GLOBAL ACADEMIC RESEARCH INSTITUTE

COLOMBO, SRI LANKA

GARI International Journal of Multidisciplinary Research

ISSN 2659-2193

Volume: 07 | Issue: 01

On 28th February 2021

http://www.research.lk

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GARI Publisher | infectious Disease | Volume: 07 | Issue: 01

Article ID: IN/GARI/ICAS/2020/105 | Pages: 54-80 (27)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 18.11.2020 | Publish: 28.02.2021

IDENTIFICATION OF ESCHERICHIA COLI AND DETECTION OF ITS ANTIBIOTIC RESISTANCE IN DRINKING WATER SOURCES FROM RAGAMA, SRI LANKA

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ABSTRACT

Every human should have the access to safe drinking water as contaminated water increase the risk of exposure to different pathogens and the development of antibiotic resistance among these pathogens result in a greatest threat to public health. Escherichia coli (E. coli) is the broadly accepted indicator of pathogenic microorganisms and fecal contamination in water sources, therefore 10 drinking water samples collected from protected/ unprotected wells and a tap-line in different areas of Ragama, Sri Lanka were used to identify E. coli by culturing on HiCrome agar, performing biochemical tests and targeting 16S rRNA, uidA, and lacY genes by PCR. Furthermore, selected E. coli colonies were used to detect E. coli resistance towards tetracycline, sulfonamide and gentamycin antibiotics by performing Kirby-Bauer disk-diffusion test and targeting tetA, tetC and sul1 genes by PCR. Out of 13 coloured colonies (blue, opaque, purple and pink) isolated from HiCrome agar, 07 showed positive results for indole test, 16S rRNA, uidA and lacY PCRs, while 02 isolates were only positive for 16S rRNA and uidA PCRs. Out of 07 selected E. coli isolates tested for antibiotic susceptibility, 06 showed tetracycline resistance whereas 03 showed sulfonamide resistance. Moreover, out of 06 tetracycline-resistant samples, 05 indicated tetA resistant genes with PCR. In conclusion, this study found these drinking water samples supported E. coli growth and the findings confirmed the presence of E. coli as well as the possibilities of other bacteria like Shigella spp. Furthermore, 06 E. coli isolates showed tetracycline-resistance and 05 of these revealed the efflux pump mechanism of E. coli through tetA genes. Additionally, 03 of these showed resistance to both tetracycline and sulfonamide, thereby exhibiting multi-drug resistance.

Keywords: Drinking water, E. coli, Antibiotic resistance

INTRODUCTION

Water is a universal solvent, which provides stability to macromolecules, membrane system and other functions inside the body and an essential molecule for metabolic processes (Humayun et al., 2015). United Nations stated that every human being should have safe, sufficient and accessible water supply specially: drinking water, as it is a human right (Fernando et al., 2016). Access to safe drinking water is a real challenge in developing countries due to poor sanitation and hygiene practices (Lyimo et al., 2016). According to World Health Organization (WHO), the death of children from diarrheal diseases increase more than 1.5 million per year. Whereas, the mortality of water-associated diseases surpass 5 million people each year and 50% from these are accounted for microbial intestinal infections (Cabral, 2010). An elevated bacterial amount in

drinking water itself has an increased risk and the existence of antibiotic resistant bacteria in these water sources make this even more complicated. In the 21st century, antibiotic resistance, which is deliberated as the 3rd largest threat to worldwide public health (Lyimo et al., 2016) increases the morbidity, mortality and healthcare expenses by limiting the efficacy of antibiotics that are debatably most effective chemotherapy developed in the 20th century (Odonkor and Addo, 2018).

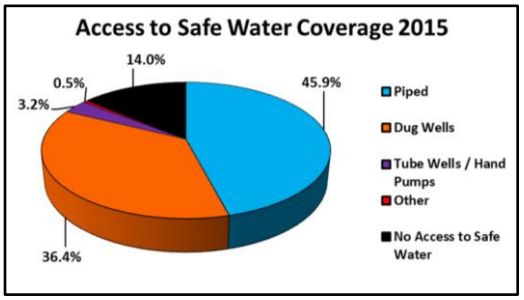

Figure 01. Overall water coverage in Sri Lanka (National water supply and drainage board, 2015)

LITERATURE REVIEW

In Sri Lanka, 94% of the population has an improved access to drinking water while the remaining 6% depend on unprotected water sources on a daily basis (WHO, 2015). Around 60% of Sri Lankan people from rural areas including the areas in Ragama use groundwater from shallow dug-wells for their drinking purpose (Mahagamage et al., 2019) (Figure 01.). The study of Arulnesan et al., (2015) showed the groundwater used for drinking is mostly contaminated due to poor protection of dug-wells, latrines built closely to the dug-wells, which are below the recommended distance (16m), poor treatment of water and seasonal variation. The geographical pattern is also a possible reason for groundwater contamination (Arulnesan et al., 2015); accordingly the groundwater in Ragama area need to be properly maintained for consumption, as the area is favorable for laterite aquifers that has low purification capacity (Figure 02).

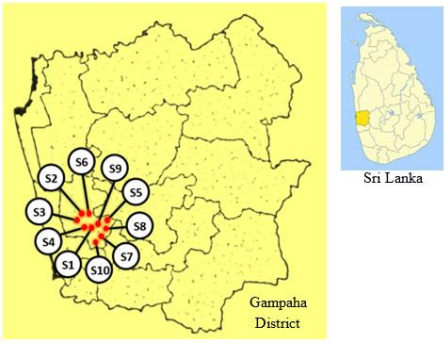

Figure 02. Water sampling locations in different areas of Ragama, Sri Lanka during the study

Water sources that lack admittance to hygienic water increased the risk of exposure to different pathogens such as Vibrio cholera, E. coli, Salmonella spp. and Shigella spp. which could cause diseases in people varying from diarrhea to severe hemolytic uremic syndrome (Fernando et al., 2016). Various wide-ranging water-borne pathogens are present in contaminated water sources, but tremendously at low concentrations (Cabral, 2010). Therefore, detection of these pathogens are quite challenging, but specifically E. coli which are gram-negative, rod shaped, facultative anaerobes (Lupindu, 2017) belong to genus Escherichia and one of the most prominent species of coliform groups, are abundant in human and warm blooded animal feces. Therefore commensal E. coli that are residing in the lower gastrointestinal tract of mammals has been used as the foremost direct microbial indicator of fecal contamination in drinking water (Fatemeh et al., 2014), besides it is indicated that 99% of pathogenic microorganisms do not exist

with the absence of E. coli in drinking water (Abbas, 2019).

The development of antibiotic resistance (AR) in these bacteria is a considerable threat to public health on a global scale (Lyimo et al., 2016). The contaminated drinking water sources shown to be major reservoirs for antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) (Lu et al., 2018), thus both commensal and pathogenic E. coli are associated with AR, since these E. coli considered being a significant reservoir for ARG (Odonkor and Addo, 2018). Hence, E. coli is a beneficial indicator for AR in bacterial communities as well as a vector for AR dissemination (Chen et al., 2017). The progression of AR is mainly linked with genetic variations encoded by bacterial plasmid and chromosomal genes or it can be due to phenotypic resistance (Amer et al., 2018). In the enterobacterial gene pool, E. coli has the ability to act as both donor and recipient for ARG, therefore can acquire ARG from environmental bacteria and can also permits its ARG to other bacteria in humans (Poirel et al., 2018) by the process of horizontal gene transfer (Chen et al., 2017). It mainly includes bacterial conjugation by mobile genetic elements: plasmids and transposons (Liyanapathirana and Thevanesam, 2016), natural transformation by naked DNA and bacterial transduction by bacteriophage (Sun, 2018) (Figure 03).

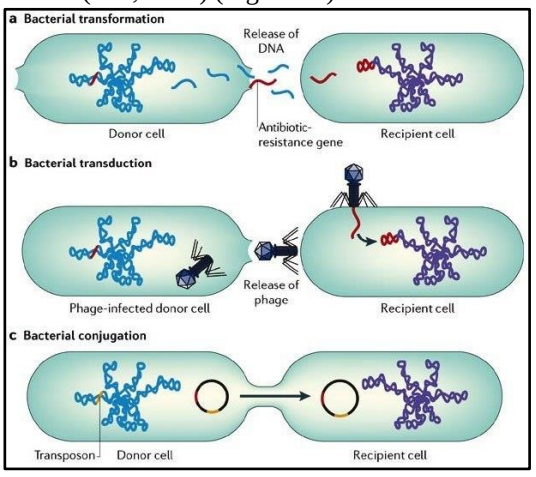

Figure 03. The horizontal gene transfer between bacteria (Furuya and Lowy, 2006)

The antibiotic susceptibility studies shown by Jayatilleka (2014) depict the antimicrobial resistance has emerged in Sri Lanka as well. The research study conducted in Kelani River, Sri Lanka by Kumar et al., (2019) revealed that almost all of the E. coli isolates in their study were resistant to more than one antibiotic and among them higher resistance was shown for older generation antibiotics specially for tetracycline and sulfonamide (Figure 04). Generally, tetracycline inhibits the protein synthesis in E. coli by averting the binding of aminoacyl-t-RNA to the 30S ribosomal subunit and E. coli obstructs this mode of action via mediating three main resistant-mechanisms (Adesoji et al., 2015). The leading resistant-mechanism in E. coli is the expulsion of tetracycline from the cytoplasm via the energy-dependent efflux pumps mediated by transporters of the major facilitator superfamily (Adesoji et al., 2015; Aminov et al., 2004). According to Poirel et al., (2018) 09 tetracycline efflux genes including tetA, tetB and tetC have been identified in E. coli that encodes for efflux-pumps. Both tetA and tetB, which are segments of non-conjugative transposons: Tn1721 [tetA] and Tn10 [tetB] are the commonest tetracycline-resistant genes in E. coli. The second mechanism comprises of the protection of ribosomes through the synthesis of ribosomal protection proteins (Aminov et al., 2004). E. coli has two genes: [tetM and tetW] encoding for these proteins and among these the presence of tetW genes were detected in Kelani River (Kumar et al., 2020). Enzyme inactivation of tetracycline has been recognized in E. coli recently by gene: tetX encoding for oxidoreductase (Poirel et al., 2018; Zibandeh et al., 2016). Sulfonamide, which is a structural analogue of p-amino-

benzoic acid (PABA), acts as a competitive inhibitor for dihydropteroate synthetase (DHPS) resulting in inhibition of folic-acid synthesis pathway. DHPS is a bacterial enzyme used for the conversion of PABA to Dihydrofolic acid in folate synthesis pathway (Byrne-Bailey et al., 2009; Zhang et al., 2009). Sulfonamide resistance is mediated by the acquirement of sul genes; sul1, sul2 and sul3, encoding alternative DHPS enzymes which are insensitive to sulfonamides or by mutational modifications in the folP genes encoding for DHPS enzyme (Byrne-Bailey et al., 2009). The sul1, which is a part of class-1 integron is the predominantly prevalent AR gene in E. coli (Poirel et al., 2018) and was also detected during the study in Kelani River (Kumar et al., 2020).

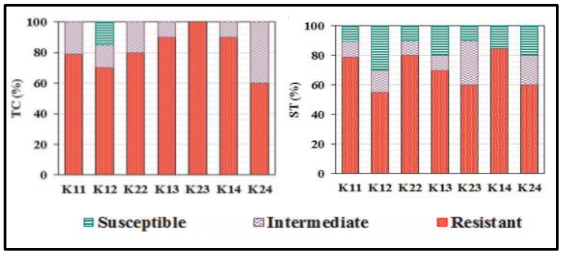

Figure 04. The percentages of tetracycline resistance (left) and sulfonamide resistance (right) detected in Kelani River, Sri Lanka (Kumar et al., 2020)

The suitability of well water for drinking has been evaluated by conducting number of studies in Sri Lanka, but limited records are available on assessing the microbiological quality of drinking water and very few records are available on ARB and ARG with related to water sources. Therefore, the aims of this study were to identify E. coli in drinking water samples using culture-based, biochemical and molecular-based techniques and to detect E. coli resistance towards tetracycline, sulfonamide and gentamycin antibiotics using culture-based and molecular-based techniques. The use of E. coli specific HiCrome agar and targeting E. coli specific uidA gene along with 16S rRNA and lacY genes in this methodology allowed better confirmation of E. coli, besides performing both Kirby-Bauer disk-diffusion test and targeting tetA, tetC and sul1 genes permitted better recognition of antibiotic resistance of E. coli in drinking water.

MATERIALS AND METHODOLOGY

Sample Collection

A total number of 10 water samples were collected into sterile glass bottles from different areas in Ragama, Gampaha District, Sri Lanka. The description of these water samples is tabulated in Table 01.

Table 01. The details of water samples

Sample Name	Location	Water source	Protected / unprotected
S1	Kadurugama	Dug-Well	Unprotected
S2	Batagama	Dug-Well	
S3	Kospalana	Dug-Well	
S4	Hapugoda	Dug-Well	
S5	Batuwatta	Dug-Well	
S6	Polpitimukalana	Dug-Well	Protected
S7	Tewatta	Dug-Well	
S8	Narangodapaluwa	Dug-Well	
S9	Walpola	Dug-Well	
S10	Ragama	Piped tap line	

Physicochemical tests

Each water sample was tested for physicochemical properties within 24hrs of collection. Temperature was measured by thermometer; chlorine concentration by chlorine strips and pH was measured using the pH meter (HANNA-HI8424).

Culturing on HiCrome agar using membrane filtration technique

A total volume of 100mL was taken from water sample and was filtered through a sterile nitrocellulose filter paper (pore size: 0.45µm, diameter: 47mm) using the membrane filtration technique and each water sample was analyzed in triplicates. Each filter paper was placed on a petri plate containing HiCrome E. coli agar (HiMedia M1295) and was incubated (GEMMYCO-IN-010) inverted at 37°C for 24hrs. E. coli ATCC 25922 was used as the positive control and autoclaved distilled water was used as the negative control.

Sub-culturing

After 24hrs incubation, each selected colony from HiCrome agar was inoculated into 10mL of Luria-Bertani (LB) broth (HiMedia) and was incubated at 37°C for 24hrs. These sub-cultures were thus used for further analysis.

Morphology analysis

The morphology analysis was done using the gram staining.

Biochemical tests

Indole test: Initially 5mL of Tryptone broth (HiMedia) was added to a clean-labeled test tube. Then the inoculation loop was dipped in the sub-cultured sample and was inoculated with the test tube containing Tryptone broth. It was then incubated at 37°C for 24hrs. After incubation, 500µL of Kovac's reagent (HiMedia) was added to each test tube and the color of ring formation was observed. The positive control was E. coli ATCC 25922 and the negative control was Staphylococcus aureus.

Citrate test: Firstly, 7mL of Simmon's Citrate agar (HiMedia) was added to a clean-labeled test tube and was kept in a slanted position until agar gets solidified. A clean cotton swab was dipped in the sub-cultured sample and was gently spread on the agar slant in the test tube. It was then incubated at 37°C for 24hrs and the color change was observed. The positive control was Staphylococcus aureus and the negative control was E. coli ATCC 25922.

DNA Extraction and visualization of genomic DNA

The bacterial DNA of newly sub-cultured samples was extracted using Promega Wizard genomic DNA purification kit according to the manufacturer's instructions (Promega, 2019). Then, 0.8% agarose (Sigma-Aldrich) gel was prepared and was placed inside the electrophoresis chamber containing 1x TAE buffer. Next, 5µL of DNA sample was mixed with 2µL of loading-dye (5x PCR buffer) and each sample was loaded into the wells separately. The gel electrophoresis (HiMedia) was run at 60V for 25mins. After 25mins, the gel was kept inside the UV-transilluminator (Life technologies: E-Gel Imager) and was connected with gel documentation system. It was then visualized under UV-base option in the gel capture application.

Identification of E. coli by PCR

PCRs were carried out targeting 16S rRNA, uidA and lacY genes for the identification of E. coli. The master mixes were prepared separately with autoclaved distilled water, PCR buffer (5X), dNTPs (10mM), MgSO4, respective forward and reverse primers (2.5µM of each) and Taq Polymerase (5units/µL). After preparing the master mix, 23.5µL of it was added to each PCR tube containing 1.5µL of respective DNA sample thereby entering a total volume of 25µL to the PCR machine (BIO-RAD Laboratories-CFX96). The positive control was E. coli ATCC 25922 and the negative control was autoclaved distilled water. The primer sequences (Table 02) and PCR conditions (Table 03) used for each PCR is given below.

Table 02. The primer sequences used for identification of E. coli

Target gene	Primer sequence (5'-3')	Target amplicon size	Reference
16S rRNA	F: GTTGTAAAGCACTTTGAGTGGTGAGGAAGG	424bp	Maheux et al., 2014
	R: GCCTCAAGGGCACAACCTCCAAG		
	F: TCCTACGGGAGGCAGCAGT	466bp	Mohommadi et al., 2003
	R: GGACTACCAGGGTATCTAATCCTGTT		
uidA	UAL (F): TGGTAATTACCGACGAAAACGG	147bp	Molina et al., 2015
	UAR: ACGCGTGGTTACAGTCTTGCG		
lacY	F: ACCAGACCCAGCACCAGATAAG	104bp	Lobersli et al., 2016
	R: TTCTGCTTCTTTAAGCAACTGGC		

Table 03. The thermo-cyclic conditions used for each PCR (Lobersli et al., 2016; Molina et al., 2015; Maheux et al., 2014)

Stage of the PCR	The conditions (temperature, time and number of cycles) followed for each primer set in PCR		
	16S rRNA primers (424bp)	*uidA* primers (147bp)	*lacY* primers (104bp)
Initial denaturation	94°C for 2mins	95°C for 3mins	94°C for 3mins
Denaturation*****	94°C for 30s	95°C for 30s	94°C for 1min
Annealing*****	55°C for 45s	58°C for 30s	58°C for 30s
Extension*****	72°C for 1min 30s	72°C for 1min	72°C for 1min
Final extension	72°C for 10mins	72°C for 10mins	72°C for 5mins
Hold	4°C for ∞	4°C for ∞	4°C for ∞
Number of cycles to be repeated (*****)	35 cycles	35 cycles	35 cycles

Afterwards, the PCR products were visualized using agarose gel electrophoresis. Initially, 2% agarose gel was prepared and was placed inside the electrophoresis chamber containing 1x TAE buffer. Then, 2µL of DNA ladder (100bp) and 2µL of each PCR product was loaded into the wells separately and the electrophoresis was run under two voltages; firstly at 45V for 40mins and secondly at 50V for 15mins. Later, the gel was kept inside the UV-transilluminator and was visualized under UV-base option in gel capture application.

Determination of antibiotic susceptibility of E. coli isolates

The sub-cultures positive for both uidA and lacY were selected and were tested for tetracycline (30µg), sulfonamide (300µg) and gentamycin (10µg) susceptibility on Mueller-Hinton agar (HiMedia) using Kirby-Bauer disk diffusion test where the

sub-cultured samples were diluted with autoclaved distilled water and the turbidity was checked with the 0.5 MacFarland standard. After the invert incubation at 37°C for 24hrs, the diameters of inhibition zones were measured following the guidelines of Clinical and Laboratory Standards Institute (CLSI 2014).

Detection of antibiotic-resistant genes using PCR

A total number of 03 PCRs were carried out for the detection of resistant genes: tetA, tetC and sul1 in E. coli. The master mixes were prepared separately for each PCR and 23.5µL from the master mix was added to each PCR tube containing 1.5µL of respective DNA sample thereby entering a total volume of 25µL to the PCR cycle. The primer sequences for each PCR are tabulated in Table 04. The thermo-cyclic conditions were as follows: for all tetA, tetC and sul1 PCRs, the initial denaturation was at 95°C for 5mins, followed by 40 cycles of denaturation at 95°C for 20s, annealing at different temperatures (tetA: 60°C for 30s, tetC: 64°C for 40s and sul1: 56°C for 30s), extension at 72°C for 30s and a final extension at 72°C for 10mins with a hold at 4°C for ∞ (Chen et al., 2013; Stoll et al., 2012).

Afterwards, the PCR products were visualized using agarose gel electrophoresis. Initially, 2% agarose gel was prepared and was placed inside the electrophoresis chamber containing 1x TAE buffer. Then, 2µL of DNA ladder (100bp) and 2µL of each PCR product was loaded into the wells separately and each electrophoresis was run under two voltages. Both tetA and tetC, were firstly run at 45V for 40mins and secondly at 50V for 15mins. Sul1 firstly run at 45V for 50mins and then at 50V for 30mins. Later, the gel was kept inside the UV-transilluminator and was visualized under UV-base option in gel capture application.

Table 04. The primer sequences used for the detection of antibiotic-resistant genes

Target gene	Primer sequence (5'-3')	Target amplicon size	Reference
TetA	F: GCTACATCCTGCTTGCCTTC	210bp	Tamminen et al., 2011
	R: CATAGATCGCCGTGAAGAGG		
TetC	F: TGCGTTGATGCAATTTCTATGC	335bp	
	R: GGAATGGTGCATGCAAGGAG		
Sul1	F: TTCGGCATTCTGAATCTCAC	822bp	Stoll et al., 2012
	R: ATGATCTAACCCTCGGTCTC		

DATA ANALYSIS

Physicochemical properties of water samples

The temperature of water samples was within 30-31°C while the pH was ranged between 5-7 and the chlorine concentration was <5ppm for each sample.

Colonies observed on HiCrome agar by membrane filtration

The colonies observed on HiCrome agar showed expected blue, and opaque colored colonies. Along with these colonies purple or pink colonies or both colors were also observed (Figure 05). All the observed and selected colonies for further analysis were tabulated in Table 05.

Gram-staining results

The particular blue and opaque colonies showed both rods and cocci bacteria in

pink color while the selected purple and pink colonies only showed pink-colored rods (Figure 02).

Biochemical test results

The indole test carried out showed pink-colored rings for 11 samples and yellow-colored rings for 02 samples (Figure 07) whereas the citrate test indicated blue slants for 10 samples and green slants for 03 samples (Figure 08).

DNA extraction results

The DNA extracted from the samples showed 11 prominent bands and 02 faint bands or no bands as shown in Figure 09.

Results obtained for molecular identification of E. coli

The DNA extracted from Promgea-kit (Figure 09) was used for all PCRs. The agarose gel (2%) images visualized after 16S rRNA, uidA and lacY PCRs are shown and detailed in Figure 10.

Table 05. The selected colonies for further identification and detection purposes

Sample Name	Triplicate	Colonies observed on HiCrome agar	Selected/isolated colonies	Sample name given for selected/isolated colonies
S1	a			S1a-Blue
	b		-	S1a-Purple
	c		-	S1a-Opaque
S2	a		-	S2b-Purple
	b			S2b-Pink
	c		-	
S3	a		-	S3b-Blue
	b			
	c		-	
S4	a			S4a-Blue
	b		-	
	c		-	
S5	a		-	S5c-Blue
	b		-	S5c-Purple
	c			
S6	a		-	S6b-Blue
	b			
	c		-	
S7	a			S7a-Opaque
	b		-	
	c		-	
S8	a			S8a-Opaque
	b		-	
	c		-	
S9	a			S9a-Purple
	b		-	
	c		-	
S10	a	-	-	-
	b	-	-	
	c	-	-	

(Colonies → -Blue Purple -Pink -Opaque, "-" Not observed/selected

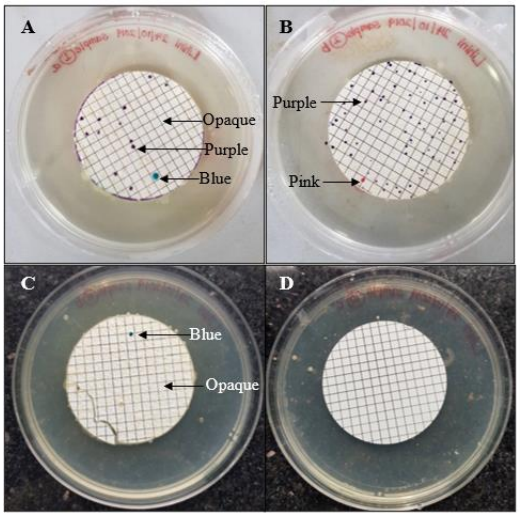

Figure 05. The water samples: 1, 2, 6 and 10 cultured on HiCrome agar. Sample-1a [A] showed blue, purple and opaque colonies while the Sample-2b [B] showed only purple and pink colonies. Both of these water samples [A and B] were collected from unprotected dug-wells. Sample-6b [C] collected from a protected dug well only showed blue and opaque colonies. Sample-10c [D] collected from the chlorinated tap-line showed no colonies on HiCrome agar.

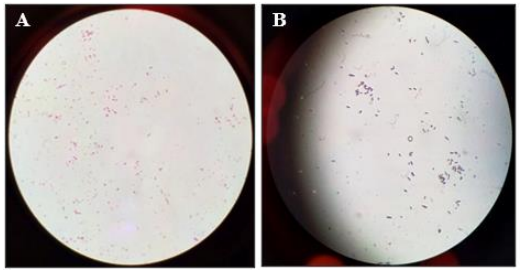

Figure 06. Gram staining of S6b-Blue [A] showed both pink rods and pink cocci while gram staining of S1a-Purple [B] showed only pink rods.

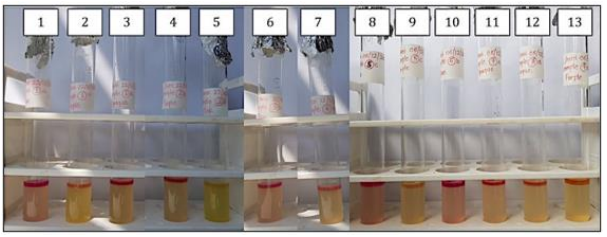

Figure 07. Indole test results. Out of the 13 samples only the S2b-Pink [5] and S5c-Purple [9] showed a yellow ring and all the other samples showed light to bright pink rings

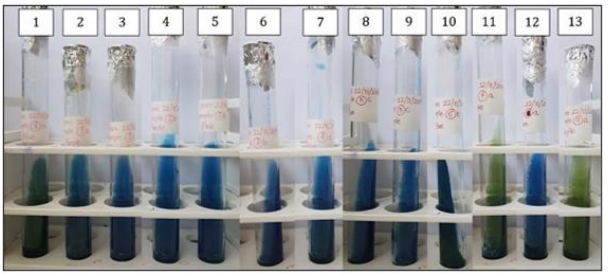

Figure 08. Citrate test results. Out of the 13 samples only the S1a-Blue [1], S7a-Opaque [11] and S9a-Purple [13] showed a green slant and all the other samples showed a clear blue slant

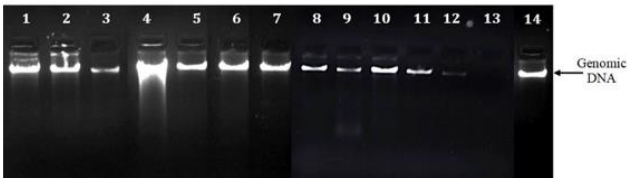

Figure 09. The genomic DNA extracted from the isolated-samples using Promega-kit indicated prominent bands for each sample (Lanes 1-11) except for S8a-Opaque (Lane 12) and S9a-Purple (Lane 13) where S8a-Opaque showed a faint band and no band was observed in S9a-Purple. Lane 14: Positive control (E. coli ATCC 25922)

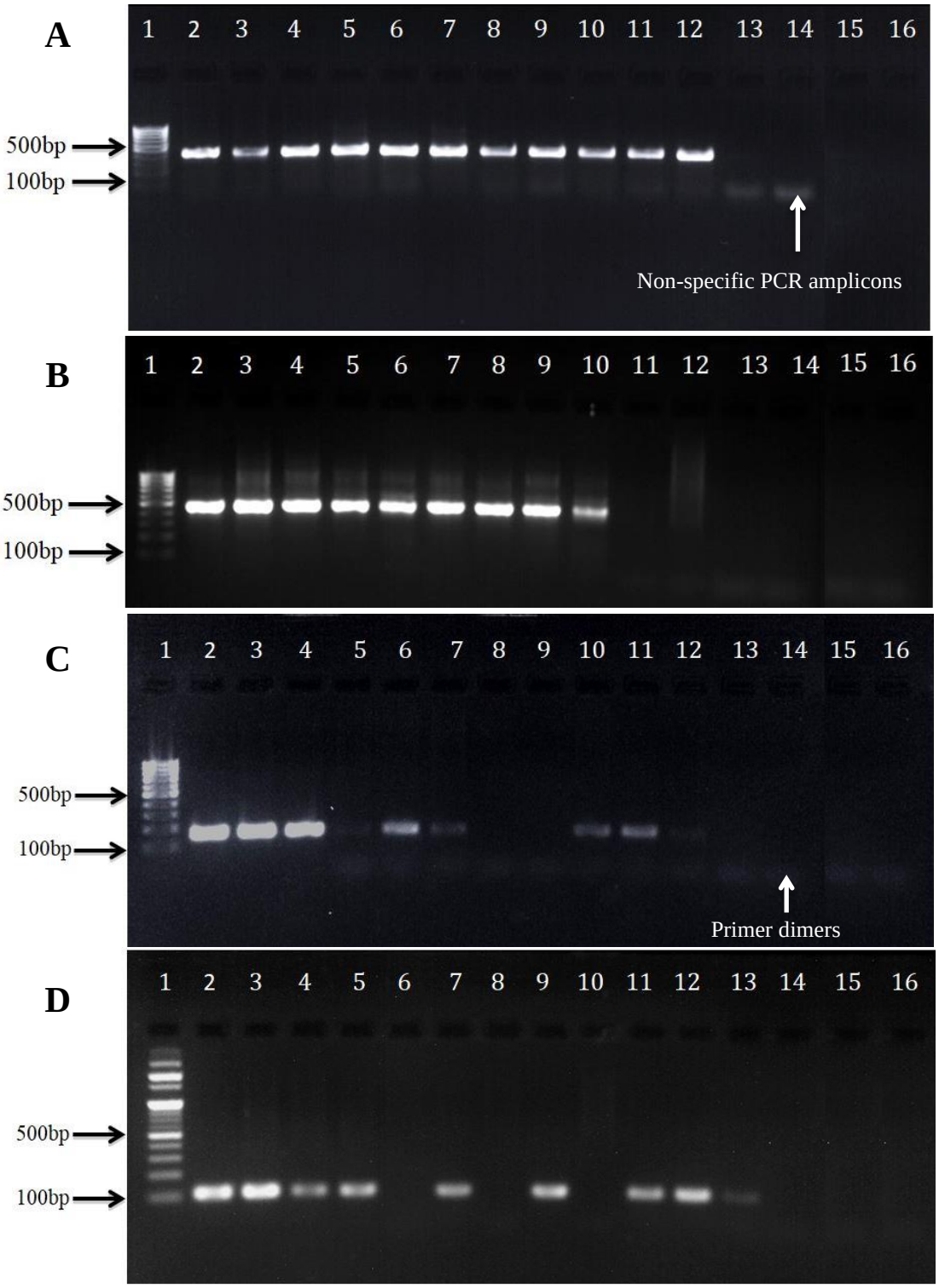
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
500bp
100bp
Non-specific PCR amplicons
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
500bp
100bp
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
500bp
100bp
Primer dimers
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
500bp
100bp

Figure 10. The agarose gel (2%) images obtained from 16S rRNA, uidA and lacY genes amplifications using 16S rRNA primers (424bp and 466bp), uidA primers (147bp) and lacY primers (104bp) are shown in [A], [B], [C] and [D] respectively. Lane 1 indicates DNA ladder (100bp). Out of 13 samples (Lanes 2-14) 09 samples showed clear bands in between 400bp and 500bp for both 16S rRNA PCRs [A and B] while 02 samples: S6b Blue and S7a Opaque showed bands only for 16S rRNA PCR (424bp). Lanes 2-4 in [C] showed intense bands whereas Lanes 5-7 and 10-12 in [C] showed faint bands in between 100bp and 200bp while other samples showed no PCR products. In [D], 09 samples showed bright to faint bands in between 100bp to 200bp range. None of the PCR amplicons were observed in the negative control (Lane 15) and also in the positive control: E. coli ATCC 25922 (Lane 16).

The summary of results obtained for the identification of E. coli is shown below.

Table 06. The summary of results obtained for the identification of E. coli

Isolated sample name	Tests performed (positive results "+" and negative results "-")						
	Gram staining	Biochemical tests		Molecular tests			
		Indole test	Citrate test	16S rRNA (424bp)	16S rRNA (466bp)	uidA (147bp)	lacY (104bp)
S1a-Blue	Pink rods and cocci	Pink (+)	Green (-)	+	+	+	+
S1a-Purple	Pink rods	Pink (+)	Blue (+)	+	+	+	+
S1a-Opaque	Pink rods and cocci	Pink (+)	Blue (+)	+	+	+	+
S2b-Purple	Pink rods	Pink (+)	Blue (+)	+	+	+	+
S2b-Pink	Pink rods	Yellow (-)	Blue (+)	+	+	+	-
S3b-Blue	Pink rods and cocci	Pink (+)	Blue (+)	+	+	+	+
S4a-Blue	Pink rods	Pink (+)	Blue (+)	+	+	-	-
S5c-Blue	Pink rods and cocci	Pink (+)	Blue (+)	+	+	-	+
S5c-Purple	Pink rods	Yellow (-)	Blue (+)	+	+	+	-
S6b-Blue	Pink rods	Pink (+)	Blue (+)	+	-	+	+
S7a-Opaque	Pink rods	Pink (+)	Green (-)	+	-	+	+
S8a-Opaque	Pink rods and cocci	Pink (+)	Blue (+)	-	-	-	+
S9a-Purple	Pink rods	Pink (+)	Green (-)	-	-	-	-

Both *uidA* and *lacY* positive samples (the samples highlighted with color) were chosen for antibiotic susceptibility test.

Results obtained from Kirby-Bauer disk diffusion susceptibility test

Out of 07 selected isolates tested on Muller-Hinton agar for antibiotic susceptibility, 06 showed resistance towards tetracycline and 03 of these were also resistant to sulfonamide. Only 01 sample was susceptible for all the antibiotics (Figure 11).

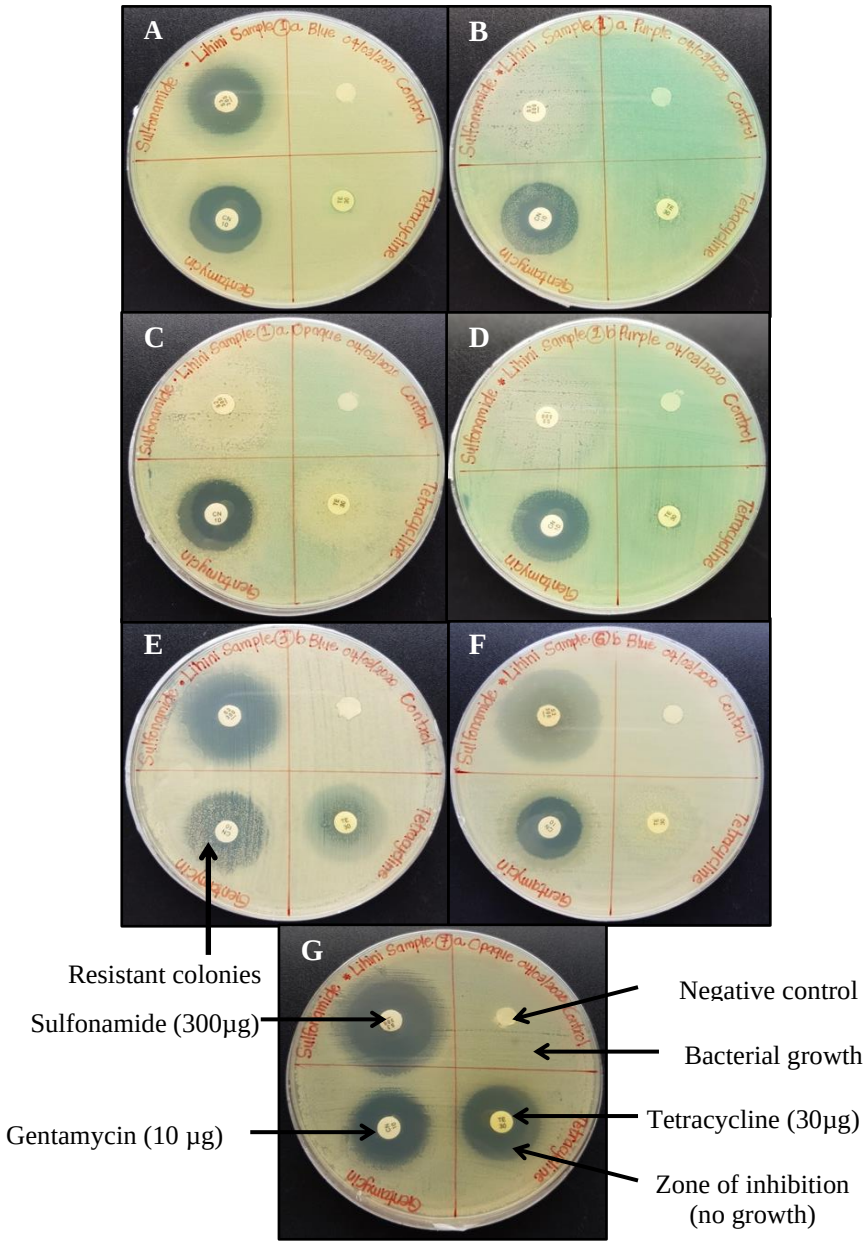

Figure 11. Kirby-Bauer disk diffusion test results on antibiotic susceptibility. S1a-Blue [A], S1a-Purple [B], S1a-Opaque [C], S2b-Purple [D] and S6b-Blue [F] showed complete resistance towards tetracycline whereas S3b-Blue [E] showed a small zone of inhibition for tetracycline with grown resistant micro-colonies in it. Only S1a-Purple [B], S1a-Opaque [C], and S2b-Purple [D] showed complete resistance to sulfonamide and others indicated clear zones of inhibitions except for S6b-Blue [F] where it showed the zone of inhibition with a smear. All the samples showed clear zone of inhibitions for gentamycin except for S1a-Purple [B] and S3b-Blue [E] where it indicated zones of inhibition with few resistant micro-colonies. S7a-Opaque [G] showed clear zones of inhibition for all 03 antibiotics.

Molecular detection results of resistance genes

The tetracycline-resistant samples: S1a-Blue, S1a-Purple, S1a-Opaque, S2b-Purple, S3b-Blue and-S6b Blue and sulfonamide-resistant samples: S1a-Purple, S1a-Opaque, S2b-Purple were tested for selected resistant genes: tetA, tetC and sul1 respectively. Out of the tested 06 tetracycline-resistant samples, 05 showed bands for tetA PCR and none of the bands were observed in tetC PCR (Figure 12). Out of the tested 03 sulfonamide-resistant samples, none of the bands were observed in sul1 PCR (Figure 13).

Figure 12. The agarose gel (2%) images obtained from the tetA and tetC genes amplification using tetA primers (210bp) [A] and tetC primers (335bp) [B]. Lane 1 indicates 100bp DNA ladder. Out of 06 samples (Lanes 2-7) in [A], 05 PCR amplicons showed bands (03 bright bands and 02 faint bands) in between 200bp-300bp along with non-specific PCR amplicons. The remaining sample: S1a-Opaque showed only non-specific PCR amplicons. No bands were observed in negative control (Lane 8) and a clear band was observed in the positive control (Lane 9). In [B], no bands were observed in samples (Lane 2-7) as well as in both negative (Lane 8) and positive (Lane 9) controls.

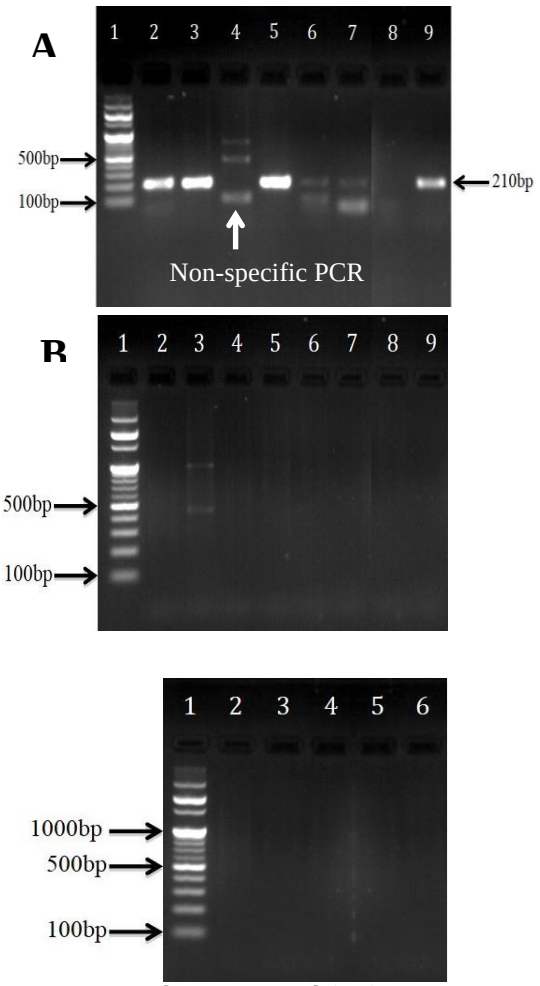

Figure 13. The agarose gel (2%) image obtained from sul1gene amplification using sul1 primers (822bp). No bands were observed in samples (Lane 2-4) as well as in both negative (Lane 5) and positive (Lane 6) controls.

The summary of antibiotic-resistance of the selected isolates is tabulated below.

Table 07. The summary of antibiotic-resistance of the selected samples

Sample name	Kirby-Bauer disk diffusion susceptibility test results				Molecular test results		
	Tetracycline (30µg)		Sulfonamide (300µg)		*tetA*	*tetC*	*sul1*
	Mean diameter (mm)	R/S	Mean diameter (mm)	R/S			
S1a Blue	0	R	20 (±0.00)	S	+	-	NP
S1a Purple	0	R	0	R	+	-	-
S1a Opaque	0	R	0	R	-	-	-
S2b Purple	7 (±0.00)	R	0	R	+	-	-
S3b Blue	10 (±0.00)	R	25 (±0.00)	S	+	-	NP
S6b Blue	0	R	28 (±0.00)	S	+	-	NP
S7a Opaque	21 (±0.00)	S	24 (±0.00)	S	NP	NP	NP

Resistant (R), Susceptible (S), Positive results (+), Negative results (-), PCR was not performed (NP)

DISCUSSION

The WHO and European Union directive have specified that E. coli or other coliform bacteria should not be detected per 100mL of drinking water as it is responsible for the rise of water-borne diseases worldwide (Brown et al., 2011). The presence of coliform bacteria indicates the contamination in drinking water sources, but mainly E. coli, which is the broadly accepted indicator to evaluate the bacteriological quality of drinking water, is used to detect the presence of fecal contamination and possible existence of pathogenic microorganisms in drinking water (Antony et al., 2016; Aung et al., 2015).

Primarily, the outcomes of physicochemical properties: temperature, pH and chlorine concentration revealed these water samples supported the growth of E. coli. Water temperature has a positive correlation to E. coli growth where it could grow-up normally in the temperature range of 20°C-40°C with an optimum growth temperature of 37°C (Islam et al., 2017; Pindi et al., 2013). The survival rates of E. coli are also temperature-dependent and ranges between 4°C-45°C (Bozaslan et al., 2016; Blaustein et al., 2012) where, higher temperatures beyond this range inhibit the growth of E. coli strains due to the denaturation of globular proteins present in its membranes whereas, lower temperatures decrease their metabolism and catalytic activity (Kumar and Libchaber, 2016; Noor et al., 2013). The pH is also important for the survival of E. coli in water as the growth-phase and metabolism of these bacteria depend on the pH homeostasis (Sanchez-Clemente et al., 2018). The optimal pH for E. coli growth is in the range of 6-8, but all the E. coli strains have the ability to grow in between pH of 4.5-9 (Gonzales et al., 2013). However, the study of Vivijs et al., (2016) discovered that E. coli is capable of surviving at extremely low pH (1.5-2.5) due to its more activity shown towards acid-resistant mechanisms. Lastly the chlorine, which is a potent oxidant that reacts with water quickly to accomplish rapid rate of killing the microorganisms present in water, by uncoupling of the electron chain and destructing nucleic acids and cell membranes of microorganisms (Shen et al., 2013; Virto et al., 2005). Liu et al., (2015) showed the

sensitivity of E. coli towards chlorine with 0.5ppm concentration, but the study of Owoseni et al., (2017) reported the recovery and survival of E. coli at low chlorine concentrations, therefore the optimum dose of 1.5ppm was proposed for the complete inactivation of E. coli. All the water samples in this study, showed temperatures as either 30°C or 31°C and pH in between the range of 5-7, proving the possibility of E. coli growth in these water samples. Nonetheless, the chlorine strips used here only detected the concentration was <5ppm in all the samples, hence it is better to use more sensitive techniques: digital colourimeter and colour wheel test kit for the specific detection of chlorine concentration in water samples.

The culturing process on HiCrome agar using membrane filtration technique trapped bacteria into 0.45µm diameter cellulose esters-containing filter papers and mostly E. coli can be trapped because they are 0.5µm in diameter and 1-2µm in length (Abbas, 2019; Bozaslan et al., 2016). Most of the studies reported that chromogenic agar has greater sensitivity, specificity and efficiency for better isolation and differentiation of bacteria in water compared to other culturing-media such as Eosin-Methylene-Blue agar and MacConkey agar (Antony et al., 2016; Akter et al., 2014), because chromogenic media use synthetic chromogenic substrates, which is based on bacterial species' enzyme activity, thus enabling to grow as colored colonies on the agar (Perry, 2017).

HiCrome E. coli agar, which is specific for the faster and accurate recovery of E. coli, is a chromogenic media, where the E. coli cells absorb the X-glucuronide substrate in the media and the intracellular β-D-glucuronidase enzyme cleaves the bond between chromogen and glucuronide, thereby liberating blue color to the E. coli colonies (HiMedia, 2015). Out of the 10 water samples cultured on HiCrome agar, 05 water samples showed blue colonies, but along with these blue colonies either purple, pink or/and opaque colonies were observed and 04 samples showed purple, pink or/and opaque colonies without blue. None of the colonies were observed in triplicates of S10 sample, which was taken from the chlorinated tap-line, indicating that the bacterial growth can be inhibited by chlorine concentration. Furthermore, the water samples taken from unprotected dug-wells showed nearly all the colony-colours whilst the samples taken from protected dug-wells showed either blue, opaque or both colonies.

The positive control only showed opaque-colonies; therefore opaque colonies were also considered as E. coli because sometimes the blue colour may not be expressed although they have the β-D-glucuronidase enzyme. The reasons remain unclear why purple and pink colonies were observed in these samples, because those two colors are possible to occur with X-GAL, ONPG, Salmon-GAL and IPTG chromogenic substrates as it is based on β-D-galactosidase activity (Lange et al., 2013; Browne et al., 2009). The only chromogenic substrate in this agar was X-glucuronide and it is based on β-D-glucuronidase activity of E. coli. This cannot be due to an error because the triplicates of each sample showed the same results; therefore, a possible reason can be due to the halogenation of indoxyl molecules present in the X-glucuronide substrate where it has a considerable effect on the color and intensity of the chromogen (Perry and Freydiere, 2007) thus expressing purple or pink colours instead of blue. The other possible reasons might be due to the fact that certain E. coli strains are not consistent with typical colony-color even if these strains belong to the same group where blue colour chromogen is liberated due to the glucuronidase activity rather than organism itself being E. coli or due to the

presence of background microorganisms other than E. coli where they can influence the colony-colour and growth of E. coli (Verhaegen et al., 2015; Lange et al., 2013).

In gram staining, the isolated samples of each culture mainly showed pink stained rods confirming the presence of gram-negative bacteria due to the counter stain with Saffranin. Pink cocci were observed with rods in some samples suggesting some mixed bacterial growth in these isolated-samples (Thairu et al., 2014).

Under biochemical tests, the indole test indicated significant results while citrate test showed debating results. The tryptophanase in E. coli can reversibly convert the tryptophan in the media into indole (Gaimster et al., 2014), which has many biological functions; therefore indole production is used to distinguish E. coli from other enteric bacteria (Han et al., 2011). Out of the 13 isolated-samples, 11 were indole-positive and the other 02 were indole-negative. Those 02 indole-negative samples (S2b-Pink and S5c-Purple) received attention because of the colony-colours, leading to an idea as in, these might not be E. coli, because 96% of E. coli strains are indole-postive, therefore these samples suggested of Shigella spp. because some Shigella spp. serotypes show indole-negative results (Rezwan et al., 2004). The citrate test was performed to detect the citrate utilization ability as a sole source of carbon in bacteria in isolated-samples, but expected results were not observed for all the samples, because typical wild-type E. coli are always negative for the citrate test (Aditi et al., 2017), but only 03 citrate-negative samples were observed while all the other samples were citrate-positive. The reason for these changes can be as a result of not maintaining the proper aerobic conditions, because aerobically citrate acts as a major substrate for tricarboxylic acid cycle, thus E. coli cannot utilize citrate due to lack of citrate transporters (CitT and CitA-CitB two-component signal transduction systems) or some strains do not synthesize transporters at all (Van-Hofwegan et al., 2016; Brocker et al., 2009). Since E. coli are facultative anaerobes, they can utilize citrate anaerobically as well, using citrate lyase/permease enzyme cleaving citrate into acetate and oxaloacetate following subsequent metabolism resulting in sodium carbonate: the alkaline product, which results in the color change of citrate agar from green to blue (Hemraj et al., 2013; Reynolds and Silver, 1983). The study of Ishiguro et al., (1978) reported though the incidence rate of citrate-positive E. coli are less, certain antigen strains (O-, K- and H-antigens) of E. coli were positive on Simmon's citrate agar and the origin of such E. coli variants are mediated by plasmids coding genes.

The above phenotypic studies could not definitely identify the isolated-samples as E. coli, hence PCR was performed for better identification of bacteria since it has a good sensitivity, specificity and yield results within a shorter time (Pavlovic et al., 2011).

Initially, 16S rRNA, which is a 1500bp large gene encoding for a catalytic RNA that is a portion of the 30S ribosomal subunit, was targeted because it is present in almost all bacteria and considered as highly conserved region within a species and among species of the same genus (Srinivasan et al., 2015). It frequently exists as a multi-gene family and the function of it has not changed over time (Suardana, 2014), therefore two sets of 16S rRNA primers that amplify the fragments of 424bp and 466bp were used, since shorter fragments <1000bp yield more sensitivity and amplify quality nucleotide sequences of the rRNA gene in E. coli (Jenkins et al., 2012). Out of 13 samples, 09 samples showed bands for both 424bp and 466bp 16S rRNA while 02 samples only showed bands for 424bp and none of the bands were observed in S8a-Opaque and S9a-Purple. The study of

Jenkins et al., (2012) reported the difficulty of identifying E. coli and Shigella spp, since they share more than 99% uniqueness in their 16S rRNA gene sequences. The uidA gene was therefore, focused as it encodes for the β-D-glucuronidase enzyme, which is observed in approximately 94% of E. coli strains (Horakova et al., 2008). A total number of 09 samples were positive for uidA and these were positive for 16S rRNA as well. The two isolates: S1a-Opaque and S7a-Opaque were belonged to this category, thereby proving that certain E. coli carry the uidA gene, but do not express the β-D-glucuronidase activity thus exhibiting as opaque colonies on HiCrome agar. This may be due to frame-shift mutations in the uidA structural gene, which can be seen in E. coli O157:H7 as well (Molina et al., 2015). The study of Bej et al., (1991) utilized different uidA primer sequences to amplify uidA region in both E. coli and Shigella spp. and revealed that both the target amino-terminal end and carboxyl end of the uidA gene are unique and conserved in E. coli as well as in Shigella spp. Interestingly, the uidA reverse primer sequence of this study is same to one of the reverse primer sequence of Bej et al., (1991) study. The problem arose with specificity, since uidA primers specific to the uidA chromosomal region also amplifies for Shigella spp. thereby not enabling to differentiate E. coli from Shigella spp. (Godambe et al., 2017), but the importance of uidA PCR is, it confirms that the isolates positive for uidA are definitely either E. coli or Shigella spp. because no other enterobacteria can encode β-D-glucuronidase phenotype with uid gene (Pavlovic et al., 2011; Cleuziat and Robert-Baudouy, 1990).

Shigella spp. are closely related to E. coli due to similarities observed in sequences between housekeeping and plasmid genes (Ud-din and Wahi, 2014). However, E. coli are well-known lactose-fermenters whilst Shigella spp. do not ferment lactose or do it so slowly (Rezwan et al., 2004). The lac operon responsible for the lactose fermentation constitutes of three genes; lacA, lacY and lacZ, where Shigella spp. are deficient in lacY gene, which encodes for the lactose permease enzyme that is essential for the transportation of lactose across cytoplasmic membranes thereby allowing the differentiation of E. coli from Shigella spp. (Awadh et al., 2018; Lobersli et al., 2016). Out of 13 isolates, 09 showed bands for lacY and 07 of them were positive for uidA as well, therefore these 07 isolated-samples, which include most of the blue, opaque, and some purple colonies, were confirmed as E. coli. Remarkably, other 02 isolates: S2b-Pink and S5c-Purple that were uidA-positive but lacY-negative, were the only isolates resulted as indole-negative; hence these two isolates might be a possibility of Shigella spp.

Both uidA-positive and lacY-positive isolated-samples were tested for antibiotic susceptibility to detect the antibiotic resistance of E. coli towards tetracycline and sulfonamide antibiotics, as it is now a growing concern worldwide (Chen et al., 2017). When the Kirby-Bauer disk diffusion test was performed, water from the Muller-Hinton agar was absorbed in to the antibiotic-disk impregnated with a known concentration and then was diffused into the surrounding agar in decreasing concentration as the distance from the disk is increased. If these antibiotic concentrations kill or inhibit the E. coli growth, no growth will be observed in the nearby area around the disk and this is symbolized as a zone of inhibition. The inhibition zone diameter is directly proportional to the antibiotic sensitivity of the isolated-sample and to the antibiotic-disk diffusion rate (Bagul and Sivakumar, 2016; Hudzicki, 2009). Out of the 07 isolated-samples, 06 were resistant towards tetracycline while 03 were resistant to sulfonamide and almost all of them showed no zone of inhibition.

Amongst the isolates: S1a-Purple, S1a-Opaque and S2b-Purple were resistant to both antibiotics exhibiting multi-drug resistance. Interestingly, S1a-Blue isolated from S1 sample was only tetracycline-resistant, whereas S1a-Purple and S1a-Opaque isolated from same S1 sample were resistant to both tetracycline and sulfonamide, thereby proving the presence of different E. coli resistant-strains in the same water sample. All the seven samples were gentamycin-sensitive and the study of Odonkor and Addo (2018) stated that E. coli strains have 90.7% of susceptibility towards gentamycin. In certain samples, there were small amount of micro-colonies growing inside the zone of inhibition, which indicated the tolerant strains of E. coli that is qualified to grow in an antibiotic concentration where other E. coli strains prevented its growth (Gefen et al., 2017).

The tetracycline-resistant and sulfonamide-resistant samples were tested for tetA, tetC and sul1 genes respectively using PCR for further confirmation. According to the results obtained, 05 samples were positive for tetA gene yielding 210bp PCR amplicons and the remaining sample showed non-specific PCR amplicons. None of the PCR yielded tetC amplicons. Both tetA and tetC are efflux genes found on large plasmids or other mobile genetic elements and often transferred via horizontal gene transfer (Poirel et al., 2018). The investigation of Zibandeh et al., (2016) reported tetA gene could be easily transferred via gram-negative bacteria, as it is commonly associated with plasmids. The research-studies of Lyimo et al., (2016) and Adesoji et al., (2015) revealed the presence of tetA, tetB and tetC in water-borne pathogens while tetA being the most prevalent gene.

Sulfonamide resistance is mainly mediated by sul1, sul2 and sul3 as these have been found in environmental bacteria, but amongst sul1 has the highest prevalence due to its location on the 3' conserved region of class-1 integrons (Byrne-Bailey et al., 2009). Unfortunately, none of the PCR amplicons were positive for sul1. A possible reason might be due to the fact that these isolated samples contain other sulfonamide genes: sul2 and sul3 or other resistant mechanisms (Zibandeh et al., 2016).

When tested for the above PCRs, the positive control, S8a-Opaque and S9a-Purple mostly yielded either non-specific PCR amplicons or no PCR products at all. The possible reasons might be due to the number of bacteria presented in the isolated-sample or the concentration of the extracted DNA from bacteria are lower than the detection limit of PCR (Walker et al., 2017; Jenkins et al., 2012), or due to high protein contamination that could interfere with Taq-Polymerase activity (Dilhari et al., 2017). Furthermore, this can be also due to primer-target mismatches during PCR, which diminish the thermal stability of the primer-target duplex, thus hindering the ability of PCR-system to amplify the template-DNA (Stadhouders et al., 2010; Bru et al., 2008).

CONCLUSION

This study was conducted to identify E. coli and detect its antibiotic resistance in drinking water collected from Ragama area and discovered that the presence of E. coli and its antibiotic resistance is high in water samples collected from unprotected dug-wells compared to protected dug-wells. Batagama and Batuwatta samples confirmed the presence of E. coli as well as the possibilities of Shigella spp. Moreover, E. coli isolates in Kadurugama, Batagama, Kospalana and Polpitimukalana samples showed tetracycline-resistance indicating tetA gene as well, while E. coli isolates in Kadurugama and Batagama samples showed resistance to both tetracycline and sulfonamide, thereby exhibiting multi-drug resistance. During the study,

HiCrome agar gave different colony-colours, which were not included in manufacturer's protocol; therefore, it would be better to use another chromogenic agar media for example Tryptone Bile X-glucuronide agar, along with HiCrome agar where both produce blue colonies for E. coli, thereby allowing better confirmation (Verhaegan et al., 2015). Moreover, the isolated-samples can be cultured again on HiCrome agar separately using streak-plate technique, because it would provide more pure colonies thereby giving better results for further tests. In biochemical tests, along with indole and citrate tests, other biochemical tests: methyl-red and vogus-proskauer can be performed. Additionally, a blank without the inoculum can be used along with positive and negative controls to check any contamination in agar media. In this study, each PCR has only taken one primer set to amplify the target gene of interest, but there are many primers designed to amplify the respective genes, therefore selecting the best primers or getting a range of primers that can amplify the target gene of interest would be better as it increases the validity of the results. Apart from tetracycline and sulfonamide, ampicillin-susceptibility can be checked since E. coli show highest resistance for ampicillin in certain studies. Besides, the same antibiotic can be tested with different antibiotic-concentrations to evaluate the E. coli-resistance level. Furthermore, the results on antibiotic-resistance can be confirmed by targeting more resistant genes: tetB, tetD, sul2 and sul3. Furthermore, the necessary water purification assays should be implemented along with proper construction of dug-wells and recommended minimum distance of 16m should be maintained within dug-wells and latrine pits to avoid contamination of water sources thus ensuring healthy and quality drinking water supply for all human beings.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor Ms. Uthpala Amarasekara at BMS, School of Science, for her advice, guidance, comments and suggestions throughout the project. Next, I would like to thank Ms. Himashi Gurudeniya and Mr. Ominda Perera at BMS, School of Science, for their advice and guidance during laboratory sessions throughout the project.

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