weber JT and Courvalin P. 2005. An emptying quiver: antimicrobial drugs and resistance. *Emerging Infectious Diseases*. **11**(6), 791.
- Okeke IN, Laxminarayan R, Bhutta ZA, et al. Antimicrobial resistance in a developing countries. Part I: recent trends and current status. *Lancet infection*. 2005;**5**:581-93.
- Canizalez-Roman A, Flores-Villaseñor HM, Gonzalez-Nuñez E, Velazquez-Roman J, Vidal JE, Muro-Amador S and León-Sicaireo N. Surveillance of diarrheagenic *Escherichia coli* strains isolated from diarrhea cases from children, adults and elderly at Northwest of Mexico. *Frontiers in microbiology*, 2016;**7**:1924.
- McLellan SL. (2004). Genetic diversity of *Escherichia coli* isolated from urban rivers and beach water. *Appl Environ Microbiol*, **70**:4658-4665.
- Casarez EA, Pillai SD, Di Giovanni GD. Genotype diversity of *Escherichia coli* isolates in natural waters determined by PFGE and ERIC PCR. *Water Res* 2007;**41**:3643-3648.
- Janezic KJ, Ferry B, Hendricks EW, Janiga BA, Johnson T, Murphy S, and Daniel SL. Phenotypic and genotypic characterization of *Escherichia coli* isolated from untreated surface waters. *The open microbiol journal*, 2013;**7**:9.
- El-Hadedy D and El-Nour SA. Identification of *Staphylococcus aureus* and *Escherichia coli* isolated from Egyptian food by conventional and molecular methods. *Journal of Genetic Engineering and Biotechnology*. 2012;**10**(1):129-135.

12. Hucker GJ and Conn HJ. Methods of Gram staining. 1923.
13. Ndung'u C, Muigai AWT and Kariuki S. Prevalence and antibiotic resistance patterns of *Escherichia coli* among hospitalised patients at thika district hospital. *East African medical journal*. 2014;**91**(6): 185-190.
14. Kirby JE, Brennan-Krohn T and Smith KP. Bringing antimicrobial susceptibility testing for new drugs into the clinical laboratory: removing obstacles in our fight against multidrug-resistant pathogens. *Journal of clinical microbiology*, 2019;**57**(12).
15. Renshaw MA, Olds BP, Jerde CL, McVeigh MM and Lodge DM. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol–chloroform–isoamyl alcohol DNA extraction. *Mol ecology resources*. 2015;**15**(1):168-176.
16. Gulitz A, Stadie J, Ehrmann MA, Ludwig W and Vogel RF. Comparative phylobiomic analysis of the bacterial community of water kefir by 16S rRNA gene amplicon sequencing and ARDRA analysis. *Journal of applied microbiology*. 2013;**114**(4):1082-1091.
17. Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M and Saigo K. Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. *Journal of bacteriology*. 1996;**178**(15): 4508-4514.
18. Sklarz MY, Angel R, Gillor O and Soares, MIM. Evaluating amplified rDNA restriction analysis assay for identification of bacterial communities. *Antonie van Leeuwenhoek*. 2009;**96**(4):659-664.
19. Betancor L, Schelotto F, Martinez A, Pereira M, Algorta G, Rodríguez MA and Chabalgoity JA. Random amplified polymorphic DNA and phenotyping analysis of *Salmonella enterica* serovar Enteritidis isolates collected from humans and poultry in Uruguay from 1995 to 2002. *Journal of Clinical Microbiology*. 2004;**42**(3):1155-1162.
20. Ram S, Vajpayee P, Tripathi U, Singh RL, Seth PK and Shanker R. Determination of antimicrobial resistance and virulence gene signatures in surface water isolates of *Escherichia coli*. *Journal of applied microbiology*. 2008;**105**(6):1899-1908.