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Author: Fathima Farha Bary Mohamed, Uthpala Amarasekara

BMS, School of Science, Sri Lanka

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IDENTIFICATION OF ESCHERICHIA COLI FROM DRINKING WATER SAMPLES IN GALLE AND THE DETECTION OF ANTIBIOTIC RESISTANT GENES

Fathima Farha Bary Mohamed, Uthpala Amarasekara

BMS, Sri Lanka

ABSTRACT

Development of antibiotic resistant bacteria in drinking water sources is a major threat because it possess harmful effects against human health. The purpose of this study is to identify Escherichia coli (E. coli) from drinking water samples in Galle and the detection of antibiotic resistant genes (ARGs). E. coli were isolated from ten different drinking water sources via membrane filtration and identified using biochemical, morphological and molecular tests. Antibiotic resistance was determined by antibiotic sensitivity test and ARGs were detected using Polymerase Chain Reaction. Bluish-green colonies were observed in membrane-filtered chromogenic plates of five out of ten water samples (Gintota, Walahanduwa, Katugoda, Kaluwella, Fort). Eleven colonies were selected from the five samples for biochemical, morphological and molecular analysis. Indole test was positive and Simmon's citrate test was negative for nine out of eleven colonies. Gram stain illustrated gram-negative pink rods mostly except for two colonies from Walahanduwa and Katugoda, which resulted in gram-negative pink cocci. All colonies were positive for 16S rRNA while ten out of eleven colonies were positive for uidA and lac Y. Thus, all samples were confirmed to have E. coli except for one sample in Kaluwella, which was lac Y negative that was confirmed to be shigella species. E. coli positive colonies were detected against tetracycline and sulfonamide resistance. All ten colonies were sensitive for sulfonamide whereas five out of ten colonies were resistance against tetracycline. Tetracycline resistant colonies were detected for tet(A) and tet(C) genes. All five colonies were negative for tet(C) while four out of five colonies were positive for tet(A). Therefore, water samples collected from Walahanduwa, Katugoda and Fort was confirmed to have E. coli resistant against tet(A).

Keywords: Antibiotic resistant bacteria, Drinking water, E. coli, Antibiotic resistant genes, Polymerase Chain Reaction

INTRODUCTION

Drinking water should be pure, free from pathogenic bacteria and safer for human health (Wolf-Baca and Siedlecka, 2019). Development of ARGs and antibiotic resistance bacteria (ARB) in drinking water is a main concern around the world since it has greater threat towards humans resulting in low treatment options (Fernando et al., 2016).

Globally, 3.2% of deaths are estimated to be due to lack of clean and unsafe drinking water, mainly in rural areas of developing countries due to poor sanitation and hygiene conditions (Chen et al., 2010). World Health Organization has stated that 3.4 million people, mostly children, die from waterborne diseases per

year (Carrillo-Gomez et al., 2019). In Sri Lanka, consumption of antibiotics has increased by 36% in-between 2000 and 2010 increasing the risk of ARB and ARGs in drinking water. Galle is a highly populated district, which receives water supply from the Gin river (figure 1) that is being polluted due to anthropogenic activities and the residential location. During heavy rainfall seasons, surrounding areas connected by drains and sewers are subjected to floods (Kumar et al., 2019). This study includes the identification of E. coli from drinking water samples in Galle and the detection of ARGs using Polymerase Chain Reaction (PCR). The significance of this project is to evaluate the transfer of ARGs to humans because long term consumption of contaminated water leads to antibiotic resistance thereby transferring it to the environment through faeces (Larson et al., 2019).

Figure 1 - Location of Gin river in Galle district (Kumar et al., 2019).

E. coli and antibiotic resistance

E. coli is a gram negative, facultative anaerobic bacilli found in gastrointestinal tracts of warm-blooded organisms as a part of normal flora (Lyimo et al., 2016). Mostly, E. coli species are ubiquitous commensals but certain strains acquires virulent genes and becomes pathogens. There are two main pathotypes of E. coli, namely, intestinal pathogens which includes enteropathogenic, enterotoxigenic and Shigatoxin-producing E. coli (O157: H7) that causes diseases such as diarrhoea, hemorrhagic colitis and hemolytic uremic syndrome, and extra-intestinal pathogens such as uropathogenic E. coli (O25:ST-131) that causes diseases like urinary tract infections, meningitis and sepsis (Osinska et al., 2017; Jafari et al., 2012).

Drinking water sources are contaminated with E. coli through animal faeces. Therefore, E. coli is used as an indicator bacteria to determine faecal contamination of water (Fatemah et al., 2014). Thus, the amount of E. coli present is used to detect the quality of water and the efficiency of water disinfection processes such as chlorination (Abada et al., 2019).

Main energy source of E. coli is glucose, thus they are unable to utilize citrate as their carbon source, under aerobic conditions. E. coli consists of the enzyme tryptophanase that decomposes the amino acid tryptophan to indole (Odonkor and Ampofo, 2013). 16S rRNA gene, which is used for bacterial identification is present in E. coli, and uidA gene that expresses the enzyme β-glucuronidase is a specific marker of E. coli species (Limayem et al., 2019; Jang et al., 2017). lac Y gene found in lac operon of E. coli strains, separates them from other coliforms such as Shigella and Salmonella (Lobersli et al., 2016).

The ability of bacteria to prevent the effects of an antibiotic is known as antibiotic resistance. Antibiotic resistance is developed in bacteria either by genetic mutations or by acquiring ARGs (Amarasiri et al., 2019) through vertical gene transfer, in which the encoding genes, situated in chromosomes or extra chromosomal plasmids are transmitted to next generation, or horizontal gene transfer, where mobile genetic elements such as conjugative plasmids, transposons and gene cassettes in integrons are exchanged among different species of bacteria (Hembach et al., 2019; Shivakumaraswamy et al., 2019). E. coli acts as a vector for the dissemination of ARGs through horizontal gene transfer (Chen et al., 2017).

Extensive use of antibiotics in hospitals and agriculture causes antibiotic resistance in bacteria through build-up of selective pressure aiding in proliferation of ARGs. Their discharge and disperse in environmental water bodies via sewage and wastewaters, due to deprived handling of industrial waste and animal faecal matter leads to contamination of surface water and groundwater by adsorption on to the soil matrix (Sanganyado and Gwenzi, 2019; Tomar et al., 2015). Usually both protected and unprotected water surfaces such as surface water (rivers, lakes) and groundwater (wells, tube wells) are used as drinking water sources. Therefore, ARGs survive drinking water treatment facilities, due to the ineffectiveness of water disinfection processes and enter household compartments through water distribution systems eventually transferring into humans causing morbidity and mortality. In humans these ARGs transfer to normal gut flora, resulting in the multiplication of ARB, thereby transferring them to the environment again through faeces and thus, the cycle continues (Mulamattathil et al., 2014; Alzahrani and Gherbawy, 2011).

Most E. coli species are resistant to antibiotics tetracycline and sulfonamide. Tetracyclines are broad spectrum antibiotics, which binds to 16S rRNA of bacterial 30S ribosomes, thereby inhibiting protein synthesis. Tetracycline resistant genes, developed in bacteria at the beginning of medical use (Markley and Wencwicz, 2018) and they are classified into four groups according to their resistant mechanisms, namely, efflux genes, enzymatic genes, ribosomal protection genes and other genes (Lu et al., 2018). The predominant forms of efflux genes, tet(A) and tet(C) acquires tetracycline resistant through efflux pump mechanism (figure 2), which alters the plasma membrane structure thereby reducing effective intracellular concentration of tetracycline (Zhang et al., 2009).

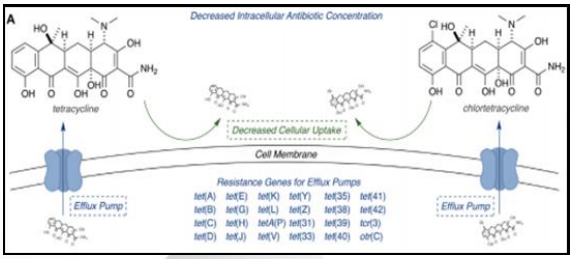

Figure 2 - Efflux pump mechanism (Markley and Wencwicz, 2018)

Sulfonamides are used in large-scale for medicine purposes. Sulfonamides inhibits DNA synthesis by acting as competitive inhibitors to para-aminobenzoic acid, thereby reducing the affinity of deoxyhypusine synthase (DHPS) enzyme coded by folP gene, in folic acid pathway of bacteria that is responsible for biosynthesis of folate used in microbial growth and thymine production (Pedrola et al., 2019; Xi et al., 2009). Sulfonamide resistance bacteria are developed due to mutations occurring in alternative forms of DHPS encoding genes known as sul genes. There are various types of sul genes, namely, sulI, sulII and sulIII based on their different mechanisms against sulfonamide resistance. The most prevalent form sulI, obtains sulfonamide resistance through target bypass mechanism in which the antibiotic is unable to bind to its target enzyme due to mutational alterations (Sanchez-Osuna et al., 2019; Gundogdu et al., 2011).

METHODOLOGY

Sample collection

Drinking water samples were collected from 10 locations in Galle (table 1).

Table 1 - Sample collection from water sources

Sample	Source	Location
1	River	Gintota
2	Well	Dadella
3	Well	Dadella
4	Lake	Walahanduwa
5	Tube well	Katugoda
6	Well	Katugoda
7	Well	Kaluwella
8	Well	Kaluwella
9	Well	Kaluwella
10	Tank	Fort

Physicochemical parameters measurement

pH, temperature and chlorine concentration of each water sample was measured using a pH meter, thermometer and chlorine test strips respectively.

Membrane filtration

From each water sample, 100ml was poured onto 0.45µm filter papers placed on Buchner funnels. Membrane filters were placed on petri plates containing 20ml of solidified HiCrome E. coli agar (HIMEDIA) using forceps. This was repeated to obtain duplicates and the petri plates were incubated at 37℃ for 24hours.

Subculture

Luria broth (LB)

Loop full of selected colonies were inoculated in falcon tubes containing 5ml of LB (HIMEDIA) and was incubated at 37℃ for 24hours.

Streak plate

Loop full of LB subcultures were quadrant streak plated on petri plates containing 20ml of solidified nutrient agar (HIMEDIA) and was incubated at 37℃ for 24hours.

Pure isolated colonies were inoculated on LB as mentioned above and was renewed every 2 weeks.

Indole test

Loop full of subcultures were inoculated on test tubes containing 4ml of tryptophan broth (HIMEDIA) and was incubated at 37oC for 24hours. Then, 0.5ml of Kovac's reagent was added into each incubated samples and the colour change was observed.

Simmon's Citrate test

Loop full of subcultures were swabbed on test tubes containing 3ml of solidified Simmon's citrate agar and was incubated at 37oC for 24hours.

Gram stain

Loop full of subcultures were transferred to slides containing drops of distilled water forming a thin smear and was heat fixed. Smear was flooded with crystal violet and was left for 1minute. Stain was washed off. Smear was flooded with gram's iodine and was left for 1minute. Stain was washed off. Smear was flooded with gram's decolourizer and was washed off immediately. Smear was flooded with safranin and was left for 45seconds. Stain was washed off. Slides were air dried and was observed through light microscope under 100x.

DNA extraction

DNA was extracted using Promega kit-based method. From overnight subcultures, 1ml was added into eppendorf tubes and centrifuged at 13000rpm for 2minutes. Supernatant was pipetted out and 600µl of nuclei lysis solution was added. Samples were vortexed and incubated at 80℃ for 5minutes in a water bath (GEMMYCO) and was cooled to room temperature. Then, 3µl of RNase solution was added and mixed by inverting the tubes for 2-5 times. Samples were incubated at 37℃ for 15minutes in a water bath and cooled to room temperature.

Then, 200µl of protein precipitation solution was added and vortexed for 20seconds. Samples were incubated on ice for 5minutes and centrifuged at 13000rpm for 3minutes. Then, 600µl of isopropanol was added into new eppendorf tubes and supernatant was transferred into it and mixed. Eppendorf tubes were centrifuged at 13000rpm for 2minutes and supernatant was poured off. Eppendorf tubes were drained on clean tissues and 600µl of 70% ethanol was added. Eppendorf tubes were inverted for several times and centrifuged at 13000rpm for 2minutes. Ethanol was aspirated and the tubes were drained on clean tissues. Pellet was allowed to air dry for 10minutes and 100µl of DNA rehydration solution was added. Extracted DNA samples were stored at 4℃.

Gel electrophoresis and visualization

Prepared 0.8% agarose gel was placed on the electrophoresis chamber (Bio-Rad) and 1x Tris-acetate-EDTA buffer was poured until gel was immersed completely. Then, 5µl of each extracted DNA sample mixed with 2µl of loading buffer was loaded into each well. Gel was allowed to run at 60V for 20minutes and amplicons were visualized through ultraviolet (UV) transilluminator (E-gel imager Life technologies).

PCR - 16S rRNA, uidA, lac Y

Forward (F) and reverse (R) primers of 16S rRNA (424bp) F: 5'-GTTGTAAAGCACTTTGAGTGGTGAGGAAGG-3'; R: 5'-GCCTCAAGGGCACAACCTCCAAG-3' (Gao et al., 2012), uidA (147bp) UAL (F): 5'-TGGTAATTACCGACGAAAACGG-3'; UAR: 5'-ACGCGTGGTTACAGTCTTGCG-3' (Molina et al., 2015) and lac Y (104bp) F: 5'-ACCAGACCCAGCACCAGATAAG-3'; R: 5'-TTCTGCTTCTTTAAGCAACTGGC-3' (Lobersli et al., 2016) were diluted and PCR master mix was prepared separately for all three genes by adding 166.875µl of distilled water, 75µl of PCR buffer (5x), 22.5µl of MgCl2, 9.375µl of dNTPs (10mM), 37.5 of forward primer (2.5µM), 37.5µl of reverse primer (2.5µM) and 3.75µl of taq polymerase into eppendorf tubes. To each PCR tube, 23.5µl of PCR master mix was aliquot followed by 1.5µl of extracted DNA sample bringing up the total volume to 25µl.

PCR tubes were placed in the PCR machine (Bio-Rad) and was allowed to run under separate PCR cyclic conditions for 16S rRNA: initial denaturation at 94°C for 2minutes, followed by 35 cycles of denaturation at 94°C for 30seconds, annealing at 55°C for 45seconds, extension at 72°C for 1minute 30seconds, and final extension at 72°C for 10minutes (Gao et al., 2012), uidA: initial denaturation at 95°C for 3minutes, followed by 35 cycles of denaturation at 95°C for 30seconds, annealing at 58°C for 30seconds, extension at 72°C for 1minute, and final extension at 72°C for 10minutes (Molina et al., 2015), and lac Y: initial denaturation at 94°C for 3minutes, followed by 35 cycles of denaturation at 94°C for 1minute, annealing at 58°C for 30seconds, extension at 72°C for 1minute, and final extension at 72°C for 5minutes (Lobersli et al., 2016).

Gel electrophoresis and visualization

Prepared 2% agarose gel was placed on the electrophoresis chamber and 1x Tris-acetate-EDTA buffer was poured until gel was immersed completely. Then, 3µl of 100bp DNA ladder was loaded into well 1 followed by 2µl of each PCR products. Gel was allowed to run at 45V for 20minutes and at 50V for 25minutes. Amplicons were visualized through UV transilluminator.

Antibiotic sensitivity test (ABST) - disk diffusion method

From overnight subcultures, 1ml was pipetted to test tubes containing 5ml of autoclaved distilled water. Turbidity of each test tube was compared and equalized with 0.5 McFarland solution, and was

swabbed on petri plates containing 20ml of solidified Muller-Hinton agar (HIMEDIA) using cotton swabs. Antibiotic disks of tetracycline (30µg), sulfonamide (300µg) and filter papers immersed in distilled water (negative control) were placed at the center of each respective divided section on petri plate using forceps. This was repeated to obtain duplicates and the petri plates were incubated at 37℃ for 24hours. Samples were categorized as resistant, sensitive and intermediate (table 2) by observing and measuring the diameters of zones of inhibition according to Clinical and Laboratory Standards Institute (CLSI).

Table 2 - Zone diameter interpretive standards (Karunaratne et al., 2011).

Antimicrobial disk	Tetracycline 30µg (mm)	Sulfonamide 300µg (mm)
Resistant	≤ 11	≤ 10
Intermediate	12 - 14	11 - 15
Sensitive	≥ 15	≥ 16

PCR – tet(A) and tet(C)

Forward and reverse primers of tet(A) (210bp) F: 5'-GCTACATCCTGCTTGCCTTC-3'; R: 5'-CATAGATCGCCGTGAAGAGG-3', and tet(C) (335bp) F: 5'-TGCGTTGATGCAATTTCTATGC-3'; R: 5'- GGAATGGTGCATGCAAGGAG-3' (Chen et al., 2013) were diluted and PCR master mix was prepared separately for both genes by adding 66.75µl of distilled water, 30µl of PCR buffer (5x), 9µl of MgCl2, 3.75µl of dNTPs (10mM), 15µl of forward primer (2.5µM), 15µl of reverse primer (2.5µM) and 1.5µl of taq polymerase into eppendorf tubes for tetracycline resistant and intermediate samples. To each PCR tube, 23.5µl of master mix was aliquot followed by 1.5µl of extracted DNA sample bringing up the total volume to 25µl.

PCR tubes were placed in the PCR machine and was allowed to run under separate PCR cyclic conditions for tet(A): initial denaturation at 95°C for 5minutes, followed by 40 cycles of denaturation at 95°C for 20seconds, annealing at 60°C for 30seconds, extension at 72°C for 30seconds, and final extension at 72°C for 10minutes and tet(C): initial denaturation at 95°C for 5minutes, followed by 40 cycles of denaturation at 95°C for 20seconds, annealing at 64°C for 40seconds, extension at 72°C for 30seconds, and final extension at 72°C for 10minutes (Chen et al., 2013).

Refer previous gel electrophoresis and visualization section.

DATA ANALYSIS

Physiochemical parameters

Table 3 - pH, temperature and chlorine concentration of water samples

Water Samples	pH	Temperature (℃)	Chlorine concentration (ppm)
1 – River (unprotected)	7.01	28.5	< 5
2 – Well (protected)	6.45	27.8	< 5
3 – Well (unprotected)	6.75	27.5	< 5
4 – Lake (unprotected)	8.13	27.6	< 5
5 – Tube well (unprotected)	7.08	27.4	< 5
6 – Well (protected)	7.04	29.0	< 5
7 – Well (protected)	6.50	28.0	< 5
8 – Well (protected)	6.80	28.0	< 5
9 – Well (unprotected)	6.90	27.0	< 5
10 – Tank (protected)	7.05	28.0	5

Membrane filtration

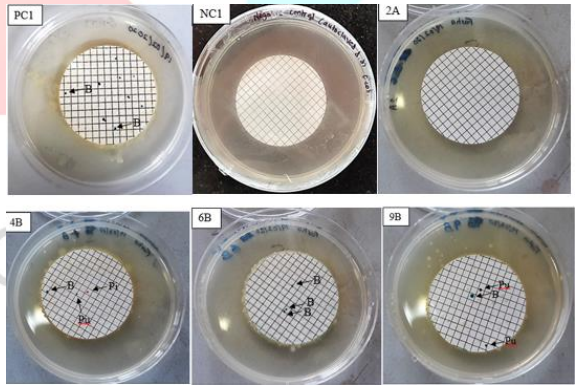

Figure 3 - Membrane-filtered plates of positive control (PC) – E. coli ATCC 25922 inoculated in distilled water:

bluish-green (B) colonies present, negative control (NC) – distilled water, drinking water sample 2: colonies absent, and drinking water sample 4, 6, 9 containing bluish-green colonies with Pink (P), Purple (Pu) and opaque colonies.

Table 4 - Sample plates showing bluish-green, pink, purple and opaque colonies. ×: colonies absent, : colonies present, (): number of colonies.

Sample plates	Bluish-green colonies	Pink colonies	Purple colonies	Opaque colonies
1A	×	×	×	
1B	(1)	×	(1)	
2A	×	×	×	×
2B	×	×	×	×
3A	×	×	×	
3B	×	×	×	
4A	×	×	(7)	
4B	(1)	(1)	(1)	
5A	×	×	×	
5B	×	×	×	
6A	(2)	×	×	
6B	(3)	×	×	
7A	×	×	×	
7B	×	×	(1)	
8A	×	×	(1)	
8B	×	×	(1)	
9A	×	×	(2)	
9B	(1)	×	(2)	
10A	×	×	×	(2)
10B	(1)	×	×	

From streak plates, eleven pure isolated colonies of interest labelled as S1: 9B bluish-green, S2: 10B bluish-green, S3: 4B bluish-green, S4: 4B pink, S5: 4B opaque, S6: 9B purple, S7: 4B purple, S8: 9B purple opaque, S9: 1B bluish-green, S10: 6A bluish-green and S11: 6B bluish-green was selected for further identification.

Morphological and Biochemical analysis

Gram stain results of isolated colonies illustrated gram-negative pink rods in S1, S2, S4, S5, S6, S7, S8, S9, S10 and gram-negative pink cocci in S3, S11.

Figure 4 Figure 5

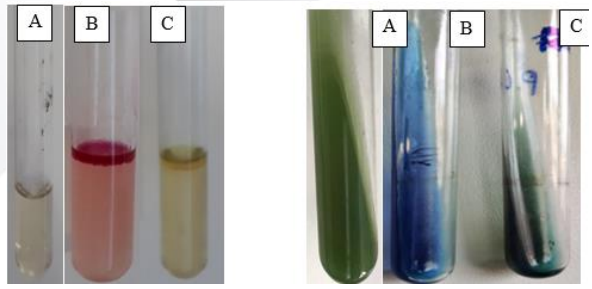

Figure 4 - Indole test. A: Blank. B: Indole positive (E. coli ATCC 25922). C: Indole negative (Staphylococcus aureus ATCC 25923).

Figure 5 - Simmon's Citrate test. A: Blank. B: Citrate positive (Staphylococcus aureus ATCC 25923). C: Citrate negative (E. coli ATCC 25922)

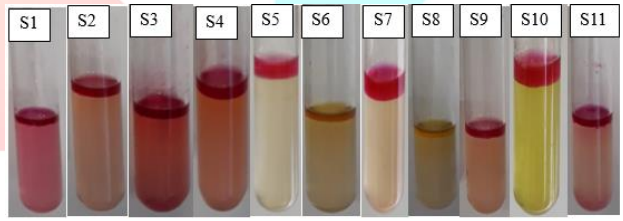

Figure 6 - Indole test results of isolated colonies. Indole positive – cherry red ring, Indole negative – yellow ring.

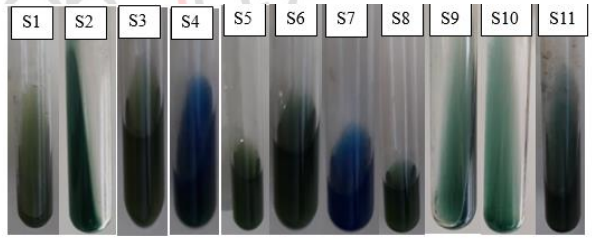

Figure 7 - Simmon's Citrate test results of isolated colonies. Citrate positive – blue, Citrate negative - green.

Molecular analysis

DNA extraction

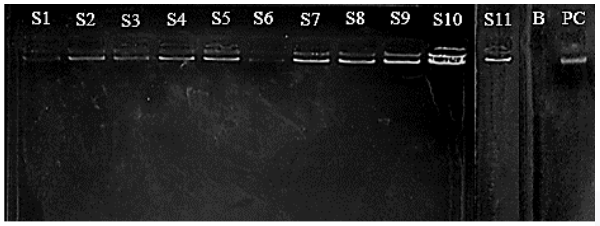

Figure 8 - 0.8% Agarose gel image for kit-based DNA extraction. S1, S6 - faint band present. S2, S3, S4, S5, S7, S8, S9, S10, S11 - clear band present. B: blank (DNA rehydration solution) - band absent. PC: positive control (E. coli ATCC 25922) - band present.

PCR – 16S rRNA, uidA, lac Y

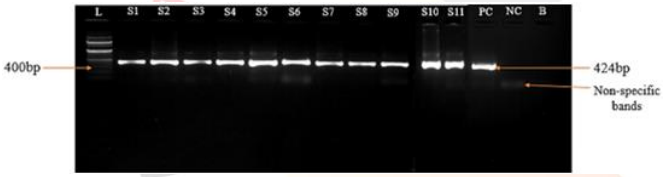

Figure 9 - 2% Agarose gel image for 16S rRNA (424bp) gene. L: 100bp DNA ladder. S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, PC: positive control (E. coli ATCC 25922) - band present. NC: negative control (Staphylococcus aureus ATCC 25923), B: blank (distilled water) - band absent.

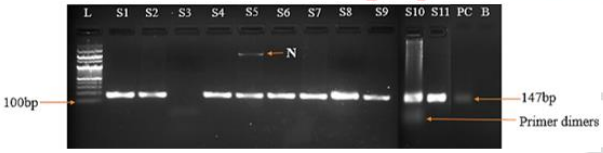

Figure 10 - 2% Agarose gel image for uidA (147bp) gene. L: 100bp DNA ladder. S1, S2, S4, S5, S6, S7, S8, S9, S10, S11, PC: positive control (E. coli ATCC 25922) - band present. S3, B: blank (distilled water) - band absent. N: non-specific amplicon.

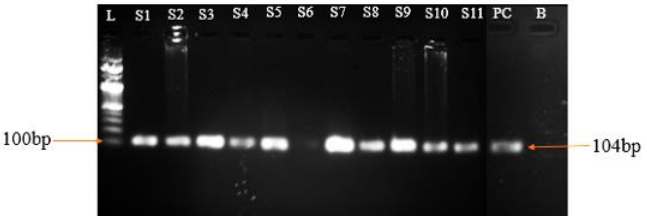

Figure 11 - 2% Agarose gel image for lac Y (104bp) gene. L: 100bp DNA ladder. S1, S2, S3, S4, S5, S7, S8, S9, S10, S11, PC: positive control (E. coli ATCC 25922) - band present. S6, B: blank (distilled water) - band absent.

ABST

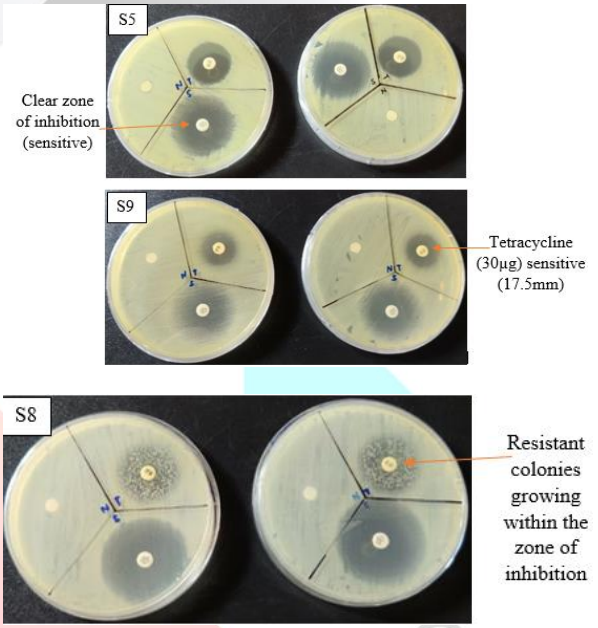

Figure 12 - Disk diffusion duplicates sensitive for both tetracycline (30µg) and sulfonamide (300µg). T: tetracycline, S: sulfonamide, N: negative control.

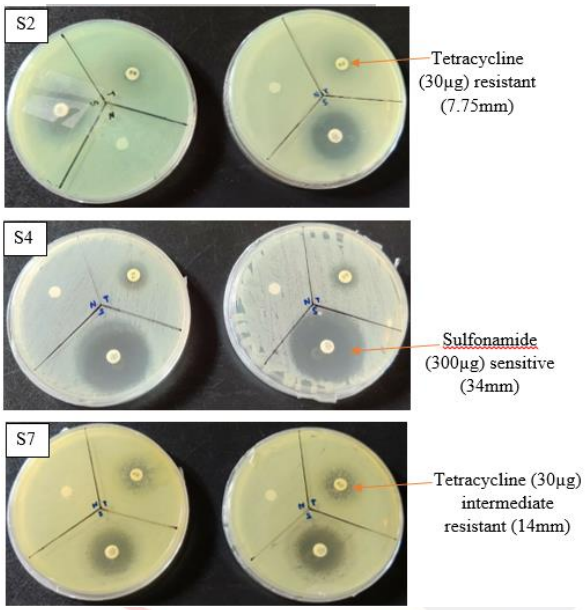

Figure 13 - Disk diffusion duplicates resistant for tetracycline (30µg) and sensitive for sulfonamide (300µg). T: tetracycline, S: sulfonamide, N: negative control.

Table 5 - Results of ABST for tetracycline (30µg) and sulfonamide (300µg). (S): sensitive, (IR): intermediate resistance, (R): resistance. (Refer table 2).

Sample	Tetracycline (mm)	Sulfonamide (mm)
S1	19.5 ±0.71 (S)	23 ±4.24 (S)
S2	**7.75 ±0.35 (R)**	25.5 ±3.35 (S)
S3	19.5 ±0.71 (S)	17 ±1.41 (S)
S4	**8.5 ±0.71 (R)**	34 ±1.41 (S)
S5	23 ±0 (S)	29.5 ±0.71 (S)
S7	**14 ±1.41 (IR)**	18 ±0 (S)
S8	21.5 ±0.71 (S)	34 ±0 (S)
S9	17.5 ±0.71 (S)	20 ±0 (S)
S10	**10 ±0 (R)**	25.5 ±0.71 (S)
S11	**8.5 ±0.71 (R)**	22 ±1.41 (S)

PCR – tet(A) and tet(C)

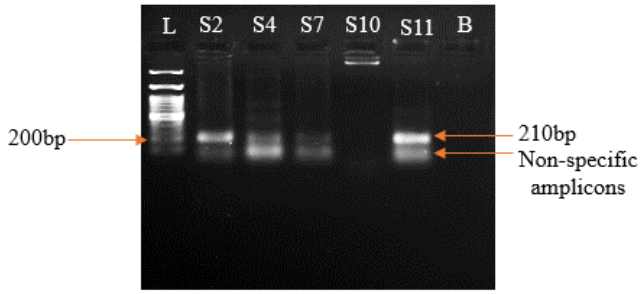

Figure 14 - 2% Agarose gel image for tet(A) (210bp) gene. L: 100bp DNA ladder. S2, S4, S7, S11 - band present. S10, B: blank (distilled water) – band absent.

No bands were observed in 2% Agarose gel image for tet(C) (335bp) gene.

Table 6 - Summary. (+): positive, (-): negative, (×): not performed

Sample	Indole	Citrate	Gram stain	Genomic DNA	16S rRNA	uidA	lacY	Antibiotic resistance	*tet*(A)	*tet*(C)
S1	+	-	Gram-pink rods	+	+	+	+	-	×	×
S2	+	-	Gram-pink rods	+	+	+	+	+	+	-
S3	+	-	**Gram-pink cocci**	+	+	-	+	-	×	×
S4	+	+	Gram-pink rods	+	+	+	+	+	+	-
S5	+	-	Gram-pink rods	+	+	+	+	-	×	×
S6	-	-	Gram-pink rods	+	+	+	-	×	×	×
S7	+	+	Gram-pink rods	+	+	+	+	+	+	-
S8	-	-	Gram-pink rods	+	+	+	+	-	×	×
S9	+	-	Gram-pink rods	+	+	+	+	-	×	×
S10	+	-	Gram-pink rods	+	+	+	+	+	-	-
S11	+	-	**Gram-pink cocci**	+	+	+	+	+	+	-

DISCUSSION

Drinking water samples were collected from both protected and unprotected water sources in Galle. E. coli can survive in temperatures ranging from 7-50℃ (Sakyi and Asare 2012), pH ranging from 4-8 (WHO, 2020) and low chlorinated effluents ranging from 0-5ppm (Owoseni et al., 2017). Physiochemical parameters of all the water samples obtained was between these ranges (table 3). Therefore, there's a high chance of E. coli being present.

Membrane filtration is used for quantitative analysis of E. coli in large volumes of drinking water (Price and Wideboer, 2017), passing through 0.45µm pores containing filter papers to retain E. coli that are small rods of 1-2µm in size (El-Hajj and Newman, 2015). Membrane filters are cultured onto selective chromogenic media (HiCrome E. coli agar M1295), which isolates E. coli by inhibiting gram-positive bacteria due to the presence of bile salts in the mixture. Bile salts elicits antimicrobial effects particularly against gram-positive bacteria via the aggregation and unfolding of cytosolic proteins and inducing pro-oxidizing shift from reduced to oxidized glutathione in bacterial cells leading to disulphide stress. E. coli consist of the cytosolic chaperone Hsp33, which helps in defending themselves against bile salts (Cremers et al., 2014). Studies has revealed that 94-96% of E. coli strains expresses the enzyme β-D-glucuronidase activity that is a sensitive and specific marker of E. coli, which separates it from other coliforms such as Salmonella and Shigella species (Perin et al., 2010). HiCrome E. coli agar detects β-D-glucuronidase enzyme activity in E. coli, which cleaves the chromogen X-glucuronide resulting in bluish-green colour colonies (Antony et al., 2016). In membrane-filtered chromogenic plates, bluish-green colonies were observed in samples 1B, 4B, 6A, 6B, 9B and 10B (figure 3). In sample 2, no colonies were observed indicating the water is pure. But pink, purple and opaque colonies were also observed in some plates (table 4). Opaque colonies occur due to lack of β-glucuronidase enzyme activity in E. coli. Salmonella enteritidis also appear as opaque colonies in HiCrome E. coli agar (Perry, 2017). Chromogenic media consist of exploited indoxylic substrates. Hydrolysis of certain specific bacterial enzymes release indoxyl molecules. Spontaneous dimerization of these indoxyl molecules in the presence of oxygen results in purple colonies. Halogenation of indoxyl molecules (5-bromo-6-chloro-indoxyl) results in pink colonies (Perry and Freydiere, 2007). Therefore, more specific tests (biochemical, morphological) were performed to identify E. coli. Selected colonies from membrane-filtered plates were inoculated in LB to promote faster growth and attain higher yield (Lessard, 2013). Pure cultures of isolated colonies on nutrient agar was obtained through quadrant streak plate technique (Sanders, 2012). Nevertheless, MacConkey agar is recommended for specific identification of isolated E. coli strains based on β-D-galactosidase activity that separates it from non-lactose fermenters (Verhaegen et al., 2015).

Indole test detects bacteria that decomposes the amino acid tryptophan to indole. 96% of E. coli species are indole positive due to the presence of tryptophanase enzyme, which converts tryptophan to indole, pyruvate and ammonia. A cherry red ring appears when indole reacts with kovac's reagent indicating a positive result (figure 4) (Liu and Summers, 2017). Most samples were indole positive whereas S6 and S8 was indole negative (figure 6). Tryptophanase enzyme in E. coli is coded by the tnaA gene located in tnaCAB operon. Functional tnaA is a tetramer composed of four active sites consisting of three

subdomains D1, D2 and D3 each, that regulates tryptophanase localization and loop-associated occlusion of the enzyme's active site. Indole negative can be due to mutations occurring in these subdomains, which blocks the enzyme's active site thereby halting the conversion resulting in yellow ring (Li and Young, 2015). Indole negative result can also be due to the presence of various indole-negative enteric bacteria like Shigella species (Rezwan et al., 2004). Simmon's Citrate test differentiates microorganisms based on their energy source, which utilize citrate as the sole carbon source. Enzyme citritase in citrate-metabolizing microorganisms, cleaves citrate to acetate and oxaloacetate, which is further degraded to pyruvate and CO2 via oxaloacetate dehydrogenase. Produced CO2 is converted to Na2CO3 shifting the media to alkaline pH, which is detected by the pH indicator bromothymol blue resulting in green to blue colour change (figure 5) (Lupindu, 2017). Most samples were Citrate negative whereas S4 and S7 was Citrate positive (figure 7). Most E. coli strains are Citrate negative because they are unable to utilize citrate as their carbon source under aerobic conditions due to lack of citrate transporters resulting in green colour (Blount et al., 2012). Few E. coli species can utilize citrate under anaerobic conditions by the expression of citT citrate/succinate antiporter due to tandem duplication in cit gene resulting in a blue colour change (Scheu et al., 2012). Citrate positive result can also be due to the presence of citrate-positive E. coli K12 strains (Hofwegen et al., 2016). Gram stain differentiates gram-negative and gram-positive bacteria via morphological analysis of their cell wall. E. coli consist of thin peptidoglycan layer with lipopolysaccharides, lipoproteins and large pores in their cell wall. Upon decolourization, peptidoglycan layer dissolves and purple stain from crystal violet is lost, thereby absorbing the colour of counter stain safranin resulting in gram-negative pink rods (Budin et al., 2012). Most of the samples were gram-negative pink rods whereas S3 and S11 was gram-negative pink cocci. E. coli consist of two major cytoskeletal proteins, divisome (tubulin-like FtsZ protein) and elongasome (actin-like MreB protein) that is important in cell shape. Inactivation of elongasome (loss of actin-like MreB protein) via mutations results in round cells (Weiss, 2013). Thus, E. coli has the ability to change its morphology from rods to cocci (L-form conversion) as a protective mechanism during lethal heat treatments by responding to stress conditions that might be due to heat fixing in gram stain (Markova et al., 2010).

Since biochemical and morphological analysis were not precise in identifying E. coli, more specific and sensitive molecular analysis of samples were performed to detect E. coli. Both clear and faint bands were observed in the gel image of Promega kit-based DNA extraction method (figure 8). Faint bands might be due to less amount of DNA concentration present in the samples that could be due to pipetting errors (Abdulamir et al., 2010). 16S rRNA gene is used to identify bacterial genera and it is 99% sequence specific for E. coli. All the samples were positive for 16S rRNA (424bp) (figure 9). Molecular amplification of 23S rRNA is recommended for E. coli species identification (Magrey et al., 2011). uidA gene is present in 97.7% of E. coli strains and 2% of Shigella species that codes for the enzyme β-glucuronidase (Molina et al., 2015). Most of the samples were positive for uidA (147bp) except for S3, which was uidA negative (figure 10) that might be due to primer not annealing to target sequence because of low primer concentration, since a bluish-green colony was observed in the chromogenic plate suggesting the gene was expressed (Godambe et al., 2017). lac Y gene is used to differentiate E. coli (lactose fermenters)

strains from Shigella (non-lactose fermenters) species (Pavlovic et al., 2011), which is found in lac operon of E. coli that codes for the enzyme lactose permease responsible in transporting lactose into the cell with the help of proton symport mechanisms (Kimanius et al., 2018). Most of the samples were positive for lac Y (104bp) except for S6 which was lac Y negative (figure 11). S6 was also indole negative that might occur due to extensive damage caused to tna operon through an insertion sequence indicating that it could be a Shigella species (Rezwan et al., 2004). Moreover, S6 was citrate negative, uidA positive and a purple colony was observed in chromogenic plate which further confirmed it to be a Shigella species (Ud-Din and Wahid, 2014). Thus, the water samples collected from Gintota, Walahanduwa, Katugoda and Fort were confirmed to have E. coli except for S6 in Kaluwella. However, due to certain mutations, various Shigella species (S.sonnei, S.dysenteriae) can ferment lactose slowly through unspecialized lactose transporters, since they consist of lac Z coding for β-D-galactosidase though they are lac Y negative (Yang et al., 2005).

Disk diffusion method of ABST is used to detect the sensitivity and resistance of bacteria to antibiotics based on the zone of inhibition according to CLSI standards (Flanagan and Steck, 2017). ABST was performed for all lac Y positive samples on Mueller-hinton agar to test E. coli resistance against tetracycline and sulfonamide. Mueller-Hinton media consist of loose agar, which allows better diffusion of antibiotics leading to truer zone of inhibition (Nassar et al., 2019), and is low in tetracycline and sulfonamide inhibitors (Hudzicki, 2016). Results indicated tetracycline resistance in S2, S4, S7, S10 and S11 (7-14mm) (figure 13) whereas all the samples were sensitive for sulphonamide (table 5). In S1, S3, S7 and S8, resistant colonies grew on zone of inhibition areas (figure 12), which might be due to the presence of resistant strains against tetracycline (Coorevits et al., 2015). PCR was performed to detect tet(A) and tet(C) genes in tetracycline resistant samples. S2, S4, S7 and S11 was positive for tet(A) (210bp) whereas S10 was tet(A) negative (figure 14), while all the samples were negative for tet(C) (335bp), which suggested tet(A) is the predominant form of tet genes present in E. coli of drinking water samples that shows resistance against tetracycline (Al-Bahry et al., 2016). S10 might be positive for other tetracycline resistant genes such as tet(B) and tet(M) (Hu, et al., 2013).

Non-specific amplicons of 200bp in 16s rRNA, 800bp in uidA and 100bp in tet(A) was observed which might be due to primer-target mismatch or because of high extension and annealing time (Green et al., 2015). High molecular weight smears were observed in 16S rRNA, uidA, lac Y and tet(A) gel images that could be due to DNA fragmentation. Primer dimers were observed in uidA gel image which may be due to addition of high primer content (Lorenz, 2012).

CONCLUSION

Drinking water samples 4, 6 and 10 collected from unprotected lake (Walahanduwa), protected well (Katugoda) and protected tank (Fort) respectively, was confirmed to have E. coli resistant against tet(A).

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